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ARTICLE

Optimization of PCR Amplification for B- and T-cell Clonality Analysis on Formalin-fixed and Paraffin-embedded Samples

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In many cases, particularly in retrospective studies, only formalin-fixed and paraffin-embedded (FFPE) tissue samples are available for molecular studies. DNA recovered from FFPE tissues generally consists of fragmented small target sequences with chemical alterations. Clonality analysis is not easy on FFPE samples, in fact, it requires even more experience than that of performed on fresh samples or is more complicated than most genomic PCR amplifications for somatic genes. In our study, we have performed a multi-parameter PCR evaluation investigating immunoglobulin heavy chain gene (IgH) and T-cell receptor gamma gene (TCRγ) rearrangements on non-purified crude lysates of FFPE samples, in order to establish the significance of different variables on performance of PCR amplification. The results showed that a slight decrease in the concentration of primers in combination with a slight increase in $MgCl_2$ and *Taq* polymerase concentrations, as well as the use diluted crude template and a standard amount of dNTPs can be the modifications of choice while adjusting IgH and TCRγ clonality tests on poor quality DNA FFPE samples. Using our improved protocol, 74% (17/23) of the tested B-cell lymphomas and 68% (31/46) of the tested T-cell lymphomas demonstrated monoclonal PCR product, proving the applicability of our optimized method. Our experience may be of help during the optimization process in technically difficult cases as well as to determine which parameters and how should be changed to minimize false-negative and false-positive results. (Pathology Oncology Research Vol 13, No 3, 209–214)

Key words: PCR optimization, B- and T-cell clonality, archived tissues

Introduction

Clonality analysis of lymphoid cell populations plays an increasing role in the primary diagnosis as well as follow-up of lymphomas and lymphoid leukemias. There are special entities which are best recognized by the aid of molecular methods, while in others these methods are required only if the immunomorphology is not decisive. In lymphoid lesions, multiple antigen receptor gene sequences can be suitable targets as clonal markers.[1-3] The recombination of antigen receptor genes results in an individual gene sequence unique for each differentiating B- or T-cell.[1-3] This rearranged pattern in the individual cell's life span does not change considerably, whereby its fingerprint-like genotype becomes utilizable. If one of these maturing or matured cells becomes malignant, this cell line becomes dominant in the overall lymphocyte population and, therefore, their individual antigen receptor gene rearrangement combination also becomes dominant in the overall rearrangement pattern at the population level. [1-3]

Southern blotting is one of the most reliable methods in detecting lymphoid cell clonality, but it is labor-intensive and requires large amounts of high-molecular-weight DNA, therefore, nowadays it is practically replaced by polymerase chain reaction (PCR) based methods. [4-7] Besides high sensitivity, speed and cost effectiveness, the great advantage of the PCR techniques is that they do not require intact DNA, therefore well suit the routine diagnostic use.[7-9] The highest sensitivity of PCR detection is

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generally expected on DNA isolated from fresh or frozen material, while using templates from FFPE tissue samples may impair performance.[7-12] Unfortunately, in the majority of the diagnostic cases and in retrospective studies, only FFPE tissue samples are available for molecular genetic studies. Although 4% formalin (10% formaldehyde solution) is an ideal fixative for traditional histomorphological examinations, it is disadvantageous for molecular studies.[13-17] Formalin fixation of the tissues is essentially based on crosslinks made by formaldehyde, resulting in the denaturation and precipitation of matrix proteins, nucleic acids and other biomolecules.[13,16] Formaldehyde facilitates a number of physico-chemical changes affecting nucleic acids, in particular DNA, including breaks, base substitutions, base losses, sequence alterations, and formations of different instable forms of DNA, and it is known to generate non-specific protein-protein and DNA-protein crosslinks as well.[13-17] Based on the above, DNA isolated from FFPE archived tissue samples contains mainly strongly fragmented, chemically modified, small – generally less than 200-300 base pairs (bp) – sequences.[13-17] The situation is even more complicated since simple, cost-effective and rapid DNA isolation methods,[17-20] employed by many routine laboratories, produce crude lysates of poor DNA quality, which may significantly affect the performance of PCR.

