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ANGELAB

A New GEnetic LABoratory for non-invasive prenatal diagnosis

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D7.3 Digital PCR system: evaluation

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1. EXECUTIVE SUMMARY OR ABSTRACT

As the digital PCR and digital RPA worked well (shown in D7.2) in D7.3 the system is validated by comparing it with spike in experiments or a commercially available digital PCR system from Bio-Rad respectively. Good correlation of data is found within the margin of error. This validates the principle shown in D7.2 for both digital PCR and digital RPA. Decontamination with commercially available decontaminants was experimentally assessed and the most promising ones identified (Task 7.4). The multiplexing capabilities are assessed and two completely new multiplexing concepts are shown (Task 7.5). A conclusion with an outlook is given at the end.

2. VALIDATION OF DIGITAL AMPLIFICATION BY COMPARISON WITH BIO-RAD COMMERCIAL DIGITAL PCR

As shown in D7.2 digital amplification was performed in the AN3 chips. The results of the digital amplification needed to be checked against an independent experiment performed in a commercially available digital PCR system. The Bio-Rad QX100 was chosen due to its use in other published research articles and its availability at University hospital of Freiburg.

In order to verify the data shown in D7.2 the same aliquot of starting material was used to perform a droplet digital PCR in a Bio-Rad QX100 machine. The numbers were compared to ensure that both yield similar results. In fact the two number sets correspond very well as can be seen in Figure 2-1 making this a promising approach. Moreover, it shows that there is no cross talk between the droplets.

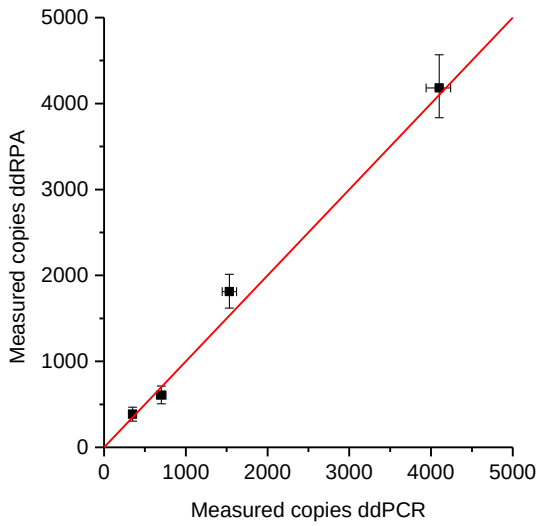

Figure 2-1 The values for ddPCR (Bio-Rad system) and ddRPA (AN3 chip) are compared. The graph shows a good correlation of both experiments. The red line is set to a slope of 1 and added for better comprehension only.

3. VALIDATION OF DIGITAL PCR REACTION BY COMPARISON WITH EXPECTED

COPY NUMBER

The ddPCR shown as proof-of-principle in D7.2 was evaluated here in more detail to prove that there is no cross-talk or inhibition in the droplet digital PCR. In order to show that the droplet digital PCR correctly counts the number of DNA molecules introduced to the mixture, an experiment with a dilution series of DNA template must be performed in order to check whether the linear fit through the data points correlates well with the expected straight line. The results can be seen in Figure 3-1.

