Master Thesis

Clonality Testing: Differences in sensitivity, specificity and application of Next-Generation-Sequencing and PCR based Methods.

A Literature Review.

Submitted by

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Abstract

Introduction: Malignant hematooncological diseases usually show a clonal T- or B-Cell receptor as the malignant lymphocytes stem from a common precursor cell. This circumstance can be used in difficult cases where differentiation between malignant and reactive is not easily done with conventional methods like histology and flow-cytometry. The Gold-Standard for B- and T-Cell clonality testing is polymerase chain reaction, using BIOMED-2/ Euroclonality approved primer sets to cover the vast majority of possible rearrangements, with subsequent capillary electrophoresis (PCR-CE). Based on the detected fragment lengths clonality can be detected. The interpretation and the sensitivity of this method shows its limitations and Next-Generation-Sequencing (NGS) is thought to be a promising technique to improve the detection of clonality.

Methods: This thesis systematically reviewed the literature of the last 23 years aiming to answer the questions which differences regarding sensitivity and specificity as well as applicability of NGS versus PCR-CE can be found and if NGS is a reasonable replacement of the Gold-Standard in clonality testing. In doing so the scope was defined using a PICOC (**P**opulation, **I**ntervention, **C**omparison, **O**utcomes and **C**ontext) framework and a systematic search of the literature, with subsequent data synthesis and quality assessment was conducted.

Results: The review of the literature showed several advantages of NGS for clonality testing like higher sensitivity and lower limit of detection. Especially the calling of the predominant sequences which can be used for following specific clones for minimal residual disease determination seems to be a major advantage of the method. The primary disadvantages of NGS for clonality testing seem to be costs and work effort compared to PCR-CE.

Conclusion: Testing T- and B-Cell clonality via Next-Generation-Sequencing contributes several new aspects for managing hematooncological disorders. Nevertheless, PCR-CE still seems to be a feasible method for clonality testing which up to now is more applicable and lower priced than NGS testing.

Zusammenfassung

Einleitung: Maligne hämatoonkologische Erkrankungen zeigen meist einen klonalen T- oder B-Zell-Rezeptor, da die zugrundeliegenden malignen Lymphozyten von einer gemeinsamen Vorläuferzelle abstammen. Dieser Umstand kann genutzt werden um in schweren Fällen, wenn eine Unterscheidung zwischen maligne und reaktiv mit konventionellen Methoden wie Histologie oder Flow-Zytometrie nicht möglich ist, weiterzuhelfen. Der Goldstandard für die B- oder T-Zell Klonalitätstestung ist die Polymerase-Ketten-Reaktion (Polymerase-Chain-Reaction, PCR) mit BIOMED-2/ Euroclonality validierten Primer-Sets und anschließener Fragmentlängenanalyse zur Klonalitätsbestimmung via Kapillar-Elektrophorese (Polymerase-Chain-Reaction and Capillary Electrophoresis, PCR-CE). Die Interpretation und die Sensitivität dieser Methodik weist jedoch Limitationen auf und Next-Generation-Sequencing zeigt sich auch in diesem Gebiet als vielversprechende Alternative.

Methodik: Diese Arbeit recherchierte systematisch die Literatur der letzten 23 Jahre, um Sensitivität und Spezifität als auch die generelle Anwendung von NGS versus PCR-CE zu evaluieren. Der Umfang der Literaturrecherche wurde definiert, indem ein PICOC-Schema (**P**opulation, **I**ntervention, **C**omparison, **O**utcomes and **C**ontext) angewendet wurde und anschließend eine systematische Literaturrecherche durchgeführt wurde. Anschließend wurden relevante Studien nach definierten Kriterien ausgewählt und bezüglich Qualität evaluiert.

Ergebnisse: Diese Literaturarbeit zeigte einige Vorteile der NGS-Technologie für die Klonalitätstestung auf. Diese betreffen vor allem die verbesserte Sensitivität und die niedrigere mögliche Nachweisgrenze. Vor allem, dass die prädominante klonale Sequenz genauer bestimmt und auch für Verlaufskontrollen genutzt werden kann, stellt einen großen Vorteil dar. Als vorrangige Nachteile sind höhere Kosten und Arbeitsaufwand zu nennen.

Conclusio: Die Klonalitätstestung mittels NGS bringt einige neue Verbesserungen mit sich, welche mit dem aktuellen Goldstandard (PCR-CE) nicht abgedeckt werden. Trotzdem scheint PCR-CE eine noch immer gut anwendbare und günstigere Variante für die Klonalitätstestung zu bieten.

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Acknowledgement

First of all, I want to thank and express my gratitude to Prof. DDr. Müllauer who was kind and patient with me and accepted the slow progress I made in writing this thesis amongst a hectic work life in the last years we all know to well.

I also want to thank my husband and my family for supporting me and listening to my constant doubts and worries in times where everything once more seemed to be overwhelming and then has not been as bad as I anticipated in the end.

Last but not least, I want to acknowledge the skills und motivation of my colleagues at the Klinik Favoriten, especially my biomedical analytics team, who are willing to accept any new analysis ideas, are determined to make it work if possible and don’t hesitate to discuss the flaws of the idea with me.

Inhalt

Abbreviations

PICOC	Population, Intervention, Comparison, Outcome, Context
DNA	Deoxyribonucleic acid
NGS	Next-Generation-Sequencing
PCR	Polymerase-Chain-Reaction
PCR-CE	Polymerase-Chain-Reaction and Capillary Electrophoresis
MRD	Minimal Residual Disease
CLL	Chronic Lymphatic Leukaemia
ALL	Acute Lymphoblastic Leukaemia
B-ALL	B-Cell Acute Lymphoblastic Leukaemia
MCL	Mantle Cell Lymphoma
DLBCL	Diffuse Large B-Cell Lymphoma
FL	Follikular Lymphoma
RQ-PCR	Real Time Quantitative Polymerase-Chain-Reaction
WHO	World Health Organisation
NK-cell	Natural Killer Cell
IG	Immunglobulin
BCR	B-Cell-Receptor
TCR	T-Cell-Receptor
IgH	Immunglobulin Heavy Locus
Igκ	Immunglobulin Kappa Locus
Igλ	Immunglobulin Lambda Locus
TCRA	TCR-alpha-chain
TCRB	TCR-beta-chain
TCRG	TCR-gamma-chain
TCRD	TCR-delta-chain
ASO-PCR	Allele specific oligonucleotide PCR

1. Introduction

Lymphoproliferative disorders form a diverse group of malignant and benign illnesses. The World Health Organisation (WHO) classification of haematolymphoid tumours in its 5th edition recognizes thirty-six different T-cell and NK-cell as well as forty-eight mature B-cell lymphoma entities. Distinguishing between a lymphoma and a benign or reactive proliferation of lymphocytes can be challenging at times as the latter quite frequently occurs in the course of infections or autoimmune processes and can mimic a malignant process. Usually, many different diagnostic modalities are used to help making the distinction. Clinic presentation, radiologic pattern or distribution of suspect lymph nodes, histomorphology and flow cytometry are some of the most important tools for lymphoma diagnosis. Integrating this abundance of information can be difficult and sometimes the results can be confusing and inconclusive as well. One good example for this is Epstein-Barr Virus infection, also known as infectious mononucleosis, which can histomorphologically be a mimicry of a Hodgkin lymphoma and is therefore recognized as a typical pitfall in haematopathology. As it is of utmost importance to distinguish malignant from benign or reactive lymphoproliferative disorders to provide the patient with the right and adequate care, the search for additional molecular marker or tests began decades ago.(1–3)

B- and T-cell lymphomas are thought to derive from at least one common precursor cell and therefore form a clonal expansion of similar atypical or malignant lymphocytes. Physiologically lymphocytes express a diversity of receptors to be equipped to recognize a variety of antigens. These different kinds of Immunglobulin- and T-Cell-Receptors, which are both described in detail below, are designed to show high affinity and specificity to distinct antigens which is necessary to adequately react to different kinds of infections. A population of benign or reactively proliferating T- and B-Cells should therefore consist of lymphocytes with a broad spectrum of receptors which would be called a polyclonal pattern. A malignant proliferation of lymphocytes in the context of lymphomas usually derives from a common cell and hence share the same type of Immunglobulin or T-Cell-Receptor. Because of this such a population of similar malignant lymphatic cells is called a clonal population (Fig. 1). Unfortunately, these two scenarios are not always the

case and clonal cells can be distributed in a polyclonal and reactive background as well as specific selection of lymphocytes due to infections for example can lead to an oligoclonal pattern of Immunglobulin- and T-Cell-Receptors.(2)

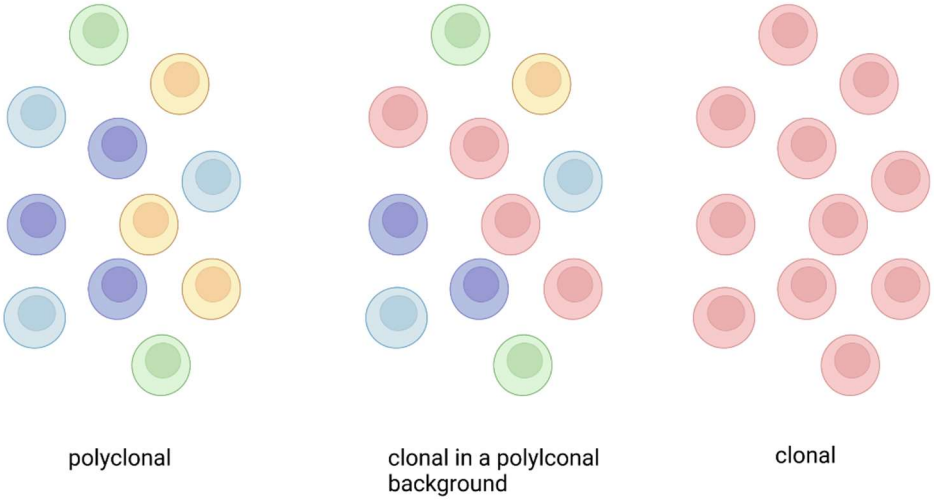

Figure 1 IG and TR diversity, Created with BioRender.com

The field of Immunogenetic Analysis was developed to study the described repertoire of Immunglobulin- and T-Cell-Receptors and soon it was discovered that this diversity of immune cells and the loss of it during the transformation to a lymphoma can be used to help solving challenging cases regarding the distinction between reactive or malignant. But the study of the immune-repertoire is not only used for the distinction of malignant or benign, its usage for the detection of minimal residual disease was discovered to be a second important field of application.(2)

For the actual testing of Immunglobulin- and T-Cell-Receptor clonality status different methods have been used in the last couple of decades. The underlying concept is always the natural genetic recombination of the genes coding for the subunits of the B- and T-Cell-Receptor which would be similar in a clonal population. In the beginning the detection of these monoclonal sequences was mainly done via Polymerase-Chain-Reaction with sequence specific primers and subsequent detection of the different product lengths via Southern Blot. Fragment analysis via capillary gel electrophoresis has become the most studied and used method of the last couple of years for the detection of clonality status. If a monoclonal population

can be detected, subsequent Sanger sequencing and the design of patient specific primers for the use of quantitative PCR can be done to follow-up on minimal residual disease. Unfortunately, this method is quite time consuming and represents a thoroughly laboratory developed process which not all laboratories can do or are willing to do. In recent years the development of Next-Generation-Sequencing and its increasing use in routine oncologic workups, has also changed the possibilities for immune-repertoire testing. In contrast to classic southern blot or capillary gel electrophoresis, which uses the detection of fragment lengths and accumulation of a distinct fragment length for the distinction between polyclonal and clonal, Next-Generation-Sequencing enables to easily sequence the recombined gene sequences of the T- and B-Cell-Receptor. Bioinformatics are then used to detect a cluster of same sequences.(2)

2. Background

As already mentioned before diversity of the T- and the B-Cell-Receptor is essential for the immune system to be able to react adequately to the abundance of antigens of viruses, bacteria and other foreign materials we are confronted with on a daily basis. Both receptors share the similarity that the needed diversity arises from somatic recombination of the genes coding for the constant and variable regions of the receptors.(4)

The following chapters address the topic of T- and B-Cell-Receptor rearrangement as well as the general concepts of clonality testing in more detail.

2.1 B-Cell-Receptor

The B-Cell-Receptor (BCR) is a transmembrane protein composed of a heavy polypeptide chain (Immunglobuline heavy chain - IgH) and a light chain (Igκ or Igλ) which both contain a constant and a variable region. The variable region (V_H) is responsible for the diversity to bind to an abundance of antigens (antigen binding site) and is consisting of a variable (V), a joining (J) and a diversity (D) region. Looking at the germline DNA segments of the Immunglobuline heavy chain (*IGH*) gene coding for the constant and variable chains of the heavy chain segments

before rearrangement has happened, numerous different gene segments for each region can be seen. About 123 to 129 Variable-, 27 Diversity- and 9 different Joining-segments have been described. After recombination of these segments the ultimate and unique VDJ transcript finally dictates the composition of the variable region of the heavy chain.(2,4)

In the beginning of the VDJ recombination a naïve progenitor B-Cell rearranges one D and J segment. Subsequent a V region joins the previously combined Diversity- and Joining-segment. In the end a productive VDJ transcript leads to the expression of a functioning variable region of the heavy chain (Fig. 2).(4)

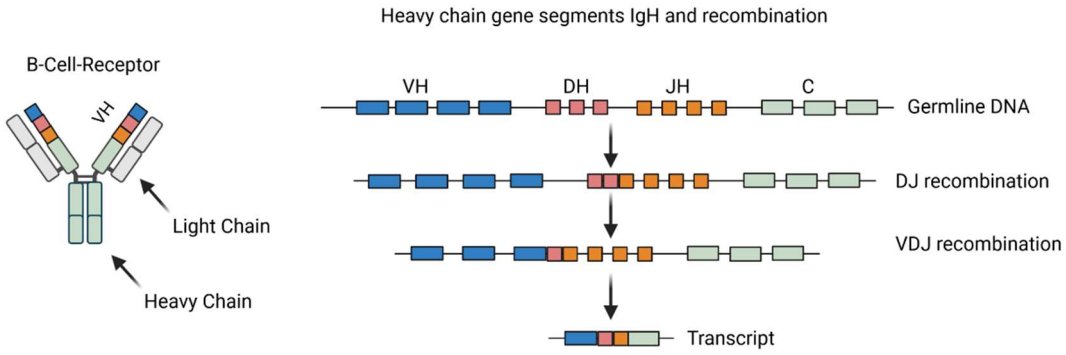

Figure 2 B-Cell-Receptor Rearrangement, Created with BioRender.com

The variable region of the heavy chain (VH) can be subdivided in seven family groups (VH1-VH7). Interestingly studies showed that some lymphoid malignancies like acute lymphoblastic leukemia seem to have a preferential recombination pattern of the B-cell-receptor with specific common family groups. The underlying reason for this is not that well understood up to now.(5)

The process of VDJ recombination happens rather randomly and therefor unproductive gene sequences are quite common. For this reason, only one-third of all recombination variants are thought to be functional and produce a B-Cell-Receptor. Usually both alleles of the *IGH* gene are recombined which can also be seen frequently in biallelic monoclonal lymphomas as well.(2)

The nomenclature of the resulting rearranged sequence includes the number of the found VH-family (e.g. VH7) as well as the selected gene segment (e.g. VH7-3).

