

Immuno-PCR technology for detection of natural human antibodies against Le^C disaccharide

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Abstract The development of an immuno-PCR assay for quantitation of low amounts of anti-glycan human antibodies is described. The sensitivity of the assay for determination of low-affinity anti-Le^C IgM has been found to be 4 ng/ml (~100 pg per sample), thus being two orders of magnitude higher compared to the conventional ELISA with the same antigen.

Keywords Immuno-PCR · Glycans · Le^C · Natural antibodies

Introduction

The antibodies against disaccharide Le^C (Galβ1-3GlcNAcβ), mainly IgM (about 80%), are detected in almost all healthy people without antigen stimulation and without variations of epitope specificity; *i.e.*, they are typical natural antibodies [1]. Affinity-isolated monospecific anti-Le^C do not interact with glycoconjugates on the surface of normal cells, though they stain breast cancer tissue [2]. Apparently, these antibodies have a surveillance function for elimination of newly formed or circulating tumor cells [3]; their specific molecular target (a glycan or glycoprotein) has not been identified yet. The level of antibodies against Le^C is rather high (about 10 μg/ml); therefore, their quantitative assay by routine enzyme-linked immunosorbent assay (ELISA) do not pose any problem.

Nevertheless, determination of these antibodies in ultra-low quantities (after gentle elution from the cells) is required for identification of the target cells and specific molecular antigens in tumor cells normally available in rather limited amounts. The same is true for other anti-glycan antibodies with obscured functions. The use of immuno-polymerase chain reaction (iPCR), with the highest known sensitivity [4], is an attractive approach to solving this problem. Despite the wide range of objects to which iPCR has been applied for the 25 years passed since its development, antibodies have rarely been in the focus of attention [5]. The following publications could be mentioned: allergen-specific IgE against mite allergens [6], tropomyosin [7], and antibodies against drugs [8]. Detection of low amounts of specific IgMs can be important for early diagnosis of Lyme disease [9] and Q-fever [10]. In the case of IgE detection in blood, sensitivities of 200 pg/ml [7] and 5.7 pg/ml [6] have been achieved; for antibodies against drugs, the sensitivity is 40 pg/ml [8].

iPCR is similar to ELISA, differing only in the last stage, where a DNA label attached to the immune complex is quantified by PCR. In this study, a conjugate of a single-stranded 60-nt oligonucleotide with streptavidin was used for iPCR. This approach to anti-Le^C detection in blood serum has a 100-fold higher sensitivity than ELISA, as well as a better linear range, thus allowing investigations where detection of low amount of antibodies is crucial.

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Materials and methods

Reagents

Chemical reagents, unless specially indicated, were obtained from Fluka (Switzerland). MaxiSorb transparent immunoplates were purchased from NUNC (Denmark).

Polyacrylamide glycoconjugates (Glyc-PAA) and affinity adsorbent were obtained from Lectinity (Russia). Complex Immunoglobulin Preparation, a therapeutic immunoglobulin mixture containing about 60% of IgG, 25% of IgM, and 15% of IgA, was obtained from Microgen (Russia). Tween 20, BSA, and casein were from Sigma (USA), biotinylated anti-human IgM antibodies were purchased from Southern Biotech (USA); streptavidin-horseradish peroxidase (Str-HRPO) conjugate, from GE Healthcare (UK); and Nunc Top Yield strips and biotin-NHS ester, from ThermoFisher Scientific (USA).

Sera

Sera of healthy subjects and patients with breast cancer were kindly provided by Prof. N.N. Tupitsyn and Prof. L.N. Liubchenko (Blokhin Cancer Research Center, Moscow).

Conjugate of DNA with streptavidin

Conjugate of DNA with streptavidin was synthesized as previously described [11, 12]. 3'- and 5'- end-biotinylated single-stranded 60-oligodeoxyribonucleotide (ODN) was obtained by direct oligonucleotide synthesis. For introduction of 3'-biotin into ODN, the corresponding solid phase for synthesis with 3'-protected biotin serinol CPG (Glen Research, USA) was used, whereas 5'-biotin was introduced using protected biotin serinol phosphoramidite (Glen Research). The streptavidin–DNA complex was prepared by mixing 10 μ M solutions of biotinylated DNA and streptavidin in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) in an equimolar ratio followed by incubation at 4 °C for 30 min. The complex obtained was stored in 50% glycerol at –70 °C.

