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BIO NEWS

No. 29 – 2008

twin.tec *real-time* PCR Plates: The bright choice

- Concentrating made easy
- Single-cell real-time PCR
- Eppendorf Technology at the **TATAA Biocenter**


Establishment of multiplex real-time PCR protocols

Establishment of multiplex real-time PCR protocols · Silverquant® microarray detection and scanning system · Influence of vessel surfaces on the recovery rate of proteins · etc.

HOLGER EGGERT, EPPENDORF AG

Eppendorf Technology at the TATAA Biocenter

Professor Mikael Kubista's vision for the TATAA Biocenter, a new institute founded in 2001 as a spin-off of the Technical University in Gothenburg, Sweden, was to set a standard in the world of DNA quantification. Operating as an independent enterprise since 2003, the TATAA has now established itself a reputation as one of the top training centers for real-time PCR applications. Besides offering basic courses in real-time PCR, the institute also runs statistics courses or training sessions on topics including immuno PCR, RNA isolation and quality control, which are attended by scientists from around the world.

Mikael Kubista

Eppendorf and TATAA Biocenter have been working closely together since 2005. TATAA employees provide seminars on real-time PCR and automation throughout the world exclusively for Eppendorf customers.

These training seminars allow participants to experience at first hand Eppendorf instruments such as the fast Mastercycler® ep *realplex* and the automated pipetting system ep*Motion*® under the guidance of an expert.

"We train our customers using state-of-the-art equipment", explains Hendrik Sjöström, Head of Marketing at the TATAA Biocenter in Gothenburg. "We are especially pleased about the fact that we were able to persuade Eppendorf to make available the automated pipetting system ep*Motion* in all European centers. This enables our participants to personally experience the positive effect automated pipetting has on the reproducibility and the statistical significance of experiments."

Alongside Gothenburg, TATAA centers have now been established in Freising (near Munich), Prague and San Francisco. Besides offering training courses, the concept of these centers also attaches great importance to research. Freising, for example, specializes in basic research.

Eppendorf's Mastercycler ep *realplex* is being used here to investigate pre- and probiotic-induced gene expressions in livestock. The automated pipetting system ep*Motion* allows high-throughput gene expression experiments to be performed with pipetting volumes as low as 2 µl.

In the TATAA center in Prague, Professor Mikael Kubista in cooperation with the new institute for Biotechnology of the Czech Academy of Sciences is establishing a national core facility for "high throughput gene expression" and "single-cell expression profiling".

Eppendorf technology is also providing major support for this venture. "The precision afforded by automated pipetting and the sensitivity of the Mastercycler ep *realplex* enable 'single cell' experiments", explains Mikael Kubista. "This research work resulted in the development of an analysis software capable of automatically reading Mastercycler ep *realplex* result files, significantly simplifying 'expression profiling'."

The TATAA center in Gothenburg specializes in the further development of real-time PCR based techniques and handling commissioned projects. Their best-known customer is NASA, who wanted to use real-time PCR to prove latent

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virus infections in astronauts. Based on the assumption that "space-related stress" increases the virus replication rate, the brief sought to establish whether hidden illnesses could break out in space.

Research by the TATAA Biocenter revealed, however, that the virus replication rate peaked in the phase prior to take-off. In reply to this finding, the NASA scientists shrugged their shoulders and remarked: "It seems our boys get a bit nervous just before take-off."

Visitors to the next real-time PCR symposium www.qpcrsymposium.com in Palo Alto, USA, will have the opportunity to see the TATAA Biocenter for themselves. Also worth mentioning is the NASA research and exhibition center that is located just around the corner.

Tip: Also see the Application Note on real-time PCR on pages 7-8 in the Application Note section of this issue!

Mastercycler® ep *realplex* • Ref. no. 204 / ep*Motion*® • Ref. no. 189

Tip

Everything's flowing smoothly

For dispensing aliquots of liquid from large supply bottles Eppendorf offers its tried and tested bottle top dispensers Varispenser®[1] and Varispenser® plus[2]. These dispensers are fully autoclavable and highly resistant to chemicals.

If you want to dispense and titrate continuously, then the Eppendorf Top Buret[3] is your first choice for more reliable results. The digital display allows an intuitive operation.

Thanks to a choice of adapters and a telescopic filling tube, the Top Buret can be used with all commonly-used bottle types.

Flexible und durable

The maintenance-friendly design of both the Varispenser and the Top Buret guarantee a long-term durability. Robustness and high-tech materials ensure a chemical resistance which is second to none. More than 100 different chemicals were tested for compatibility with the bottle dispensers.

The result is astounding: From acetone to calcium chloric to hydrochloric acid – none of these chemicals impairs the functionality of the products.

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[2] ChemSaver Dispenser
[3] Brinkmann Bottletop Buret
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What is needed to measure a difference with real-time PCR?

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Abstract

Real-time PCR is today the prime technology to measure differential expression [1]. Results are robust and data reliable. But what sets the limits?

In this application note we describe the Power test and its use in experimental design to estimate the number of replicates needed in order to detect a certain difference in gene expression. We also apply the Power test on experimental data generated by manual and automated pipetting.