Similar to the described recombination of the variable region of the heavy chain, the variable region of the kappa (κ) and lambda (λ) light chain rearranges as well, except that the gene segments of the light chain do not contain a Diversity (D) part.(4)

2.2 T-Cell-Receptor

The T-Cell-Receptor (TCR) is also constructed of a heterodimer of polypeptides. This heterodimer usually consists of an Alpha (α) and a Beta (β) transmembrane polypeptide chain. In a minority of cases the T-Cell-Receptor is composed of a Gamma (γ) and a Delta (δ) chain. These polypeptide chains contain variable regions similar to the B-Cell-Receptor which are rearranged in the maturation of the T-Cell as well.(4)

While the TCR-β-chain (*TCRB*) and the TCR-δ-chain (*TCRD*) are composed of a Variable-, a Diversity- and a Joining-region, the TCR-α-chain (*TCRA*) and the TCR-γ-chain (*TCRG*) only have V- and J-segments which are recombined as well.(4)

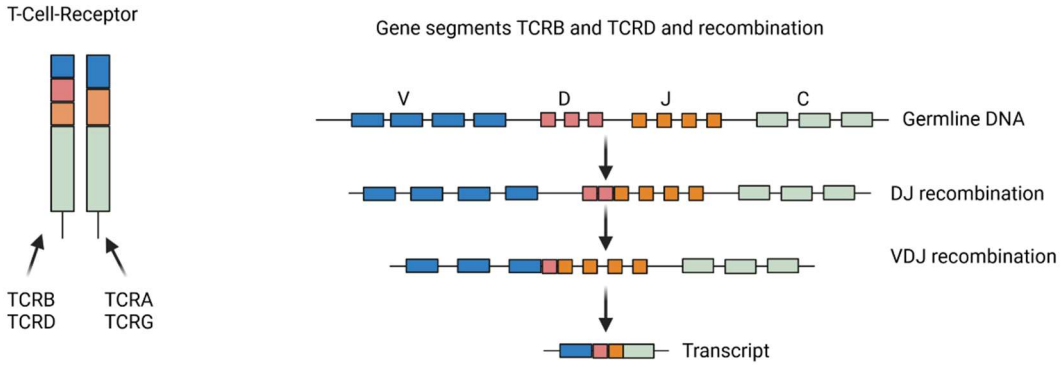

Figure 3 T-Cell-Receptor Rearrangement, Created with BioRender.com

2.3 General Concepts of Clonality Testing

Initially Southern Blot technology was used to analyse the diversity of V(D)J recombination of the T- and B-Cell-Receptor. Slowly capillary electrophoresis replaced southern blotting as a gold standard for clonality testing. Recently Next generation sequencing seems to be a promising method for the detection of monoclonal T- or B-cells.(2,6)

The BIOMED-2/ EuroClonality multiplex PCR protocols containing the primer sets which showed to achieve the most sensitive results, are commonly used for clonality testing. Most Next-Generation-Sequencing Assay designs were developed on the basis of these primer sets as well.(2,6)

2.4 Polymerase Chain Reaction and Capillary Electrophoresis

The underlying concept of the usage of PCR amplification and fragment analysis via capillary electrophoresis is that the rearranged V(D)J segments of TCR and BCR are not only unique regarding their sequences but also concerning the fragment length of these sequences. As previously mentioned, a benign lymphocyte population is usually quite diverse which can also be seen in their abundance of different BCR and TCR receptors (Fig. 1). A PCR amplification and subsequent fragment analysis of such a lymphocyte population would show a polyclonal pattern with a variety of different sized fragments, usually displaying a Gaussian distribution (Fig. 7, 8, 11).

Since the beginning of BCR and TCR clonality testing one of the main issues to overcome was the development of adequate primer sets which would be appropriate to recognize all or the majority of possible rearrangements. To do so, the European BIOMED-2 network, which was renamed to EuroClonality consortium, conducted several studies to guarantee an assay with a high detection rate. Early PCR protocols and subsequent fragment analysis showed the problem of frequently false negative tests due to a rearrangement in the lymphoma population which was not included in the primer set or false positive results due to the non-uniform interpretation.(6)

Looking closer at the VDJ rearranged gene sequence of the Immunglobuline heavy chain, different regions can be seen. The variable region contains three framework regions (FR1, FR2, FR3) which are preceded by a leader region. The consensus of

the European BIOMED-2 network concluded that a cocktail of primer sets distributed in three tubes (Tube A, Tube B, Tube C) targeting different variants of the FR1, FR2 and FR3 regions combined with a JH reverse primer set covers the majority of possible rearrangements and achieves the most sensitive results for *IGH* monoclonality testing.(7) (Fig. 4)

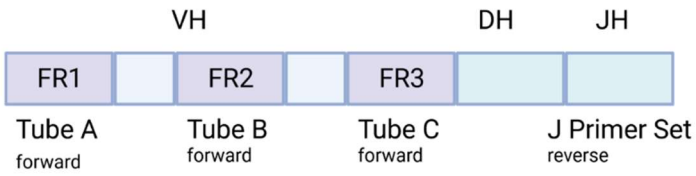

Figure 4 IGH Primer Set, Created with BioRender.com

After VDJ recombination, somatic hypermutation is the next physiological step to increase the diversity of the B-Cell-Receptor to adapt to different kinds of antigens. This process increases the variants of sequences of the *IGH* locus considerably and is one of the reasons *IGH* monoclonality testing fails. To increase the sensitivity of the detection of monoclonality of post germinal center, and therefore hypermutated, lymphomas, testing of the light chain genes was introduced. Especially monoclonality testing of the *IGκ* gene showed to improve the detection rate significantly and additional consortium approved primer sets were developed as well.(8) (Fig.5)

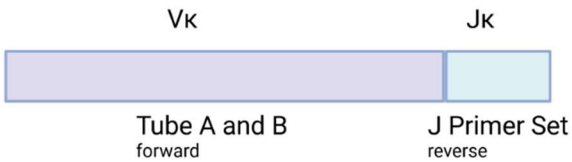

Figure 5 IGK Primer Set, Created with BioRender.com

For the planning of primer sets for TCR clonality testing the whole rearrangement process for all possible subunits of the T-cell-receptor (TCR-alpha, TCR-beta, TCR-gamma and TCR-delta) was taken into account. The recombination of the different genes physiologically happens in a hierarchical order – at first *TCRD* rearranges, followed by *TCRG*. If both rearranged end results are productive a functional TCRγδ receptor is expressed. Subsequently the *TCRB* locus rearranges and lastly *TCRA* rearrangement occurs. If both transcripts are productive a TCRαβ receptor is expressed. Interestingly almost all T-Lymphocytes with a TCRγδ receptor expressed on the cell surface also show a rearranged *TCRB* gene and nearly all TCRαβ expressing T-lymphocytes show a rearranged *TCRG* gene as well. Taking this into account standardized primer sets for *TCRG* and *TCRB* gene loci were developed to simplify the needed primer range and to maximize the sensitivity for different T-cell-lymphoma entities, regardless which receptors are actually expressed on the surface. (Fig. 6) Different studies confirmed that this approach is indeed feasible and the combination of *TCRB* and *TCRG* gene testing achieves higher sensitivity.(9)

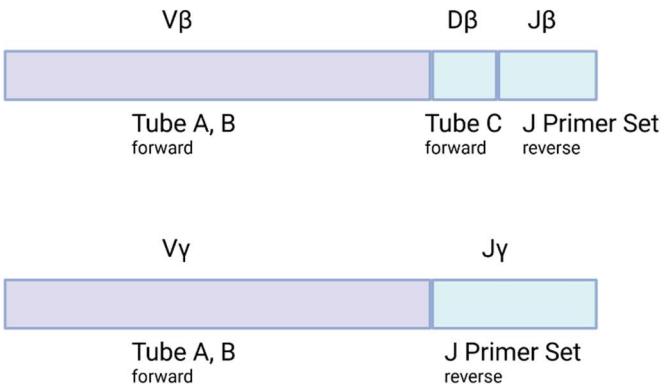

Figure 6 TCR Gamma and Beta, Created with Biorender.com

Regarding the method of clonality testing via fragment analysis itself, the first step is to amplify the DNA, which was obtained from different materials, with the previously described primer assays. If the DNA stems from formalin fixed material, the EuroClonality consortium recommends for the fragment analysis to run a duplicate assessment with different DNA amounts. Usually, if there is enough DNA concentration to begin with, there should be one assessment with 50 ng and one

with 200 ng. This should be done to recognize possible PCR inhibitors on the one hand and one the other hand to rule out pseudoclonality, which can often be misleading. Pseudoclonality usually occurs if the DNA input is too low or if the potentially malignant lymphocyte infiltrate is sparse. In this case a specific sequence can amplify preferentially and cause a seemingly dominant peak in the fragment analysis. Using different DNA concentrations can help to identify such a pseudoclonal peak.(10)

Interpretation of the gained fragment analysis results can be a challenge in itself. The EuroClonality consortium published several recommendations for the interpretation, defining fragment ranges for each primer set, where peaks should be considered valid and trying to standardize the definition of monoclonal and polyclonal as much as possible.(10) Looking at figures 7 to 13 the results of clonality analysis using GeneMapper can be seen. In the first row the monoclonal control, which is provided with the assay, shows a representative peak. In the second row a polyclonal control pattern can be seen. The third and fourth row represent the different DNA concentrations, 50 and 200 ng respectively. Looking at figure 7, 8 and 11 a different fragment pattern for polyclonality can be seen. This stems from the different primer sets which are used – in this case *IGH* Tube C, *IGK* Tube B and *TCRG* Tube A. The last row represents a negative control (H_2O) where no peaks should be seen to rule out contamination. Figures 9 and 12 show monoclonal distribution of DNA-fragments, defined as a dominant peak which is reproducible. Older interpretation guidelines tried to specify which height the peak should have to be called monoclonal and this resulted in instructions like “three fold the height of the third tallest fragment-peak in the polyclonal background”. The EuroClonality consortium recommends that such instructions should be used cautiously because following it strictly could cause false negative and positive results. The interpretation of the fragment analysis results should be done taking the histology and other clinical information into account if possible. In cases where the peaks seem to be not as straight forward as represented in figures 7, 8, 11, 9 and 12, the interpretation sometimes remains a little subjective.(6,10)

Another interesting aspect of interpretation of fragment analysis for clonality is the distinction of biclonality and biallelic monoclonality as shown in figures 10 and 13. Usually both alleles of the *IGH* and *TCR* gene undergo rearrangement as described

above. Finding two dominant peaks in the fragment analysis therefor usually represents one monoclonal lymphocyte population with biallelic monoclonality. A biclonality, defined as two distinct monoclonal lymphocyte populations would be rare and difficult to distinguish with fragment analysis alone.(6)

