SUPPLEMENTAL MATERIAL

Michaela Svachova, Martin Tichy, Patrik Flodr, Jana Steigerova, Zdenek Kolar, Jan Bouchal. Clonality testing of lymphoproliferative disorders in a large cohort of primary and consultant biopsies (doi: 10.5507/bp.2017.006)

Suplemental Methods

DNA isolation

Total genomic DNA from FFPE biopsy samples was extracted from 5 µm sections (number of sections depended on the size of the examined tissue) after deparaffinisation in xylen using the Puregene® DNA isolation Kit (Gentra Systems, Minneapolis, USA) according to our previously published protocol[25]. After isolation, DNA was rehydrated in 20 µl Hydration Solution and then DNA yield and quality were confirmed by spectrophotometric analysis on POWER Wave XS (BIOTEK®Instruments, INC., USA). To ensure amplifiable DNA from paraffin-embedded material, a special set of control gene PCR primers was used resulting in a ladder of four fragments (100, 200, 300 and 400 bp) (recommended by BIOMED-2 guidelines), (Suppl. Fig. 1). Product 600 bp is not used due to common degradation of DNA.

Primers and PCR conditions

The PCR is an extremely sensitive and highly specific method used for the detection of target DNA sequences, IgH and TCR gene rearrangements. DNA is amplified with a series of consensus primer pairs (Generi Biotech, CZ). The primer sequences[15,26] are shown in Suppl. Table 1.

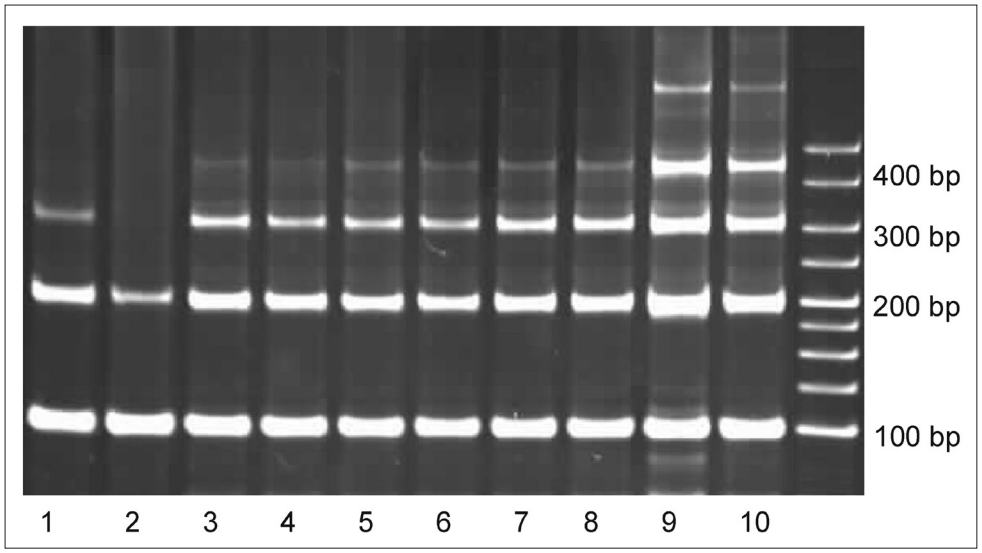
To evaluate TCR gene rearrangements, T-cell receptor-γ chain (TCR-γ) gene rearrangement is frequently investigated because it occurs at an early stage of T-cell development[9]. TCRG genes are classical PCR clonality targets and are useful in all T-cell malignancies of the TCRγδ and TCRαβ lineage[15]. In our laboratory, analysis using set of primers Vγ11-Jγ11 that produce bands ranging between 70 and 110 bp was performed[27,28]. Molecular analysis of the TCRB genes is also an important tool for the assessment of clonality in suspected T-cell proliferations. An incomplete Dβ-Jβ rearrangement has been investigated in this study. The analysis using sets of primers Tβ D1-Tβ J2 and Tβ D2-Tβ J2 was performed. The PCR products range from 55 bp to 100 bp (ref.[26,28]).

For complete IgH gene VH-JH rearrangements, consensus oligonucleotide sets of primers were used to anneal to the three conserved regions called framework areas (FR1 – FR3) within hypervariable complementarity-determining regions (CDR1 – CDR3) (ref.[4,29,30]). According to the BIOMED-2 guidelines[15], three multiplex sets of VH primers complementary to sequences of FR1, FR2, FR3 regions and to JH regions were used to amplify rearranged DNA. The PCR products range from 310-360 bp for VH FR1-JH, 250-295 bp for VH FR2-JH and 100-170 bp for VH FR3-JH.