Although PCR-based clonality analyses are not novel methods, there is still paucity of published studies that analyze the necessary changes in the PCR parameters while amplifying DNA templates isolated from FFPE tissue samples. In our study, we have performed a multi-parameter PCR evaluation investigating immunoglobulin heavy chain (IgH) gene and T-cell receptor gamma (TCRγ) gene rearrangements on non-purified crude lysates of FFPE samples, in order to establish significance of different variables on performance of PCR amplification. We discuss how these parameters can be changed in "fine-tuning" of clonality PCR. Our experience may be of help during the optimization process in technically difficult cases, as well as in determining which parameters and how should be changed to minimize false-negative and false-positive results.

Material and Methods

Tissue samples

To evaluate the parameters and the efficacy of the PCR amplification on DNA templates isolated from FFPE samples, cases were retrieved from the archives of the Laboratory of Tumor Pathology and Molecular Diagnostics, Institute for Biotechnology, Bay Zoltan Foundation for Applied Research. Five hyperplastic lymphoid tissues (3 lymph nodes, 2 hyperplastic tonsils), 3 B-cell lymphomas (1 diffuse large B-cell lymphoma, 1 marginal zone B-cell lymphoma, 1 cutaneous B-cell lymphoma), and 3 T-cell lymphomas (1 mycosis fungoides, 2 nodal peripheral T-cell lymphomas) were used in the optimization phase.

The refined PCR clonality protocol was tested in 23 B-cell lymphoma cases (9 diffuse large B-cell lymphomas, 10 extranodal and nodal marginal zone B-cell lymphomas, and 4 follicular B-cell lymphomas), and 43 T-cell lymphoma cases (12 mycosis fungoides, 4 cutaneous anaplastic large cell lymphomas, and 26 nodal peripheral T-cell lymphomas). The cases selected were previously fully characterized by immunohistochemistry.

DNA isolation

Our study was based on the utilization of crude DNA lysates made from FFPE tissue sections. Briefly, 1-4 pieces 10-μm-thick paraffin sections per case were cut into sterile 1.5 ml Eppendorf tubes with clean „low profile" microtome blades. To avoid contamination, the microtome blades were cleaned with ethanol and slipped, using a new surface after cutting each case. The number of sections cut into the Eppendorf tubes was determined by the size of the biopsy: 4 sections of a skin biopsy or 1 section of a block of 1 cm in diameter were cut. In the case of biopsy size in between, we used 2 sections per tube. The sections within the Eppendorf tube were dewaxed by washing twice for 10 minutes in xylene and once in absolute ethanol (1-1 ml). The sample was centrifuged after each step at 13,000 rpm for 3 minutes and, after removing the supernatant, the pellets were carried over. After washing with ethanol, the pellets were air-dried in a thermostat at 56ºC. The dried precipitate was resuspended in 300 μl lysis buffer (50 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.0), 30 μl proteinase K solution (20 mg/ml, MBI Fermentas Life Sciences) was added, and it was incubated for 18 hours at 56ºC. Afterwards, if the sample was not cleared, an extra 20 μl proteinase K solution was added, and the sample was incubated for a further 3 hours. Finally, proteinase K was inactivated by boiling for 10 minutes and the supernatant was used as template.

Primers

Our PCR study was focused on the investigation of IgH gene and TCRγ gene rearrangements. For the IgH studies, the VH-FR3 5'-ACACGGCYSTGTATTACTGT-3' forward degenerated primer corresponding to the framework-3 (FR3) variable region of the gene, and the JH 5'-CCTGAGGAGACGGTGACC-3' reverse primer corresponding to the joining region of the gene were utilized.[3,4] Using these primers, the expected size of the PCR product is between 70-120 bp. For the TCRγ studies, the $Vγ_{11}$ 5'-TCTGGRGTCTATTACTGTGC-3' forward primer was used, which is complementary with the Vγ1-8 variable region of

the gene, and the $J_{\gamma 12}$ 5'-CAAGTGTTGTTCCACTGCC-3' reverse consensus primer, which is complementary with the $J\gamma_{1\text{-}2}$ joining region of the gene.[3,8] This primer combination generates 75-95-bp-long PCR products.[8]