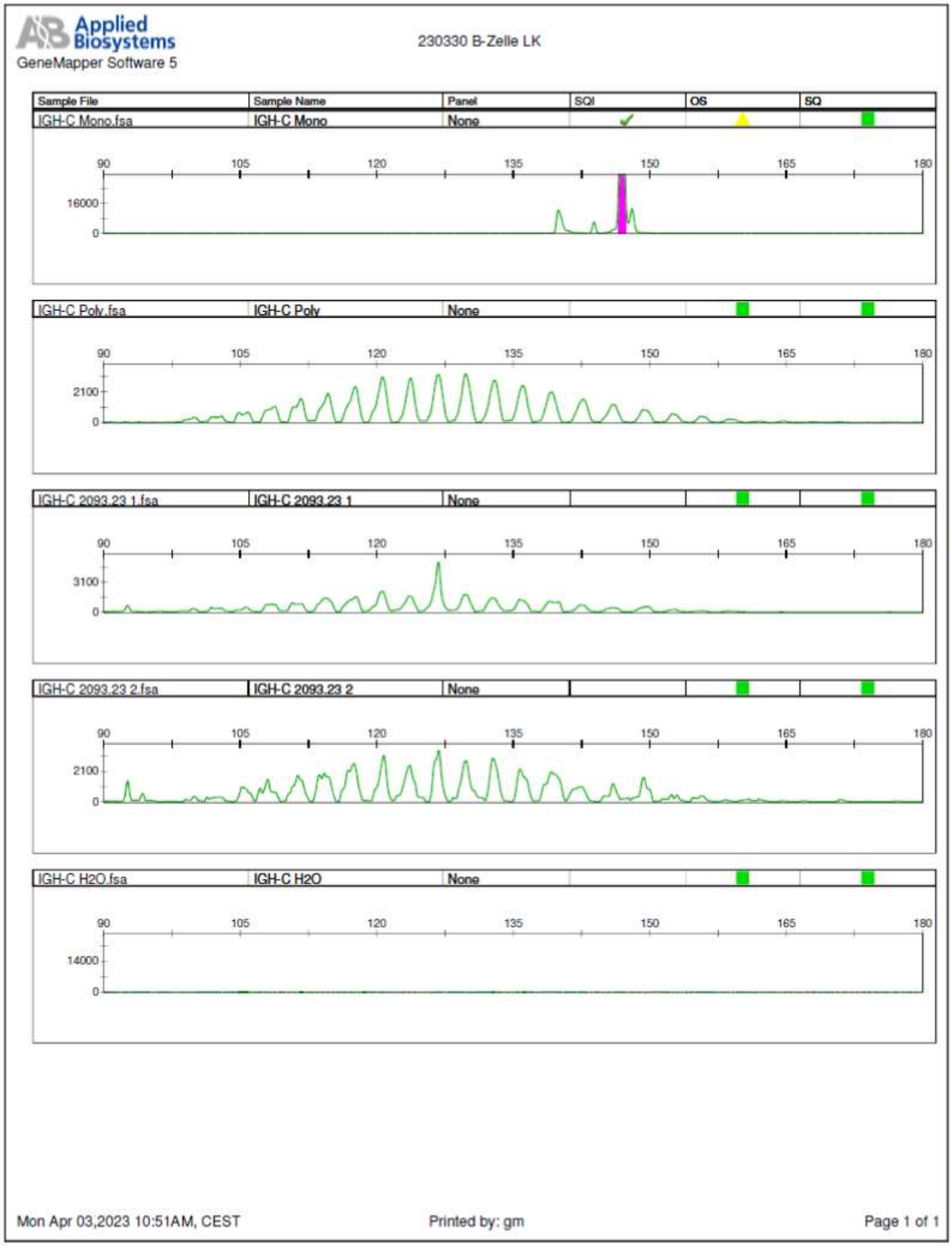

Figure 7 IGH polyclonal example, created at the Klinik Favoriten

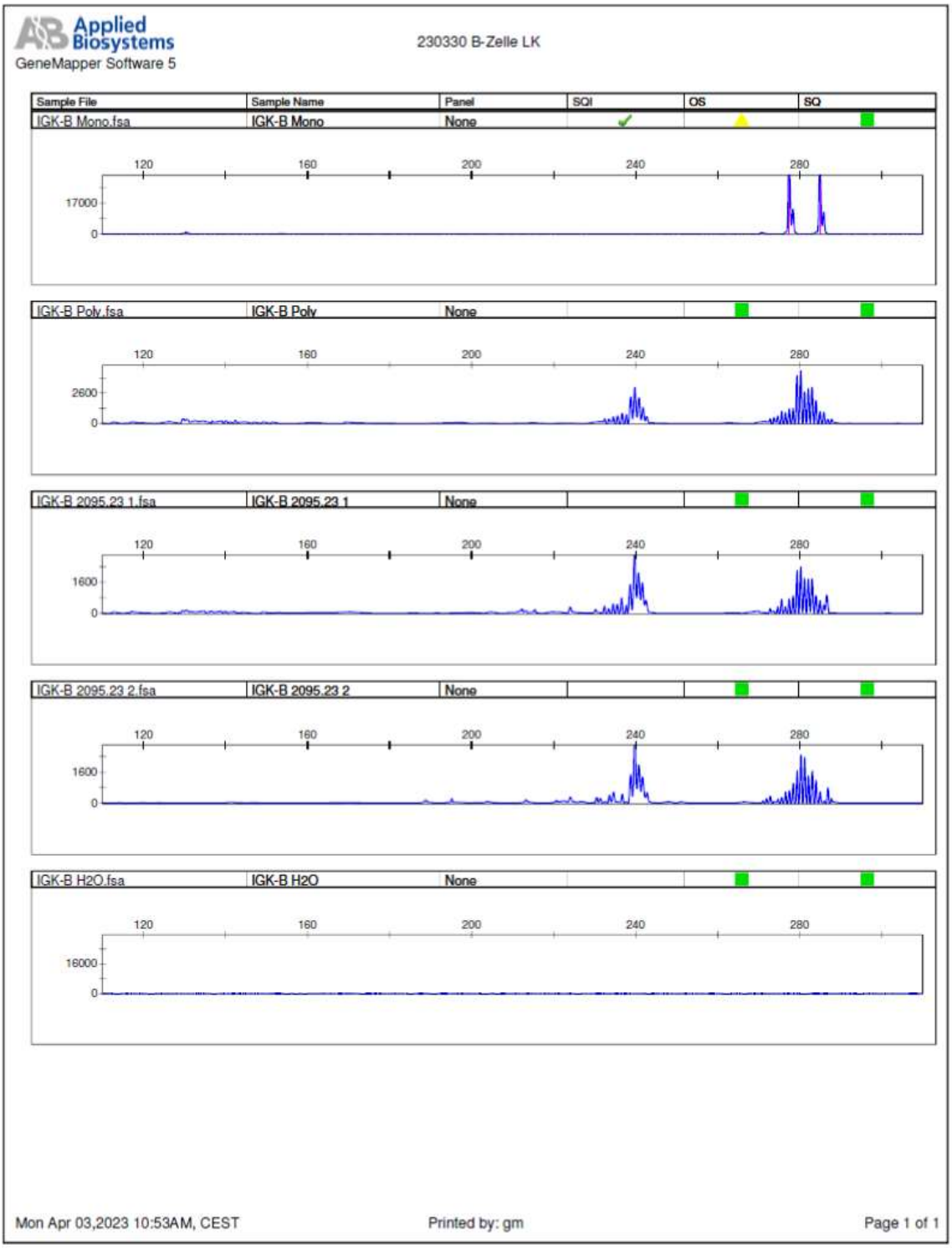

Figure 8 IGK polyclonal example, created at the Klinik Favoriten

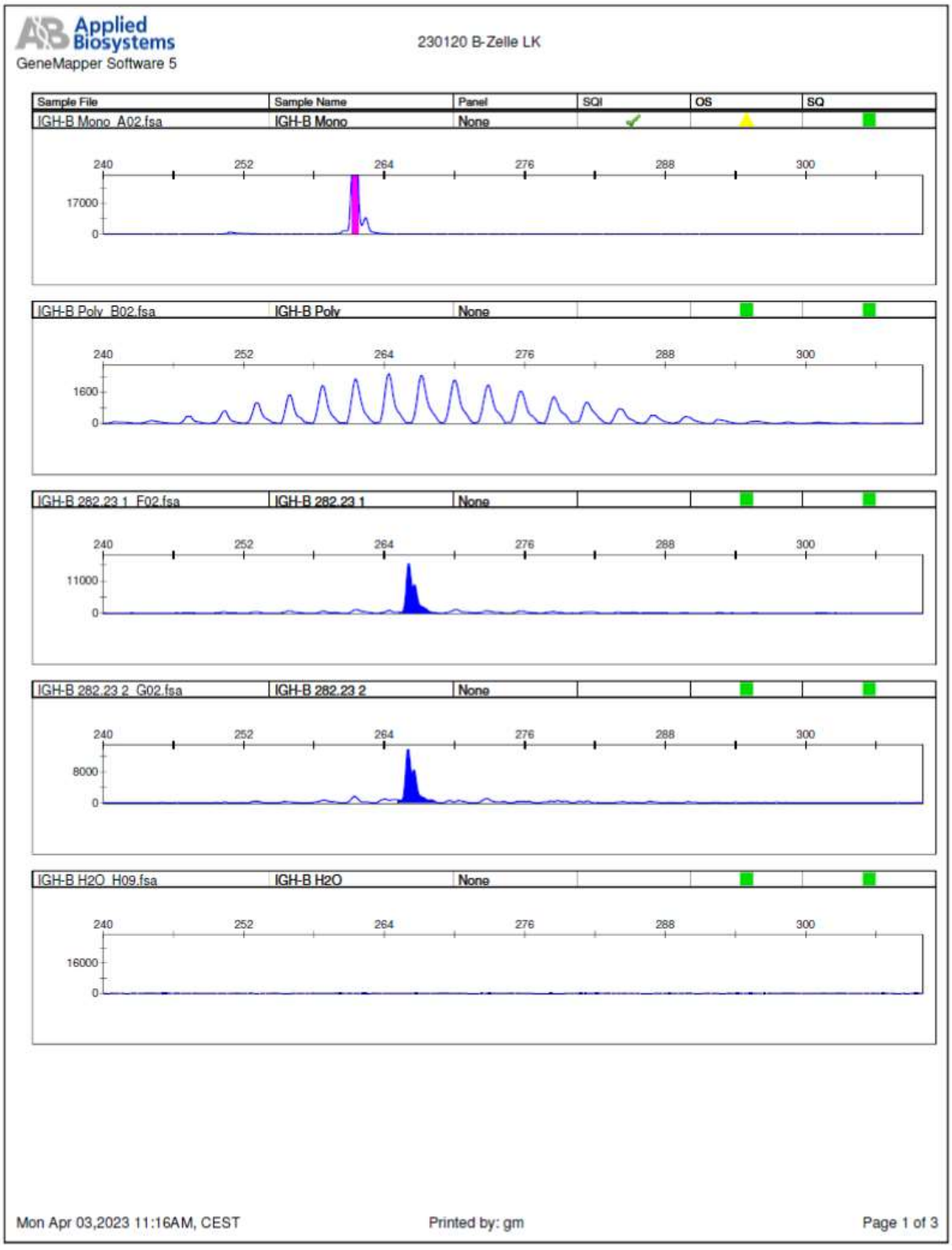

Figure 9 IGH monoclonal example, created at the Klinik Favoriten

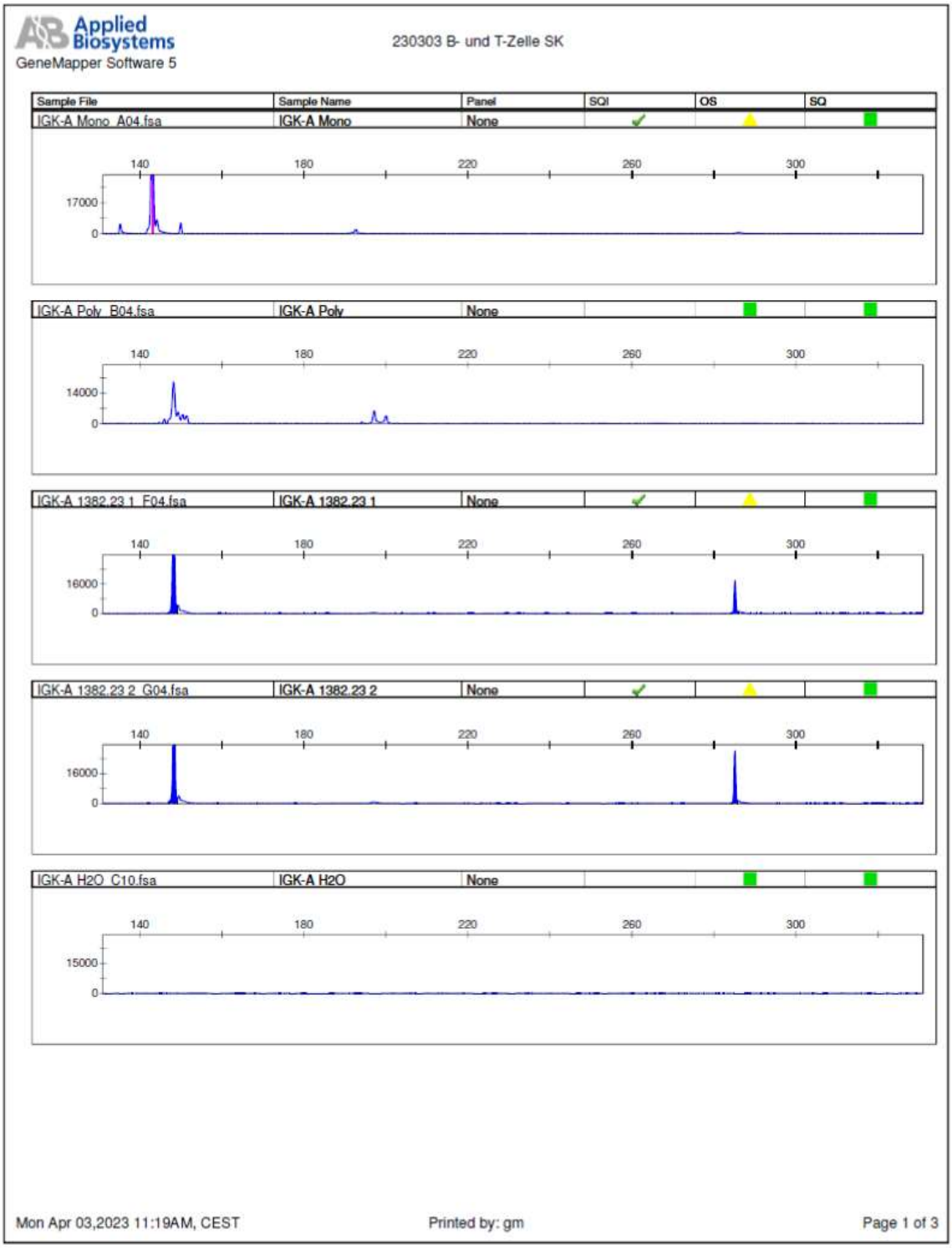

Figure 10 IGK biallelic monoclonal example, created at the Klinik Favoriten

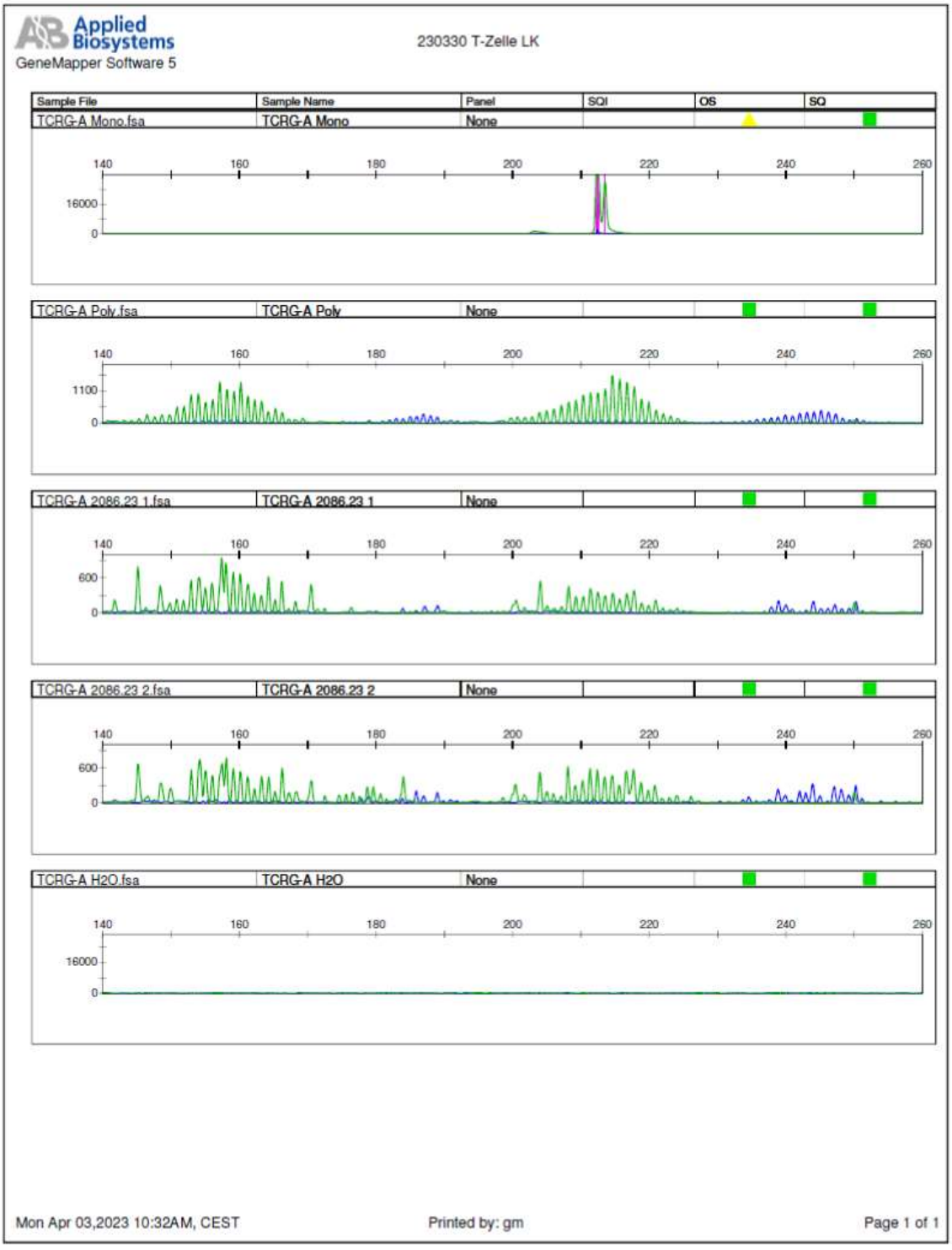

Figure 11 TCRG polyclonal example, created at the Klinik Favoriten

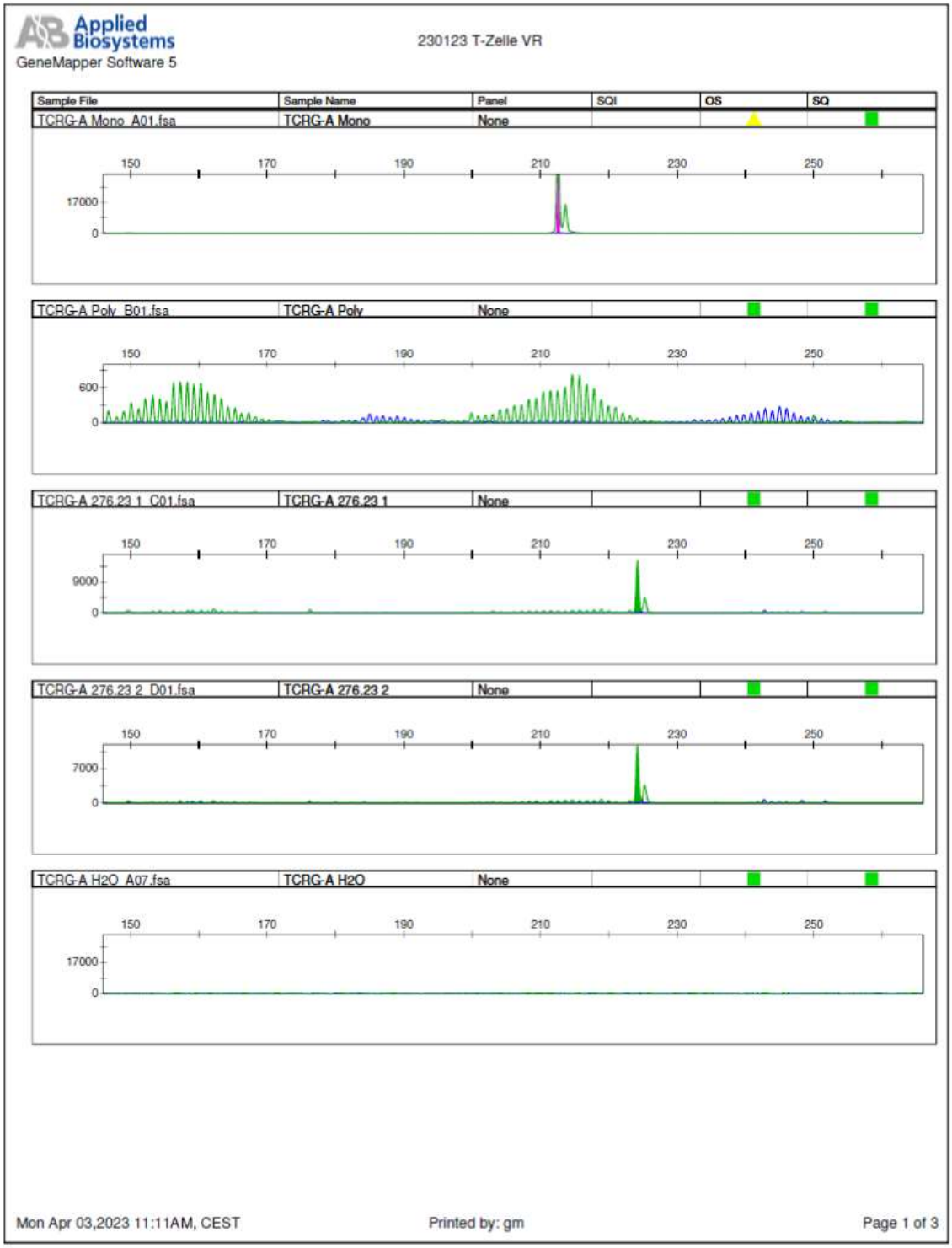

Figure 12 TCRG monoclonal example, created at the Klinik Favoriten

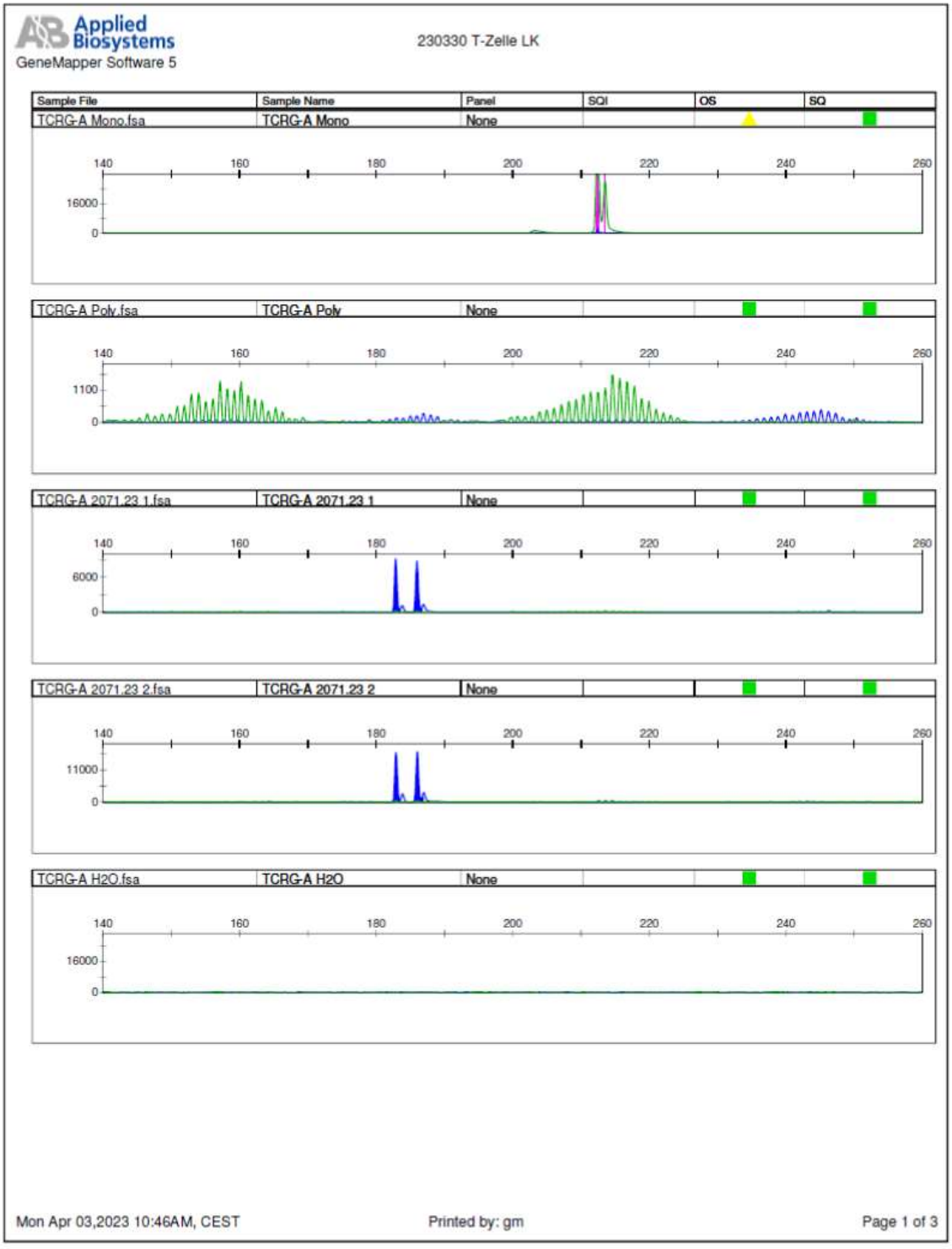

Figure 13 TCRG biallelic monoclonal example, created at the Klinik Favoriten

2.5 Next-Generation-Sequencing

The implementation of Next-Generation-Sequencing (NGS) in many different routine diagnostics also made an impact on lymphocyte clonality testing with many studies in the last decade regarding feasibility and usage of different assays as well as bioinformatics.(11)

In general similar primer assay sets are used as described above. Depending on the NGS platform which is used (usually Illumina or IonTorrent) different primer sets are available at the moment with some vendors not providing *TCRG* assays for IonTorrent for example. After the initial library preparation and sequencing, usually a vendor provided, or laboratory developed Bioinformatic software is used for interpreting the data. Interpretation for NGS clonality testing is less standardized than the gold standard of fragment analysis. Commonly the most dominant sequence, identified by quantity of read counts in regard to the background reads, quite similar to the interpretation of fragment analysis, is evaluated.(2)

In the last couple of years commercially available diagnostic kits appeared which led many laboratories to questioning the advantages of implementing the technique and if switching from fragment length based to sequence based analysis of clonality should be done. This thesis aims to review the literature of the last 23 years regarding these questions.

3. Aim of the study

