Applications

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Improved reproducibility and sensitivity in real-time PCR with Eppendorf® twin.tec *real-time* PCR plates*

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Abstract

In this Application Note, the influence of various consumables on the results of real-time PCR is described. In comparison to transparent micro test tubes, the white wells of the Eppendorf twin.tec *real-time* PCR plates* result in an improved amplification of the fluorescence signal and a reduced influence of the thermoblock on the reflection of the signal. These effects in turn lead to increased reproducibility and improved sensitivity in real-time PCR experiments.

Introduction

In PCR, consumables made from polypropylene are mainly used today, as this material is capable of forming especially thin-walled and well-proportioned micro test tubes that ensure a rapid and consistent temperature transfer from the thermoblock to the sample. In addition, the material is distinguished by low binding properties with respect to proteins and nucleic acids, so that the reaction components are completely available for efficient PCR.
Since the introduction of real-time PCR, the requirements with respect to the components have become stricter. This is the case both for reagents and disposables. Transparent micro test tubes, which are used frequently, can only increase the fluorescence signal to a limited extent. In addition, due to the permeability of the material, the fluorescence signal can be reflected from the thermoblock of the real-time PCR device, and thus have an interfering influence on the fluorescence signal.

Through the addition of titanium dioxide to the polypropylene, the fluorescence signal is considerably increased by the reflection against the walls of white wells. In addition, the fluorescence signal is enhanced more evenly, because the interfering reflection of the thermoblock is significantly reduced. This is proven by a considerable improvement of the reproducibility with respect to replicate samples, while the background noise of the baseline is also reduced. This makes it possible to measure an increase in the fluorescence much earlier, and can, depending upon the threshold value setting, result in lower C_t values, amounting to improved sensitivity of the real-time PCR experiment.

* Eppendorf owns protective rights under European Patent EP 1 161 994, US Patent 7,347,977

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Materials and Methods

The following plates were evaluated within a comparative experiment:

Eppendorf twin.tec PCR plate* with clear wells
Eppendorf twin.tec PCR plate* with frosted wells
Eppendorf twin.tec *real-time* PCR plate* with white wells
Competitor plate A with white wells
Competitor plate B with white wells

For the comparison of the various plates, all preparations were pipetted with the same automated pipetting station ep*Motion*® 5070, and were processed with the same real-time PCR system Mastercycler® ep *realplex*^{4} S. The following PCR system was used in a SYBR Green application:

PCR target: 108 bp, fragment from lambda DNA
Forward primer (600 nM): cgcacaggaactgaagaatg,
Reverse primer (300 nM): ccgtcgagaatactggcaat,
Template: lambda DNA (Roche)

A tenfold dilution series of the lambda DNA was manually created for a range of 100 – 1x10^7 copies for each reaction preparation. In order to exclude the influence of potential pipetting inaccuracies, all additional components were added to the various DNA concentrations.

These mini-master mixes were pipetted into the respective wells of a plate in 6 replicates of 20 µl each with the help of the Eppendorf ep*Motion* 5070. The plates were then heat-sealed with Eppendorf Heat Sealing Film in order to prevent evaporation. Following this, the plates were centrifuged for 1 min at 500 x g and real-time PCR was carried out with the following program:

95°C 2 min
95°C 10s
60°C 30s
} 40x

Results and Discussion

In comparison to a plate with frosted wells (semi-transparent), the absolute fluorescence signals in the twin.tec *real-time* PCR plates* are strengthened more than tenfold due to the reflective properties of titanium dioxide (Fig. 1a). Equally good signal improvements could also be observed in comparison to clear wells (data not shown). In addition to the considerably stronger signals, the enlarged amplification plots (Fig. 1b) show that the replicates of the respective DNA concentrations are amplified much more homogeneously than in transparent wells. In these, the fluorescence is reflected not only onto the walls of the wells, but also onto the thermoblock. In addition, in the event that the reaction vessel is not evenly and completely in contact with the thermoblock, the fluorescence signal will be additionally dispersed due to the refraction index of air.