PCR amplification and gel electrophoresis

PCR amplification was performed in 0.2 ml thin-walled PCR tubes (AHN), using 50 µl master mix running in an *Eppendorf Mastercycler® personal* PCR thermal cycler. The standard PCR parameters, the modifications were tested against, were as follows: 1x 200 mM PCR buffer containing $(NH_4)_2SO_4$ (MBI Fermentas Life Sciences), 100 µM dNTP (MBI Fermentas Life Sciences), 1.5 mM $MgCl_2$, 100-100 pmol/ml of each primers, 1 µl DNA template and 1.5 U recombinant *Taq* polymerase (MBI Fermentas Life Sciences). The PCR amplification consisted of 35 cycles. The first cycle started with a 5-min denaturation step, and *Taq* polymerase was added during this step. Temperature and timing parameters of the cycles were as follows: denaturation – 30" at 95ºC, annealing – 30" at 56°C (IgH) or at 54°C (TCRγ), extension – 30" at 72°C, last extension – 10' at 72°C. To compensate for the reduced efficiency, we considered modifying the cycling parameters other than increasing the number of cycles and lengthening the duration at each temperature within the cycle. The primer, dNTP, $MgCl_2$ and template concentrations as well as the volume of the *Taq* polymerase were tested. The analysis included step-by-step gradual adjustment of a single variable or optional adjustment of parameters in pairs (*Table 1*), until the best results were obtained. DNA template replaced with distilled water served as a negative control. The PCR products were run on 10% polyacrylamide gel using *Mini-PROTEAN®3* (Bio-Rad Laboratories) electrophoresis set and $AgNO_3$ staining[21] for visualization. DNA detection based on the reduction of silver proved to be approximately one hundred times more sensitive for products of less than 200 bp than the ethidium-bromide- or SYBR green-based fluorescence detection methods. This excellent sensitivity makes it especially useful in the monitoring of PCR-based antigen receptor gene rearrangement studies.[21]

***Table 1.* Values of the PCR variables tested in the study**

Template DNA [dilution of 1 µl template]: 1; 1:2; 1:5; 1:10; 1:100; or 2 µl 1x template
Primers [pmol/reaction]: 10; 50; 100; 125; 175; 250; 375; 500
Taq polymerase [U/reaction]: 1.5; 2.0; 2.5
$MgCl_2$ [mM/reaction]: 0.875; 1.0; 1.125; 1.25; 1.375; 1.5; 1.675; 1.75
dNTP [µM/reaction]: 50; 100; 200; 400

Results

The role of the primer concentrations was investigated first, without modifying any other parameters. The use of 10 pmol primers/reaction in the IgH FR3-JH amplification revealed a weak product, while 50 pmol primers/reaction proved to be the optimal concentration. The higher concentrations showed clear-cut dominant monoclonal products, but the weaker intensity amplicons of the polyclonal B-cell populations were suppressed, which may lead to false positive interpretation of the results (*Fig. 1*). The increasing primer concentration raised the product intensity in the monoclonal cases, but higher non-specific amplification was also detected. The TCRγ Vγ1-8/Jγ1-2 amplification optimally required 10 pmol primers/reaction concentrations, while the higher concentrations resulted in a strong background reaction, which even suppressed the expected monoclonal product.

Using crude lysates, their DNA content cannot be measured correctly, therefore the role of template DNA concentrations was investigated in a dilution experiment. In combination with the above optimal primer concentrations, the 1 ml undiluted lysate/reaction added showed the best results in IgH FR3-JH reaction, while the 1:2, 1:5, 1:10 and 1:100 template dilutions revealed a reaction product with gradually weakening visibility. Two ml undiluted template/reaction added showed increased non-specific background activity. The monoclonal TCRγ product was best seen with 1:10 template dilution, while the lowest reproducibility was found when undiluted lysate was utilized.