3.1 Objectives

This review aims to assess the literature of the last 23 years regarding fragment analysis based and NGS-based methods for identifying B-Cell-Receptor and T-Cell-Receptor clonality for evaluation of malignancy or for evaluation of minimal residual disease (MRD) in the setting of routine diagnostic procedures.

The goal of this literature review is to determine the usability and advantages as well as disadvantages, if there are any, of Next-Generation-Sequencing compared to the gold standard method of fragment analysis. This review should help routine diagnostic laboratories to decide whether the relatively new application of Next-Generation-Sequencing for clonality testing is feasible and justifies the higher costs in contrast to fragment analysis.

The review tries to answer the following questions:

- Which differences regarding sensitivity and specificity of the two methods can be found in the literature?
- Which differences can be found concerning:
    - application
    - used patient material (type of material, DNA or RNA, amount, etc.)
- Which advantages and disadvantages of both methods can be found in the literature?

4. Methods

4.1 Defining the scope of the literature review

Further defining the research question and the scope of this literature review a PICOC (**P**opulation, **I**ntervention, **C**omparison, **O**utcomes and **C**ontext) framework was used.(12) This framework was adapted to fit this specific topic at hand.

Table 1 PICOC Schema of the aim of the literature review

Population	Acute lymphoblastic leukemia or Mycosis Fungoides or Multiple Myeloma or other hematological malignancy
Intervention or Exposure	Clonality Testing via Next Generation Sequencing
Comparison	Clonality Testing via PCR based methods/ Fragment Analysis
Outcome	Sensitivity Specificity Evaluation of minimal residual disease Evaluation of other advantages/ disadvantages of the methods
Context	T- and B-Cell Receptor clonality for determination of malignancies

4.2 Search terms

Prior to the actual literature search in databases like Pubmed and OVID, search terms and associated MESH (Medical Subject Headings) terms were defined.

