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Educational no. 4: PCR-based methods

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Summary For decades, polymerase chain reaction (PCR) has been playing a fundamental role in hematology. Not only in diagnostics, but also in follow-up and therapeutic decision-making, PCR impacts the treatment of patients with neoplastic diseases. In this educational, commonly used PCR methods are explained. In addition, the strengths and weaknesses of PCR in the clinical setting are illustrated.

Keywords Polymerase chain reaction · Measurable residual disease, MRD · Hematology · Neoplasms · Therapy

Introduction

Polymerase chain reaction (PCR) revolutionized clinical diagnostics. The principle, first described in 1987 [1], is as simple as it is brilliant: The genetic region of interest is determined by short synthetic oligonucleotides, so-called primers, and the DNA sequence in between (usually 150–500 base-pairs long) is synthesized (amplified) by a thermo-stable enzyme (DNA polymerase) through cycles of well-defined temperatures in a device called the Thermocycler. The reaction leads to a doubling of the product each cycle and an exponential multiplication of the target sequence. Under optimal conditions, a single target DNA molecule is sufficient for a positive PCR result.

However, the outstanding sensitivity of PCR is significantly reduced if similar DNA sequences are co-amplified in the same tube, which is the case in clonality testing (IG/TCR rearrangements), chimerism analysis (STR) or wildtype/mutated DNA (e.g., FLT3-ITD).

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PCR methods frequently used in clinical diagnostics

In principle, the target sequence has to be DNA. Hence, if the expression of a certain gene is of interest, mRNA has to be transcribed into complementary DNA (cDNA) through an enzyme called "reverse transcriptase" (RT). Therefore, PCR for expression analysis is called "RT-PCR." To increase sensitivity and specificity, a second PCR run with primers located within the first primer pair on the target sequence is called "nested-PCR." After PCR, the products have to be visualized, usually via electrophoresis on the basis of their size. These classic endpoint PCRs allow for assessing positive or negative results and for differences in the size of PCR products (e.g., FLT3-ITD, STR) (Fig. 1).

Quantitative PCR (Q-PCR) enables the quantification of PCR products via fluorescence measurement during synthesis in real time. This can be an intercalating dye that binds to every DNA molecule, or fluorescent-tagged oligonucleotides, binding specifically to the PCR product during synthesis. The more target sequence in the sample, the earlier the exponential phase of PCR reached, hence the lower the value of the cycle-threshold number (C_T, defined as the cycle number at which the PCR amplification curve crosses a predefined threshold). This allows for an absolute quantification (e.g., number of leukemic cells), if genomic DNA is the target sequence, because each cell contains two DNA molecules. In the case of QRT-PCR, the relative amount of the cDNA is determined. The C_T of the target gene is rated in comparison with that of a control gene (constant expression per cell), and results are usually reported as a percentage relative to the control gene [2]. Particularly in the quantification of fusion transcripts like BCR/ABL in Philadelphia chromosome-positive leukemia, QRT-PCR is commonly used. It allows for the measure-