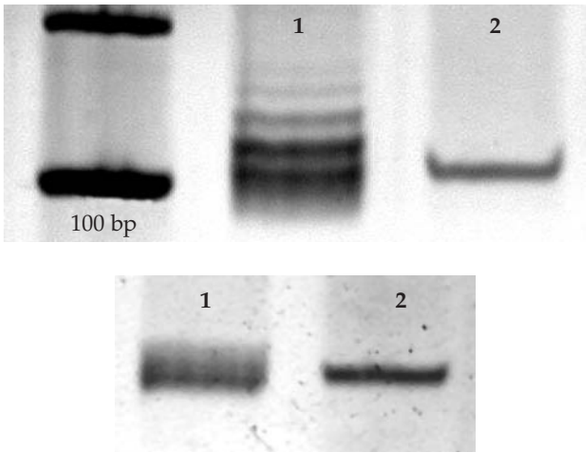

Figure 1. *The role of primer concentration in IgH FR3-JH amplification.* ***(A).*** *In the case of the optimal 50 pmol/reaction concentration, the polyclonal (1) and monoclonal product (2) can be readily discriminated.* ***(B).*** *The increase in the primer concentration to 100 pmol/reaction can slightly suppress the fainter amplicons of the polyclonal product (1) which may lead to false positive evaluation, since it becomes similar to the dominant band of the clear-cut monoclonal product (2).*

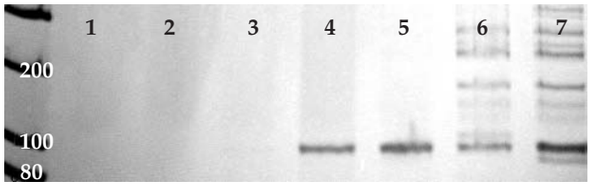

Figure 2. *The role of $MgCl_2$ concentration in IgH FR3-JH amplification. (1) 0.875, (2) 1.0, (3) 1.125, (4) 1.25, (5) 1.375, (6) 1.5, (7) 1.675 mM $MgCl_2$ concentrations with 1.5 U Taq polymerase. The dominant monoclonal product occurs in the concentration range of 1.25-1.675 mM and is accompanied with background amplification in the 1.5 mM and 1.675 mM $MgCl_2$ concentrations.*

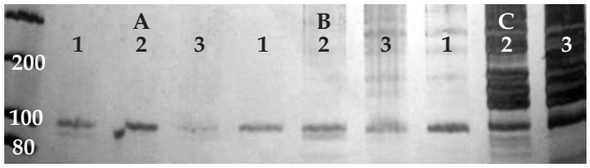

Figure 3. *The relationship between $MgCl_2$ and Taq polymerase concentration in monoclonal IgH FR3-JH gene rearrangement. A: 1.0 mM, B: 1.375 mM, C: 1.75 mM $MgCl_2$; (1) 1.5 U, (2) 2.0 U, (3) 2.5 U Taq polymerase. The increased amount of the Taq polymerase is able to compensate for the lower $MgCl_2$ concentration, while generates background amplification in the optimal $MgCl_2$ concentration.*

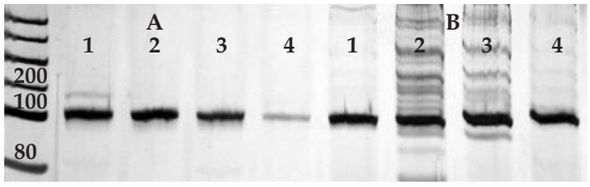

Figure 4. *Evaluation of the effect of dNTP concentration on monoclonal IgH FR3-JH gene rearrangement. A: 100 pmol/reaction primer concentration: (1) 50 μM, (2) 100 μM, (3) 200 μM, (4) 400 μM dNTP concentration. At standard primer concentration, the higher dNTP concentration blocks the reaction. B: 250 pmol/reaction primer concentration: (1) 50 μM, (2) 100 μM, (3) 200 μM, (4) 400 μM dNTP concentration. The elevated primer concentration compensates for the inhibitory effect of the higher dNTP concentrations, but is accompanied by increased background amplification.*

Testing the $MgCl_2$ concentration with pair-wise modification with *Taq* polymerase, we found that 0.875 mM $MgCl_2$ concentration was insufficient for the optimal reaction. The 1.0-1.25 mM $MgCl_2$ concentrations were sufficient to detect monoclonal product only if we increased the *Taq* content, but raising the amount of the enzyme resulted in a high background activity. Our investigations revealed the 1.375-1.75 mM $MgCl_2$ concentration as the optimal range, while 2.0 mM or higher concentrations again resulted in a higher background activity (*Fig. 2*). Higher $MgCl_2$ concentrations could be compensated by adding less *Taq* polymerase or more dNTP. Low, 1.0 U/reaction *Taq* polymerase volumes could not be compensated even with higher $MgCl_2$ concentrations. The 1.5 U *Taq* polymerase combined with less than 1.0 mM $MgCl_2$ concentration was capable to provide a weak reaction product, while the 2.0 U or higher enzyme volumes could compensate the low $MgCl_2$ concentrations with regard to the monoclonal product, but significantly raised the non-specific amplification activity as well (*Fig. 3*).