To cover all the topics of this literature review, predefined in the PICOC schema, the following search terms were used:

Table 2 Search and MESH terms of the review

Search term	**MESH terms**(13)
Next Generation Sequencing	High Throughput Nucleotide Sequencing Nucleotide Sequencing, High-Throughput Sequencing, High-Throughput Nucleotide Next-Generation Sequencing Next Generation Sequencing Sequencing, Next-Generation Illumina Sequencing Sequencing, Illumina Ion Torrent Sequencing Sequencing, Ion Torrent Ion Proton Sequencing Sequencing, Ion Proton Deep Sequencing Sequencing, Deep High-Throughput RNA Sequencing High Throughput RNA Sequencing RNA Sequencing, High-Throughput Sequencing, High-Throughput RNA Massively-Parallel Sequencing Massively Parallel Sequencing Sequencing, Massively-Parallel

	Pyrosequencing High-Throughput Sequencing High Throughput Sequencing Sequencing, High-Throughput High-Throughput DNA Sequencing DNA Sequencing, High-Throughput High Throughput DNA Sequencing Sequencing, High-Throughput DNA
Clonality	Clonality Clonal Clonalities Clonally
Acute lymphocytic leukemia	Precursor Cell Lymphoblastic Leukemia Lymphoma Leukemia, Acute Lymphoblastic Acute Lymphoblastic Leukemia Leukemia, Lymphoblastic Leukemia, Lymphoblastic, Acute Leukemia, Lymphocytic, Acute Lymphoblastic Leukemia Lymphoblastic Leukemia, Acute Lymphoblastic Lymphoma Lymphocytic Leukemia, Acute Acute Lymphocytic Leukemia Leukemia, Acute Lymphocytic Lymphoma, Lymphoblastic Acute Lymphoid Leukemia Leukemia, Acute Lymphoid Lymphoid Leukemia, Acute Leukemia, Lymphoid, Acute Leukemia, Lymphocytic, Acute ALL, Childhood Childhood ALL

Multiple myeloma	Multiple Myelomas Myelomas, Multiple Myeloma, Multiple Myeloma, Plasma-Cell Myeloma, Plasma Cell Myelomas, Plasma-Cell Plasma-Cell Myeloma Plasma-Cell Myelomas Myelomatosis Myelomatoses Plasma Cell Myeloma Cell Myeloma, Plasma Cell Myelomas, Plasma Myelomas, Plasma Cell Plasma Cell Myelomas Kahler Disease Disease, Kahler Myeloma-Multiple Myeloma Multiple Myeloma-Multiples
Amplified Fragment Length Polymorphism Analysis	AFLP Analysis AFLP Analyses Analyses, AFLP Analysis, AFLP
Mycosis fungoides	Mycosis fungoides
Haematological neoplasms	Hematologic Neoplasm Neoplasm, Hematologic Hematologic Malignancies Hematologic Malignancy Neoplasms, Hematologic Hematological Neoplasms Hematological Neoplasm Neoplasm, Hematological

	Malignancies, Hematologic Malignancy, Hematologic Blood Cancer Blood Cancers Cancer, Blood Hematological Malignancies Hematological Malignancy Malignancy, Hematological Hematopoietic Neoplasms Hematopoietic Neoplasm Neoplasm, Hematopoietic Neoplasms, Hematopoietic Hematopoietic Malignancies Hematopoietic Malignancy Malignancy, Hematopoietic
B-Cell-Receptor	Membrane Bound Immunoglobulin Bound Immunoglobulin, Membrane Immunoglobulin, Membrane Bound Membrane-Bound Immunoglobulins Membrane Bound Immunoglobulins Surface Immunoglobulins Surface Immunoglobulin Immunoglobulin, Surface Receptors, Antigen, B Cell Antigen Receptors, B-Cell Antigen Receptors, B Cell Receptors, B-Cell Antigen B-Cell Antigen Receptors B Cell Antigen Receptors B-Cell Antigen Receptor Antigen Receptor, B-Cell B Cell Antigen Receptor Receptor, B-Cell Antigen

	Immunoglobulins, Membrane-Bound Immunoglobulins, Membrane Bound Immunoglobulins, Surface
T-Cell-Receptor	T-Cell Antigen Receptor Antigen Receptor, T-Cell Receptor, T-Cell Antigen T Cell Antigen Receptor T-Cell Receptors Receptors, T-Cell T Cell Receptors T-Cell Receptor Receptor, T-Cell T Cell Receptor Receptors, T-Cell Antigen Receptors, T Cell Antigen T-Cell Antigen Receptors Antigen Receptors, T-Cell Antigen Receptors, T Cell
PCR	Polymerase Chain Reactions Reaction, Polymerase Chain Reactions, Polymerase Chain PCR Inverse PCR PCR, Inverse Inverse Polymerase Chain Reaction Nested Polymerase Chain Reaction Nested PCR PCR, Nested Anchored PCR PCR, Anchored Anchored Polymerase Chain Reaction
Cutaneous T Cell Lymphoma	Lymphoma, T Cell, Cutaneous T-Cell Lymphoma, Cutaneous

	T Cell Lymphoma, Cutaneous Cutaneous T-Cell Lymphoma Cutaneous T Cell Lymphoma Cutaneous T-Cell Lymphomas Lymphoma, Cutaneous T-Cell Lymphomas, Cutaneous T-Cell T-Cell Lymphomas, Cutaneous Granulomatous Slack Skin Slack Skin, Granulomatous

4.3 Databases and sources

For the conduction of the search PubMed® as well as OVID® online database was used. In OVID the following resources were used – Books, Journals, EBM Reviews - ACP Journal Club 1991 to January 2023, EBM Reviews - Cochrane Central Register of Controlled Trials January 2023, Reviews - Cochrane Database of Systematic Reviews 2005 to February 22, 2023, EBM Reviews - Cochrane Clinical Answers February 2023, EBM Reviews - Cochrane Methodology Register 3rd Quarter 2012, EBM Reviews - Database of Abstracts of Reviews of Effects 1st Quarter 2016, EBM Reviews - Health Technology Assessment 4th Quarter 2016, EBM Reviews - NHS Economic Evaluation Database 1st Quarter 2016, Embase 1974 to 2023 February 24, Ovid MEDLINE(R) and Epub Ahead of Print, In-Process, In-Data-Review & Other Non-Indexed Citations, Daily and Versions 1946 to February 24, 2023.

4.4 Search strategy

The search was divided in three main parts. In an **initial scoping search** of the literature search terms were defined after reading several studies relevant to the topic of this review.

Using the previously listed search terms and associated MESH terms ten **main searches** were done each on OVID® and Pubmed® respectively.

Table 3 Search description

Search	Search term combinations (MESH terms are not listed)
#1	(clonality) AND (hematological neoplasms)) AND (high-throughput sequencing)) AND (pcr)
#2	(next Generation Sequencing) AND (fragment analysis)) AND (hematological neoplasms)
#3	(next generation sequencing) AND (fragment analysis)) AND (clonality)
#4	(next Generation Sequencing) AND (bcr)) AND (clonality)
#5	(next-generation sequencing) AND (b-cell-receptor)) AND (clonality)
#6	(next generation sequencing) AND (t-cell-receptor)) AND (clonality)
#7	(next generation sequencing)) AND (acute lymphocytic leukemia)) AND (clonality)
#8	(next generation sequencing) AND (mycosis fungoides)) AND (clonality)
#9	(next generation sequencing) AND (cutaneous t cell lymphoma)) AND (clonality)
#10	(next generation sequencing) AND (multiple myleoma)) AND (clonality)

An abundance of topic relevant and not relevant literature was found using the ten described search term combinations. The bibliography was then narrowed down by reading the title and abstract using predefined inclusion criteria.

The most important inclusion criterion was relevance to the topic of this review defined by the PICOC schema. Studies including a comparison of NGS and PCR based methods were preferred but studies individually examining just one of these methods were also included. Regarding the publishing date of the literature, studies older than 2000 were not included which means the relevant literature of a span of 23 years was examined. The included study designs were experimental studies and proof of concept studies. Poster presentations were also accepted.

After narrowing down the bibliography each of the studies was read and once more evaluated regarding the inclusion criteria.

The third stage of the search strategy included a **bibliography search** of the included studies to identify literature relevant to the topic of this review which was previously not found using the search term combinations.

4.5 Data synthesis and Quality assessment

For Data collection and synthesis an Excel based form was created. This form divided the criteria and data which should be evaluated in different groups.

In regard to quality characterization of the included literature different criteria were used for quantitative studies, which represent a major part of the included literature, and qualitative studies.

Quality criteria for quantitative studies were as follows:

- *Reference:* was there a comparison of the tested method with a gold standard reference method (e.g., histology, flowcytometry, PCR-CE)
- *Method:* were the used methods described intelligible for the reader (e.g., used platform, panel, bioinformatics regarding Next-Generation-Sequencing or PCR-CE)
- *Power:* have there been 10 or more samples in the described testing of the method

Quality criteria for qualitative studies were as follows:

- *Transparency*: is the used method to get to these insights documented and comprehensible
- *Inter-subjectivity*: is the obtained subjective data discussed and reflected on
- *Documentation of the literature review:* is there a clear documentation of the method of the literature search

Concerning the methods of the reviewed literature, data regarding the sample quantity and which sample type was used, and details of the used NGS- and/ or PCR-CE was extracted. This included the following main characteristics: platform (e.g., Illumina, IonTorrent, (NGS) or Genetic Analyzer 3500, ABI3730) (PCR-CE)), panel or primer-sets, bioinformatics and used criteria to do the evaluation of the gained data.

Finally, the following additional data was extracted from the included studies if applicable: sensitivity of NGS or PCR-CE method, advantages and disadvantaged of the used method, quantity of used DNA, time consumption of the method.

For simplification of the data evaluation the literature was divided in different discussion topic groups (MRD, FFPE, description of the available methods, Pro/ Con NGS, Pro/ Con PCR-CE, TCR, BCR, ALL, multiple myeloma). It is necessary to say that one study could be included in several of these topic groups.

5. Results

Using the search, quality and data analysis criteria mentioned above three main data groups could be identified:

- **Data Group A:** B- and T-Cell-Receptor Clonality Testing via Next-Generation-Sequencing.
- **Data Group B:** B- and T-Cell-Receptor Clonality Testing via PCR-CE.
- **Data Group C:** Minimal-Residual-Disease Testing via Next-Generation-Sequencing.

5.1 Data Group A

This Data Group includes literature that evaluated B- and T-Cell-Receptor Clonality Testing via Next-Generation-Sequencing. Five studies included data concerning both T-Cell-Receptor and B-Cell Receptor testing. Three studies only evaluated the use of Next-Generation-Sequencing for B-Cell-Receptor Testing. In contrast more literature could be found regarding T-Cell-Receptor testing in the context of mycosis fungoides. Seven studies fulfilling all inclusion criteria could be identified for this topic. Altogether 15 studies evaluating the use of Next-Generation-Sequencing for clonality testing were included in this study.

The included studies were published between 2014 and 2022, with the main peaks of relevant literature in 2015, 2019 and 2021 (Figure 1).

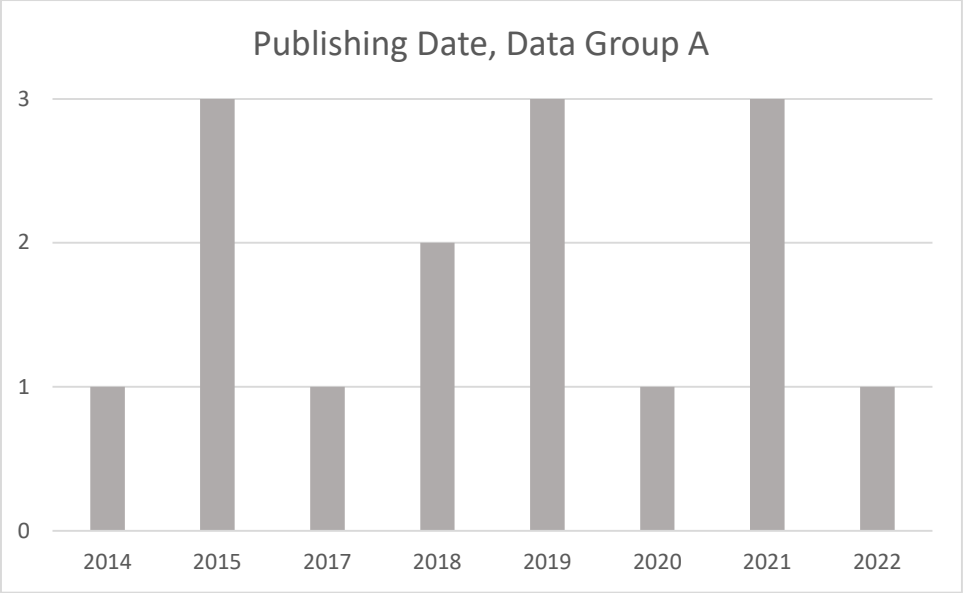

Figure 14 Publishing Date, Data Group A

The 15 included studies were published in ten different journals. Three of them were published in the Journal of Molecular Diagnostics (Figure 2).

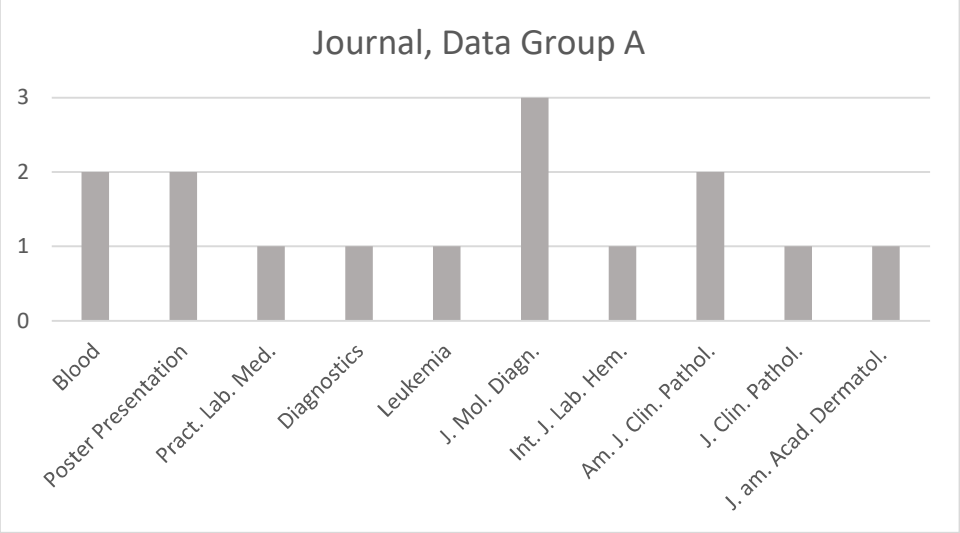

Figure 15 Journal Type, Data Group A

Data-Group A: B- and T-Cell-Receptor Clonality Testing, Next-Generation-Sequencing