Isolation of anti-Le^C antibodies

Complex Immunoglobulin Preparation (35 mg/ml, 200 mg per 1 ml of adsorbent) was dissolved in PBS (pH 7.3) and applied to a column with Le^C-PAA-Sepharose 6FF equilibrated with the same buffer; the column was washed with PBS, 75 cm/h. Bound specific antibodies were eluted with 0.2 M TrisOH/0.5 M NaCl, pH 10.2, 15 cm/h, with immediate neutralization of the eluate with 2 M Glycine-HCl (pH 2.5) to final pH 7.3–7.5. All the buffers used contained 0.02% NaN₃. Monitoring of chromatography was performed using a UV detector (Bio Rad, USA), 280 nm. Antibodies were concentrated by centrifugation in plastic test tubes equipped with membrane filters with a molecular weight cut-off of 100 kDa (100 kDa MWCO, Millipore, USA) at 4 °C, 3000 g, to a volume of 0.3–0.9 ml. The concentration of antibodies was measured at 280 and 260 nm. The antibodies were stored at 4 °C for up to 6 months or at –20 °C for longer periods of time.

Sera not containing antibodies against Le^C

Individual serum samples were diluted in PBS to a total protein concentration of 35 mg/ml and processed as described above. The breakthrough fraction was collected immediately after elution of free volume. The lack of antibodies against Le^C in the breakthrough fraction was confirmed by re-chromatography/concentrating on an affinity adsorbent. This material is referred to as negative serum (*NegS*). Starting serum (positive serum, *PosS*) was used as a control.

Sera with known content of anti-Le^C antibodies

Antibodies against Le^C isolated from Complex Immunoglobulin Preparation as described above were added to *NegS* to concentrations of 100, 30, 10, 3, 2, and 1 μ g/ml. Samples with different contents of anti-Le^C antibodies were used to plot the calibration curve. Aliquots were stored at –20 °C.

Biotinylation of antibodies

Antibodies (1.5 mg/ml) were transferred to 0.1 M sodium carbonate buffer, pH 8.5, using centrifuge test tubes equipped with membrane filters with a molecular weight cut-off of 100 kDa at 4 °C, 3000 g, and a 1 mg/ml biotin succinimide ester solution in DMSO was added (0.1 of the total volume). Reaction was carried out for 2 h at room temperature and constant stirring, followed by transfer of the antibody solution to PBS. Nativity of biotinylated antibodies was confirmed by ELISA comparison with starting antibodies by means of tests for interaction with a set of glycans including Le^C [13].

Determination of anti-Le^C antibodies by ELISA

MaxiSorp plates were coated with Le^C-PAA (5 μ g/ml, 100 μ l/well) in 0.05 M sodium carbonate buffer (pH 9.6) at 37 °C for 1 h. The plates were blocked with 3% BSA in 0.15 M PBS for 1 h and washed with PBS containing 0.1% Tween (PBST). The analyzed samples in serial double dilutions (100 μ l/well) in PBS containing 0.3% BSA were applied onto the plate and incubated for 1 h at 37 °C. The plates were washed three times with PBST buffer, incubated with biotinylated anti-IgM antibodies (1:2000, PBS, 0.3% BSA) for 1 h at 37 °C and washed three times with PBST. Then, the plates were incubated with streptavidin–horseradish peroxidase (1:1000, PBS, 0.3% BSA) for 1 h at 37 °C and washed three times with PBST. A color reaction was performed during incubation in the presence of a buffer saline containing 0.1 M sodium phosphate, 0.1 M citric acid, 0.04% *o*-phenyldiamine, and 0.03% H₂O₂ for 30 min. The reaction was stopped by addition of 50 μ l of 1 M H₂SO₄. The absorbance was measured using a plate spectrophotometer (Victor², WALLAC) at 490 nm. Δ OD was calculated as follows: Δ OD = OD (Le^C-PAA) – OD (PAA).

