Molecular hematology

Qualitative to quantitative techniques

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ABSTRACT

Over the last decade molecular diagnostics technology has developed dramatically from the most laborious, time-consuming southern blot methodology through the revolution of polymerase chain reaction (PCR) technology to the most reliable, fast, and contamination free molecular analyzer, the real-time quantitative-PCR. The Section of Hematology, Department of Pathology and Laboratory Medicine at King Faisal Specialist Hospital and Research Center has shared this experience during the last 10 years with more than 6,546 samples submitted for the analysis of different gene rearrangements, fusion gene transcripts and gene mutations including Ig heavy chain gene rearrangement for B-cell malignancies, T-cell receptor gamma chain gene rearrangement for T-cell malignancies, BCR/ABL-P210 and P190 fusion gene transcripts, for chronic myeloid leukemia and Philadelphia positive acute lymphoblastic leukemia, PML/RARα fusion gene for promyelocytic leukemia, AML1/ETO for acute myeloid leukemia (AML-M2) with t(8;21), CBFB/MYH11 for AML ($M4E_0$) with inv (16), BCL-2 for follicular lymphoma, and BCL-1 for mantle cell lymphoma. Hence, most molecular assays are qualitative in nature, quantitative assays are deemed necessary in the monitoring and follow-up of minimal residual disease in leukemia and lymphoma, and proved in our experience to serve as an essential tool to confirm complete remission (CR) post-chemotherapy and bone marrow transplantation, and to detect signs of early relapse for proper clinical intervention. In this manuscript, we retrospectively review our experience in molecular hematology and propose our recommended guidelines at King Faisal Specialist Hospital and Research Center.

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Molecular techniques are important tools in the diagnosis, classification, and follow-up of hematological malignancies. While in most lympho-proliferative or leukemic infiltrations adequate morphological assessment and immunophenotyping are sufficient to establish the diagnosis, a minority of difficult cases will require molecular investigation for definitive diagnosis; the utility of this approach being well established. Laboratories with the required expertise and, which participate in relevant quality assurance programs should perform molecular tests. Molecular testing has 3 main roles and several subsidiary roles in the setting of lymphoma/leukemia diagnosis. The main indications for molecular testing include the establishment of monoclonality where this has not been achieved by morphology and immuno-phenotyping, to aid in correct lymphoma and leukemia classification, and to detect minimal residual disease (MRD) post chemotherapy and bone marrow transplantation (BMT).[1-5] Several molecular techniques are available for clinical practice including southern blotting, polymerase chain reaction (PCR), fluorescence in situ hybridization, gene expression profiling using complimentary DNA microarray technology and real-time quantitative PCR, (RQ-PCR).[6-9] The purpose of this manuscript is to provide an overview of our experience with different molecular

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techniques during the last 10 years (1994–2003) at the Department of Pathology and Laboratory Medicine, the Section of Hematology, Molecular Hematology sub-section, King Faisal Specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia.

DNA extraction. High molecular weight DNA/ RNA were isolated from fresh bone marrow aspiration and peripheral blood (PB) by DNAZOL (DNA) and TRIZOL (RNA). The DNA from paraffin embedded lymph node, and skin biopsy specimens was extracted by standard proteinase K digestion and organic extraction procedures.[10]

Southern blotting. In this technique, DNA is extracted from cells or tissues and is cut at specific sites by incubation with 3 restriction endonucleases available (Bam HI, Eco RI, Hind III). The DNA fragments are then separated by electrophoresis, in agarose gel, and the fragments transferred to a nitrocellulose membrane by vacuum blotting. After transfer, the DNA is fixed to the membrane and specific fragments are detected using a P^{32} labeled probe (a DNA sequence corresponding to the gene of interest). Positive and negative controls and a marker on either side of the samples of interest are provided to demonstrate positive and negative results and also to estimate the size of fragments. One percent (1%) sensitivity control is also provided.[11]

