

QuanTyper™-48



QuanTyper™-48 is the first real-time thermal cycling instrument utilising SuperConvection™. This patented technology, invented and developed by AlphaHelix Molecular Diagnostics AB, improves thermal cycling processes such as PCR* and makes the instrument both faster and more sensitive than conventional thermal cyclers.

- 40 cycle qPCR including melt analysis in 15 minutes
- 48 samples per run
- Quadruplex
- 20 - 200 µl volume range
- Rapid and unique HRM analysis

Background

Several attempts have been made to speed up the PCR process, mostly by reducing reaction volumes and/or using specialized reaction vessels such as thin capillaries and cuvettes (Wittwer *et al* 1989). The limitations for speeding up ramping rates in such systems are currently set by the time it takes to homogenize the temperature in the reaction mixture, a process driven by the convection at normal gravity, 1 x g.

Heating and cooling of a reaction mixture creates density (temperature) variations within the fluid that the gravity will eventually equilibrate, if waiting long enough. It is important for the outcome of the PCR process that the entire sample reaches temperature equilibrium, preferable as fast as possible, before moving to the next target temperature. If not, poor template denaturation, mispriming and other side-reactions will occur that have adverse effects on specificity and yield (Wittwer *et al* 1991). Up to now, using a larger

reaction volume than 100 µl for PCR has been considered futile due to the lower amplification efficiency seen in larger reaction volumes. Larger reaction volumes are hampered by slower ramp rates as a result of inefficient energy transfer and poor homogenization of the fluid within the reaction tube.

To overcome these problems a novel, rapid and sensitive real-time thermal cycler, QuanTyper-48, has been developed. QuanTyper-48 combines fast ramping and cooling with high-speed centrifugation to generate an increased g-force, which induces rapid homogenization of the entire reaction volume in a process called SuperConvection. The increased g-force acts upon the colder and denser subset of the fluid whereby the temperature equilibrates at a much faster rate than previously possible, in volumes ranging from 20 to 200 µl (Mårtensson *et al* 2006). SuperConvection improves the performance of any amplification process based on thermal cycling, e.g. PCR, real-time PCR and cycle sequencing, as well as demanding High Resolution Melting (HRM) applications.

QuanTyper-48 also features a sophisticated control system for in-tube temperature measurements of the actual reaction temperature. This allows for careful and accurate monitoring of reaction temperature and the degree of homogenisation.

Features and benefits of QuanTyper-48

- **Sensitivity; run 100 – 200 µl reactions for improved sensitivity.**
 - Important when only a few target molecules are present.
 - Makes it possible to dilute the effect of inhibitors potentially present in the sample.
 - Improved mixing increases the number of molecular interactions.

- A larger reaction volume reduces the problem of amplicon re-annealing, thereby allowing more PCR product per volume to be formed.

- **Speed; 40 cycles of amplification (20 µl samples) ready within 15 minutes.**

- Saves time and enables more runs per day.
- Increased specificity due to rapid and precise temperature homogenization (reduces side-reactions).

- **Flexibility**

- Vast working range, from 20 to 200 µl reactions.
- Multiplex with up to four different targets (quadruplex).
- A multitude of filter positions enables detection of almost any combination of dye-labelled molecules. *Custom filter configurations available upon request.*

- **Temperature uniformity and detection accuracy; the revolving format minimizes sample to sample variations.**

- Allows accurate melting point and HRM analysis.
- A single detector ensures uniform fluorescence detection of all samples. Thus, there is no need for internal (passive) calibrators, e.g. ROX.

qPCR applications

While in conventional thermal cyclers homogenization occurs by convection at normal gravity (1 x g), with superconvection homogenization occurs at 3000 x g. This, together with extreme heating and cooling, gives instant homogenization of the samples and previously unachievable temperature ramping rates that lead to more efficient amplification and time savings.

Example #1: Large volume PCR

SuperConvection now makes >100 μ l PCRs a realistic and advantageous choice. In particular when:

- **Only a few target molecules are present in the sample.** Using more of the sample equals more target molecules.
- **The presence of inhibitors influences the result.** Running the same amount of sample DNA in a larger volume of reaction mixture dilutes the effect of inhibitors.