Immuno-PCR: optimization of the amount of the coating antigen Le^C

The technology used was based on our previous studies using iPCR [11, 12]. Nunc Top Yield Strips (ThermoFisher Scientific, USA) were coated with Le^C-PAA at various concentrations in 0.05 M sodium carbonate buffer (pH 9.6) (50 µl/well) at 4 °C overnight. Pure buffer saline was used as a negative control. Wells were blocked with a solution containing 1% BSA in TBS (100 µl/well) for 1 h at room temperature and washed three times with TETBS (20 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, 0.1% Tween 20, pH 7.5). Biotinylated anti-Le^C antibody (50 µl/well) (0.6 µg/ml, TBS, 0.1% BSA) was added, the mixture was incubated for 1 h at room temperature, and the wells were washed four times for 2 min with TETBS. Then, a 5 pM solution of DNA complexed with streptavidin (50 µl/well) in TETBS containing 0.1% BSA was added, and the mixture was incubated for 1 h at room temperature. After incubation, the wells were washed 13 times with 100 µl TETBS as follows: four washings for 10 s followed by nine washings with alternating intervals of 5 min/10 s. The wells were then washed three times for 30 s with TBS and dried. The amplification mixture (50 µl/well) was added to the wells, after which mineral oil (20 µl/well) was added, and real-time polymerase chain reaction (RT-PCR) was carried out. PCR was performed using a DTprime real-time PCR machine (DNA-Technology, Russia). The reaction profile was as follows: initial denaturation at 94 °C for 5 min followed by 40 cycles of annealing and elongation at 60 °C for 15 s and denaturation at 94 °C for 5 s. Fluorescence measurement (FAM channel) was done in each cycle during the annealing and elongation steps. The PCR results were analyzed using the software supplied with the detection thermocycler. For all samples, the mean quantification cycle (C_q) values and standard deviations (SDs) were calculated. The limit of detection (LOD) was calculated as follows: LOD = [average (40-C_q) control without antigen + 3 * SD (40-C_q) control without antigen]. The normalized values (ΔC_q) were calculated as follows [ΔC_{q(i)} = Average (40-C_q)_(i) - LOD]. Samples with ΔC_q smaller than or equal to zero were considered negative; the ones with this value higher than zero, positive. Only normalized values were used in comparing different experiments.

iPCR: detection of antibodies against Le^C in sera

Le^C-PAA and PAA in 0.05 M sodium carbonate buffer (1 µg/ml, 50 µl/well) was coated onto Nunc Top Yield strips at 4 °C overnight. A blocking solution (1% BSA, 1% casein, and 1 µM oligonucleotide for blocking, OB, (5'-A-ACG-CCA-CCG-CGA-GCA-GGA-GCA-GGA-3')) in TBS; 100 µl/well) was added, the mixture was incubated for 1 h

at room temperature and washed three times for 1 min with 100 µl/well TETBS.

The serum samples were diluted with TBS (1:500), placed into the wells (50 µl/well), and incubated for 1 h at room temperature. The calibration curve was plotted using samples containing known concentrations of anti-Le^C. After incubation, the wells were washed with TETBS four times for 2 min, biotinylated anti-human IgM (1:6000, TBS, 0.1% BSA, 50 µl/well) was added, and the wells were incubated for 1 h at room temperature. After incubation, the wells were washed four times for 2 min with TBS. The subsequent steps with addition of streptavidin–DNA complex and amplification were carried out as described above. After PCR, the difference between the C_q for each sample added to the wells coated with glycan-free PAA C_q (PAA)_(i) (background) and Le^C-PAA was calculated: ΔC_{q(i)} = C_q (PAA)_(i) - C_q (Le^C-PAA)_(i). Samples with ΔC_q smaller than or equal to zero were considered negative and those with this value higher than zero, positive. Only ΔC_q values were compared in different experiments.

