Ranque-Hilsch Vortex Tube Thermocycler for fast DNA amplification and real-time optical detection

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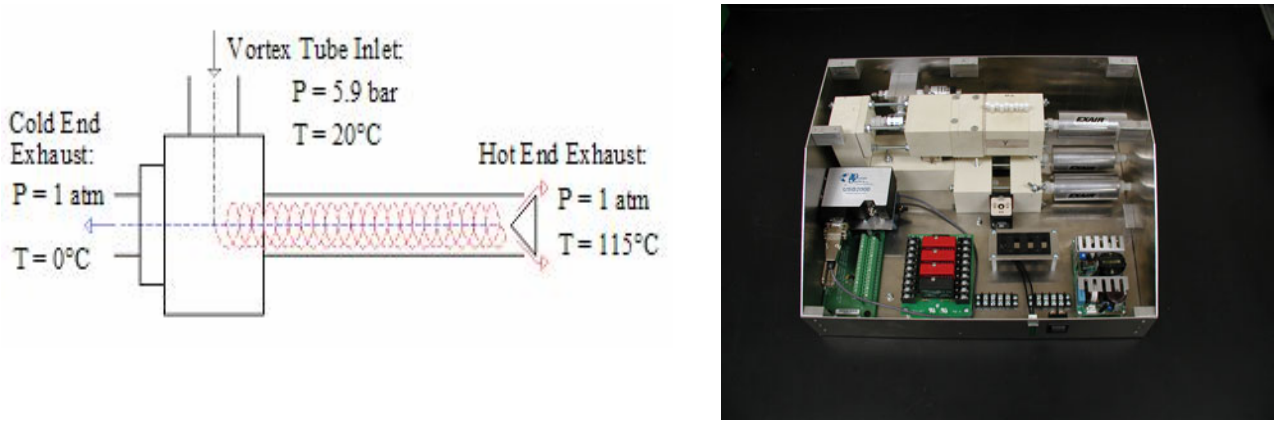
1. Introduction

The Polymerase Chain Reaction (PCR) is a powerful and sensitive enzymatic technique used to exponentially increase the number of copies of a specific sequence of template DNA. The PCR process includes three steps: sample preparation, DNA amplification, and detection. After preparation, the sample mixture (includes DNA, primers, and polymerase) is allotted into a desirable sample size (typically 1 to 100 μL) for amplification. The amplification step consists of thermally cycling the sample for N cycles (~35) between specific denaturation, annealing, and elongation temperatures; typically 90-95°C, 50-65°C, and ~72°C, respectively. Theoretically, one cycle will double the existing amount of DNA, but in practice the PCR process can be described in terms of overall efficiency (Y), where $X=(1+Y)^N$ is the DNA amplification yield. Typical PCR amplification has an efficiency of 70% and requires approximately 35 cycles for 10^8-fold amplification. Gel electrophoresis is the standard for product detection. However, real-time detection can be achieved by measuring the fluorescence of dye/DNA complexes throughout the amplification stage of the reaction. This drastically reduces total assay time by eliminating the need for an individual step for product detection. Real-Time PCR also provides the required optical measurements used in quantitative PCR analysis.

There are many types of PCR machines used in practical applications. However, these devices may generally be classified into two categories: *robotic devices* which move the DNA samples to the heat; and *thermocyclers* which bring the heat to the samples. Robotic devices, such as Stratagene's ROBOCYCLER™ (Strategene, La Jolla, CA), move tubes containing PCR reaction samples to and from a series of water baths, which are heated to different temperatures. Although robotic thermocyclers can be useful in certain applications, they are incapable of high-speed PCR due to their rates of heating and cooling (approximately 2 min/cycle). The two basic types of thermocyclers are programmable heat blocks and forced hot-air thermocyclers. In commonly employed heat block thermocyclers, the amplification stage consists of cycling the temperature of the samples using computer-controlled heat blocks. These devices typically require hours of operation time due to the slow heating and cooling processes. Forced hot air thermocyclers, such as the LightCycler™ (Roche Diagnostics Corporation, Germany) have drastically reduced typical amplification time by eliminating the large thermal mass of heat blocks and utilizing convection heat transfer between air and thin walled capillary tubes. PCR amplification consisting of 30 cycles can be performed in as little as ~10 to 30 minutes.