The experience was that the higher accuracy in automated dispensing greatly reduces the number of required replicates to achieve a certain discrimination and thus saving valuable time and money.

Introduction

Most scientists are familiar with the comparison of the response of a single marker gene in two groups. In real-time PCR the data are first pre-processed to account for inter-run variations, variations in extraction yields, PCR efficiencies, etc. [2]. Optionally the calculated relative quantities are converted to logarithmic scale to obtain Normal (= Gaussian) variation of the data.

The mean and the standard deviation (SD) of the expression are calculated for each of the two groups and are compared by performing a t-test. The comparison produces a probability (p) value representing the likelihood of observing a difference between means of identical populations.

This value is at least as large as the difference actually observed. This so called 2-tailed test assumes no previous knowledge regarding the groups and both can have higher expression. For didactic purposes we will use the 1-tailed test here.

The 1-tailed test is appropriate when we already know which of the groups will show higher expression if there is a difference. A common case is comparing the effect of a stimulating substance with untreated control. Before conducting a study one should also set a confidence level. This should be based on the consequences of making erroneous inference based on the test result. A common, yet arbitrary confidence level is 95 %. Let us see how this translates into practise.

SD of the CT values of technical replicates depends on factors such as reaction volumes, dispensing, RT and extraction performance. A value of around 0.25 CT, which corresponds to a coefficient of variation (CV) of 1 % at a CT of 25, is quite typical.

Fig. 1 shows a Normal distribution centred at 0 with SD = 0.25. The vertical line drawn at CT = 0.41 dissects the Normal distribution in such a manner that 95 % of the area is to the left and 5 % is to the right of the decision line. This is the decision line at 95 % of the confidence level. If the enhancer has no effect and we perform 100 independent comparisons, 95 of them are expected to show CT < 0.41 and 5 CT 0.41.

Hence, the risk is 5 % that a measurement of an enhancer with no effect will, due to confounding variation, show a difference in expression that will be interpreted as significant. This is the so-called type I error, or false positive.

But, what if there is a difference? Will we be able to detect it? To address this we must specify the difference we wish to detect. Assuming we are interested to determine whether a gene is present in one or two copies in a genome, this then constitutes a 2-fold difference which corresponds to CT = 1.

The case is illustrated in Fig. 2. It shows two Normal distributions with maxima separated by one CT. The distribution centred on zero is the same as before. So is the decision line.

The other Normal distribution shows how measured CTs between a sample containing one copy and a sample containing two copies per genome are expected to vary due to confounding effects. The decision line intersects also the second Normal distribution. 1 % of its area is to the left of the decision line and 99 % is to the right. Hence, using a threshold of CT = 0.41, 5 % of cases having a single copy per genome will be misinterpreted as having two copies (false positive) and 1 % of the cases when the genome contains two copies will be misinterpreted as containing only one copy (false negative). The ability to detect the difference, which is 100 % – 1 % = 99 %, is known as the power of the test.

What if we are interested in a smaller difference, for example, detecting a trisomy? The difference between two and three copies corresponds to CT = 0.58.

Fig. 1

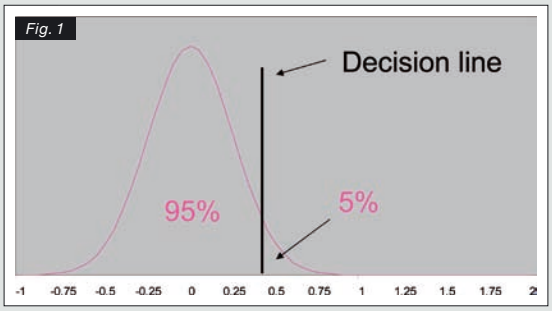
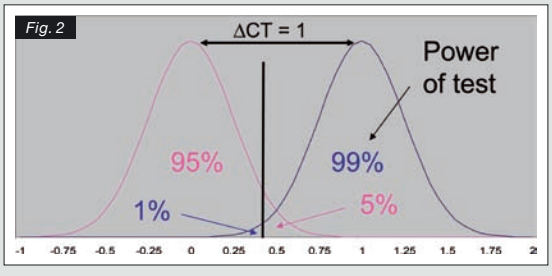


Fig. 2



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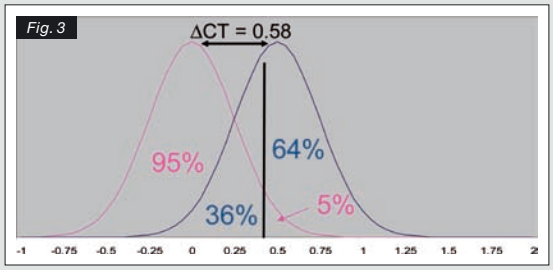
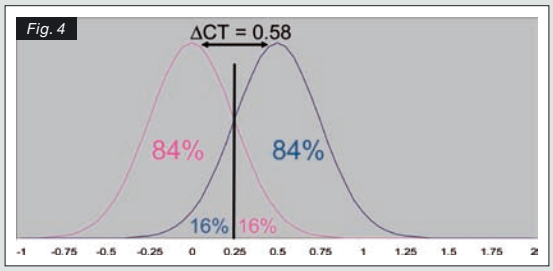
What is needed to measure a difference with real-time PCR?