Results and discussion

Optimization of iPCR for detection of natural IgM

The advantage of the iPCR technology is also its potential drawback, because PCR permits detection of single DNA molecules in the sample [14], thus leading to the risk of a false positive signal. Nevertheless, numerous published iPCR studies report on a complete lack of DNA amplification in the

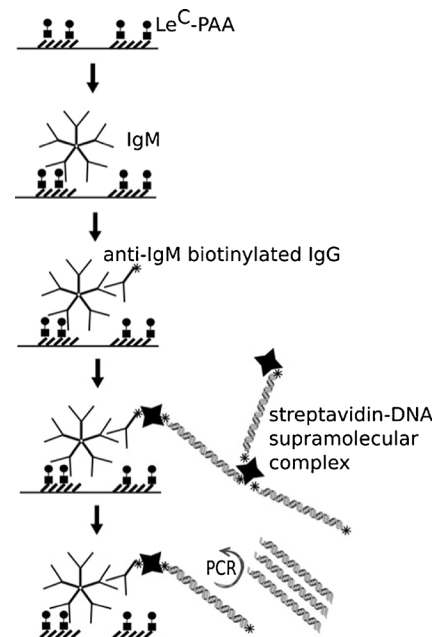


Fig. 1 Schematic presentation of the iPCR approach to detection of anti-Le^C IgM

Table 1 Variants of blocking solutions and corresponding iPCR results

Blocking agent	ΔCq (<i>NegS</i>)	ΔCq (<i>PosS</i>)
BSA (1%)	-0.5 ± 0.1	0.9 ± 0.1
BSA (5%)	0.5 ± 0.1	1.0 ± 0.2
BSA + 1 μ M OB	0.5 ± 0.1	2.4 ± 0.2
BSA + 1 μ M OB + gelatin (1%)	-0.5 ± 0.1	1.5 ± 0.1
BSA + 1 μ M OB + casein (1%)	0.1 ± 0.1	2.8 ± 0.2

samples actually free of the analyte. However, the signal in negative samples is absent even after 25, 35, 40, and 52 amplification cycles [15–18]. According to our experience, negative control samples give the amplification signal after 30–32 cycles, which correlates with the results of other studies [19–22]. Therefore, reduction of false amplification requires special attention to the optimization process. When iPCR was performed in the ‘sandwich’ format [11, 12], we used Nunc Top Yield Strips and bovine serum albumin as a blocking agent. In this study, a range of steps have been taken to optimize our iPCR protocol, namely, selection of the concentrations of all reagents, the plastic and blocking agents, and, especially, the washing procedure. A schematic presentation of the immuno-PCR approach is shown in Fig. 1.

Blocking agent Has been selected under the following conditions: 1 μ g/ml Le^C-PAA or 1 μ g/ml PAA overnight at 4 °C, blocking for 1 h, application of *NegS* and *PosS* (in dilutions of 1:100 to 1:1000). The variants of the blocking solution and the corresponding results are shown in Table 1. The oligonucleotides used contained no more than three nucleotides complementary to the DNA label. The key factors for selection of the optimal composition were (1) the minimum ΔCq value for *NegS* as an indicator of nonspecific sorption of serum components and (2) the maximum ΔCq value for *PosS*. In

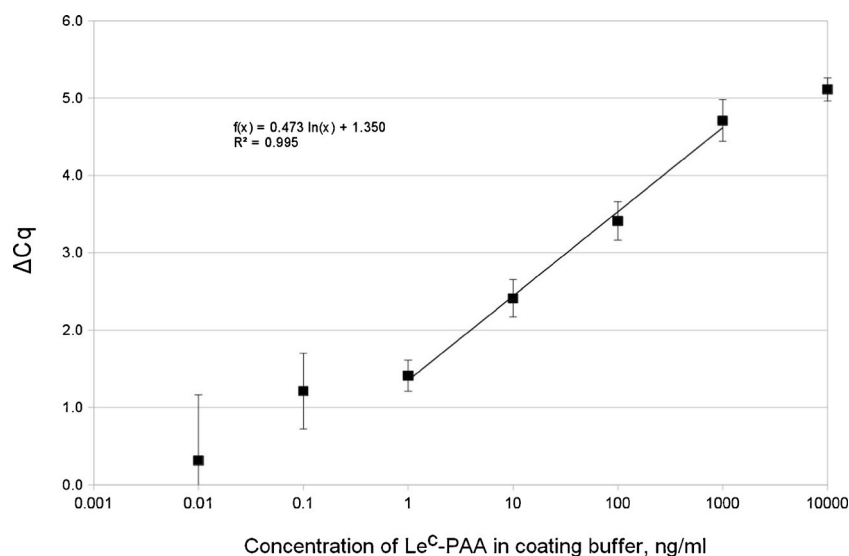
subsequent experiments, the BSA (1%) + 1 μ M OB + 1% casein combination was used.