Figure 1 shows how a 200 μ l PCR run, using the same amount of DNA as a 20 μ l PCR run, rescues fluorescence detection (using a *LightUp* probe) and amplification of bovine faecal samples.

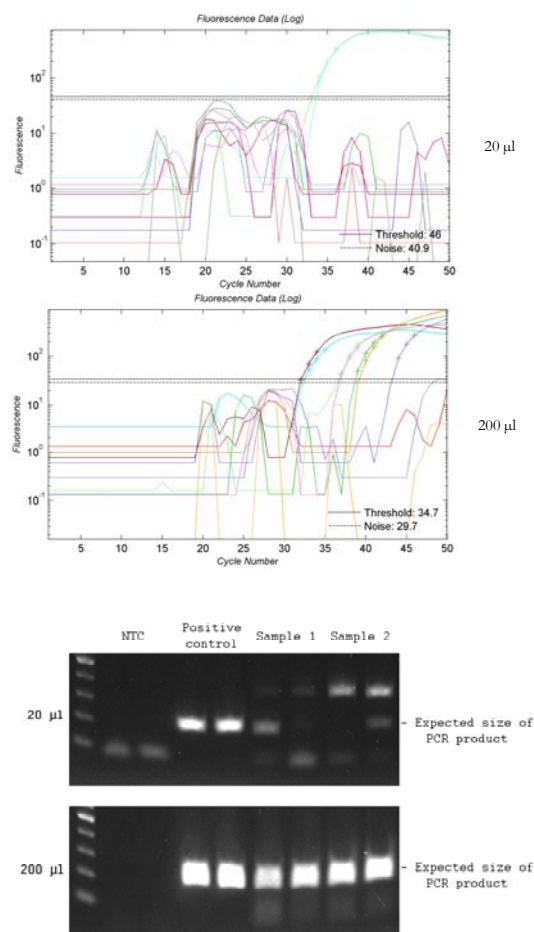


Figure 1 Differences in amplification efficacy between the same samples run in 20 vs. 200 μ l PCR reaction volumes, respectively. Upper panel shows real-time fluorescence detection, and the lower panel shows the same samples

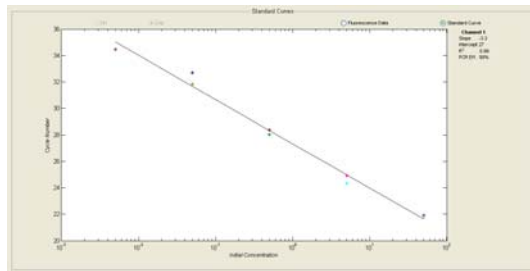
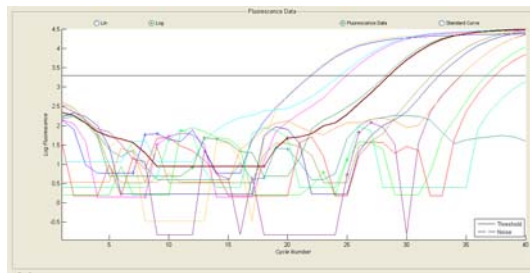
subsequently analysed by 2% agarose gel electrophoresis.

Example #2: 'Normal' volume PCR (20 μ l)

The positive effect of SuperConvection also makes it feasible to run very fast and accurate qPCR assays at the 'normal' 20 μ l scale, using standard 0.2 ml tubes. This is possible without compromising sensitivity or specificity, reducing run times to minutes instead of hours.