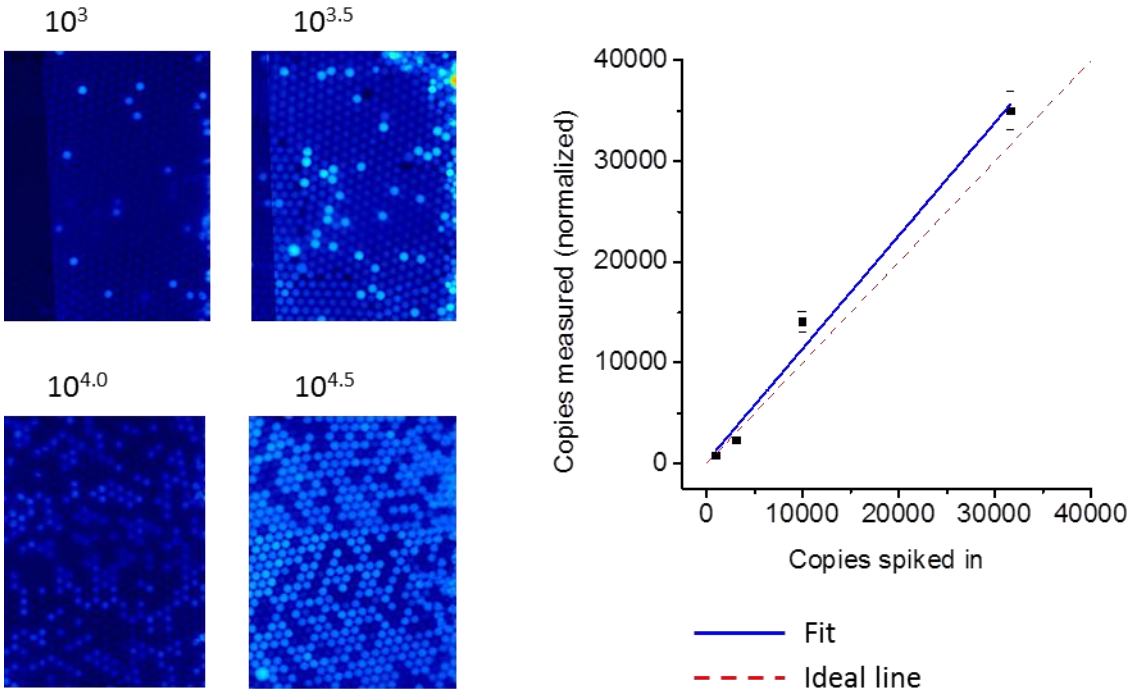

Figure 3-1 Validation of digital droplet PCR. 4 dilution steps with DNA at different concentrations were prepared. The diluted DNA templates were mixed with PCR master mix consisting of polymerase, additives, primers and probes for the template. The pictures on the left show the four independent experiments (false color fluorescent images, cropped). The graph on the right shows the amount of copies that was spiked into the sample (x-axis) and the number of copies measured (normalized) on the y-axis. The black squares show the value after Poisson correction while the black lines correspond to the 1σ-error margine provided by Poisson calculations. The blue solid line shows the least-square fit (calculated with Origin 9.0) while the dashed red line shows the ideal line that would be expected in an experiment without any error sources.

The experiment was performed by spiking in a defined number of DNA templates (4 dilution steps) into 4 aliquots of common PCR mix. The aliquots (20 µl) were emulsified by the developed droplet generation method and cycled in a shallow chamber with a temperature protocol adjusted to the microfluidic chip (taking into account thermal conductivity, thermal mass etc.). After cycling, fluorescent images were taken with a fluorescence scanner. In these images positive and negative droplets were counted. These values were fed into an excel sheet developed by us to automize Poisson calculations. The excel sheet gives back the amount of copies measured plus an upper and lower 1σ-boundary. The experiment corresponds very well with the expected numbers. This allows the conclusion that no cross talk or inhibition take place during ddPCR or in the read out. Slight

deviations can be explained by inevitable errors such as sampling errors, Poisson errors, pipetting errors etc.

4. DECONTAMINATION EXPERIMENTS

DNA strands from external sources, i.e. DNA that was not initially present in the sample, can corrupt sample analysis leading to false results. Often, these contaminating DNA strands originate from previous amplification reactions. It is thus necessary to have a measure to avoid and reduce contamination in the laboratory and in possibly contaminated reusables.