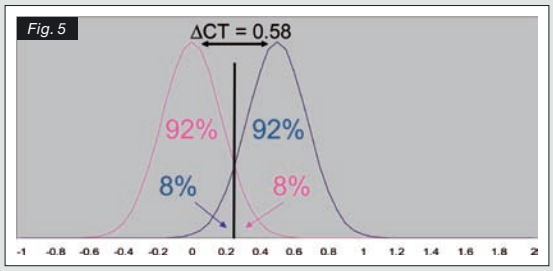
The case is illustrated in Fig. 3, with the decision line at CT = 0.41. The false positive rate is still 5 %, but the false negative rate is now 36 %. The power of the test is only 64 %. Guided by the Power test we may decide to move the decision line. Changing threshold to CT = 0.29, the decision line intersects the two Normal distributions in such a manner that the rates of false negatives and false positives are both 16 % (Fig. 4).

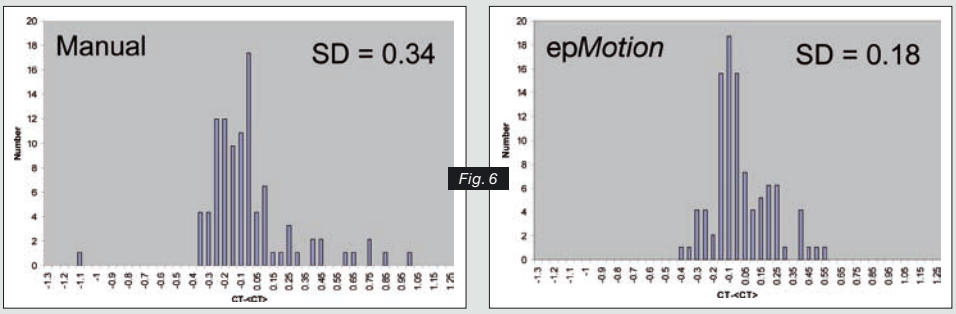
We learn from this exercise that by moving the decision line we can improve our chances to make the correct decision when there is a difference. But this is at the expense of decreasing our chances to make the correct decision when there is no difference.

What if both false negatives and false positives rates are too high?







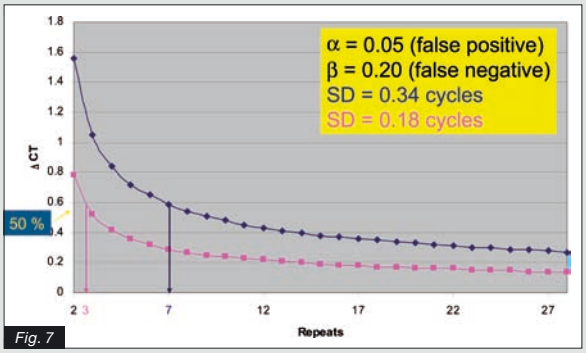


We can use replicates to improve accuracy. If confounding variation is technical we use technical replicates and if it is biological we use biological replicates (increase the number of studied subjects).

The precision of measured CT value is given by the standard error, SE, which is SD divided by the square root of the number of replicates.

$$SE = \frac{SD}{\sqrt{N}}$$

Hence, if we double the number of samples SE will decrease by a factor of 1.41. The case is shown in Fig. 5. The decreased SE narrows the two Normal distributions, reducing their overlap. The rates of false positives and false negatives are now 8 %. Increasing the number of replicates even more improves our ability to distinguish a normal case from a trisomy further.



Results and discussion

Using Power test we can optimize the cost performance of our experiment by designing it with minimum number of replicates necessary to reach requested confidence and power. To do this we must estimate the SD of our assay. Fig. 6 shows variation of 96 replicates of an assay developed at TATAA Biocenter when performed either manually by a technician or using the Eppendorf ep*Motion*® automated dispenser. From the replicates we estimate for this assay SD = 0.34 when performed manually and SD = 0.18 when performed with the ep*Motion*. The purpose of this study was to detect a 50 % difference in the amount of target and we could accept 5 % false positives and 20 % false negatives. With Power test we can calculate the number of replicates needed to reach the required accuracy. Since we have only estimates of SD we must use t-distributions instead of Normal distribution. For this we use the GenEx software from MultiD Analyses (www.multid.se). The result in Fig. 7 shows that when dispensing manually, seven replicates are needed, whereas when using the ep*Motion* three replicates suffice.

Literature

[1] The Real-Time Polymerase Chain Reaction, M. Kubista, J.M. Andrade, M. Bengtsson, A. Forootan, J. Jonak, K. Lind, R. Sindelka, R. Sjöback, B. Sjögreen, L. Strömbom, A. Ståhlberg, N. Zoric, *Molecular Aspects of Medicine* (2006) 27, 95-125

[2] The Prime Technique. Real-time PCR data analysis. M. Kubista, R. Sindelka, A. Tichopad, A. Bergkvist, D. Lindh, A. Forootan. G.I.T. *Laboratory Journal* 9-10, pp 33-35 (2007)

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