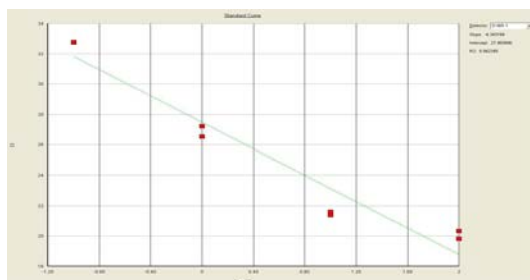
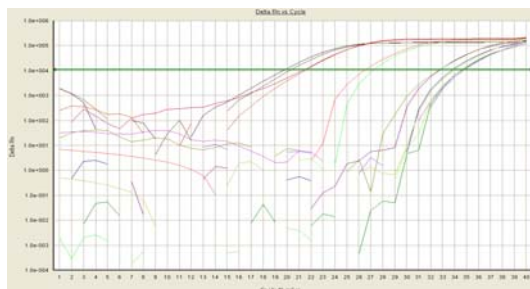
Figures 2a and 2b compare qPCR data generated side-by-side on QuanTyper-48 and ABI 7500 instrument, respectively. Identical samples were run in duplicate on each instrument. PCR reaction mixes contained SYBR Green I for real-time detection. Melting point analysis was used to discriminate between specific and non-specific amplification. As seen in Figure 2a, the result from the QuanTyper-48 run shows growth curves and Ct values with a more even and correct distribution. The standard curve also shows a higher PCR efficiency compared to the ABI 7500 instrument in a run that took just 14 minutes to complete on QuanTyper-48. Interestingly, there were also differences in the quality of product formation (specificity), as shown in Figure 2b, where non-specific bands are visible in most ABI 7500 samples. In addition, the sensitivity was better with QuanTyper-48, as can be seen in the last wells (corresponding to the most diluted samples). Here, one of the two duplicate samples amplified a single gene copy in QuanTyper-48, while in ABI 7500 neither of the two samples amplified.

Raw data from QuanTyper-48
(total run time 14 minutes)



Standard curve QuanTyper-48

Raw data from ABI 7500
(total run time 1 hr 40 minutes)



Standard curve ABI 7500

Figure 2a Platform comparison of amplification efficacy between identical 20 μ l samples run on QuanTyper-48 vs. ABI 7500. The PCR template was human DNA, prepared from blood, and 10-fold serially diluted from 50 ng down to 5 pg (5 pg roughly corresponds to one human haploid copy of DNA). Amplicon length is 115 bp.

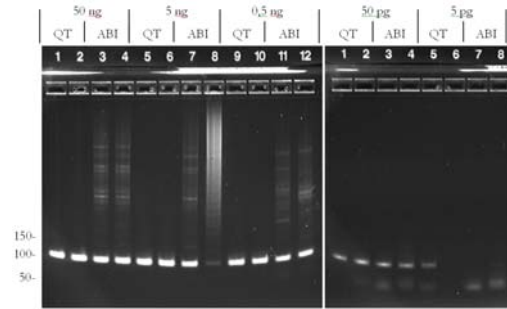


Figure 2b The amplified samples were separated on a 2% agarose gel (E-gel, Invitrogen). QuanTyper-48 produces clean PCR products with sensitivity down to one single gene copy.

HRM analysis applications

The rotor-based format of QuanTyper-48 ensures uniform heating and cooling. The sophisticated in-tube temperature measurement system, in combination with SuperConvection, takes High-Resolution Melting (HRM) analysis to another level in terms of speed and accuracy. HRM analysis in QuanTyper-48 takes only a couple of minutes without compromising performance. Analysis of the raw data, including different degrees of normalization, differentiation and temperature shift compensations, is carried out before presenting the data to the user. Challenging applications such as analysis of Class 4 SNPs (Table 1, SNP classes, defined by Venter et al 2001) is quickly and accurately resolved using QuanTyper-48, as illustrated in Figure 3.

Table 1 The four different SNP classes

SNP class	Base change	Temp shift	Distribution in human genome
1	C/T or G/A	>0.5° C	64%
2	C/A or G/T		20%
3	C/G		9%
4	A/T	<0.2° C	7%

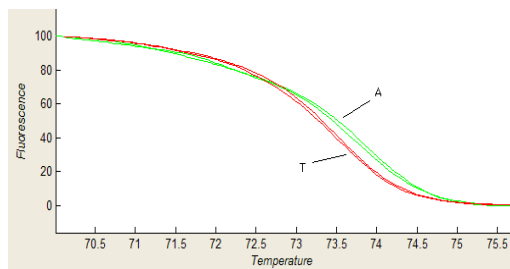


Figure 3 High-resolution melting analysis of a single A/T SNP variation within a 50 bp amplicon. Two amplicons, differing only by a single nucleotide pair (A/T and T/A, respectively), were subjected to HRM analysis using QuanTyper-48. Following PCR amplification in the presence of saturating DNA dye SYTO9, the samples were heated at a rate of 0.1° C/s with continuous acquisition of fluorescence data. After normalization of fluorescence data using the dedicated HRM software, the two genetic variants could be readily distinguished. (Primer sequences kindly provided by Dr. Marie Allen, Uppsala University, Sweden.)