We thus examined the degradation efficiency of different decontamination solutions. In order to measure degradation efficiency instead of a simple removal of possibly still functional DNA, we aimed to do the decontamination process in a closed system. This prevents washing away or adsorption of possibly intact DNA. Therefore, 1 µl dsDNA (3.7 x 108 copies/µl) was added to 9 µl decontamination solution. After 60 minutes of decontamination, 90 µl sodium phosphate buffer (30 mM) were added to dilute the decontamination solution. A PCR was performed using 1 µl of the dilute decontamination solution as template material in a total of 10 µl PCR mix. PCR mix consisted of 1x Finnzymes DyNAmo Flash Probe qPCR Kit master mix (Biozym, Hessisch Oldendorf, Germany), 300 nM primers and 200 nM probes (Biomers, Ulm, Germany) and PCR-grade water. The reactions were spiked with 10^4 copies of lambda phage DNA (Sigma Aldrich, Schnelldorf, Germany) to ensure that no residues of decontaminants inhibit PCR.

Results showed valid amplification conditions for all diluted decontamination solutions, except DNA Exitus Plus, where the amplification of spike-in control DNA was affected. Decontamination with DNA Remover or HCl led to a decrease of amplifiable DNA by a factor of about 2 or 100, respectively (see Figure 4-1). After decontamination with DNA Zap or bleach, no residual DNA could be detected. However, the high limit of detection must be considered when assessing the results. With a limit of detection of about 10 copies per PCR reaction and a dilution factor of 1:100 when transferring the sample to the PCR mix, only copy number above 1000 could be detected in the decontamination solution. The results give a first hint on decontamination efficiencies, but further experiments should be made with our digital amplification system to improve the limit of detection.

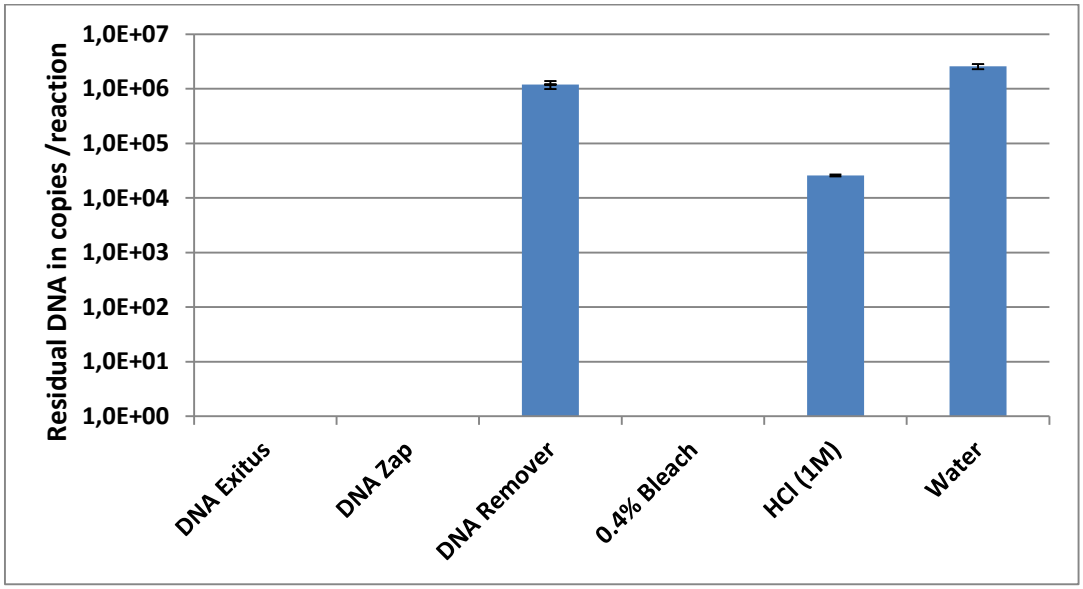

Figure 4-1: Measurement of remaining DNA in the respective decontamination solution. Since only 1 % of the decontamination solution was analyzed per PCR reaction, a 100-fold higher amount of DNA must be considered in the decontamination solution. DNA Exitus affected the amplification of spike-in control even after dilution. DNA in water was not decontaminated and is a reference for decontamination efficiency.