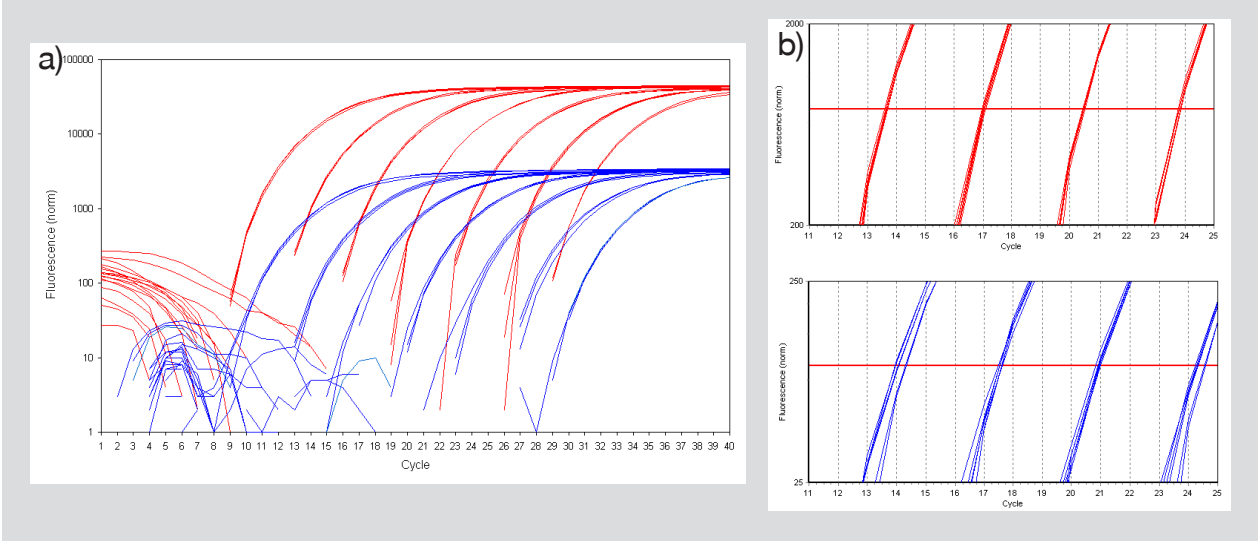

Figure 1: Comparison of fluorescence signals and reproducibility of replicates
a) A lambda serial dilution of 100 to 1x10^7 copies per reaction was amplified with SYBR Green in twin.tec PCR plate* with frosted wells (blue) and twin.tec *real-time* PCR plate* with white wells (red). **b)** The exponential phase of 6 replicates each (1000 – 1x10^6 copies) are displayed in enlarged amplification plots.

This influence is prevented by the white wells of the twin.tec *real-time* PCR plates*.

For purposes of most effective comparability with various consumables, the standard deviations for each 6 replicates were averaged over several log steps and compared (Fig. 2).

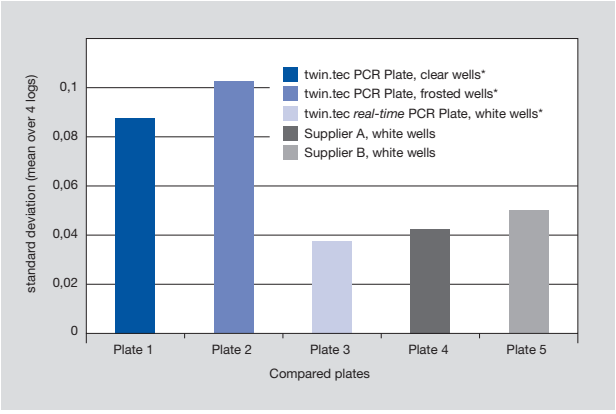

Figure 2: Mean standard deviation over a range of 4 logs
The standard deviation of 6 replicates each was calculated at 1000 to 1x10^6 copies per reaction at a time. The values were averaged afterwards.