As far as the nucleotide concentrations are concerned, at least 50 μM dNTP was found to be necessary for visible monoclonal reaction products, while the 100 μM of dNTP proved to be the optimal concentration. The higher dNTP concentrations with standard primer concentration proved to be inhibitory, while an increasing non-specific background activity was experienced if the higher dNTP concentrations were combined with higher primer concentrations (*Fig. 4*).

Using the improved protocol, 74% (17 out of 23) of the tested B-cell lymphoma cases and 68% (31 out of 46) of the tested T-cell lymphoma cases demonstrated monoclonal PCR product (*Figs. 5 and 6*).

Discussion

Nowadays, PCR-based clonality examinations are among the routine methods of hematopathology, and the demand is rising to perform more tests on FFPE tissue samples. One of the most active research areas in molecular pathology is retrospective studies on archival tissue samples. The template from FFPE samples, more or less independently from the extraction method, contains variable quantities of different substances, namely genomic DNA, fragmented DNA originated from genomic DNA, crosslinked non-usable DNA, depurinated nucleic acid, fragmented RNA, protein/peptide fragments, metallic- and other contaminating, and chelating agents. Keeping the above in mind, clonality analysis is not easy on FFPE samples, in fact, it requires even more experience than that of performed on fresh samples, and is more complicated than most genomic PCR amplifications for somatic genes. In our study, we have analyzed multiple PCR variables in order to establish which of them influence most the IgH FR3-JH and the TCRγ Vγ1-8/Jγ1-2 PCR amplifications on non-purified DNA isolated from FFPE samples.

Theoretically, there are two ways to improve PCR amplification from inadequate template DNA, namely modification of the DNA extraction method or, alternatively, optimization of the PCR running parameters. In our study, we have used a simple proteinase K-based lysis and the produced raw DNA was tested as template. Although this raw lysate may contain chemical and peptide inhibitors that

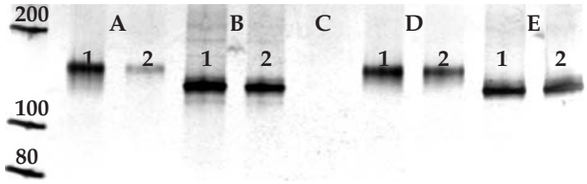

Figure 5. *Optimized IgH FR3-JH protocol tested in B-cell lymphoma cases. A, B, D, E: monoclonal products; C: negative control (no template). (1) template dilution 1:10; (2) template dilution 1:100. Monoclonality is established if a dominant band of the same size is consistently demonstrated with both 1:10 and 1:100 template dilutions.*

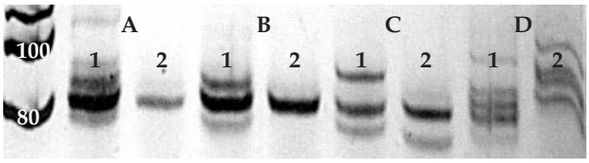

Figure 6. *Optimized TCRγ protocol tested in T-cell lymphoma cases. A, B, C: monoclonal pattern; D: polyclonal pattern. (1) template dilution 1:10; (2) template dilution 1:100. Dominant reproducible band within the expected size range is indicatory for monoclonal rearrangement in lanes A, B, and C. Polyclonal pattern is seen in lane D with multiple inconsistent sized bands.*

influence the PCR reaction, we have chosen to use no further DNA purification, since these procedures are labor-intensive, time-consuming, increase the risk of contamination, and may cause further dilution of the target DNA. According to our and others' experience,[18-20] crude DNA isolations can be well suited for clonality testing, nevertheless they possess disadvantages, including inappropriate OD reading due to protein remnants and other contaminations.

The modification of the thermocycling parameters by increasing the number of cycles and lengthening the duration at each temperature within a cycle seems to be the simplest way to compensate for the reduced PCR efficiency on poor quality template. Our previous experience revealed that an enhancement in annealing temperature by 2-3°C is preferable to decrease the likelyhood of the random priming, while increasing the number of the cycles above 35 could not improve the performance sufficiently.