Until then, bleach (0.4 %) or DNA Zap is recommended to decontaminate possibly contaminated reusables, devices and workbenches. The decontamination solution should be allowed to react for at least 5 minutes before the surface is washed or wiped with DI water. However, compatibility of the decontamination reagents with the surface should be tested to prevent corrosion.

5. NEW MULTIPLEXING CONCEPTS

Multiplexing of amplification reactions is used to increase the number of targets that can be detected in one reaction, thereby saving sample, time and materials. Currently the degree of multiplexing (i.e. number of targets that can be detected in one reaction) is mostly limited to the number of colors the reader can discriminate. State-of-the-art fluorescence readers are able to distinguish four colors with six being the absolute maximum. In order to further increase this number the concept of “monochrome multiplexing” is used. This enables the user to distinguish two targets in the same color channel. Two new concepts are shown below, monochrome multiplexing by photo bleaching and monochrome multiplexing by thermal bleaching.

Another degree of freedom is opened by not only using frequency (color) and intensity of the fluorescent signal but extending the system to the time regime. The concept of bleaching refers to the inherent property of most dyes to loose fluorescence intensity over time when irradiated at the right wavelength. This loss of intensity, however, is not equally fast for all dyes and can differ greatly. It is therefore possible to distinguish two dye molecules of the same color attached to two different probes by their bleaching properties. In order to do this a PCR is performed, the sample is scanned afterwards to get a first set of information of the fluorescence intensity. It is then bleached by irradiation with light and scanned again to yield a second set of information. By comparing both of these it is possible to deduce which sample was amplified in which reaction volume (compare Figure 5-1).

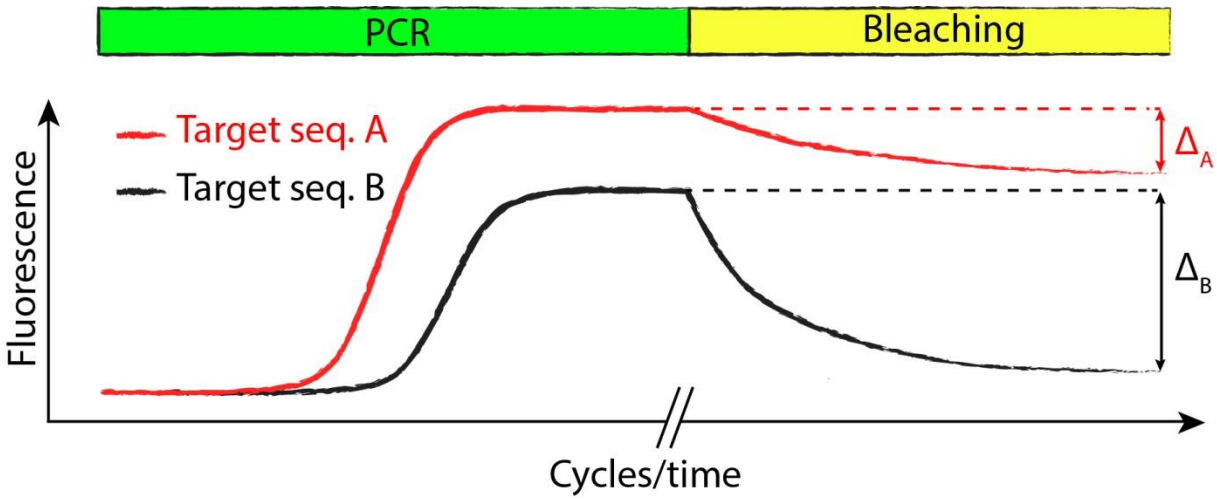

Figure 5-1: Schematic of the investigated monochrome multiplexing concept. Two target sequences (A and B) are amplified by duplex PCR. Target specific hydrolysis probes labeled with fluorophores that are identical in their emission wavelength, but different in their photobleaching behavior, are dequenched during formation of amplification products. Both show a signal increase in the same fluorescence channel. The final fluorescence level can be the same or different (as depicted here). After PCR an external light source is switched on and the fluorescence intensity decreases due to photobleaching. For slowly bleaching dyes such as Atto647N and Atto488 the relative decrease is small (Δ_A), for fast bleaching dyes such as Cy5 and FAM the relative decrease is large (Δ_B).