Some of the most recent publications describe PCR results using natural convection processes. For example, Krishnan *et al.*[1] used a Rayleigh-Benard convection cell to amplify 315 ng of DNA in a 35μL sample with a reaction time of 1.5 hours. The amplification and reaction time reported is comparable to standard PCR thermocyclers. Braun *et al.*[2] amplified 500 pg of bacteriophage λ-DNA in a 20μL sample 10^5- fold within 25 minutes of assay time.

The heating and cooling performance is the primary concern in all automated thermocyclers. The innovation of the thermocycler described in this paper relies on taking advantage of the natural heating and refrigeration capacities of the Ranque-Hilsch Vortex Tube. A vortex tube is a device that produces two gas streams of significant temperature difference from a single source of compressed gas. An attractive feature of the vortex tube is its reliability – it operates without any moving parts or electrical components. The inlet gas pressure is typically in the range of two to eleven atmospheres and enters a cylindrical container tangentially through one or more ports. After entering the cylinder, the gas spirals down the length of the tube (see Figure 1 (a)) where it meets a control valve. According to the valve setting, a fraction of the gas (x) will exit the tube at an outlet temperature up to about 110ºC warmer than the inlet temperature of the gas. The remaining gas fraction (1-x) exits at the other end with a maximum temperature difference of about 70 ºC lower than the initial gas temperature. The outlet temperatures can be varied according to the absolute pressure drops across the tube, the type of gas used, and with the control valve that changes the fraction of gas exiting at each end. Figure 1 illustrates vortex tube operation for one set of flow conditions.



(a) ***(b)***

Figure 1: (a) Ranque-Hilsch Vortex Tube conditions for basic thermocycler operation. (b) The vortex tube thermocycler.

The vortex tube was discovered by George Ranque[3] and a systematic explanation of its performance was achieved by Rudolph Hilsch.[4] However, since that time there has been dispute over the numerous theoretical and experimental approaches used to describe the physical aspects of the vortex tube, now called the Ranque-Hilsch Effect. More recently, these explanations vary from the effects of acoustic streaming in swirling flow researched by Kurosaka[5] to the characterization of the vortex tube as a natural heat pump due to a secondary circulation.[6] Ahlborn and Gordon[7] have quantified the performance of the vortex tube as a thermodynamic machine. Figure 1(b) shows a vortex tube thermocycler that we have built.

2. Ranque-Hilsch vortex tube thermocycler

The majority of vortex tube applications are associated with the tube's refrigeration capacity. The Ranque-Hilsch Vortex Tube Thermocycler presented in this paper is novel in that it uses a vortex tube within a system. Computer control and pneumatic flow valves are employed to use both the hot and cold exhaust streams as necessary to heat/cool the PCR samples. The compact design of the thermocycler gives it overall dimensions of approximately 12 in x 8 in x 6 in. It also has very limited dependence on electricity and can be controlled using a laptop computer. The entire device is based on the simplicity and reliability of the vortex tube and effectively cycles three, 20 μL, sample capillaries within the range of PCR typical temperatures.

The device is entirely dependent on the performance of the vortex tube (Exair Inc., Cincinnati, OH), so its operational parameters were optimized. The compressed air supply used is capable of delivering room temperature air at a flow rate of approximately 3.2 x 10^{-3} m^3/sec (6.8 SCFM) at a gage pressure of approximately 5.9 bar (85 psi). The first step in optimizing the vortex tube is to size the generator, a flow restriction device, to correspond with the available air supply. The appropriate generator should maximize the volumetric flow rate, while providing enough resistance to create a large pressure drop across the tube. The tube operates most efficiently at inlet pressures of about 5.5 – 7.5 bar (~80-110 psi).

The exit temperatures are variable according to the ratio of gas exiting at each end. Typically, the exit temperature difference at each end is inversely related to the mass fraction of gas exiting. For this application, experimentation concluded that the hot gas temperature must exceed 100°C to ensure the sample can still reach the denaturation temperature after experiencing minimal heat loss throughout the system. To obtain the minimum increase in hot gas temperature of 80°C, the vortex tube must be set so the fraction of hot gas exiting is low, approximately 20% of the inlet flow. The resulting cold flow exits the vortex tube at about 0°C. Accordingly, the tube becomes more effective during the cooling process and sample heating becomes the rate-limiting step.