During formalin fixation, protein-DNA crosslinks, breakage of the DNA strand, and alterations of DNA sequences occur.[13-17] The DNA fragmentation does not only affect the sequence of interest, but may produce random oligonucleotides in the nucleic acid debris. The crude lysate may contain a large number of small fragments that are not long enough to allow efficient amplification, but compete with the primers or even with the template.[6,14] This may lead to inhibition of the specific PCR amplification. Adjusting the primer concentrations, we found that there is no significant increase in the primer requirement using crude lysate as template. This finding may be surprising, since one could expect requirement of higher primer concentration to compensate for inhibition due to the presence of oligonucleotide debris, but it can be explained by the low template DNA concentration or by the ability of these oligonucleotides to compete with the template too, acting rather like general silencers of the PCR amplification. The primer excess, on the other hand, favored the formation of secondary primer structures, leading to mispriming and random annealing with reduced production of the desired amplicons.

The dilution of the crude lysate template may theoretically reduce the hampering effect of the nucleic acid debris or other chemical contaminants. We found no significant improvement in IgH FR3-JH amplification with diluting the template, but gradually weakening PCR reactions. Nevertheless, we still suggest conceivable benefit of template dilution in the case of FFPE samples with potentially high content of inhibitors,[22] but careful consideration of the results are necessary to avoid false positive dominant products which may be experienced while diluting templates from samples with low B-cell content.[22,23] The TCRγ Vγ1-8/Jγ1-2 PCR, on the other hand, benefited from the template dilution, which suggests that the inhibitory contaminants present in the crude lysate can be, at least partly, compensated in this way.

$MgCl_2$, an unavoidable co-factor of PCR, plays a dual role in the amplification reaction. The Mg^{++} ions are necessary for *Taq* polymerase in the elongation step of the primed DNA.[24] It also contributes stabilizing the nucleic acids involved in the reaction, forming complexes with denatured single-stranded template DNA, primers, and free dNTPs.[24] Its absence results in insufficient nucleic acid interactions, and thereby weakens the amplification. We found that no significant change of $MgCl_2$ concentration is required for the use of crude lysate templates.

Although *Taq* DNA polymerase[24] has higher error rate than other thermostable polymerases used in PCR, it is less sensitive than other polymerases and less likely to fail, therefore *Taq* polymerase is the enzyme of choice for amplification from poor quality templates. The crude lysate may contain a large number of 3'-end of small fragments that are not long enough to allow efficient amplification, but may serve as abortive PCR template and compete for the enzyme.[6,14] Even if they do not produce any visible amplicons, the overall intensity of the specific reaction may be jeopardized. Supposing the presence of inhibitory contaminants, we could expect higher enzyme requirement for templates of inadequate quality. In our FFPE clonality assays, we found that apparently a minor increase (from 1 U/reaction to 1.5 U/reaction) of *Taq* polymerase was necessary to improve amplification quality. Added more enzymes resulted in stronger amplification, but with decreased specificity due to tuned up non-specific amplification as well.

Our FFPE clonality assays demonstrated a relative insensitivity to dNTP content showing close to optimal amplification in a wide concentration range. However, the dNTP excess may have inhibitory effect through the binding of the Mg^{++} ions; this can be counterbalanced with higher primer concentrations.

Using crude lysate as template with our improved protocol, we were able to detect high monoclonality rates: 74% (17 out of 23 cases) in the B-cell lymphomas and 68% (31 out of 46) in the T-cell lymphomas tested. These high rates concur with the figures reported using DNA extracted from fresh/frozen tissues[25,26] or using multiple primer combinations,[3] proving the applicability of our optimized method.

In conclusion, our results showed that a slight decrease in primer concentration in combination with a slight increase of $MgCl_2$ and *Taq* polymerase, as well as using diluted crude template and standard amount of dNTPs can be the modifications of choice while adjusting IgH and TCRγ clonality tests on poor quality DNA form FFPE samples. Our experience suggests that the empirical adjustment of the PCR parameters in FFPE clonality analyses may be of great value in understanding the trends which can be of help during the optimization process in technically difficult cases, as well as in determining which parameters and how should be changed to minimize false-negative and false-positive results.

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