Material for adsorption of glycoconjugates We compared Nunc Top Yield Strips (Thermo Scientific Cat. No. 248909), Thermowell 96 Well Polycarbonate PCR Microplate (Corning Costar Cat. No. 6511), Robostrip PC clear (AJ Roboscreen), and Greiner Thermoquick polycarbonate plates (Greiner Cat. No. 651570). Nunc Top Yield Strips and Robostrips, developed specially for iPCR, combine a high polycarbonate adsorption capacity with a convenient shape suitable for PCR thermoblocks. Earlier, we observed no differences between Greiner Thermoquick polycarbonate plates and Nunc Top Yield Strips [12]. When polycarbonate plates for PCR Corning Costar 65,111 and Greiner Thermoquick polycarbonate plates 671,570 were used for detection of antibodies, the results were similar to those for Nunc Top Yield Strips and Robostrips, though with lower values of ΔCq . Therefore, Nunc Top Yield Strips were used in this study.

It should be noted that for iPCR detection of antibodies Nunc Top Yield Strips were also used before [6, 10]. There are also alternative approaches, *e.g.*, enzymatic or thermal DNA depletion from antibody followed by transfer of a part of the mixture to PCR test tubes [23, 24] or performing several PCR cycles in Nunc Top Yield Strips and further transfer of the mixture to the PCR test tubes [18].

Coating concentrations Of Le^C-PAA varied in the range presented in Fig. 2.

The minimum concentration of Le^C-PAA at which the signal significantly exceeded the background was found to be 100 pg/ml. A plateau was reached at concentrations higher than 1 μ g/ml; this value was selected for subsequent experiments.

Fig. 2 Dependence of the PCR signal (ΔCq) on the concentration of Le^C-PAA in the coating buffer saline

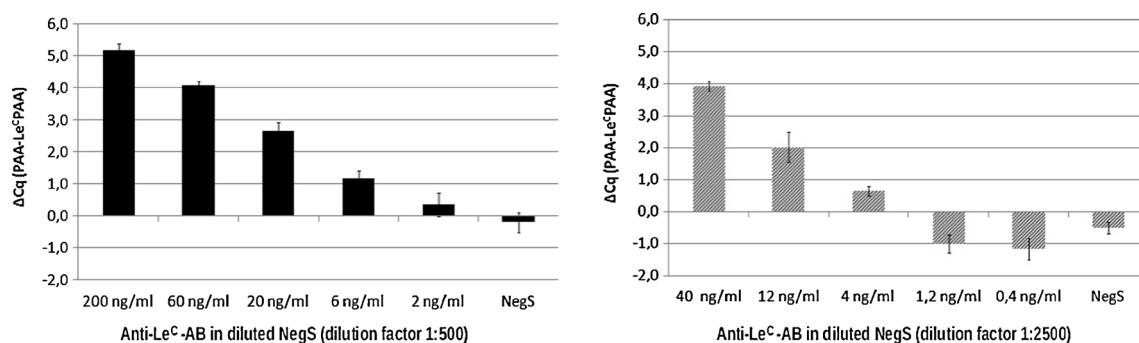


Fig. 3 Comparison of the iPCR detection of anti-Le^C antibodies in serum samples diluted 1:500 and 1:2500. The samples contained predetermined amount of the antibodies

Titer of secondary (biotinylated) anti-human antibodies

The working dilution titer of the antibodies varied from 1:3000 (as in ELISA) to 1:9000. At the dilution of 1:6000, the ΔC_q value for positive samples did not differ from that obtained at the dilution of 1:3000. At the dilution of 1:9000, ΔC_q for the *PosS* sample decreased by 0.5–1 cycle due to an increase in C_q (Le^C-PAA). Therefore, the dilution range from 1:3000 to 1:6000 was used.

Washing procedure It should be noted that deviation from the complicated scheme of washing (see Materials and Methods) impair the results. Numerous washings, especially at the last stage (before adding the amplification mixture), are essential for iPCR. A number of authors [10, 23] note that the use of an automated washer decreases the background signal and improves the reproducibility.

