Quantification of PCR products using microparticles and flow cytometry

N. Wedemeyer¹
E. Pascher¹
U. Cassens²
W. Göhde¹

¹ Institut für Strahlenbiologie, Westfälische Wilhelms-Universität Münster, Robert-Koch-Str. 43, 48149 Münster, Germany
wedemey@uni-muenster.de
² Institut für Transfusionsmedizin, Westfälische Wilhelms-Universität Münster, Domagkstr. 11, 48149 Münster, Germany

Summary. The analysis of genetic biomarkers has become an important tool in clinical diagnostics. This includes the identification of disease-related genetic alterations, the detection of pathogenic infective germs on DNA or RNA level and the quantification of the expression of marker genes indicating an altered physiological status. It has been previously described that the combination of polymerase chain reaction (PCR), microparticles and flow cytometry represents a universal platform technology for the routine analysis of such biomarkers. Here we demonstrate the applicability and flexibility of this technology by means of various applications. The quantification of interferon γ (IFNG) mRNA in irradiated white blood cells is shown as well as the detection of latent infections with cytomegalovirus (CMV). Besides the quantification of single amplification products, the flow cytometric assay is also capable of analysing products of a multiplex PCR. As an example, we describe the identification of spontaneous deletions in the genome of a hybrid cell line using a co-amplified gene (RABI) essential for the cell survival as an internal control.
Furthermore, we show that the use of a green laser (532 nm, 50 mW) substantially increased the sensitivity of the assay compared to conventional flow cytometers using a 488 nm (25 mW) laser. We conclude that the analysis of PCR products using microparticles and flow cytometry fulfils the criteria of clinical routine diagnostics regarding (i) sensitivity, (ii) specificity, (iii) reproducibility and (iv) automatibility.

Key words: microsphere, interferon γ, cytomegalovirus, CD59, A5 hybrid cell line

Flow cytometry is an automated tool for the detection of fluorescent cells or particles with a broad range of applications in routine clinical diagnostics. Traditionally, it is used for immunophenotyping, e.g. of cells of the haematopoietic system, by using fluorescence-labelled antibodies raised against cell surface proteins (usually CD proteins) [1] or for the measurement of the DNA content of tumour-relevant cells after intercalation of fluorescent dyes into the DNA [2]. In combination with microparticles, latex or polystyrene beads measuring a few micrometers, flow cytometric analysis was also enabled at the molecular level. As long ago as the early 1980s, soluble proteins (human IgG) were detected by flow cytometry [3]. In the meantime the combination of flow cytometry and microparticles has been used to adapt various molecular biological methods including the study of protein interactions [4], the identification of differentially expressed genes [5, 6] or the detection of single nucleotide polymorphisms (SNPs) [7, 8]. Additionally, the quantification of amplified DNA fragments (PCR products) has been described by various groups [9-12]. A common feature of these methods is the need to mark the PCR products with two different labels. The first label, usually digoxigenin (DIG), is incorporated into the amplicons for subsequent dyeing using fluorescently labelled antibodies, and the second label (usually biotin - BIO) is required to immobilize the PCR products selectively to microparticles (coated with streptavidin). This step is essential since the flow cytometer is capable of detecting only fluorescence dyes that are located on a larger structure, for example fluorescently labelled antibodies bound to the cell surface or fluorescently labelled amplicons on microspheres. However, these techniques have not prevailed over comparable techniques such as ELISA or TaqMan technology especially because of time- and labour-intensive hybridisation and antibody labelling steps (at least 2 hours). A simple and more rapid technique was recently developed [13]. Instead of bringing together both DNA labels by hybridising two single labelled DNA strands, oligonucleotides labelled with DIG and BIO respectively were used directly for amplification. These double-labelled amplification products enable simultaneous binding to microparticles coated with anti-DIG antibodies and fluorescence labelling using streptavidin-R-phycocerythrin (figure 1). Finally, the mean fluorescence intensity per bead is determined by flow cytometry. Using this 25-min assay, PCR products down to 0.4 fmol, corresponding, for example, to 40 pg of a 163 bp amplification product, were detected.

In addition to the examination of products from a single PCR, the method is also applicable to the simultaneous analysis of multiple amplicons as shown recently for competitive PCR products [14]. In order to quantify target and competitor PCR products separately, amplification products are labelled during PCR with DIG and dinitrophenol (DNP) respectively to enable a selective capture of each PCR product using microparticles coated with anti-DIG or anti-DNP antibodies and their separate quantification after fluorescence labelling in the flow cytometer.