To test whether two different targets can be distinguished in a duplex reaction with readout in only one color channel, real-time PCR reactions were performed. Two probes with dyes of comparable excitation and emission wavelength (644/669 nm for Atto647N and 650/670 nm for Cy5) but different bleaching behavior were used. Amplification of DNA targets dequenched one of the dyes above. The decay rates of the dequenched dyes upon irradiation with excitation light differ significantly as can be seen from Figure 5-2. The Cy5 and FAM signal pass a 0.8 threshold after only 22 and 49 seconds respectively, whereas the Atto647N and Atto488 signal take 126 and 155 seconds respectively to reach a level of 0.8 and stabilize at around 0.75. The same effect can be seen when using the dyes FAM and Atto488. The visible decay in the signals of Atto647N and Atto488 might be connected to the photobleaching of background of Cy5 and FAM probes respectively. The dyes are quenched by dark quenchers with a quenching efficiency of <100%. The fluorescence emission of the dye that is not quenched by the quencher is termed “unquenched” fluorescence, opposed to “dequenched” fluorescence light which is emitted after the quencher has been removed from the hydrolysis probe. The unquenched fluorescence emission by Cy5 and FAM respectively decreases when irradiated because of photobleaching.

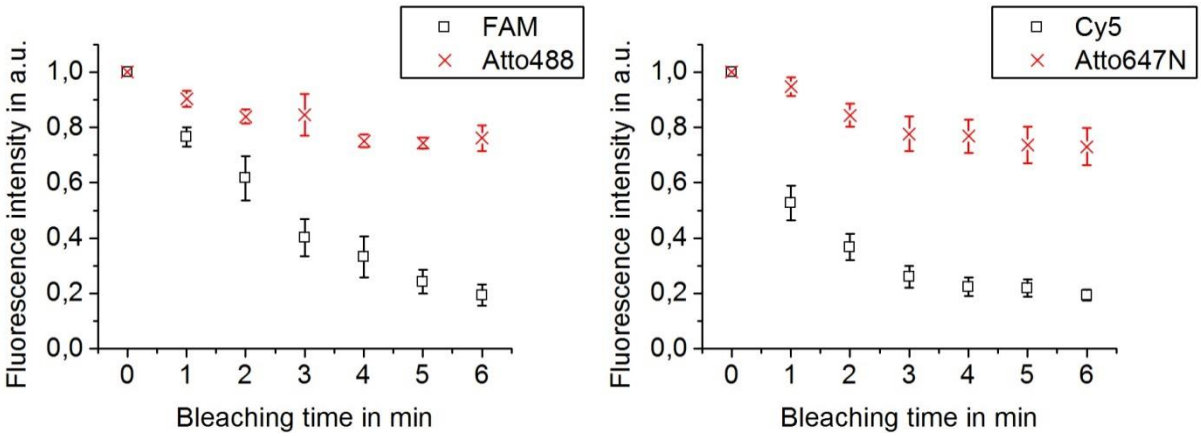

Figure 5-2: Differences in bleaching of FAM and Atto488 or Cy5 and Atto647N labeled dequenched probes. Comparison of fluorescence intensity decay of completed PCR reactions upon irradiation by white LED light. Four different hydrolysis probes labeled with FAM, Atto488, Cy5 and Atto647N were dequenched during PCR. After 4-6 minutes of bleaching a large difference in remaining fluorescence intensity can be seen in both systems. This enables to distinguish probes labeled with dyes of similar emission wavelength (FAM/Atto488 and Cy5/Atto647N).

The concept was also applied to digital PCR as can be seen from Figure 5-3.