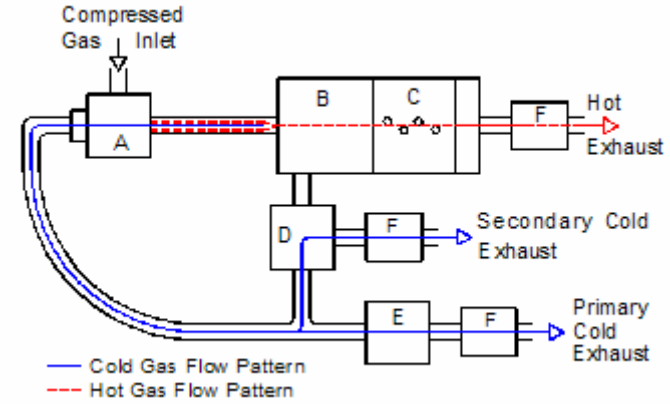


Figure 2: Flow pattern for sample heating: A. Ranque-Hilsch Vortex Tube, B. Vortex Flow Conditioner, C. Sample Chamber, D. Cold Flow Valve, E. Cold Release Valve, F. Exhaust Muffler.

The setup of the thermal cycling device is designed to optimize the convective heat transfer to the sample capillaries during both the heating and cooling processes (Fig. 2). The temperatures of the PCR samples are indirectly measured via a thermal sensor placed in an additional capillary tube. This signal is continually recognized within the computer

programming, which in turn controls the status of two electronically actuated pneumatic valves. Both of these valves are used to direct the cold air flow within the system according to the sample temperature change required.

The system heats the samples by only allowing the hot air to pass through the sample chamber as shown in Figure 2. The three-way cold flow valve is closed, while the two-way cold release valve is open. This valve configuration provides two exhausts for all of the cold air produced by the vortex tube to exit the system with negligible back pressure. Meanwhile, the air exiting the hot end of the vortex tube flows across the samples located in the sample chamber before exiting the system via the sample chamber exhaust. The vortex tube is adjusted so the hot gas temperature is approximately 115°C and the corresponding volumetric flow rate of hot gas produced is approximately 6.4×10^{-4} m^3/s (1.4 SCFM). The machine can heat the samples at an average rate of approximately 3.2°C/sec.

The transition from heating to cooling must be performed without interrupting the operating vortex tube by stopping flow on the cold side. Therefore, the cold flow valve is opened before closing the cold release valve. Adequate cooling is achieved under the configuration where all of the cold and hot gases mix in the vortex flow conditioner before reaching the sample chamber. Since the mass flow rate of cold gas is about four times greater than the hot gas, it is overwhelming and the resulting stream works to cool the samples to the annealing temperature. The cold gas is approximately 5°C before reaching the two valves and the cold/hot gas mixture is typically around 30°C. The samples are cooled at an average rate of 16°C/sec.

When a temperature hold is included in a PCR protocol, the programming logic uses set temperature limits and temperature vs. time slope measurements to control the status of the cold flow valve and keeps the sample chamber at the desired temperature. In many cases, holds are not necessary at the denaturation and elongation temperatures. Under these circumstances, overshooting and undershooting is minimized through feedback control from the previous thermal cycle. This enables the machine to make heating/cooling transition adjustments within a PCR amplification to maximize performance by accurately reaching the desired target temperatures.

Another important factor is the temperature deviation between the samples. The vortex flow conditioner is the first step in minimizing sample temperature discrepancy by effectively mixing the hot and cold gases to provide nearly isothermal inlet flow conditions. The arrangement of sample capillaries then becomes the primary concern. The staggered arrangement (Fig. 2) reduces the effect of the first capillary shadowing the subsequent ones. Also, the sample spacing is minimized to reduce the effect of heat loss to the chamber walls. The thermal sensor is placed in the downstream capillary position to ensure the other three capillaries have reached the desired temperatures. The temperature variance is most important at the denaturation and annealing temperatures. When these are set at 90 and 56°C respectively, the maximum temperature a sample reaches is 91.5°C while the minimum is 55°C. The other capillaries are between these limits and the desired temperature.