Polymerase chain reaction. ***Immunoglobulin gene rearrangement.*** Enzymatic amplification of the IgH gene was performed in a Perkin Elmer GeneAmp PCR System 9600 using a single V_H primer homologous with a highly conserved sequence near the 3' end of FR3 in conjunction with a single consensus J_H primer. Briefly, the reaction mixture contained 0.5 ug of DNA; 10 mM of Tris HC1, pH 8.3; 200 uM of each dNTP; 50 mM KCl; 1.5 mM $MgCl_2$; 10 pmol/µL of each primer and 1 U Taq polymerase (Fast start Taq; Roche) in a final volume of 25 µl. The consensus V_H and J_H primers were synthesized by Gulf Biotech (Portland, Oregon, USA). The sequences were V_H 5' CTG TCG ACA CGG CCG TGT ATT ACT G 3' and J_H 5' AAC TGC AGA GGA GAC GGT GAC C 3'. The reaction mixture was subjected to 40 cycles of PCR following an initial 10 minute denaturation step at 94^{0}C. Each cycle consisted of a 30 second denaturation step at 94^{0}C, a 30 second annealing step at 60^{0}C, and a one minute elongation step at 72^{0}C. The last cycle was followed by a 15 minutes elongation step at 72^{0}C. Ten microliters of PCR amplified product was resolved by electrophoresis on a 6% polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet light. A discrete band is seen in clonal B-cell processes within the predicted size range of 90 to 160 base pairs.[12] All samples were subjected to amplification using primers for the erb-B2 gene to confirm that amplifiable DNA was present.

T-cell receptor gene rearrangement. Enzymatic amplification was performed in a Perkin Elmer GeneAmp PCR System 9600 using consensus primers for T-cell receptor gamma chain V regions, and a single consensus primer for the J region as described previously with minor modifications. Briefly, the reaction mixture contained 0.5 ug of DNA; 10 mM of Tris-HCl, pH 8.3:200 uM of each dNTP; 50 mM KCl; 1.5 nM $MgCl_2$; from 7-10 pmol/µL of each primer and 1 u Taq Polymerase (Fast start Taq; Roche) in a final volume of 25 ul. The consensus TCRγ chain V and J primers were synthesized by Gulf Biotech (Portland, Oregon, USA). Sequences were:

Vγ (1-8)II5' ACCAGGAGGGGA AGGCCCCACAG3';
Vγ9 5' GGAAAGGAATCTGGCATTCCG3';
Vγ10 5' AATCCGCAGCTCGACGCAGCA 3';
Vγ 11 5' GCTCAAGATTGCTCAGGTGGG 3';
Vγ 12 5' CCTCTTGGGCACTGCTCTAAA 3';
Vγ1/2 5' ACCTGTGA CAACAAGTGTTGTTC 3'

The reaction mixture was subjected to 40 cycles of PCR following an initial 10 minute denaturation step 94^{0}C. Each cycle consisted of a 30 second denaturation step at 94^{0}C, a 30 second annealing step at 62^{0}C and a one minute elongation step at 72^{0}C. The last cycle was followed by a 15 minute elongation step at 72^{0}C. Ten ml of PCR amplified product was resolved by electrophoresis on a 6% polyacrylamide gel, stained with ethidium bromide and visualized under ultraviolet light. A discrete band(s) is seen in clonal T-cell processes within the predicted size range of 190-260 base pairs.[13] All samples were subjected to amplification using primers for the erb-B2 gene to confirm that amplifiable DNA was present.

BCL-2 major breakpoint region. Enzymatic amplification of the BCL-2 major breakpoint region rearrangement was performed in a Perkin Elmer GeneAmp PCR System 9600 using a oligo-nucleotide primer of a region 5' to the BCL2 MBR and a single consensus J_H primer. The 5' BCL2 MBR primer was synthesized on a applied Biosystem, the DNA synthesizer and the J_H primer was synthesized by Gulf Biotech. The sequences were BCL-2 MBR 5' GAG AGT TGC TTT ACG TGG CCT G 3' and J_H 5' AAC TGC AGA GGA GAC GGT GAC C 3'. The reaction mixture contained 10 mM of Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.00% W/V gelatin; 200 uM of each dNTP; 20 pmol/µL of MBR primer and 10 pmol/µL JH primer; 1 U Taq polymerase (Fast start, Roche) and 0.5 µg of DNA in a final volume of 25 µl. The mixture was subjected to 40 cycles of PCR

following an initial 10 minutes denaturation step at 94^{0}C. Each cycle consisted of a 30 second denaturation step at 94^{0}C, a 30 second annealing step at 62^{0}C and a one minute elongation step at 72^{0}C. The last cycle was followed by a 15 minutes elongation step at 72^{0}C. Ten microliters of PCR amplified product was resolved by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and visualized under U/V light. A discrete band of approximately 200 bp is expected for positive results.[14]