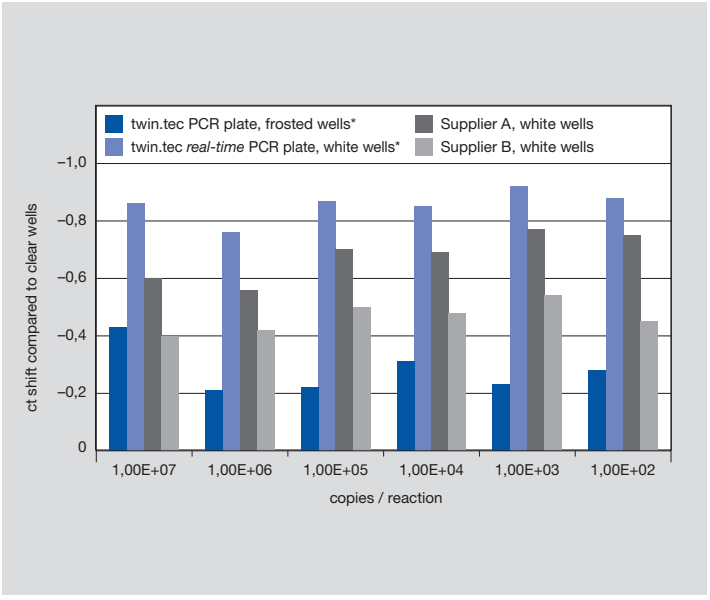

Figure 3: C_t shift improvement
C_t values which were obtained in twin.tec PCR plates* with clear wells were set as equal to 1 for all examined DNA concentrations. The C_t shift improvement of all other plates was compared to the twin.tec PCR plates* with clear wells.

While the transparent wells have a mean standard deviation of 0.09 and 0.1, the reproducibility of the replicates can be improved with the twin.tec *real-time* PCR plates* to a standard deviation of less than 0.04. White plates of other manufacturers also reduced the standard deviation, down to 0.05.
The consistent enhancement of the fluorescence signal by the white polypropylene also improves the signal-to-noise ratio of the measurement, thus supporting the earlier differentiation of baseline and point of increasing fluorescence. The determination of C_t values generally takes place in the exponential increase of the amplification curve. The threshold value for the determination of C_t values is thus very often set to the default of ten-fold standard deviation of the baseline. This evaluation therefore requires a qualitatively good baseline with a low noise level. The comparison shown in figure 3 was carried out with the help of this threshold value setting. It was thereby shown that the plate with clear wells generated the highest C_t values. These were set as equal to 1 for all examined DNA concentrations and viewed in relation to the C_t shift of all other tested plates.

It thereby became clear that frosted wells also offer a minor improvement of the C_t values in comparison to completely clear wells. In contrast, white wells improve the C_t values for all DNA concentrations by up to 0.92. This increases the sensitivity of the assay by a factor of nearly 2, assuming an amplification efficiency of 100 %. The white plates of other manufacturers also show an improvement of the C_t values in comparison to clear wells. However, the C_t shift of alternative white well plates lies at a maximum of 0.77 and 0.54, respectively.

Conclusion

The use of Eppendorf twin.tec *real-time* PCR plates* can increase the sensitivity and the reproducibility of real-time PCR experiments. This offers the greatest advantage for real-time PCR systems with low fluorescence or with small reaction volumes, which can lead to a reduction of the signal. As a result of the improvements shown here, the use of white wells can be of advantage in the analysis of samples, especially those with low nucleic acid concentrations.

Ordering Information

Product	Description	Order no. International	Order no. North America
twin.tec® *real-time* PCR Plate 96, skirted*	white frame	0030 132.513	951022015
	blue frame	0030 132.505	951022003
	black frame	0030 132.521	951022027
twin.tec® *real-time* PCR Plate 96, semi-skirted*	white frame	0030 132.548	951022055
	blue frame	0030 132.530	951022043
	black frame	0030 132.556	951022067
Mastercycler® ep *realplex*^{4} S	with silver block and 4 emission filters	6302 000.601	950020318
ep*Motion*® 5070	Automated pipetting system	5070 000.000	960000005
Heat Sealing Film	10 x 10 units	0030 127.650	951023060
Heat Sealer		5390 000.024	951023078

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