Study	Sample Size/ Type	NGS Panel	Bioinformatics	DNA Input	Sensitivity / Concordance to Reference Method
B- and T-Cell-Receptor Testing					
Next Generation Sequencing (NGS) Based IGH and TCR Clonality Assays Provide Excellent Specificity and Sensitivity for Routine Clonal Characterization and Monitoring of Lymphoproliferative Disorders. *Arcila et al. Blood (2017)*(14)	534 samples (Blood, Bone Marrow and FFPE tissue samples) CLL, plasma cell neoplasms, B-ALL, low-grade B cell lymphoma, T-cell lymphoma NOS, DLBCL, FL, HCL	LymphoTrack® IGH + TRG - assays (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®) and In-House validated Software (LymphoClone)	N/A	Sensitivity NGS: 94% (502/ 534) Sensitivity PCR-CE: 89% (475/534) Reference – lymphoma classification incorporating histology, flow-cytometry und molecular test results
Validation and clinical implementation of next generation sequencing for routine IGH and TCRG clonality assessment. *Arcila et al. AMP Meeting (2015)*(15)	160 samples (Blood, Bone Marrow and FFPE tissue samples) 92 B cell neoplasms, 34 T cell neoplasms, 34 Non-clonal samples	LymphoTrack® IGH + TRG - assays (Invivoscribe®) Illumina MiSeq Platform	Invivoccribe® software (LymphoTrack®)	Minimum input of 50 ng	94% concordance to PCR-CE Reference – PCR-CE
Validation and interpretation of IGH and TCR clonality testing by Ion Torrent S5 NGS for diagnosis and disease monitoring in B and T cell cancers. *Lay et al. Practical Laboratory Medicine (2020)*(16)	195 samples (Bone Marrow, Fresh lymph node, FFPE, Fluid, Blood) T- and B-cell neoplasms	LymphoTrack® IGH (FR1, FR2, FR3) + TRG - assays (Invivoscribe®) IonTorrent Platform	Invivoscribe® software (LymphoTrack®)	Minimum input of 50 ng	IGH concordance PCR-CE and NGS in 62,7% TCR concordance PCR-CE and NGS in 66,7% Reference – PCR-CE
LymphoTrack Is Equally Sensitive as PCR GeneScan and Sanger Sequencing for Detection of Clonal Rearrangements in ALL Patients. *Paulsen et al. Diagnostics (2022)*(17)	78 ALL samples B-ALL, T-ALL	LymphoTrack® IGH (FR1), IGK + TRG, TRB - assays (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	Minimum input of 50 ng, average input of 150 ng	96-100% detection rate Reference – PCR-CE
Validation of Next Generation Sequencing Based Clonality Assays for Diagnosis and Minimal Residual Disease Tracking in Lymphoid Malignancies. *Wu et al. Blood (2021)*(18)	40 samples (patient samples not further described, cell-lines) ALL, MM, CLL, lymphoma, BP-CML	Customized BCR/TCR clonality assay (IGH, IGK, IGL, TCRB, TCRG) NovaSeq 6000 System	Customized bioinformatic pipeline	N/A	Above 90% positive detection rate Reference – not specified

B-Cell-Receptor Testing Only					
Next-generation sequencing of immunoglobulin gene rearrangements for clonality assessment: a technical feasibility study by EuroClonality-NGS. *Scheijen et al. Leukemia (2019)*(19)	14 FFPE samples and human peripheral blood as well as tonsillar tissue as healthy control samples (amount not specified) B cell lymphoma	EuroClonality-NGS IGH, IGK primers IonTorrent Platform	ARResT/ Interrogate	Minimum input of 40 ng	71-100% detection rate Reference - PCR-CE
Establishment of Immunoglobulin Heavy (IGH) Chain Clonality Testing by Next-Generation Sequencing for Routine Characterization of B-Cell and Plasma Cell Neoplasms. *Arcila et al. J Mol Diagn (2019)*(11)	1189 samples (blood, bone marrow, FFPE, fresh tissue samples) B-cell neoplasms	LymphoTrack® IGH (FR1, FR2, FR3, Leader), IGK assay (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®) and In-House validated Software (LymphoClone)	Minimum input of 25 ng	initial assessment clonality detection rate >97% Reference – lymphoma classification incorporating histology, flow-cytometry und molecular test results
Next-Generation Sequencing Based Clonality Assessment of Ig Gene Rearrangements. A Multicenter Validation Study by EuroClonality-NGS. *Van den Brand et al. J Mol Diagn (2021)*(20)	209 samples (FFPE, frozen tissue, blood) B-cell neoplasms, reactive lymphoproliferation, inconclusive cases	EuroClonality-NGS IGH, IGK primers IonTorrent Platform	ARResT/ Interrogate	Minimum input of 40 ng	Concordance PCR-CE and NGS in 98% Sensitivity NGS: 96% Sensitivity PCR-CE: 95% Reference – histology consens review of cases
T-Cell-Receptor Testing Only					
Validation of a Next-Generation Sequencing–Based T-Cell Receptor Gamma Gene Rearrangement Diagnostic Assay. *Ho et al. J Mol Diagn (2021)*(21)	101 samples (FFPE, blood, bone marrow)	LymphoTrack® TRG assay (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	Minimum input 50 ng, Mean input 173,3 ng	Sensitivity 73% / 91% (NGS/ PCR-CE) Specificity 91% / 75% (NGS/ PCR-CE) Accuracy 82% / 83% (NGS/ PCR-CE) Reference – clinicopathologic diagnosis
Next-Generation Sequencing for Detection of Clonal TRG Gene Rearrangements Shows Improved Specificity and Positive Predictive Value Compared to Fragment Analysis Using BIOMED-2 Primers and Capillary Electrophoresis. *Ewalt et al. Invivoscribe Poster Presentation (2015)*(22)	58 samples (FFPE) T-cell lymphoproliferative disorders, reactive tissues, B-cell lymphoma	LymphoTrack® TRG assay (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	N/A	Sensitivity 79-89%/ 86% (NGS/ PCR-CE) Specificity 97-100%/ 83% (NGS/ PCR-CE) Reference – clinicopathologic diagnosis
Evaluation of Next-Generation sequencing based clonality analysis of T-cell receptor gamma gene rearrangements based on a new interpretation algorithm. *Nollet et al. Int J Lab Hem (2019)*(23)	121 samples (blood, bone marrow, fresh lymphnodes, skin biopsies, FFPE) Reactive T-Cell lymphocytosis, T-Cell neoplasms, B-Cell	LymphoTrack® TRG assay (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	Minimum input of 50 ng	78% concordance to PCR-CE* Reliable result for FFPE samples in 94,4% Reference - PCR-CE *only fresh samples were taken into account

	neoplasms, MDS, graft vs. host disease				
The Value of T-Cell Receptor γ (TRG) Clonality Evaluation by Next-Generation Sequencing in Clinical Hematolymphoid Tissues. *Kansal et al. AM J Clin Pathol (2018)*(24)	41 samples (blood, bone marrow FFPE) benign cases, T-cell neoplasms, B-cell neoplasms	LymphoTrack® TRG assay (Invivoscribe®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	Minimum input of 50 ng	NGS for TRG clonality diagnostic in 100%
Role of high-throughput sequencing in the diagnosis of cutaneous T-cell lymphoma. *Rea et al. J Clin Pathol (2018)*(25)	100 samples (skin biopsies, blood) Reactive, atypical lympoid infiltrate reactive, Atypical lympoid infiltrate clonal, Mycosis fungoides	TCRB ImmunoSEQ assay Platform N/A	N/A	N/A	Sensitivity 68%/ 72% (NGS/ PCR-CE) Specificity 100%/ 88% (NGS/ PCR-CE) Accuracy 84%/ 80% (NGS/ PCR-CE) Reference – clinicopathologic diagnosis
T-cell clonality assessment by next-generation sequencing improves detection sensitivity in mycosis fungoides. *Sufficool et al. J am acad Dermatol (2015)*(26)	35 samples (FFPE) Mycosis fungoides	TCRG via consensus primers targeted Vg2 to Vg11 and JP1, JP, J1, JP2, and J2 Illumina MiSeq Platform	FASTX toolkit collapse function to determine the most frequent sequences, Alignment to IMGT Database	100 ng	Sensitivity 85%/ 44% (NGS/ PCR-CE) Reference - histology
A Comparison of Deep Sequencing of TCRG Rearrangements vs Traditional Capillary Electrophoresis for Assessment of Clonality in T-Cell Lymphoproliferative Disorders. *Schumacher et al. AM J Clin Pathol (2014)* (27)	48 samples (peripheral blood, bone marrow, FFPE) T-cell neoplasms and non neoplastic samples	TCRG Panel Ion PGM and MiSeq Platform	Custom software package "TCRDriver"	N/A	Sensitivity NGS 89% Specificity NGS 100% Reference – PCR-CE
Abbreviations: CLL – chronic lymphocytic leukemia; B-ALL – B cell acute lymphocytic leukemia; DLBCL – diffuse large B cell lymphoma; FL – follicular lymphoma; HCL – hairy cell leukemia; N/A – not applicable; PCR-CE – Polymerase chain reaction and capillary electrophoresis; MM – multiple myeloma; BP-CML – blast phase chronic myeloid leukemia; T-ALL T cell acute lymphocytic leukemia; MDS – myelodysplastic syndrome					

5.2 Data Group B

This Data Group includes literature that evaluated B- and T-Cell-Receptor Clonality Testing via Polymerase Chain Reaction and subsequent Capillary Electrophoresis to analyse the profile of fragment length frequencies. Four studies included data concerning both T-Cell-Receptor and B-Cell Receptor testing. Four studies only evaluated the use of this method for B-Cell-Receptor Testing. Three studies tested PCR-CE and T-cell clonality analysis. Altogether 11 studies evaluating the use of Next-Generation-Sequencing for clonality testing were included in this study.

The included studies were published between 2006 and 2021 with a main peak around 2007 (Figure 3).

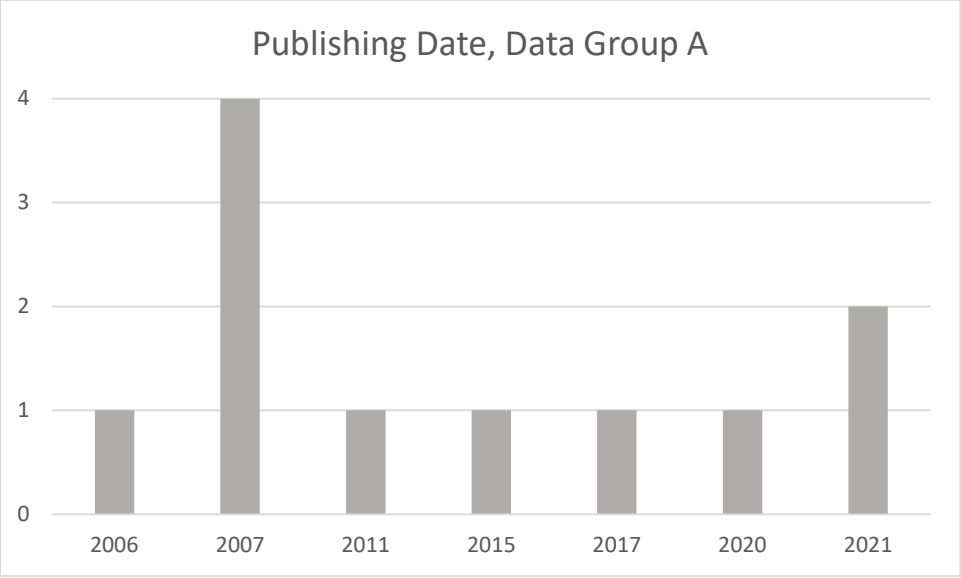

Figure 16 Publishing Date, Data Group A

The 11 included studies were published in eight different journals. Three of them were published in the Journal of Molecular Diagnostics (Figure 4).

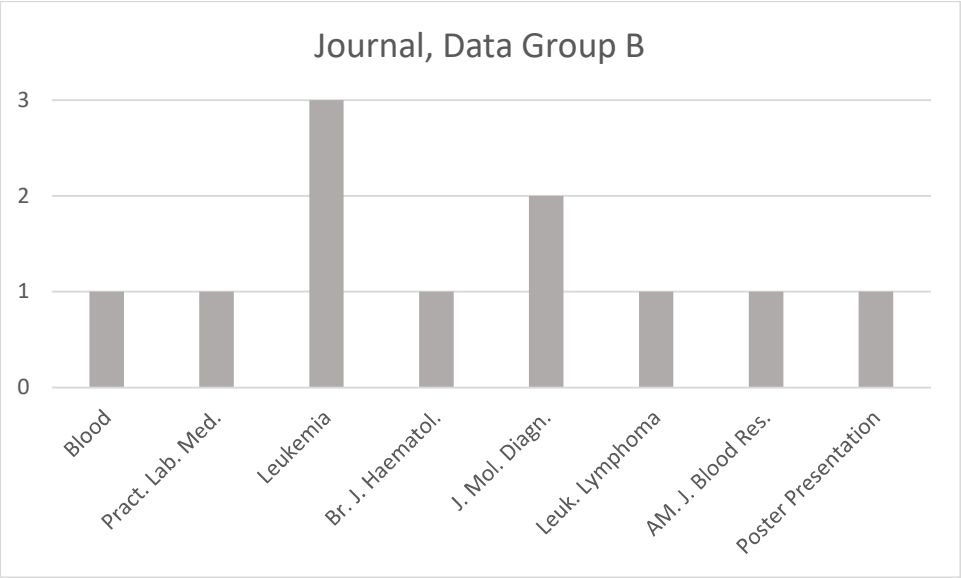

Figure 17 Journal Type, Data Group B

Data-Group B: B- and T-Cell-Receptor Clonality Testing, PCR-CE

Study	Sample Size/ Type	Assay	Assessment Criteria	DNA Input	Sensitivity / Concordance to Reference Method
B- and T-Cell-Receptor Testing					
Next Generation Sequencing (NGS) Based IGH and TCR Clonality Assays Provide Excellent Specificity and Sensitivity for Routine Clonal Characterization and Monitoring of Lymphoproliferative Disorders. *Arcila et al. Blood (2017)*(14)	534 samples (Blood, Bone Marrow and FFPE tissue samples) CLL, plasma cell neoplasms, B-ALL, low-grade B cell lymphoma, T-cell lymphoma NOS, DLBCL, FL, HCL	Invivoscribe® Standard CE IGH and TRG Assay	N/A	N/A	Sensitivity NGS: 94% (502/ 534) Sensitivity PCR-CE: 89% (475/534) Reference – lymphoma classification incorporating histology, flow-cytometry und molecular test results
Validation and interpretation of IGH and TCR clonality testing by Ion Torrent S5 NGS for diagnosis and disease monitoring in B and T cell cancers. *Lay et al. Practical Laboratory Medicine (2020)*(16)	195 samples (Bone Marrow, Fresh lymph node, FFPE, Fluid, Blood) T- and B-cell neoplasms	TCRG, IGH FR1/2/3 Assay (Invivoscribe®) ABI 3130 or 3500 GeneMapper Software	BIOMED-2 guidelines	N/A	IGH concordance PCR-CE and NGS in 62,7% TCR concordance PCR-CE and NGS in 66,7% Reference – PCR-CE
Polymerase chain reaction-based clonality testing in tissue samples with reactive lymphoproliferations: usefulness and pitfalls. *Langerak et al. Leukemia (2007)*(28)	106 samples (Fresh frozen tissue) Reactive tissue lesions	IGH, IGK, IGL/ TCRB, TCRG, TCRD multiplex PCR BIOMED-2 (Invivoscribe®) GeneScan Analysis	Clonality defined as unequivocal clinal Ig/TCR bands/ peaks, present in multiple PCR reactions Low-Level clonality defined as detection of a predominantly weak IGH/TCR peak in a polyclonal background	N/A	Polyclonal 75% (79/106) Low-Level Clonal 15% (16/106) Clear monoclonal 10% (11/106) – potentially false positive result Reference – histopathological diagnosis
A practical strategy for the routine use of BIOMED-2 PCR assays for detection of B- and T-cell clonality in diagnostic haematopathology. *Liu et al. Br J Haematol (2007)*(29)	441 samples (Fresh frozen and FFPE)	BIOMED-2 reactions for IG and/ or TCR (Invivoscribe®) Gel-Electrophoresis	Polyclonal – ill-defined smears of varying size range Clonal – one or more reproducible, discrete band	50-200 ng	Sensitivity B-cell neoplasms 96% Sensitivity T-cell neoplasms 98% No significant difference between FFPE and fresh frozen Reference – histopathological diagnosis