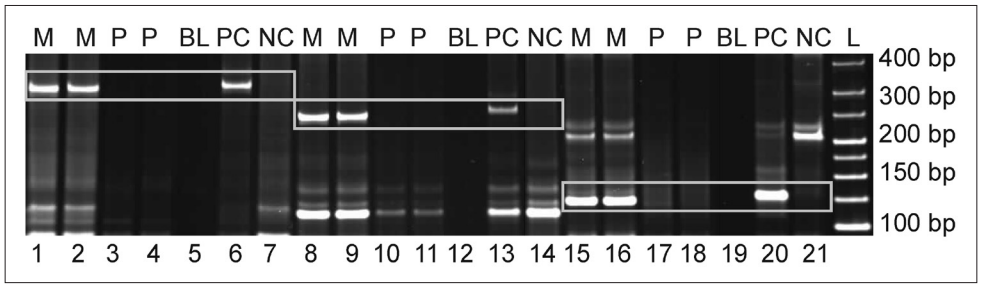
To eliminate false-positive results, heteroduplex analysis was used to analyze the PCR products for discrimination between monoclonal lymphoid cells with identical junctional regions (homoduplexes) and polyclonal lymphoid cells with highly diverse junctional regions (heteroduplexes) (ref.[31]). The heteroduplex technique is rapid, simple and cheap, and uses double-stranded PCR products that are denatured at 95 °C for 5 min and subsequently reannealed by rapid cooling at 4°C for 60 min to induce duplex formation[15,32]. Electrophoresis of the homoduplexes in a 6% polyacrylamide gel results in a single band within a predictable size range, whereas the heteroduplexes form a smear at a higher position[15].

Supplemental Table 1. Sequences of primers (ref.[15,26]).

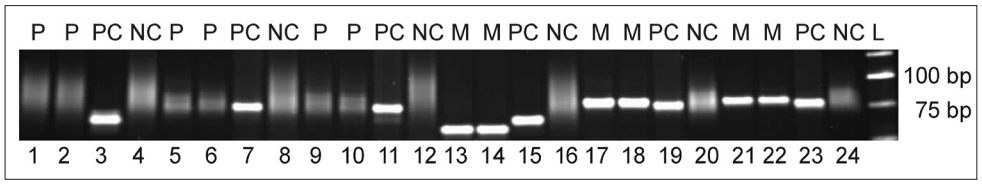
IgH genes	Sequences of primers
VH1-FR1	5' GGCCTCAGTGAAGGTCTCCTGCAAG 3'
VH2-FR1	5' GTCTGGTCCTACGCTGGTGAAACCC 3'
VH3-FR1	5' CTGGGGGGTCCCTGAGACTCTCCTG 3'
VH4-FR1	5' CTTCGGAGACCCTGTCCCTCACCTG 3'
VH5-FR1	5' CGGGGAGTCTCTGAAGATCTCCTGT 3'
VH6-FR1	5' TCGCAGACCCTCTCACTCACCTGTG 3'
VH1-FR2	5' CTGGGTGCGACAGGCCCCTGGACAA 3'
VH2-FR2	5' TGGATCCGTCAGCCCCCAGGGAAGG 3'
VH3-FR2	5' GGTCCGCCAGGCTCCAGGGAA 3'
VH4-FR2	5' TGGATCCGCCAGCCCCCAGGGAAGG 3'
VH5-FR2	5' GGGTGCGCCAGATGCCCGGGAAAGG 3'
VH6-FR2	5' TGGATCAGGCAGTCCCCATCGAGAG 3'
VH7-FR2	5' TTGGGTGCGACAGGCCCCTGGACAA 3'
VH1-FR3	5' TGGAGCTGAGCAGCCTGAGATCTGA 3'
VH2-FR3	5' CAATGACCAACATGGACCCTGTGGA 3'
VH3-FR3	5' TCTGCAAATGAACAGCCTGAGAGCC 3'
VH4-FR3	5' GAGCTCTGTGACCGCCGCGGACACG 3'
VH5-FR3	5' CAGCACCGCCTACCTGCAGTGGAGC 3'
VH6-FR3	5' GTTCTCCCTGCAGCTGAACTCTGTG 3'
VH7-FR3	5' CAGCACGGCATATCTGCAGATCAG 3'
JH cons	5' CTTACCTGAGGAGACGGTGACC 3'
TCR γ genes	
Vγ 11	5' TCT GG(AG) GTC TAT TAC TGT GC 3'
Jγ 11	5' CAA GTC TTG TTC CAC TGC C 3'
TCR β genes	
Tβ D1	5' CAA AGC TGT AAC ATT GTG GGG AC 3'
Tβ D2	5' TCA TGG TGT AAC ATT GTG GGG AC 3'
Tβ J2	5' AGC AC(GCT) GTG AGC C(GT)G GTG CC 3'
Control genes	
100A	5' GCCCGACATTCTGCAAGTCC 3'
100B	5' GGTGTTGCCGGGAAGGGTT 3'
200A	5' TGTTGACTCGATCCACCCCA 3'
200B	5' TGAGCTGCAAGTTTGGCTGAA 3'
300A	5' TGCGATGTGGTCATCATGGTG 3'
300B	5' CGTGTCATTGTCGTCTGAGGC 3'
400A	5' CCGCAGCAAGCAACGAACC 3'
400B	5' GCTTTCCTCTGGCGGCTCC 3'



Supplemental Fig.1. Example of the control gene PCR analysis of the DNA extracted from paraffin-embedded tissues (lymphatic node) using different fixing agents (lanes 1-8) and frozen tissue (lane 9-10). Fixatives: lane 1-2, formalin, showing reduced amplifiability; lane 3-4, Fine Fix, (Bamed, CZ); lane 5-6, sodium chloride; lane 7-8, ethanol.