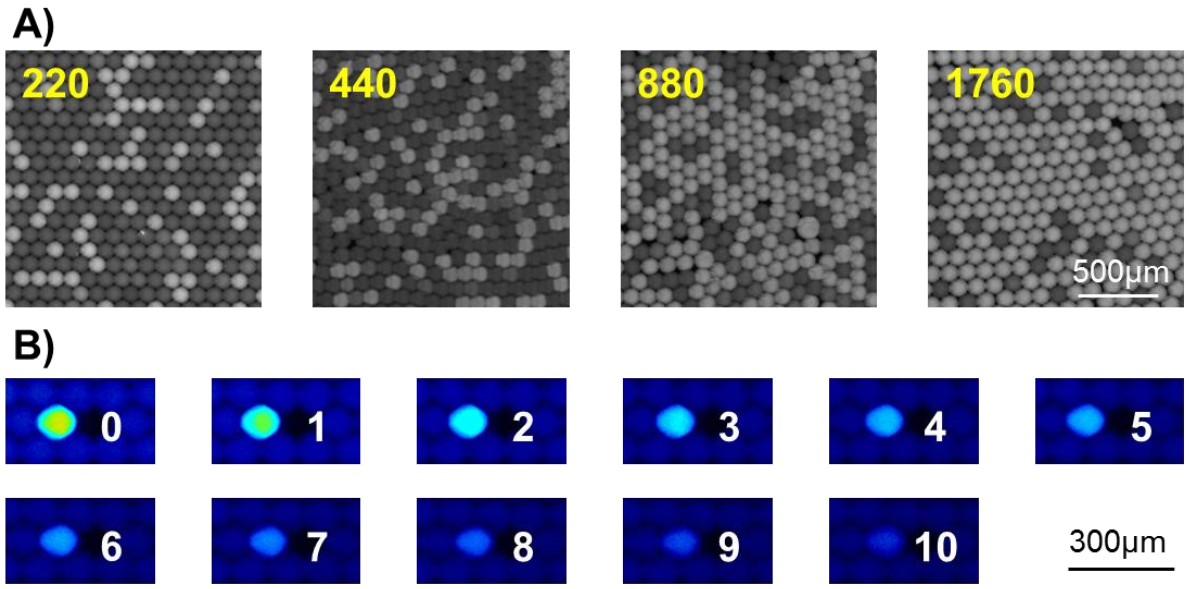

Figure 5-3: Photobleaching in droplet digital PCR. A) Micrographs taken after 10 minutes of irradiation with white LED light. All bright droplets contain Atto488 dequenched hydrolysis probes since FAM fluorescence intensity is reduced to background level after 10 minutes of bleaching. The yellow numbers refer to the expected number of *E. coli* targets per µl. B) Fluorescence intensity decay of a droplet with dequenched FAM probe over the course of 10 minutes (white numbers).

Another approach developed by us also increases the multiplexing degree within one wavelength. Again another degree of freedom is used, temperature. The concept of monochrome multiplexing by temperature uses the inherent property of fluorescent

molecules and quencher to change their fluorescence intensity and quenching efficiency with temperature. The increase or decrease of fluorescence intensity depends on the structure of the fluorophore and is independent of the emission wavelength. This makes it possible to distinguish two fluorophores that emit at a similar wavelength by the temperature dependence of their fluorescence intensity. This approach does not use melting curve analysis and is independent thereof. After (or during) the PCR the fluorescence intensity is measured at two or more different temperatures and the ratio allows to deduce which target is being amplified (compare Figure 5-4).