Here we present three further applications of clinical relevance: analysis of IFNG expression after in vitro irradiation (100 kV X-ray) of whole blood, detection of latent CMV infection in blood donor probes, and the identification of spontaneous deletions of the human CD59 gene in the A5 human-hamster hybrid cell line. Additionally, in order to improve the flow cytometric assay a novel fluorescent dye and a novel excitation laser were tested.

Material and methods

RNA preparation and reverse transcription

Total RNA was isolated from human lymphocytes of 3 mL in vitro irradiated whole blood (EDTA) probes using the RNeasy kit (QIAGEN). The in vitro irradiation and isolation ofuffy coat cells was as described previously [14]. Reverse transcription of 100 ng total RNA, pre-heated at 65 °C for 5 min, was performed using the OMNISCRPT kit (QIAGEN) as described. Since exon-exon overlapping primers were used (for IFNG), previous DNase I treatment was renounced.
**DNA isolation**

DNA was isolated from whole blood and from CD59 negative A5 cells (isolation of A5 as described in detail previously by Wedemeyer et al. [15]) using the DNeasy kit (QIAGEN) as recommended by the supplier.

**Polymerase chain reaction**

PCR was carried out in a 25 µL volume containing Taq-DNA polymerase (1 unit per 25 µL PCR; QIAGEN) mixed with 1 µL Taq Start antibody (CLONTECH) as recommended by the supplier, 10 pmoL of each primer (IFNG: 5’-GCAGGTCATTCAGATGTAGC-3’ and 5’-GACATTCAAGTCAGTTACGA-3’; PBGD: 5’-ACACAGCCTACCTTCCAAGCGAGCCCAT-3’ and 5’-CCCTGTGGTGACATAGCAATGATTTC-3’, nested amplification 5’-TGCTAGAATACGACGACACGCAAG-3’ and 5’-ACTTCCAGGACCGTACCCTT-3’; CMV gH: first amplification 5’-AGCCTCATCATCACCCAGACGGACA-3’ and 5’-GTCCACGACGCAGTCTAATTCC-3’, nested amplification 5’-TGCTAGAATACGACGACACGCAAG-3’ and 5’-ACTTCCAGGACCGTACCCTT-3’; CMVUL93: first amplification 5’-GGCAGCTATCGTGACTGGGA-3’ and 5’-GATCCGACCCATTGTCTAAG-3’, nested amplification: 5’-TTAGCGCGTGACCTGTTACG-3’ and 5’-TCTAAGTTATTACGCAGTCCG-3’; CD59: 5’-GGAGTGGAAGTATACCACAAG-3’ and 5’-GCAAGCCTAATGAGGATTACAG-3’; RAB1: 5’-CTGCTGCTAAAATCTGCCTCC-3’ and 5’-CTAACCAACCACGGGAAACGA-3’) and 0.4 µL RT-sample or 100 (deletion assay) or 500 (CMV detection) ng of DNA. After heat denaturation at 94 °C for 4 min, 30 to 40 cycles at 92 °C/0.5 min, 60 °C/0.5 min and 72 °C/1 min were carried out. Final polymerisation was done for 5 min at 72 °C. Amplifications were performed in the Mastercycler (Eppendorf).

**Flow cytometric assay**

Before capturing the PCR products, antibody-coated magnetic particles [14] were washed twice in 500 µL 3 x binding buffer (15 mM Tris, pH 7.5; 1.5 mM EDTA; 150 mM NaCl) using a magnetic holder (Promega). After washing, 1 µL beads were incubated with 1 mL of a purified PCR product (purification as previously described by Wedemeyer et al. [13] and 1 µL of 90 μg/µL streptavidin-R-phycocerythrin (Sigma) by rotating at room temperature for 15 min. Finally, the beads were washed with 500 µL PBS plus 0.04% Tween 20, and resuspended in 1.5 mL TE.

**Results**

Quantification of gene expression

Quantitative PCR is of increasing importance to clinical diagnostics. In particular, the determination of expression levels of disease-associated genes is of interest since the genome and proteome projects reveal thousands of hitherto unknown genes and their relevance to certain disorders. One main field of application of quantitative PCR is the analysis of cytokine expression. These hormone-like factors are responsible for communication between immune cells in response to stress factors, for example irradiation. Here we show the dose-dependent increase of the IFNG mRNA level in whole blood cells of 9 healthy probands after in vitro irradiation with 100 kV X-ray. For this kind of analysis it is essential to normalize the data by using a housekeeping gene with constitutive expression as internal control. In this case, the porphobilinogenase desaminase (PBGD) gene, which was previously demonstrated to be expressed highly homogeneously in all kinds of human cells [16], was used. As shown in figure 2, an increase of IFNG expression was determined for all probands over all applied doses. Although one probe (a) revealed an above-average increase, most of the data were consistent, demonstrating the feasibility and reproducibility of the method.