Reverse transcriptase PCR (RT-PCR) BCR/ABL-P210: BCR/ABL. Total cellular RNA was prepared from blood or bone marrow buffy coats and stored at –70^{0}C until assayed.[15] The cDNA was synthesized using Promega's Reverse Transcription System (Madison, Wisconsin, USA). The 20 µl reaction mixture contained 5 mM MgC12, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1 mM each dNTP, 25U ribonuclease inhibitor, 7.5U AMV reverse transcriptase, 12.5 ng Random primer and 1000 ng sample RNA in RNase-free water. The reaction mixture was incubated at 42^{0}C for 60 minutes, then, heated at 95^{0}C for 5 minutes. The tubes were held briefly at 4^{0}C until the PCR step. The 3' primer sequence for reverse transcription of BCR/abl is 5'–GGT ACC AGG AGT GTT TCT CCA GAC TG-3'. A PCR reaction mixture was prepared with the 5' primer sequence 5'-GAG CGT GCA GAG TGG AGG GAG AAC A-3'. After an initial 10 minute denaturation step at 94^{0}C, PCR was performed in a Perkin Elmer Gene Amp PCR System 9600 by 30 cycles of 94^{0}C 30 seconds, 66^{0}C 30 seconds, 72^{0}C 1 minutes followed by a 15 minute extension at 72^{0}C. Ten microliters of amplified product were electrophoresed in a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. A band seen at either 430 or 500 base pairs is interpreted as positive. All samples were subjected to amplification using primers for the actin gene to confirm that mRNA was present. A band is always present at approximately 200 bp.[16]

Nested PCR: BCR-ABL p210. Nested PCR is performed for the detection of minimal residual disease. In a total reaction volume of 20 µl containing 10 mM TRIS, 50 mM KCL, 1 mM $MgC1_2$, 0.16 mM of each dNTP, 0.5 pmol of each primer (B2A 5'-TTC AGA AGC TTC TCC CTG ACA T-3' and CA3 5'-TGT TGA CTG GCG TGA TGT AGT TGC TTG G-3') and IU of Faststart Taq polymerase (Roche), 2 µl of first round PCR product is added, and subjected to an initial denaturation of 94^{0}C for 10 minutes, followed by 30 cycles of denaturation at 96^{0}C for 30 seconds, annealing at 64^{0}C for 50 seconds and extension at 72^{0}C for 1 minute. A final extension is performed at 72^{0}C for 15 minutes. The PCR product is visualized under UV light after electrophoresis in a 1.5% agarose gel. Positive samples yield a band at 38 bp or 456 bp.[17]

Reverse transcriptase PCR (RT-PCR) PML-RARα. Total cellular RNA was prepared from blood or bone marrow buffy coats and stored at –70^{0}C until assayed. The cDNA was synthesized using Promega's Reverse Transcription System (Madison, Wisconsin, USA). The 20 µl reaction mixture contained 5 mM MgCl2, 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1 mM each dNTP, 20U ribonuclease inhibitor, 15U AMV reverse transcriptase, 250 ng oligo (dT) primer and 100-200 ng sample RNA in RNase-free water. The reaction mixture was incubated at 42^{0}C for 15 minutes, then heated at 95^{0}C for 5 minutes. The tubes were held briefly at 4^{0}C until the PCR step. A PCR reaction mixture was prepared with the 3' primer sequence 5' – ACC GAT GGC TTC GAC GAG TTC-3' and the 5' primer sequence 5'–AGC CCT TGC AGC CCT CAC AG-3'. After an initial 10 minutes denaturation step at 94^{0}C, PCR was performed in a Perkin Elmer Gene Amp PCR System 9600 by 40 cycles of 94^{0}C 1 minute, 55^{0}C 1 minute, 72^{0}C 1 minute followed by a 15 minute extension at 72^{0}C. Ten microliters of amplified product was electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. A band seen at 220 base pairs or 550 and 694 base pairs is interpreted as positive.[18] All samples were subjected to amplification using primers for the actin gene to confirm that mRNA was present.

Nested PCR: PML-RARα. Nested PCR is performed for the detection of minimal residual disease. In a total reaction volume of 20 ul containing 10 mM TRIS, 50 mM KCL, 1 mM, $MgC1_2$, 0.16 mM of each dNTP, 0.5 pmol of each primer (AB12 5' -AAT ACA ACG ACA GCC CAG AAG-3' and AB13 5'-CTC ACA GGC GCT GAC CCC AT-3') and IU of Faststart Taq polymerase (Roche), 2 ul of first round PCR product is added and subjected to an initial denaturation of 94^{0}C for 10 minutes, followed by 30 cycles of denaturation at 94^{0}C for 30 seconds, annealing at 62^{0}C for 30 seconds and extension at 72^{0}C for 1 minute. A final extension is performed at 72^{0}C for 15 minutes. The PCR product is visualized under UV light after electrophoresis in a 1.5% agarose gel. Positive samples yield a band at 390 bp (long form or variant). Short form first round PCR product is not amplified in the nested PCR. A 220 bp band will be presented after the first round of cycling in patients who are positive for the short form of PML-RARA.[19]