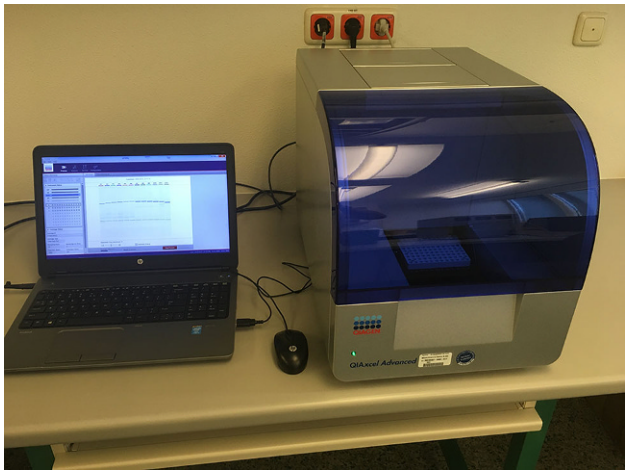

Fig. 1 Analysis of endpoint PCR products

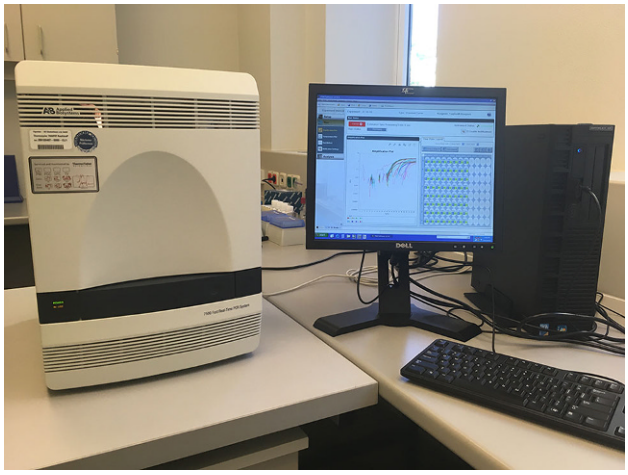

Fig. 2 Q-PCR device

ment of changing gene expression during therapy, but provides no information about the absolute number of leukemic cells in the sample (Fig. 2).

Both in classic as well as in Q-PCR, multiple targets can be amplified in parallel by using a multitude of primers, called "multiplex-PCR." In addition, small DNA-mutations can be detected and amplified by the use of allele-specific oligonucleotides as primer, which are adapted to the mutated sequence (ASO-PCR, e.g., JAK2 V617F Q-PCR).

In digital PCR (dPCR) each single DNA molecule is amplified inside a separated space (partition). This can be small wells or water-in-oil-emulsion droplets in nano- and picoliter volumes (ddPCR), depending on the platform used. If the target sequence is in the partition, a fluorescence signal is generated (positive or negative, hence digital), and at the end of the run each positive well or droplet is enumerated [3]. Because PCR is separated in partitions, the DNA sequences do not interact among each other, leading to a theoretically higher sensitivity. Importantly, the sensitivity of dPCR mainly depends on the number of partitions in the system and has to be proven for each PCR assay. However, the main advantage of dPCR is the possibility to quantify directly (number of positive partitions) without the need of standard curves.

Application in clinical diagnostics

Even if PCR is a powerful technique, pre-analytical considerations are essential: RNA in living cells is degraded over time, therefore long-term transportation of samples should be avoided (e.g., BCR/ABL monitoring). DNA is stable and sending samples at room temperature is possible (e.g., monitoring of IG/TCR rearrangements in patients with acute lymphoblastic leukemia, ALL). In addition, excellent RNA and DNA preparations as well as cDNA synthesis are a prerequisite for good PCR results.

Reproducibility and standardization of PCR assays is an important subject in clinical diagnostics. An example is the quantification of IG/TCR rearrangements with patient-specific PCR assays in ALL: In the EuroMRD consortium of nearly 60 laboratories worldwide, PCR is standardized to an outstanding extent, and members are evaluated in annual quality-control rounds. However, numerous publications of QRT-PCR for fusion genes or NPM1 mutations in acute myeloid leukemia (AML) revealed similar results, even without standardization of assays, pointing to the robustness of PCR if performed in experienced laboratories.

In the context of measurable residual disease (MRD) in leukemia, stochastics have to be seriously considered: If a sample is expected to contain a single leukemic cell, according to Poisson distribution the probability of each to contain none or one leukemic cell is predicted to be 37%, with decreasing probabilities for more than one cell [4]. This has eminent impact on PCR testing and interpreting in clinical laboratories:

- Each test has to be done in triplicate at least.
- Sample volume is important (at least 10–20 ml peripheral blood, 2–5 ml bone marrow of the first aspirate).
- Fluctuations of quantitative results at low levels should not be overestimated.
- A negative result of a previously positive-testing patient, as well as the contrary, has to be confirmed with a second sample taken several weeks later, before any clinical intervention is performed.

Although a single target DNA molecule is sufficient to give positive results, the sensitivity of PCR assays in MRD is defined less by the reaction itself than by the quality of samples and the number of cells analyzed.

Conflict of interest O. Zach declares that he has no competing interests.

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