HRM analysis with QuanTyper-48 can also be used to automatically genotype samples as shown in Figure 4. A prerequisite is that samples with known genotypes are included in the run in order to be able to make an accurate genotype assignment of the unknown samples.

Position	Name	Genotype	Confidence
<input type="checkbox"/>	1 B14 1 HV I	undefined	82.781
<input type="checkbox"/>	2 B14 2 HV I	undefined	84.934
<input checked="" type="checkbox"/>	10 B20 1 HV I	aaa	97.36
<input checked="" type="checkbox"/>	11 B20 2 HV I	aaa	99.906
<input checked="" type="checkbox"/>	12 B20 3 HV I	aaa	100
<input checked="" type="checkbox"/>	13 C2 1 HV I	aaa	99.837
<input checked="" type="checkbox"/>	15 C2 3 HV I	aaa	97.264
<input checked="" type="checkbox"/>	16 C5 1 HV I	aaa	99.591
<input checked="" type="checkbox"/>	17 C5 2 HV I	aaa	96.859
<input checked="" type="checkbox"/>	18 C5 3 HV I	aaa	97.626
<input checked="" type="checkbox"/>	19 B14 1 HV II	ccc	100
<input checked="" type="checkbox"/>	20 B14 2 HV II	ccc	99.819
<input checked="" type="checkbox"/>	21 B14 3 HV II	ccc	99.493
<input checked="" type="checkbox"/>	22 B15 1 HV II	ccc	99.941
<input checked="" type="checkbox"/>	23 B15 2 HV II	ccc	93.258
<input checked="" type="checkbox"/>	24 B15 3 HV II	ccc	94.709
<input checked="" type="checkbox"/>	25 B18 1 HV II	ccc	97.138
<input checked="" type="checkbox"/>	26 B18 2 HV II	ccc	91.705
<input checked="" type="checkbox"/>	27 B18 3 HV II	ccc	95.007

Figure 4 Genotyping analysis using standards (see position 12 and 19) with known genotypes. The unknown samples are assigned a confidence score related to the level of agreement with the standards (100 indicates a perfect match).

QuanTyper-48 in cycle sequencing amplification procedures

While much effort has been made to speed up the PCR process, very little has

been done to speed up the linear cycle sequencing amplification process used for Sanger sequencing. In fact, the recommendation regarding the thermal ramping rate for a common cycle sequencing protocol (ABI BigDye Terminator v3.1 Cycle Sequencing Kit) is not to exceed 1° C/s. Such slow ramping leads to run-times of several hours for this amplification step alone. However, Figure 5 shows data that indicates that these slow protocols are no longer necessary when using QuanTyper-48. Excellent Sanger sequencing data was obtained even though the cycle sequencing amplification process was shortened to <20 minutes for 25 cycles.

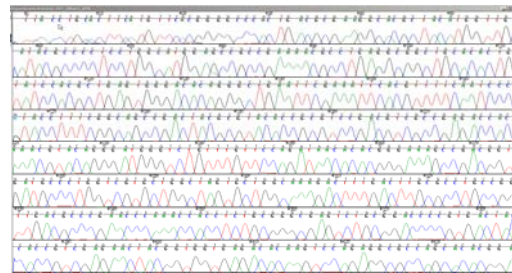


Figure 5 Printout showing the first 400 bases of an FMDV sample (Foot and Mouth Disease Virus) prepared using QuanTyper-48. One microliter of the PCR reaction was transferred directly (i.e. without purification) to a sequencing mix (ABI BigDye® Terminator v3.1 Cycle Sequencing Kit) containing the same forward primer as used in the PCR and then amplified using QuanTyper-48. Total cycle time was 18 minutes for 25 cycles. (PCR samples kindly provided by Alia Yacoub, Swedish Veterinary Institute, Uppsala, Sweden.)