Influence of serum components In the iPCR assay, as well as in ELISA, it is necessary to reduce matrix effects for serum samples. Large amounts of serum components in the sample lead to a decrease in the signal-to-background ratio, mostly by increasing the background signal. Dilution of the samples improves the signal-to-background ratio; however, excessive sample diluting leads to a decrease in sensitivity. In order to find a balance, serum samples with a known content of specific antibodies diluted 1:100 to 1:2500 were compared. The ΔC_q value and the minimum detectable concentration of anti-Le^C were evaluated. The best results were obtained for serum with an initial specific antibody content of 3 $\mu\text{g/ml}$ at a dilution of 1:500. Equal results were obtained with a concentration of 10 $\mu\text{g/ml}$ at a dilution of 1:2500 (Fig. 3). At a dilution of 1:100, a high background signal ($C_q\text{PAA}$) was observed, the ΔC_q value did not exceed 2 even for samples with a high content of antibodies (data not shown); therefore, serum proteins seem to influence the results of iPCR at this dilution. Thus, since 500- to 1000-fold dilutions were suitable for detection of IgM antibodies in serum samples, a 500-fold dilution of sera was used in subsequent studies.

Detection of anti-Le^C antibodies in samples of breast cancer patients

The optimized iPCR protocol was used for determination of anti-Le^C IgM antibodies in sera of patients with diagnosed breast cancer and their comparison with the samples from healthy donors. As before, *NegS* and reconstructed samples with a known content of the antibodies were used for plotting the calibration curve. Deviations of the ΔC_q values did not exceeded 0.2 cycles, thus showing a good reproducibility. The concentration of anti-Le^C antibodies in the serum samples studied fell within the interval 3–60 $\mu\text{g/ml}$. It should be noted that the level of anti-Le^C antibodies determined by iPCR was higher ($p \leq 0.0005$, one-way ANOVA performed using the QtiPlot software) in serum samples from healthy donor ($n = 45$; $31.3 \pm 4.3 \mu\text{g/ml}$) than in samples of serum from patients with diagnosed breast cancer ($n = 38$; $17.0 \pm 4.1 \mu\text{g/ml}$, Fig. 4). These results correlate with our previous ELISA data on similar cohorts [2].

Minimum quantity of antibodies against Le^C detectable by iPCR

In order to find the lowest detection limit, we used “reconstructed” samples prepared from anti-Le^C-free serum (see Materials and Methods) by adding an exact amount of affinity-isolated antibodies of this specificity. The minimum concentration of antibodies reliably detectable by iPCR in

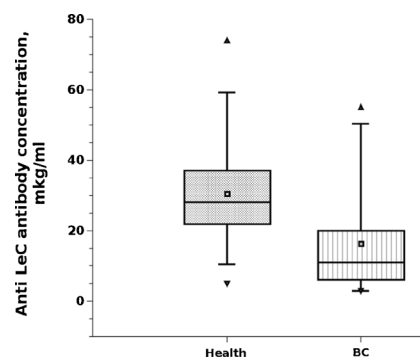
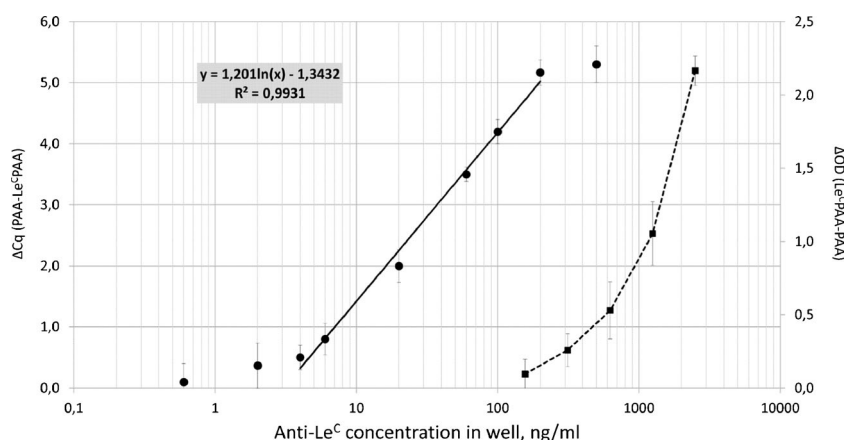