**Figure 1.** Simplified diagram of the flow cytometric assay to quantify PCR products.
**Flow cytometric detection of latent CMV infection**

Because of its high sensitivity, the method is especially suitable for the detection of DNA sequences with low copy numbers. One important field of application is the diagnosis of latent infection with pathogenic viruses. For example, about 40 to 70% of the European population is latently infected with cytomegalovirus (CMV), usually without clinical complications [17]. However, the transfusion of CMV-contaminated blood components might lead to a severe syndrome in immunodeficient patients. The in-line depletion of leukocytes from blood components prior to transfusion is now demanded and may distinctly reduce the rates of transfusion-associated infections by CMV. However, the remaining risk for transmission of CMV by leukocyte-depleted blood components is currently not known. Therefore, the examination of blood donors or at least of blood components assigned to immunosuppressed patients regarding latent virus infection would be desirable and helpful. Compared to classical techniques, such as ELISA and microscopy, PCR shows higher sensitivity and rapidity at lower costs. However, one substantial difficulty in detecting latent CMV infection by PCR is the low copy number of viral genomes in blood cells. Only 0.01 to 0.012% of all mononucleated cells are expected to contain viral sequences [18]. Therefore, up to 2 mg of total DNA must be added as template per PCR to enable an adequate number of viral copies. Furthermore, a nested PCR is required to increase the amplification efficiency [19]. We tested 110 healthy blood donors for latent CMV infection in a blinded case control study by amplification of the highly conserved CMV-specific glycoprotein H (gH) gene and the flow cytometric assay. 4 probes were unequivocally determined as CMV-positive in two independent analyses (figure 3). However, in a parallel study 18 probes were shown to be serologically positive. This means that only 22% of all putative infections were positive in both studies. Although 18 probes were found to be positive in a comparative study using an already established and accepted CMV PCR assay [19, 20], only 6 (33%) were consistent with serological data (not shown). Thus, two-thirds of all positive-tested probes were estimated as false-positive. We conclude that, particularly in the detection of latent CMV infection, the establishment of the PCR parameters (eg oligonucleotides or cycling conditions) affects the sensitivity and the specificity more than the detection system, since specifically generated PCR products could be reliably detected even at low concentrations using our flow cytometric assay.

**Identification of spontaneous deletions in the human/hamster A1 hybrid cell line**

Studying mutational events at autosomal loci with regard to carcinogenesis requires great experimental effort since many genetic alterations are stable and have no measurable effect on cellular functions. Therefore, in vitro models using hemizygous marker genes, such as the X-chromosome linked HPRT gene, have been proposed for determination of the mutation frequencies and qualities and thus the risk potential of exposed substance or irradiation. One extensively used model for studying the distribution of genetic alterations is the human-hamster A1 hybrid cell line [21]. This cell line contains a standard set of hamster chromosomes plus a single human chromosome 11 which confers the expression of the cell surface protein CD59.

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**Figure 2.** Determination of IFNG expression in human lymphocytes after X-irradiation. Total RNA was isolated from nine blood probes from healthy probands after *in vitro* irradiation (different doses of 100 kV X-ray). After reverse transcription, 40 cycles of IFNG PCR were performed, and in parallel 35 cycles of a PBGD (internal control) PCR. Subsequently PCR products were analysed by flow cytometry. The logarithm of the quotient of the mean fluorescences (MF) was plotted against the doses.
Cells comprising mutations in the gene can be identified and selected by their loss of the CD59 antigen using specific antibodies. We studied the quality of the CD59 mutation in cells which had spontaneously lost the antigen. For this purpose, we used a gene of the hamster genome (RAB1), a small GTPase essential for the survival of the cell, as a co-amplified internal control. For a separate analysis, PCR products of the RAB1 gene were labelled with DNP and BIO, and the PCR products of the CD59 gene with DIG and BIO. Using microparticles coated with anti-DNP and anti-DIG antibodies, we were able to distinguish PCR products of CD59 and RAB1 in a single run.

**Figure 3.** Analysis of 110 healthy blood donors regarding latent CMV infection. Lymphocytes isolated from 5 mL blood (EDTA) were used for DNA preparation. 500 ng of DNA were employed for nested gH PCR (25 µL). The PCR products were detected by flow cytometry. Probes obtaining a mean fluorescence below 12 (10% of the maximal measured fluorescence; pointed line) were defined as CMV-negative.