B-Cell-Receptor Testing Only					
Next-Generation Sequencing-Based Clonality Assessment of Ig Gene Rearrangements. *Van den Brand et al. J Mol Diagn (2021)*(20)	209 samples (FFPE, frozen tissue, blood) B-cell neoplasms, reactive lymphoproliferation, inconclusive cases	BIOMED-2 multiplex PCRs GeneScan Analysis	EuroClonality/BIOMED-2 guidelines	N/A	Concordance PCR-CE and NGS in 98% Sensitivity NGS: 96% Sensitivity PCR-CE: 95% Reference – histology consens review of cases
Application of BIOMED-2 clonality assays to formalin-fixed paraffin embedded follicular lymphoma specimens: superior performance of the IGK assays compared to IGH for suboptimal specimens. *Halldorsdotis et al. Leuk Lymphoma (2007)*(30)	40 samples (FFPE) Follicular lymphoma	BIOMED-2 multiplex PCRs 2100 Bioanalyzer	Clonal – single DNA peak of greater that 15 relative fluorescence units	N/A	IGH - false negative 65% (26/40) IGK – false negative 8% (3/40) – concluded to be more robust for testing of follicular lymphoma Reference – histopathological diagnosis
Use of IGK gene rearrangement analysis for clonality assessment of lymphoid malignancies: a single center experience. *Mannu et al. Am J Blood Res (2011)*(31)	59 samples (FFPE) B-cell lymphoproliferative disorder	BIOMED-2 multiplex IGK assay (after failure of IGH assay) GeneScan analysis	Clonal – 1 or 2 peaks Oligoclonal – 3 to 5 peaks Polyclonal – Gaussian distribution	100 ng	Sensitivity 83% Specificity 80% Marginal zone lymphoma and follicular lymphoma most frequently tested false negative with IGH
Validation of immunoglobulin gene rearrangement detection by PCR using commercially available BIOMED-2 primers. *McClure et al. Leukemia (2006)*(7)	266 samples (211 fresh samples, 55 FFPE) normal, reactive, neoplastic	BIOMED-2 IGH and IGK primer Capillary electrophoresis in an automated genetic analyzer	Clonal – clearly predominant peak in an acceptable size range	N/A	Sensitivity ranging depending on entity from 82 to 100% plasma cell neoplasms 95% mantle cell lymphoma 86% DLBCL 82%
T-Cell-Receptor Testing Only					
Validation of a Next-Generation Sequencing–Based T-Cell Receptor Gamma Gene Rearrangement Diagnostic Assay. *Ho et al. J Mol Diagn (2021)*(21)	101 samples (FFPE, blood, bone marrow)	TRG Gene Clonality Assay for ABI Fluorescence Detection (Invivoscribe®) ABI GeneAmp 9700 GeneMapper	Clonal peaks – peaks which are at least two-fold higher than the polyclonal background (maximum 2 peaks) Oligoclonal – three or more peaks Polyclonal – no clonal peaks as described above	1000 ng	Sensitivity 73% / 91% (NGS/ PCR-CE) Specificity 91% / 75% (NGS/ PCR-CE) Accuracy 82% / 83% (NGS/ PCR-CE) Reference – clinicopathologic diagnosis

Next-Generation Sequencing for Detection of Clonal TRG Gene Rearrangements Shows Improved Specificity and Positive Predictive Value Compared to Fragment Analysis Using BIOMED-2 Primers and Capillary Electrophoresis. *Ewalt et al. Invivoscribe Poster Presentation (2015)*(22)	58 samples (FFPE) T-cell lymphoproliferative disorders, reactive tissues, B-cell lymphoma	PCR-CE for TRG rearrangement No further details	N/A	N/A	Sensitivity 79-89%/ 86% (NGS/ PCR-CE) Specificity 97-100%/ 83% (NGS/ PCR-CE) Reference – clinicopathologic diagnosis
Powerful strategy for polymerase chain reaction-based clonality assessment in T-cell malignancies Report of the BIOMED-2 Concerted Action BHM4 CT98-3936. *Brüggemann et al. Leukemia (2007)*(9)	188 samples (fresh or frozen samples) T-cell-malignancies	BIOMED-2 multiplex PCR tubes for TCRB, TCRG, TCRD	N/A	N/A	Using a combination of TCRB and TCRG testing resulted in ≥2 clonal peaks in 95% of all clonal cases

Abbreviations: CLL – chronic lymphocytic leukemia; B-ALL – B cell acute lymphocytic leukemia; DLBCL – diffuse large B cell lymphoma; FL – follicular lymphoma; HCL – hairy cell leukemia; N/A – not applicable; PCR-CE – Polymerase chain reaction and capillary electrophoresis; MM – multiple myeloma; BP-CML – blast phase chronic myeloid leukemia; T-ALL T cell acute lymphocytic leukemia; MDS – myelodysplastic syndrome; Mycosis fungoides - MF

5.3 Data Group C

This Data Group includes literature that evaluated the use of T-Cell and B-Cell Next-Generation-Sequencing for the evaluation of minimal residual disease.

Eight studies were included in this group mainly concerning the minimal residual disease testing of B-ALL and T-ALL. Two of the eight studies also investigated the use of NGS B-Cell-Receptor clonality testing for minimal residual disease identification in Multiple Myeloma cases.

The included studies were published between 2012 and 2022 with a main peak around 2019 (Figure 5).

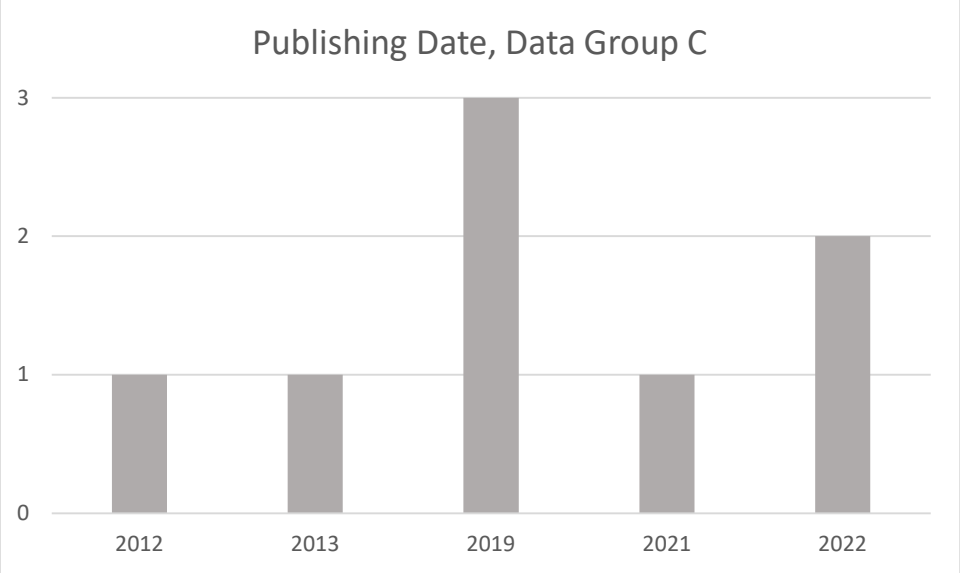

Figure 18 Publishing Date, Data Group C

The 8 included studies were published in seven different journals. Two of them were published in the Leukemia Journal (Figure 6).

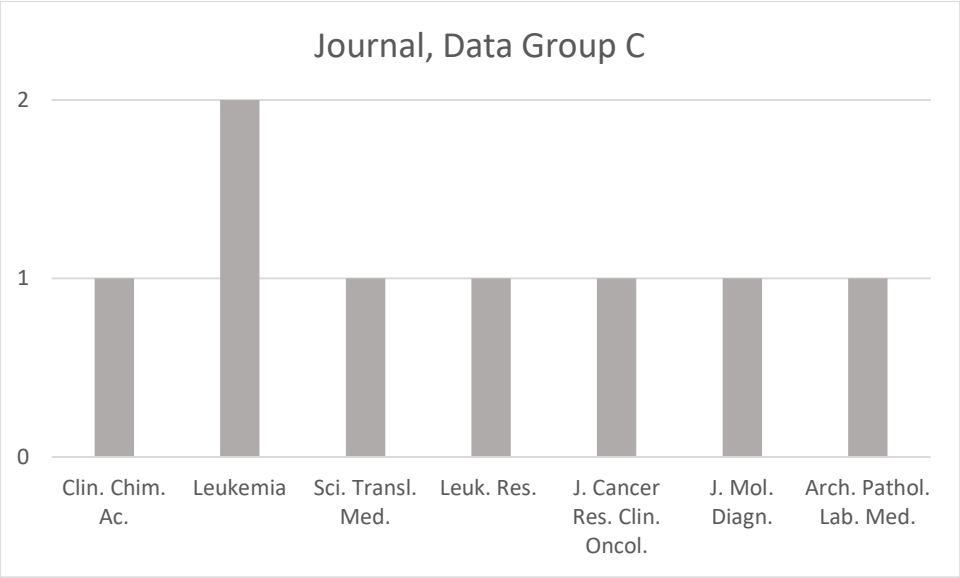

Figure 19 Journal Type, Data Group C

Data-Group C: Minimal-Residual-Disease Testing, NGS					
Study	**Sample Size/ Type**	**Assay**	**Bioinformatics**	**DNA Input**	**Concordance to Reference Method/ Results**
Considerations for monitoring minimal residual disease using immunoglobulin clonality in patients with precursor B-cell lymphoblastic leukemia. *Jo et al. Clin Chim Ac (2019)*(32)	74 samples (bone marrow of 47 patients) B-ALL	IGH FR1/2/3, IGK assay Panel (LymphoTrack®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	50 ng	Initial diagnosis 100% (47/47) All patients with persistent remission showed negative or <0,01% MRD of Ig clonality in NGS MRD in NGS was significantly associated with survival outcome
Next-generation sequencing and real-time quantitative PCR for minimal residual disease detection in B-cell disorders. *Ladetto et al. Leukemia (2013)*(33)	378 samples (55 patients, peripheral blood, bone marrow) ALL, MCL, MM	LymphoSIGHT method IGH, IGK, TCRB, TCRD, TCRG	N/A	N/A	Concordance NGS/ Real Time Quantitative Polymerase-Chain-Reaction (RQ-PCR) high (R=0.791, p<0,001) Conclusion – NGS has a sensitivity equivalent to RQ-PCR Reference: RQ-PCR
Highthroughput sequencing detects minimal residual disease in acute T lymphoblastic leukemia. *Wu et al. Sci Transl Med (2012)*(34)	43 paired samples (blood; initial samples and day 29 after treatment) B-ALL	TCRB, TCRG Illumina HiSeq platform	N/A	N/A	Initial diagnosis (NGS) 31/ 43 TCRB clonal 27/ 43 TCRG clonal Detection of minimum residual disease 25/ 35 (NGS) 13/ 35 (flow cytometry) Reference – multiparametric flow cytometry
Next-generation antigen receptor sequencing of paired diagnosis and relapse samples of B-cell acute lymphoblastic leukemia: Clonal evolution and implications for minimal residual disease target selection. *Theunissen et al. Leuk Res (2019)*(35)	42 paired samples (bone marrow, peripheral blood; initial diagnosis and relapse)	Sequenta, IGH, IGK, TRG, TRD, TRB Illumina HiSEQ platform	PRecISe Clonal Analysis (PRISCA)	120-180 ng	NGS – ≥ 2 major clonal rearrangements per sample at initial diagnosis was detectable 91% rearrangements detected by PCR could also be detected by NGS NGS detected 114 additional rearrangements – seen as minor subclones Initial large index clones could be detected in 84% of relapse samples (stability, NGS) – RQ-PCR showed stability only in 71% Reference – RQ-PCR
Standardized next-generation sequencing of immunoglobulin and T-cell receptor gene recombinations for MRD marker	50 samples (10 patients)	Self-designed primers IGH, IGK, TCRB, TCRG, TCRD	ARResT/ Interrogate	N/A	NGS – detection of 259 clonal rearrangements / Sanger sequencing identified 248

identification in acute lymphoblastic leukaemia; a EuroClonality-NGS validation Study. *Brüggemann et al. Leukemia (2019)*(36)	B- and T-ALL	Illumina MiSeq			NGS provides 4% more detectable rearrangements than reference method Better performance in presence of polyclonal background Reference – conventional PCR plus Sanger sequencing of clonal products
Clinical application of next-generation sequencing-based monitoring of minimal residual disease in childhood acute lymphoblastic leukemia. *Mai et al. J. Cancer Res. Clin. Oncol. (2022)*(37)	236 paired samples (64 patients, bone marrow obtained before start of treatment, on day 19 and at end of induction) B- and T-ALL	Self-designed primers, IGH, IGL, TRG, TRB Illumina platform	N/A	N/A	Positive MRD detection via NGS - 57,5% of B-ALL - 80% of T-ALL (higher than detection rates achieved with RQ-PCR or Flow Cytometry) Reference – RQ-PCR
Ig Gene Clonality Analysis Using Next-Generation Sequencing for Improved Minimal Residual Disease Detection with Significant Prognostic Value in Multiple Myeloma Patients. *Ha et al. J Mol Diagn (2021)*(38)	312 samples (bone marrow) Multiple Myeloma	IGH FR1, IGK assay Panel (LymphoTrack®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	200-400 ng	Detection rate: initial sample 96,7/ 95,4% (PCR/ NGS) monitoring sample 44,7/ 70,3% (PCR/ NGS) remission sample 33,4/ 60,3% (PCR/ NGS) Reference – PCR-CE
Interlaboratory Analytical Validation of a Next-Generation Sequencing Strategy for Clonotypic Assessment and Minimal Residual Disease Monitoring in Multiple Myeloma. *Medina et al. Arch Pathol Lab Med (2022)*(39)	101 patients (bone marrow, collected at diagnosis and early follow up) Multiple Myeloma	IGH FR1/2/3, IGK assay Panel (LymphoTrack®) Illumina MiSeq Platform	Invivoscribe® software (LymphoTrack®)	50 ng (at diagnosis) 650 ng (follow up)	Detection rate initial sample 99/ 100% (PCR and Sanger/ NGS) NGS: Median sensitivity of 0,001% Flow Cytometry: Median sensitivity of 0,002% Reference – PCR/ Sanger, Flow Cytometry