Fig. 4 Concentration of anti-Le^C in healthy donor sera (Health) and patients with diagnosed breast cancer (BC)

Fig. 5 Dependence of the ΔCq (solid line) and ΔOD (dashed line) on the concentration of antibodies against Le^C. ●, iPCR; ■, ELISA



reconstructed sera was found to be 2 $\mu\text{g/ml}$, *i.e.* 4 ng per sample (one well). It was calculated as the difference of the ΔCq at the tested concentration of antibodies from the ΔCq for *NegS* (serum without addition of antibodies) $\pm$ MSR (Fig. 5, solid line). Note that, at a concentration of antibodies higher than 100 $\mu\text{g/ml}$, the plot reaches a plateau. Analysis of the same samples with the known content of antibodies by ELISA (Fig. 5, dashed line) showed that the minimum detectable value was 300 ng per sample (one well). Figure 6 represents typical amplification curves obtained for different ranges of antibodies detected by iPCR in this study.

The iPCR detection level reached is not so outstanding as in the case of antigens, which is not surprising, because the affinity of natural IgM antibodies against glycans (the typical K_{DS} fell in the range from 10^{-6} to 10^{-4} M) is several orders of magnitude lower than that for other antigens.

According to the literature data, the sensitivity of iPCR detection of antibodies exceeds that of ELISA detection by a

factor of 50–1500, as exemplified by assays of completely different antibodies irrespective of the conjugate type used, such as Tus-*Ter* lock, biotin–streptavidin, and covalent conjugates [6–10].

Concluding remarks

The iPCR-based determination of low-affinity anti-glycan IgM antibodies has displayed a good reproducibility and a rather high analytical sensitivity. Because anti-Le^C immunoglobulins are typical representatives of natural human antibodies with respect to affinity, isotype composition (mainly IgM) [3], and genesis, other biologically significant anti-glycan antibodies, especially those with low blood contents, can be quantitated similarly. Of special note is the reliable determination of low amounts of antibodies, which is important for detection of cell targets of natural human antibodies. The value of 4 ng corresponds to $\sim 10^8$ molecules of IgM. If the number of cell targets capable of elution from one cell does not exceed 10^4 , for identification of antibodies against tumor-associated carbohydrate antigens (TACA), the iPCR method will require 10^4 – 10^5 tumor cells, which is quite a reasonable amount. At the same time, the sensitivity achieved still does not permit the expected applicability of iPCR to detection of immunoglobulins bound to circulating tumor cells, which could be isolated in amounts only several dozen per experiment even by modern methods of cell sorting. Therefore, solution of this problem requires further increase in the sensitivity of iPCR.

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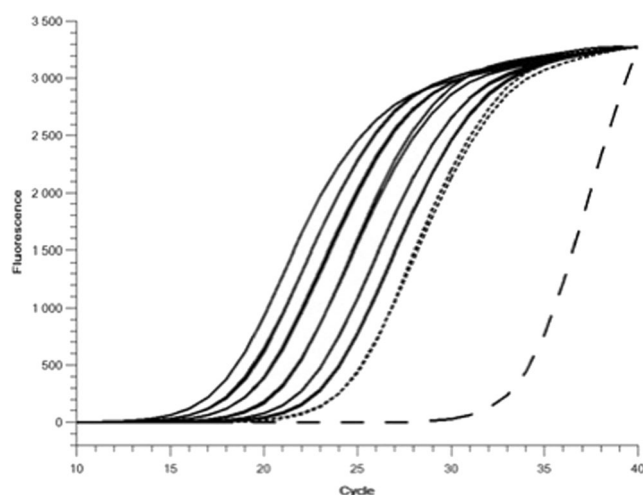


Fig. 6 Amplification curves for different ranges of antibodies detected by iPCR. Solid lines are the amplification curves for samples with known amounts of specific antibodies (wells coated with Le^C-PAA); dotted lines are negative control curves (wells coated with glycan free PAA); dashed line is PCR negative control curve (without adding the streptavidin–DNA complex)

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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