The instrument

The heart of the instrument is the centrifuge that operates at 5000 rpm. A circular infrared (IR) heater circumvents the centrifuge and the samples (see Figure 6). When the IR heater is raised, and lit, it separates the 'reaction chamber' from the cooled air outside, thus facilitating fast temperature ramping rates. Once the IR heater is turned off, and lowered, the pre-cooled air enters the 'reaction chamber' and cools the samples. Fans are used to

further increase the cooling efficiency. An in-tube temperature measurement system is used to accurately monitor sample temperature.

AlphaHelix 0.2 ml sample tubes are designed and manufactured according to very strict specifications to ensure trouble-free operation in QuanTyper-48.



Figure 6 The rotating samples are heated using a circular high-energy IR source.

Software

The QT-48 software runs on Windows® XP and is delivered pre-installed on the personal computer supplied with the system. The QT-48 software is used to define the run parameters, in the 'set-up view'. A 'rotor view' is seen during the run, where the progress is shown together with on-screen fluorescence raw data for the samples, as illustrated in Figure 7.

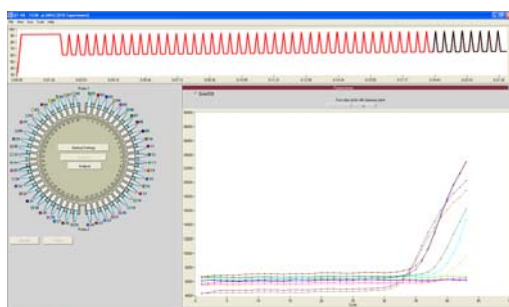


Figure 7 The upper graph shows the progress of the run in a dynamic temperature graph and the lower graph shows the fluorescence signals from the samples in real time.

After the qPCR run, the QT-analysis software will present Ct values for all samples that exhibited amplification. Noise and threshold levels are easily

adjusted in either a linear or logarithmic plot. Amplification efficiency is calculated and a standard curve will be plotted if two or more standards were included in the run. Figure 8 shows fluorescence data and Ct values and the position of the noise and threshold levels.

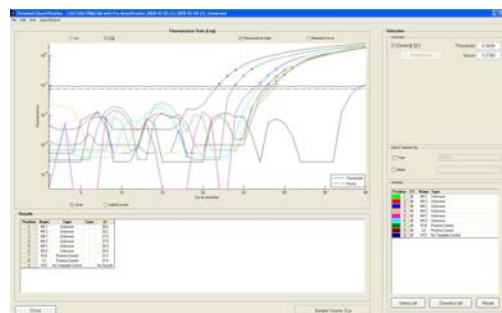


Figure 8 The upper graph shows the log fluorescence data with noise and threshold level settings. Ct values for each sample are presented in the lower left window.

QT-Analysis includes both standard melting point analysis as well as HRM analysis (depending on instrument configuration). HRM can be used to genotype samples, as shown in Figure 9.

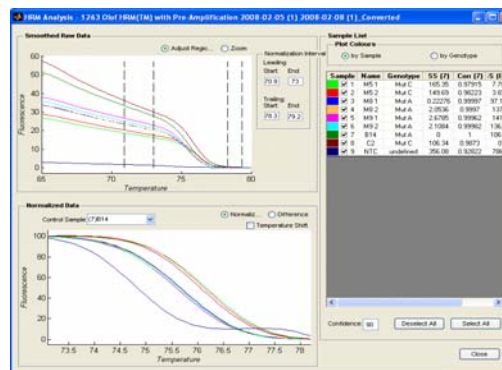


Figure 9 The upper graph shows the raw data with two specified normalization intervals while the lower graph shows the normalized data that is the basis for the final genotyping analysis.

Printout

Printouts are defined in a protocol using a web browser-based interface (e.g. Internet Explorer®). Figure 10 shows an example of a sample table generated as part of an Analysis Report.

The screenshot shows a 'Manual Melt Analysis Report' with a table of PCR products. The table has columns for 'Position', 'Name', 'Type', 'Batch Type', and 'Tm (°C)'. The products are listed in pairs for each channel (Green and Yellow), with names like 'B14.1 HV.1', 'B14.2 HV.1', etc., and Tm values ranging from approximately 80.0 to 85.8 °C.

Figure 10 An example of a sample table, with annotated Tm values, generated as part of an Analysis Report for printout.