Real-time quantitative-polymerase chain reaction. The PCR was performed using a final volume 20 µl mastermix containing 1X buffer, dATP, dCTP, dGTP, dUTP and Taq polymerase); 4 mM $MgCI_2$, 0.25 pM of each 3' and 5' fluorescent hybridization probes (synthegen) 0.5 pM of each 3' and 5' oligo- nucleotide primer. Amplification

occurred in a 3 step cycle procedure (denaturation, 94^{0}C-10 minutes, 95^{0}C, 2 s, ramp rate 20^{0}C/s; annealing 64^{0}C, 10 s, ramp rate 20^{0}C/s; and extension 72^{0}C, 26 s, ramp rate 2^{0}C/s) for 45 cycles. The following primers were used for amplification: b2a2, b3a3, b2a3, primers B2A and NA4-. Sequences of the probes have been selected empirically using the recommendations of the manufacture. Amplification, fluorescence detection, and post-processing calculations were performed using the light cycler apparatus (Roche Diagnostics). A 5' hybridization probes were labeled with fluorescein, 3' probes with LC Red640. Fluorescein is excited by blue light (470 nm) and emits green light at a slightly longer wave length. If the 2 dyes are in close proximity (namely after co-hybridization of the 2 oligonucleotide probes to the target sequence), the energy emitted by the fluorescein excites the LC Red640, which then emits red fluorescent light (640 nm). Fluorescence was measured after each annealing step and expressed as the ratio between fluorescence at 640 nm (designated F2) and at 530 nm (background, designated F1). The fluorescence signal was plotted against the cycle number for all samples and external standards. These standards consisted of serial dilutions (10-^{1} to 10-^{7}) of K562 cell line in normal RNA. Initially, the crossover point was determined for each standard dilution, namely, the point at which the signal rose above the background level. The higher the initial number of starting molecules, the earlier the signal appears above the background. A standard curve for each run was constructed by plotting the crossover point against the log. The amount of target molecules in each sample was then calculated automatically by reference to this curve. Results were expressed a ratio of abnormal cDNA to normal cDNA.[20]

Over the last 10 years (1994–2003) the Diagnostic Molecular Hematology Section of the Department of Pathology and Laboratory Medicine at King Faisal Specialist Hospital and Research Center processed 6,546 samples for molecular testing to detect different gene rearrangements and translocations. This included the detection of the immunoglobulin heavy chain gene rearrangement (IgH), T-cell receptor gamma chain gene rearrangement (TCRγ chain), BCR/ABL-P210 for chronic myeloid leukemia (CML), BCR/ABL-P190 for acute lymphoblastic leukemia (ALL), PML-RARα fusion gene for acute promyelocytic leukemia (PML), BCL-2 for follicular lymphoma and BCL-1 for mantle cell lymphoma. **Table 1** shows the different available molecular tests at the Molecular Hematology Section of the Department of Pathology and Laboratory Medicine. **Table 2** shows the total number of specimens performed every year with a noticeable increase of molecular testing requests by Clinical Hematology and Oncology services. **Table 3** shows the total number of different molecular tests performed during the last decade. Using primers for the immunoglobulin heavy chain gene from the framework number 3 (FR3), more than 80% of B-cell lymphoproliferative disorders were detected using PCR. T-cell clonality was detected using 3 sets of primers in more than 90% of T-cell lymphoproliferative disorders including T-cell ALL and cutaneous T-cell lymphoma. Over the last 3 years the BCR/ABL P190 and P210 detection were transferred into the real-time quantitative RQ-PCR technique with a cut-off value of less than 1/10,000 (10-^{4}) sensitivity rate of detection using the light cycler technology. A comparison between the commercially available kit for BCR-ABL (ROCHE Diagnostics, Mannheim, Germany) and in house prepared primer mixture used for real-time PCR demonstrated a lower sensitivity (4/57 cases false negative) for the commercial kit in addition to its inability to discriminate between different BCR-ABL fusion genes (BCR/ABL P210 verses BCR/ABL P190). Qualitative RT-PCR for the detection of PML-RARα fusion gene was also started and simply analyzed using available commercial kits (ROCHE Diagnostics, Mannheim, Germany). However, this kit has shown a lower sensitivity (2/23 cases false negative) and lack of discrimination between the short and long isoforms of the fusion transcripts, therefore, it has been replaced by an in house prepared primer mixture. The quantitative procedure for BCR/ABL-210 was evaluated in different bone marrow and peripheral blood (PB) samples and showed full consensus, therefore, PB samples are recommended for MRD follow-up **Table 4.** The recommended guidelines for molecular studies in our laboratory are summarized in **Table 5.**