**Figure 4.** Flow cytometric detection of spontaneous deletions of CD59 gene in the human/hamster A L hybrid cell line. DNA from individual, immunologically CD59 negative cell clones were analysed by a duplex PCR using RAB1 (control) and CD59 (target) specific primers. As controls, DNA from CHO cells (hamster), human lymphocytes and intact A L cells were used. Amplification products were immobilised using microparticles coated with anti-DNP or anti-DIG antibodies. After fluorescence labelling the microparticles were analysed in the flow cytometer. The mean fluorescence of each microparticle population is given.
anti-DIG antibody respectively, PCR products were separately immobilized and simultaneously labelled with streptavidin-R-phycoerythrin. The fluorescence intensity of each microparticle population was determined by flow cytometry. Each PCR was highly specific as demonstrated by means of control DNAs: Using DNA isolated from hamster cells (CHO) provided only a signal of the anti-DNP particles, for human DNA only a signal of the anti-DIG particles, and for non-mutated A5 cells at both particle groups fluorescence signals appeared (figure 4). The determined frequency of spontaneous deletions of 68% was observed, which is in agreement with previous studies [22].

**Novel fluorescent dyes and excitation lasers**

One possible means of improving the flow cytometric assay is to use better fluorescent dyes. Today, different fluorescent dyes conjugated to streptavidin are available, including a double dye comprising phycoerythrin and Cy5, to enable fluorescence resonance energy transfer (FRET). This allows excitation using the normal 488 nm laser and an emission at 670 nm (red light). In this fluorescence range the signal-to-noise ratio is optimal on account of low autofluorescence. However, compared to the previously used phycoerythrin the fluorescence intensity is slightly higher (figure 5a) but cannot significantly raise the sensitivity.

More promising is the use of more efficient excitation lasers, for example, a green laser exciting at 532 nm (50 mW), which is available for the mini flow cytometer CyFlow™ (CyTecs, Görlitz, Germany).

**Discussion**

During the past three years the combination of microparticles and flow cytometry has been frequently adapted for molecular biology [23]. The microparticles were applied as a surface for molecular reactions, comparable with nylon membrane or microtiter wells. As the latter, microparticles can be coated covalently with a broad range of biomolecules including nucleic acids, proteins or carbohydrates. The manifold approaches demonstrate the universal applicability of this platform. As examples, real time studies of DNA repair enzymes and the analysis of protein phosphorylation should be mentioned [24, 25]. Moreover, as demanded by clinical diagnostics, the use of paramagnetic particles enables automated handling of thousands of probes in parallel. However, paramagnetic particles with a homogeneous shape and size, as would be needed to obtain a convincing distribution of fluorescent signals, are not yet available. The gating of a certain group of particles (e.g. monomers) in the flow cytometer means a waste of PCR product, microparticles and dye.

Flow cytometry is known to be a highly sensitive detection system. In contrast to other fluorescent reading devices, flow cytometers can distinguish between particles differing in size, shape or fluorescence. This allows the simultaneous analysis of biomarkers using differently labelled or sized microparticle populations. Recently, a microsphere-based system (FlowMetrix™, Luminex Corp.) was established for the simultaneous analysis of up to 100 different reactions using flow cytometry. As described for other assays, microspheres coated with hybridisa-

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**Figure 5.** Comparison of different fluorescent dyes and excitation lasers. **a.** The amount of DIG- and BIO-labelled PCR products (CMV gH) was immobilized using microparticles coated with anti-DIG antibody. Simultaneously captured PCR products were labelled using streptavidin-phycoerythrin or streptavidin-phycoerythrin-Cy5. The mean fluorescence was determined by flow cytometry. **b.** A dilution series was made of a CMV gH PCR product ranging from 10 ng (0.1 pmol) to 10 pg (0.1 fmol). After binding and staining the amount of captured PCR products per microparticle was determined with the PAS III flow cytometer (blue laser) and the CyFlow (green laser) in parallel.
tion probes are used to selectively bind fluorescently labeled nucleic acids [26]. We have shown that as few as two differently coated microparticles are adequate for reliable quantification of a single target sequence plus an internal control. Beyond this, the current improvements in flow cytometry will prospectively not only increase the sensitivity, as demonstrated for the novel green excitation laser, but also enable high throughput analysis. Better compatibility of flow cytometers with existing robotic systems is required to permit a fully automated flow cytometric assay. This assumes, however, that besides detection, also cell isolation and probe preparation have to be adapted for paramagnetic microparticles. In particular for virus detection, the isolation of only virus-infested cells, for example the CD14 positive for latently CMV-infected donors, might additionally increase the sensitivity of the assay.

References