Supplemental Fig. 2. Examples of PCR analysis of IgH gene rearrangements using three multiplex sets of primers (VH FR1, lane 1-7; VH FR2, lane 8-14; VH FR3, lane 15-21). Lanes PC: Positive control (B-cell lymphoma). Lanes NC: Negative control (reactive lymph node). Lane BL (blank): PCR amplification without DNA template. Lane M: Samples with monoclonal rearrangements. Lane P: Polyclonal samples. Lane L: Ladder. The patient samples were analyzed in duplicate.



Supplemental Fig. 3. Examples of PCR analysis of TCR gene rearrangements using three pairs of primers (Vγ-Jγ, lane 1-4 and 13-16; Tβ D1-J2, lane 5-8 and 17-20; Tβ D2-J2, lane 9-12 and 21-24). Lanes PC: Positive control (T-cell lymphoma). Lanes NC: Negative control (reactive lymph node). Lane M: Samples with monoclonal rearrangements. Lane P: Polyclonal samples. Lane L: Ladder. The patient samples were analyzed in duplicate.

FFPE samples have a relatively high detection rate of clonality, however when DNA is fragmented, it may not efficiently be amplified potentially producing false-negative results. To reduce false-negative results, amplification of multiplex control genes was used resulting in a ladder of four fragments (mentioned above). DNA samples from B and T lymphoma cases with a well-established clonal rearrangement were employed as monoclonal controls. Water was included as a negative control in all reactions to monitor contamination of PCR reactions.

Polymerase chain reaction

To improve the sensitivity of clonal IgH and TCR rearrangement analysis, heat-activated thermostable DNA polymerases were used. These polymerases can eliminate the production of non-specific reaction products such as primer-dimers and mis-primed products[10].

Reaction conditions for TCR:

The reaction mix (25 µl final volume) consisted of template DNA (50-500 ng), 10× ImmoBuffer and 50 mM

$MgCl_2$ Solution (Bioline, USA Inc.), 100 mM dNTP Mix (Promega Madison, USA), 0.1 mM of each primer, 1 U IMMOLASE™ DNA Polymerase (providing both 5'→3' polymerase and 3'→5' exonuclease proofreading activities) (Bioline, USA Inc.) and distilled water. Cycling conditions for TCR: Pre-incubation at 95 °C for 10 min was necessary to activate the enzyme. The PCR programme consisted of 35 cycles, denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, extension at 72 °C for 15 s, and final extension at 72 °C for 10 min.

Reaction conditions for IgH:

The reaction mix (50 μL final volume) was consisted of template DNA (50-500 ng), 10× Thermo-Start HP Buffer and 25 mM $MgCl_2$ (ABgene® UK), 100 mM dNTP Mix (Promega Madison, Wi, USA), 10 μM of each primer, 2 U Thermo-Start Taq DNA Polymerase (providing 5'→3' polymerase and exonuclease activities but lacks 3'→5' exonuclease proofreading activities) (ABgene®, UK) and distilled water. Cycling conditions for IgH: Pre-incubation at 95 °C for 15 min was necessary to activate the enzyme. The PCR programme was consisted of 35 cycles, denaturation at 95 °C for 45 s, annealing at 60 °C for 60 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min.

Reaction conditions for control PCR:

The reaction mix was the same as for IgH, only final volume was 25 μL. Cycling conditions for control PCR: Pre-incubation at 95 °C for 15 min was necessary to activate the enzyme. The PCR programme was consisted of 35 cycles, denaturation at 95 °C for 20 s, annealing at 56°C for 30 s, extension at 72 °C for 60 s, and final extension at 72 °C for 5 min. All reactions were performed in a T-*personal* Combi thermocycler (Biometra, Germany) and Mastercycler gradient (Eppendorf AG, Germany).

Post-amplification steps and gel electrophoresis

After heteroduplex analysis, the PCR products were diluted with loading buffer and with appropriate molecular weight marker HyperLadder V (Bioline, USA Inc.) loaded onto 6% PAGE gel and run at 140 V for 60-75 min. The gels were washed in a strengthening solution (glycerine, 5%; ethanol, 35%; water, 60%) for 30 min and than in a dye solution containing GelRed™ Nucleic Acid Stain (Biotium, USA) for 40 min. The PCR products were then visualized on an ultra-violet light illuminator GBox HR-Imaging system (Syngene, UK). The analysis of PCR products on polyacrylamide gels rather than agarose gels is essential to provide sufficient resolution and also enhances detection of dominant bands within background smears (Suppl. Fig. 2, Suppl. Fig. 3) (ref.[33,34]).