Technical specifications

General data

Dimensions: 78 x 47 x 73 cm (WxHxD)

Weight: Approx. 135 kg

Power supply: 220 VAC / 16 A

Power consumption: Idle 300 W, average 2.4 kW, max. 3.5 kW

Noise level: <72 dBA

Operating temperature ambient: 18° C to 32° C

Centrifugation: 5000 rpm (approx. 3100 x g)

Sample capacity

Number of samples per run: 48 (full rotor)

Sample volume: 20 µl, 50 µl, 100 µl, 200 µl

Real time detection system

Optical system: Fixed path length

Detection type: Photomultiplier (PMT) with 16-bit A/D-converter

Data acquisition capacity: Approx. 4000 measures / second

Cumulative data acquisition time per channel: Approx. 1.5 seconds

Detection limit: <1 nM fluoresceine

Multiplex capacity: Two configurations: Duplex or Quadruplex (see below)

Excitation system: LED based, with a fixed excitation filter per LED

Emission filters: Band pass filter

Duplex configuration

Channel	Excitation filter	Emission filter	Example fluorophores
Green	470 ±10 nm	530 ± 15 nm	FAM™, SYBR® Green I, LC Green® plus, SYTO®9, SYTO®13
Yellow	525 ±5 nm	555 ± 5 nm	JOE™, TET™, VIC®, SYTO®82

Quadruplex configuration

Channel	Excitation filter	Emission filter	Example fluorophores
Green	470 ±10 nm	530 ± 15 nm	FAM™, SYBR® Green I, LC Green® plus, SYTO®9, SYTO®13
Yellow	525 ± 5 nm	555 ± 5 nm	JOE™, TET™, VIC®, SYTO®82
Orange	575 ± 5 nm	610 ± 5 nm	CAL Fluor Red 590®, ROX™, Texas Red®
Red	625 ± 5 nm	660 ± 5 nm	Cy®5

Custom configuration with up to 10 excitation and 12 emission filters in total is available upon request.

Thermal performance

General

Pre-run hold temperature: 8° C – 12° C

Temperature working range:

40° C to 98° C @ 20 µl and 50 µl

50° C to 98° C @ 100 µl and 200 µl

Temperature resolution: 0.1° C (smallest programmable resolution)

Melt gradient rate range: 0.1° C/s to 1.0° C/s (in steps of 0.1° C)

HRM analysis gradient rate range: 0.1 - 0.2° C/s

Accuracy of (air) temperature at thermal equilibrium: Standard deviation 0.01° C at 92° C, 72° C and 50° C, measured 60 seconds after clock start.

Maximum heating rate: 8° C /s (in-tube measurement).

Maximum cooling rate: 12° C/s (in-tube measurement).

Typical cycle times

2-step protocol: Cyclic from 95° C hold for 0 s; to 60° C hold for 5 s; back to 95° C.

@ 20 µl:	19.5 s
@ 50 µl:	22.5 s
@ 100 µl:	37.5 s ¹
@ 200 µl:	51 s ¹

A 40 cycle run according to the 2-step protocol takes ≤13 minutes @ 20 µl.

3-step protocol: Cyclic from 95° C hold for 0 s; to 58° C hold in 3 s; to 72° C hold for 3 s; back to 95° C.

@ 20 µl:	24 s
@ 50 µl:	30 s
@ 100 µl:	42 s ¹
@ 200 µl:	57 s ¹

A 40 cycle run according to the 3-step protocol takes ≤16 minutes @ 20 µl.

¹Specification valid for 24 samples.

Typical HRM gradient time

0.1° C/s from 70° C to 95° C takes 4 minutes.

References

1. Wittwer CT et al (1989) "Automated polymerase chain reaction in capillary tubes with hot air". *Nucleic Acid Res.* 17:4353-4357
2. Wittwer CT et al (1991) "Rapid cycle DNA amplification: time and temperature optimization". *BioTechniques* 10:76-83
3. Mårtensson G et al (2006) "Rapid PCR amplification of DNA utilizing Coriolis effects". *Eur Biophys J* 35:453-458
4. Venter et al (2001) "The sequence of the human genome". *Science* 291:1304-1351

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