The real-time detection is conducted by monitoring the fluorescence in one of the capillaries as the reaction progresses. The reading is taken once per cycle during the heat to denaturation step at a specified read temperature. When the capillary reaches the user designated temperature a blue light source (LS450, Ocean Optics, Dunedin, FL) shines light at 470 nm into the capillary where it reacts with the dye/double stranded DNA complexes. The fluorescence emitted by these complexes is then recorded by a CCD array based

spectrometer (USB2000, Ocean Optics) designed for operation within the VIS/NIR range of wavelengths (approx. 370 – 1050 nm).

3. Experimental procedure

The performance of the Ranque-Hilsch Vortex Tube Thermocycler is demonstrated using a 96 bp λ-DNA amplicon. The sensitivity of the machine and the ability to monitor a PCR reaction real-time was tested by varying the initial DNA concentrations from 0.25 to 100 fg. Reaction progress was monitored by measuring the fluorescence emitted at 520 nm by SYBR Green bound to double-stranded DNA complexes upon excitation at 470 nm. This dye binds to all double-stranded DNA and can not distinguish between a specific PCR amplicon and primer-dimer artifact. Therefore, careful design of primers, optimum reaction conditions, and a Hot-Start PCR technique is very crucial when using SYBR Green for Real-Time PCR.

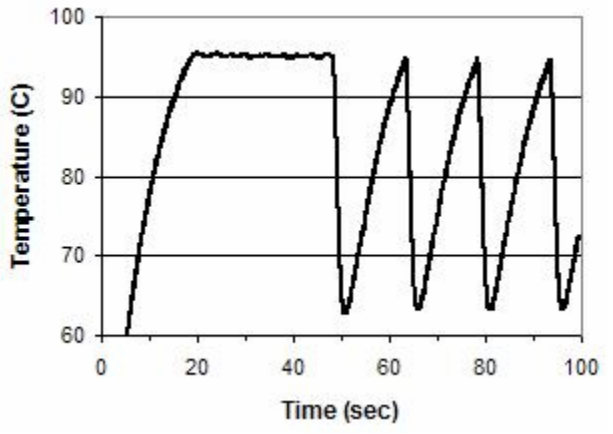
The oligonucleotide primers described by Braun *et al.* [2] were used to amplify the target λ-DNA fragment. The Primer sequences were 5'-GATGAGTTCGTGTCCGTACAACTGG -3' and 5'-GGGCAATCAGTTCATCTTTCGTCATGG-3' with melting temperatures of approximately 61ºC and 62ºC, respectively. The reaction mix (20 µl) consisted of 600 nanomolar of each primer, 100 fg to 0.25 fg of λ-DNA (Fermentas Inc., Hanover, MD), 5 mM Magnesium Sulfate, 200 µM dNTPs, 1X SYBR Green I (S7563, Molecular Probes), 600 µg/mL BSA, and 0.8 unit of KOD Hot Start Polymerase (Toyobo Co. Ltd, Japan) in 1X KOD Hot Start Polymerase Buffer.

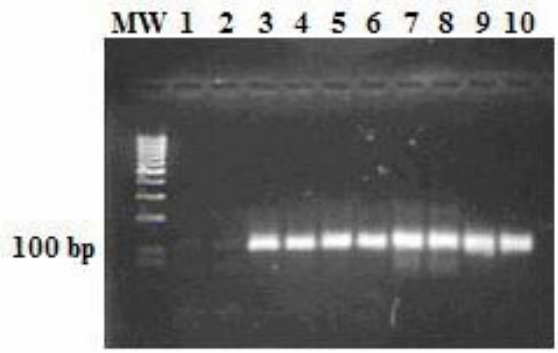
The speed of the Ranque-Hilsch Vortex Tube Thermocycler is best utilized in combination with a fast enzyme, such as KOD Hot Start Polymerase. This enzyme requires an initial 30 second hot start exceeding 90ºC to become fully activated. During this period, thermolabile antibodies bound to the DNA polymerase are inactivated, as described by Mizuguchi *et al*.[8]

Therefore, each reaction consisted of initial activation of the enzyme at 95ºC for 30 seconds, followed by 45 cycles of 95ºC for 0 sec, 62ºC for 0 sec, and 72ºC for 0 sec. There are no holding times required for the denaturation and annealing steps since they happen almost instantaneously. Elongation occurs within a temperature range surrounding 72ºC. Therefore, a large portion of the ramp time from the annealing to the denaturation temperature is utilized for elongation. If a fast enzyme such as KOD polymerase is used (approximately 300 nt/sec at 72ºC), it is possible to copy up to 1,000 bp during the heating stroke, without pausing at 72ºC. The amplification consisted of 45 cycles to demonstrate machine sensitivity to 0.25 fg of bacteriophage λ-DNA (~5 copies). Photon counting was carried out once per cycle using a 500 msec integration time when the sample reaches 80ºC while heating to the denaturation temperature. Samples were also analyzed by 2.5% agarose gel electrophoresis in TAE buffer. The DNA fragments were visualized using 312 nm UV light and photographed with Polaroid Type 667 film.