The development of clinically useful molecular assays is spawned largely by the dissection of rather specific translocations that essentially define distinct disease entities, and the exploitation of the physiologic process of antigen receptor gene rearrangementin lymphoid cells. Different molecular techniques have different advantages, and limitations in different phases of disease diagnosis and follow-up.[21] All clinical laboratories started a decade ago with the Southern Blot technique, which proved with time that it has its major limitations including a low sensitivity threshold, the requirement of radioactivity, or the need for fresh/frozen tissue, in addition to being a very lengthy procedure delaying results and management of patients.[22] This prompted investigators to develop an alternative molecular diagnostic approach, achieved by the discovery of the PCR, which revolutionized molecular hematology and molecular diagnostics in general. Its advantages over the conventional Southern Blot included the rapidity of assay, the amenability to automation, no need for radioactivity, the need for a very small amount of extracted DNA, and its sensitivity, which might reach up to 10-^{6} or 10-^{8}

Table 1 - Available molecular hematology tests at King Faisal Specialist Hospital and Research Center, Department of Pathology and Laboratory Medicine.

Molecular hematology test	Sub-heading	Findings
Leukemia		
BCR/ABL- P210	t(9;22)	Chronic myeloid leukemia
BCR/ABL- P190	t(9;22)	Acute lymphoblastic leukemia
PML/RARα	t(15;17)	Promyelocytic leukemia (M3)
AML1/ETO	t(8;21)	Acute myeloid leukemia (M2)
CBFB/MYH11	inv(16)	Acute myeloid leukemia (M4E0)
Lymphoma		
IgH chain gene rearrangement		B-cell malignancies
T-cell receptor γ chain gene rearrangement		T-cell malignancies
BCL-2 t(14;18)		Follicular lymphoma
BCL-1 t(11;14)		Mantle cell lymphoma
Thrombophilia		
Factor V Leiden (G1691A) mutation		
Prothrombin (G20210A) mutation		

Table 2 - Molecular testing of total specimens from 1994-2003.

Year	Total specimens
1994	98
1995	292
1996	438
1997	518
1998	267
1999	584
2000	915
2001	1048
2002	984
2003	1402

Table 3 - Different molecular tests performed between 1994-2003.

Molecular test	Number of specimens
IgH gene rearrangement	1479
TCR gene rearrangement	1400
BCR/ABL P210	2078
BCR/ABL P190	121
PML/RARα	529
Saved DNA/RNA	1508
FV Leiden	139
Miscellaneous	231

Table 4 - Bone marrow versus blood samples for BCR/ABL gene detection.

Bone marrow	Blood
31/100	33/100
31/10	45/10
21/100	25/100
75/10	65/10
25/100	23/100

Table 5 - Recommended guidelines for molecular studies.

Diagnosis	Assay
New acute leukemia	Save DNA/RNA
New CML	Baseline quantitative BCR/ABL-P210 in addition to Karyotyping
New pediatric ALL	BCR/ABL-P190
New adult ALL	BCR/ABL-P210 and P190
New PML (M3)	PML/RARα (Light Cycler)
New AML (Non-M3)	If positive do, Nested-RT-PCR AMLI/ETO, CBFB/MYH11, BCR/AbL P210
Follow-up CML	Quantitative BCR/ABL-P210 on PB <1/10,000 follow with Nested RT-PCR
Follow-up PML (M3)	PML/RARα (Light Cycler)