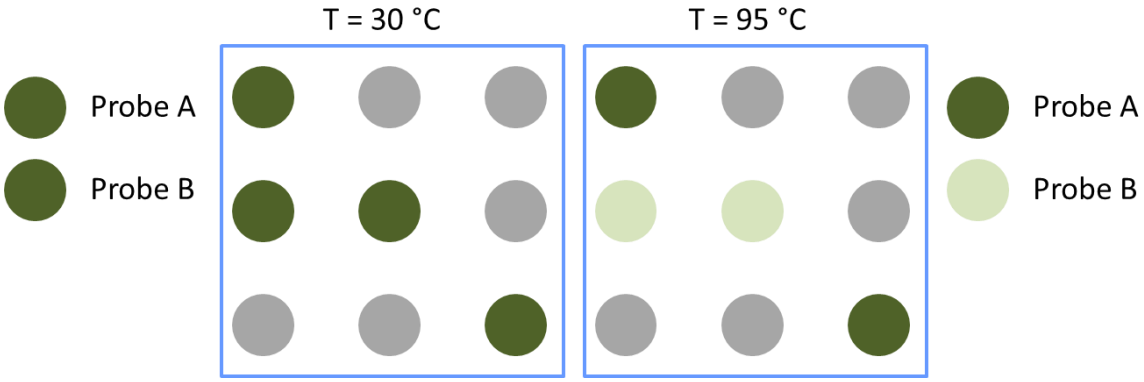

Figure 5-4: Monochrome multiplexing by temperature: The first measurement is performed directly after PCR at 30 °C. Subsequently the sample is heated to 95 °C and measured a second time. The fluorophore of probe B loses its fluorescence intensity quickly compared to the fluorescence of probe A.

The experimental results show that it is possible to distinguish all three cases (only target A present, only target B present, both targets present) as can be seen from Figure 5-5.

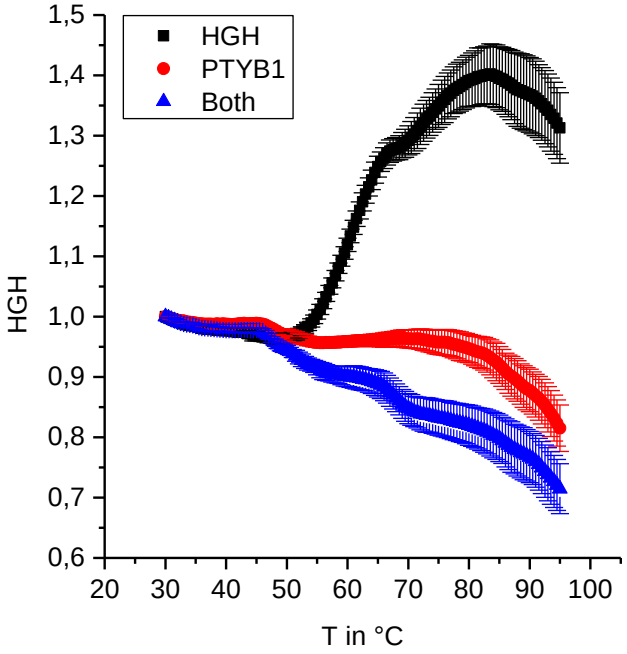

Figure 5-5 Three kind of duplex PCRs were performed. These contained only template HGH DNA, only PTYB1 DNA or both DNA respectively. The probes targeting HGH were labeled with Cy5, the probes targeting PTYB1 were labeled with Atto647N. After the PCR the fluorescence intensity of the completed PCRs were monitored over a temperature range from 30 to 95 °C.

These results are very promising, as they show that the concept of monochrome multiplexing by temperature was successfully used in a duplex PCR system. This enables the user to expand the multiplexing capability without the need for additional equipment. The concept can be performed on any real-time PCR instrument and with any hydrolysis probe PCR system. The time needed for the additional measurement is minimal, since measurement will be performed at two different temperatures only as opposed to the full temperature range shown in the experiments above. For the monochrome multiplexing approach a patent is being prepared while a manuscript for publication waits application for the patent and will be handed in after application.

6. CONCLUSION AND OUTLOOK

We showed that our digital amplification is yielding results comparable to these of commercially available machines but has many advantages over the current state-of-the-art. New multiplexing concepts designed by us enable us to increase the degree of multiplexing without interfering with the standard reaction protocols and standard biochemistry. The concepts are therefor of great relevance to the ANGELab project and can hopefully be further improved with the use of quantum dots or quantum dot rods currently developed by CAN.