4. Results and discussion

The total reaction time, which includes the 30 second hot start and subsequent 45 cycles between the denaturation, annealing, and elongation temperatures, was less than 12 minutes. Figure 3 (a) shows the temperature vs. time profile for the hot start and initial 3 cycles of the PCR reaction. Figure 3 (b) shows that positive results were obtained for each initial concentration of λ-DNA while the negative controls (no initial λ-DNA) remained negative. This gel picture was used to conduct amplification and efficiency calculations to demonstrate machine sensitivity. An initial DNA amount of 0.25 fg produced an amplification of 2.7×10^{11}-fold, which corresponds to an overall efficiency of 79.5%.





(a) *(b)*

Figure 3: PCR reaction 0 to 100 fg of 96 bp λ-DNA amplicon. (a) Temperature vs. time profile includes 30 second hot start followed by initial 3 cycles of the total 45 cycles (only 10 shown) programmed for 95, 62, and 72ºC without temperature holds at these temperatures. (b) Gel results of same run. MW: 100 bp molecular weight marker, Lanes 1 & 2: negative control, 3 & 4: 0.25 fg DNA, 5 & 6: 1 fg DNA, 7 & 8: 10 fg DNA, 9 & 10: 100 fg DNA.

The corresponding Real-Time PCR spectrometer data is shown in Figure 4. The PCR reaction is deemed successful when the intensity increases beyond an established threshold value defined as 150% of the baseline intensity. The number of cycles necessary to reach the threshold value increases as the initial DNA concentration is reduced. In the absence of primer-dimer artifacts, the negative control should not exceed the threshold value. Quantitative PCR is possible by calculating the initial concentration of DNA using the number of cycles necessary for the intensity to surpass the threshold value. The 0.25 fg DNA sample surpassed the threshold value 8 minutes into the reaction at cycle number 34.

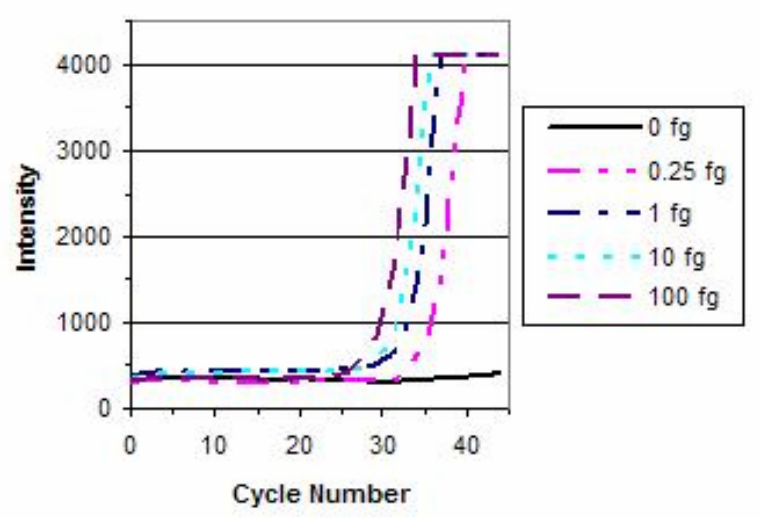


Figure 4: Spectrometer intensity vs. cycle number for increasing initial DNA concentrations.

Concluding, the Ranque-Hilsch Vortex Tube Thermocycler is capable of performing rapid PCR with real-time optical detection. It produces significant reaction yields with very small amounts of initial DNA. Positive results have been obtained from numerous tests amplifying a wide variety of DNA sources. Fluorescence data obtained throughout the reaction minimizes total assay time and can be used for quantitative PCR. Besides direct applications, the speed and sensitivity of this device enables it to be used as a scientific instrument for basic studies such as PCR assembly and polymerase kinetics.

5. Acknowledgements

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6. References

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