CML - chronic myeloid leukemia,
ALL - acute lymphoblastic leukemia, PML - promyelocytic leukemia,
AML - acute myeloid leukemia, PB - peripheral blood,
RT-PCR - reverse transcriptase-polymerase chain reaction

using the nested RT-PCR.[23-25] The PCR assays are either qualitative or quantitative. Most molecular diagnostic assays are qualitative in nature; namely, they simply detect the presence or absence of a particular genetic event such as a monoclonal immunoglobulin or T-cell receptor gene rearrangements or the presence of chromosomal translocation such as BCR/ABL or PML-RARα. Quantitative assays are used to quantify accurately the PCR product in the setting of MRD detection and monitoring. These include limiting dilution studies competitive PCR and RQ-PCR. Since non-quantitative RT-PCR analysis gives only limited information, several groups have developed quantitative or semi-quantitative RT-PCR assays that enable the kinetics of the residual BCR/ABL transcripts to be monitored overtime in patients with CML after allogenic stem cell transplantation or Imatinib Mesylate (glivec) therapy.[26-27] Over the last 3 years, we have established a similar real-time RT-PCR approach for detection and quantification of BCR/ABL fusion transcripts using the light cycler technology. A comparison between the commercially available kit for BCR/ABL and the in house prepared primer mixture used for real-time PCR showed a lower sensitivity (4/57 cases false negative) and specificity for detection of different BCR/ABL fusion genes (BCR/ABL P210 versus BCR/ABL P190). Therefore, the in house prepared primer mixtures for the above-mentioned fusion genes became the method of choice in our laboratory. This is in addition to the lower sensitivity (2/23 cases false negative) and specificity in detecting different short and long isoforms of PML/RARα fusion gene performed using pre-prepared kit developed by Roche Diagnostics, Mannheim, Germany. Therefore, all new cases of PML (M3) in our laboratory are subjected to polyacrylamide gel electrophoresis to detect the different isoforms due to their reported importance in the management of this disease with all-trans retinoic acid therapy. The major advantages of this methodology are: the amplification and product analysis are performed in the same reaction vessel, avoiding the risk of contamination, the results are standardized by the quantification of housekeeping genes and the complete PCR analysis takes less than 60 minutes.[28] Molecular analysis in hematological malignancies has major advantages over the conventional methodology including morphology, cytochemistry, immunophenotyping, and karyotyping. However, it is essential to understand the number of limitations of molecular testing, which can be fully categorized into false positive and false negative. There is an ever-expanding list of leukemia or lymphoma associated translocations detected by sensitive PCR or RT-PCR techniques in normal individuals with neither concurrent nor subsequent development of malignancy.[29] The biologic significance of these is uncertain. Although they do suggest that many of these translocations are indeed necessary but not sufficient for the full neoplastic phenotype, therefore from the clinical laboratory prospective the presence of such translocations in the normal population should elicit some questions, but certainly not undue concern. The reasons for this include the fact that many of these have been detected only with hypersensitive often nested RT-PCR reactions, detecting this fusion genes at levels so low (10^{-6} to 10^{-8}) that they are unlikely to be relevant in the context of MRD testing where 10^{-4} appears to be the usual degree of sensitivity required. However, in our laboratory all CML cases with < 1/10,000 transcript levels will be subjected to nested RT-PCR and only a negative result confirms complete molecular remission. Such an approach is still controversial in the clinical setting.[30] Molecular results with PB and bone marrow were concordant in all cases indicating that either tissue may be used for residual disease monitoring in CML. This result confirms similar reported data.[31] False negative PCR molecular genetic studies of antigen receptor gene rearrangements can be due to technical or biologic factors and these are significant problems in diagnostic molecular hematology laboratories. Technical factors are usually an analytical compromise related to the design of the clinical assay. In our laboratory, a single V_H primer homologous with a highly conserved sequence near the 3'of FR3 in conjunction with a single consensus J_H primer is chosen for detection of the IgH gene rearrangement with only an 80% prediction rate.[32] An example of biological factor is nicely demonstrated by the high level of false negative rate in detection of t(11;14), (BCL-1 in mantle cell lymphoma), which has more than a 50% false negative rate related to heterogeneity of break points of the BCL-1 locus other than those involving the major translocation clusters.[33-35] Therefore, clonality does not always equate with malignancy, nor does its absence necessarily indicate a benign process. This fact is also supported by the presence of the T-cell receptor gene rearrangement in general conditions including immune reconstitution after BMT.[36] This emphasizes the point that it is essential to remain cognizant of the variably frequent pitfalls and caveats of molecular analysis, the later validate the notion that from a diagnostic laboratory perspective molecular analysis is the one tool the results of which always need to be integrated into the appropriate pathological and clinical context to optimally harness their value.

We also conclude that quantitative real-time PCR (RQ-PCR) with hybridization probes is a rapid, sensitive, contamination free and reliable method in monitoring of MRD in leukemia, lymphoma, and other malignancies that are characterized by specific molecular markers.

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