Abbreviations: CLL – chronic lymphocytic leukemia; B-ALL – B cell acute lymphocytic leukemia; DLBCL – diffuse large B cell lymphoma; FL – follicular lymphoma; HCL – hairy cell leukemia; N/A – not applicable; PCR-CE – Polymerase chain reaction and capillary electrophoresis; MM – multiple myeloma; BP-CML – blast phase chronic myeloid leukemia; T-ALL T cell acute lymphocytic leukemia; MDS – myelodysplastic syndrome; Mycosis fungoides – MF; Mantel cell lymphoma - MCL

6. Discussion

This review of the literature of the last 23 years concerning the use of Next-Generation-Sequencing for B- and T-Cell-Receptor clonality testing shows that several smaller but also bigger studies with up to 1189 included samples using different sample types like blood, bone marrow, formalin-fixed-paraffin-embedded tissue or fresh frozen tissue do exist which usually focus on determining sensitivity, detection rate or the use of different interpretation algorithms. Many of the studies compared the results with the gold standard of Capillary Electrophoresis. Some of the studies examined the use and application of NGS alone. (11,14,14,16–20,22–27,38)

6.1 Platforms, Assays and Bioinformatics

Looking at the studies of Data Group A (B- and T-Cell-Receptor Clonality Testing, Next Generation-Sequencing) it can be seen, that the predominant used NGS-platform was the Illumina MiSeq platform. Nine of the 15 included studies used this platform for their sequencing. Three studies were done using an IonTorrent platform (Thermo Fisher Scientific). In one study of 2021 Wu et al used an Illumina NovaSeq6000 System.(18) One other study of Schumacher et al used both Ion PGM (Thermo Fisher Scientific) as well as a MiSeq platform for their sequencing.(27) Lastly, Rea et al did not specify further which platform was used in their testing.(25)

Regarding BCR and TCR clonality testing the previously mentioned variable regions are sequenced and the percentages of all found sequence variants are called using different bioinformatic tools. For this approach targeted either amplicon or capture-based Next-Generation-Sequencing is most suitable which can be done using both predominant NGS platforms, Illumina MiSeq as well as Thermo Fisher Scientific IonTorrent.(4,40)

As mentioned above Schumacher et al used both the IonTorrent (PGM) as well as the Illumina MiSeq platform in their study published in 2014 to sequence the *TCRG* gene and compared the data of both NGS-platforms. As a reference for clonality they used the results from PCR-CE. In the comparison of 14 cases sequenced on each platform they encountered the well-known higher error rate especially in

homopolymer regions of the semiconductor IonTorrent sequencing method. This resulted in the detection of many subclones who varied just in one to two bases in highly repetitive regions. This subclones where therefore excluded from their further analysis. Both platforms where able to detect the same predominant clones especially in monoclonal samples. In polyclonal samples the order of the percentages of the found subclone sequences was not always the same.(27)

Amplicon based assays from Invivoscribe® for BCR as well as TCR NGS-clonality testing were the most common used as they are also the market leader regarding PCR-CE clonality assays which were developed using the recommendations of EuroClonality/ BIOMED2.(6) Nine out of the 15 included studies used assays from Invivoscribe®. Two studies by EuroClonality used self-developed primers based on the previously developed and standardized BIOMED-2 PCR-CE primers and evaluated the usability for Next-Generation-Sequencing.(19,20) Three studies used customizes panels(18,26,27) and one used the TCRB ImmunoSEQ assay(25).

Twelve of the included NGS based clonality studies tested for T-Cell-Receptor clonality. All but one included the *TCRG* gene with the one exception only sequencing *TCRB* via the ImmunoSEQ assay. As mentioned in the background section, *TCRG* rearranges quite early and is also almost always present in TCRαβ expressing T-lymphocytes. Because of this *TCRG* proofed to be a reliable target for clonality testing using PCR-CE in the past. Different studies also confirmed that the combination of *TCRB* and *TCRG* gene testing achieves even higher sensitivity.(9) In the development of NGS TCR assays *TCRG* was the primarily used target which can be seen in the used assays of the included studies. *TCRB* based assay are following in recent years and the question remains if like in PCR-CE testing a combined *TCRG* and *TCRB* testing in Next-Generation-Sequencing should be done to increase sensitivity here as well.(24,41)

As mentioned in the study of Nollet et al, testing 85 fresh and 36 FFPE samples regarding TCRG clonality, Bioinformatics and defining the interpretation algorithms is a crucial point for clonality testing via NGS as well as via CE-PCR.(23) Of the 15 included studies seven used the Invivoscribe® provided software LymphoTrack® alone to evaluate the data. Six used customized bioinformatic software tools or online available databases, like ARRest/ Interrogate or the international

ImMunoGeneTics information system® (IMGT), for interpreting the sequencing data. Two studies, both done by Arcila et al, used a combination of Invivoscribe® and an in-house validated software. Three studies described their interpretation algorithm and potential pitfalls more in detail.(11,23,27)

Nollet et al described in their study, published in 2018, the usual interpretation algorithm of the LymphoTrack® software. Following these interpretation recommendations Clonality can be assumed if the most prominent sequence shows 2,5% of total reads (assuming that the sample has at least 20 000 reads in total) and is two-fold the frequency of the third most frequent sequence. If less than 20 000 reads are acquired for the sample, the cut-off is raised to 5%. Nollet et al described several flaws of this interpretation algorithm, one being that low-level monoclonal sequences are determined as “no evidence of clonality” as well as if two clonal sequences are found, the percentage of the third most frequent sequence would not be reliable for comparison. Furthermore, they stated that pseudoclonality can occur, quite similar like in PCR-CE, if only few lymphocytes are present in the sample. Their proposed multi step alternative interpretation algorithm includes that a duplicate analysis of each sample is done and that each is evaluated separately. They defined as primary evidence for monoclonality that the predominant sequence acquires at least 2% of total reads with the prerequisite that the sample has at least 1000 total reads. Secondly the data of a potentially monoclonal sample is evaluated top down regarding the decrease of frequencies. In a potentially monoclonal sample the registered frequencies of the other sequences should decrease two-fold each step down. Lastly, they established two additional criteria for clonality, one being that the found potentially clonal sequence should be four times higher than the most frequent background sequence and it should show a fraction of 4,5% or higher of all reads. If just one of these criteria is met, the sample is determined as polyclonal with a minor clonal lymphocyte population. If both are met the distinction between monoclonal and oligoclonal is done regarding the number of sequences which show a frequency four times greater that the background. If more than two can be seen, the sample is determined oligoclonal. In comparison with the gold standard PCR-CE they concluded that especially the duplicate analysis of each sample contributes to more reliable results.(23)

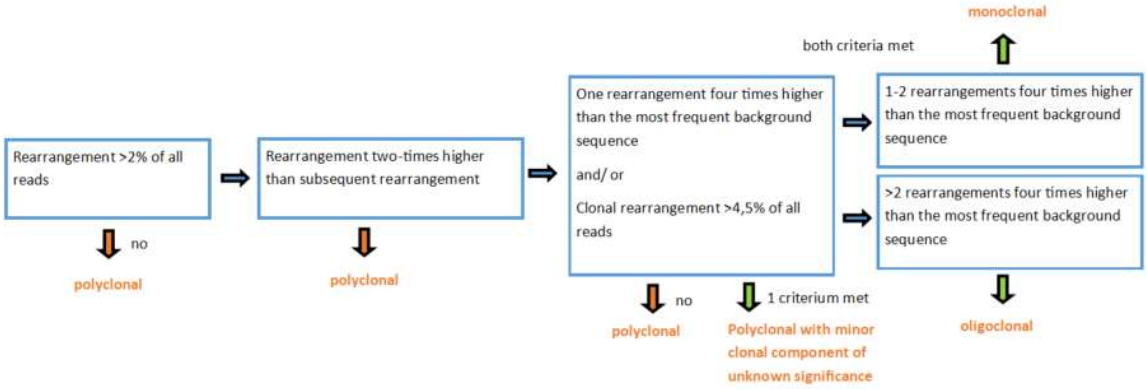

Figure 20 Interpretation algorithm suggested by Nollet et al(23)

Schumacher et al described in their study a quite similar but not so extensive approach of an alternative interpretation algorithm. They defined two criteria which must be met to consider a sample clonal. The first criterion is, that the top one or two sequences are four times higher in frequency than the third most predominant sequence. Secondly the most frequent sequence should show at least a frequency of 4,5% of the total reads. Only if both criteria are met the sample will be considered monoclonal, if just one criterion is achieved the sample is seen as “indeterminate”.(27)

Arcila et al used an in-house developed bioinformatics pipeline additionally to the LymphoTrack® software and adapted the interpretation recommendations of LymphoTrack® as well. If more than 100 000 total reads could be achieved monoclonality was considered if a sequence showed more than 2,5% of total reads and 10 times the read count of the polyclonal background. If only 30 000 to 50 000 total reads where given a sequence had to harbour more than 5%, as well as 20 times the read count of the background for being considered a clonal sequence. Arcila et al also stated several pitfalls and problems regarding the interpretation of the data, one of them being a dominant sequence with less than 2,5% of total reads but 20 times more reads than the background. In this case they considered that due to low tumor content a false negative result could be at hand and recommended a second testing. Another pitfall mentioned concerns the detection of multiple dominant but slightly different sequences. In this case they considered that due to sequencing errors or due to ongoing somatic hypermutation small differences in the

sequences occur and recommended to add percentages of these slightly different sequences if they seem closely related. Another example for a potentially pitfall of false positivity would be multiple monoclonal signals in a sparse background. In this case they stated that the pattern should be considered oligoclonal and if possible, a repeat testing with higher DNA input should be done.(11)

Concerning DNA input, not all of the included studies stated clearly which amount of DNA they used. 10 of the 15 in Data Group A included studies listed in their articles which DNA amount was used for library preparation. The minimum DNA input ranged from 25 ng up to 100 ng with six studies using at minimum of 50 ng DNA for their library preparation which is concordant with the Invivoscribe® recommendations of minimal input.(41)

Arcila et al conducted a dilution sequence and DNA input studies to determine the limit of detection of a clonal recombination and minimum DNA input. They stated that 2,5% clonal DNA in a wildtype background could be detected and that a minimum input down to 25 ng DNA did not significantly influence the detection of a clonal rearrangement. The DNA input study was done on a specimen where flow cytometry confirmed that the clonal B-Cell population would be 10% of all contained B-cells.(11)

Ho et al also did a dilution series using a commercially available clonal control and stated that the detection of clonality could be achieved consistently using a dilution as low as 0,1% in their approach of T-cell clonality testing. For a diagnostic specimen they concluded that the detection limit of clonal cells should be set at 5% clonal cells in a wildtype background. For diagnostic samples they used 50 to 250 ng DNA input with an average of 173,3 ng.(21)

As seen in Data Group C higher amounts of DNA were used in multiple studies regarding the use of Next-Generation-Sequencing in the context of minimal residual disease detection as more DNA input is necessary for detecting the low percentage of a remaining clone under therapy. More on this topic below.

6.2 Sensitivity and Specificity

Regarding sensitivity and specificity many of the in Data Group A included studies differ quite significantly in their approach of determining the true positive and true

negative rates, using different platforms, assays as well as references, making a comparison difficult. Six of the fifteen included studies used clonality testing via the gold standard of PCR-CE as a reference of “true” clonality. Seven used a mixture of clinicopathological diagnosis consensus as a reference. Two studies did not clarify in their article which reference they used.

In one of their studies Arcila et al used 534 blood, bone marrow and FFPE specimens for initial diagnosis of B-cell as well as T-cell clonality via PCR-CE and NGS methodology. As a reference they used a clinicopathological consensus incorporating flow-cytometry. Using this approach they determined the limit of detection to be 1% clonal DNA in a polyclonal background for NGS testing and 5-10% for PCR-CE. In 94% of cases they were able to detect the clonal sequence via NGS testing compared to 89% via PCR-CE.(14)

Looking at a similar approach by Ho et al, who only included T-cell receptor clonality testing, quite different results can be seen. They also used clinicopathologic diagnosis as a reference and compared NGS and PCR-CE testing. In their study they only used FFPE samples. Regarding Sensitivity NGS was only able to detect 73% of all clinicopathological as clonal determined cases. In comparison PCR-CE showed a clinical sensitivity 91%. In their study NGS testing showed better results concerning specificity compared to PCR-CE with a true positive rate of 91% compared to 83% which could be achieved via PCR-CE testing. Nevertheless the overall accuracy between NGS and PCR-CE showed high concordance.(21)

Lay et al used the results from *IGH* and *TCRG* PCR-CE clonality testing of 195 samples (bone marrow, fresh tissue, FFPE, fluid and blood) as a reference and examined the concordance to Next-Generation-Sequencing. Regarding *IGH* clonality analysis via NGS and PCR-CE a concordance of 62,7% could be shown. For *TRG* testing a concordance of 66,7% could be shown between the two methods. In the disconcordant cases NGS was able to detect monoclonal or specific polyclonal sequences which could not be shown via PCR-CE.(16)

Nollet et al tested FFPE and fresh samples regarding *TCRG* clonality via NGS and PCR-CE as well. In their study a concordance could be found in 78% of all fresh samples. Interestingly in their study testing on FFPE material could be done successfully in most cases (34/36) via NGS but only in less than half of the cases

via PCR-CE (16/36), even if results of total reads in NGS testing were overall lower than in fresh samples. As FFPE samples are known to be more difficult to work with because of DNA fragmentation, this seems to be an interesting outcome.(23)

To describe one more study of Data Group A in more detail, Van den Brand et al tested 209 samples (FFPE, frozen tissue and blood) regarding *IGH* and *IGK* clonality using histology consens review as a reference. In their study they were able to show a concordance of 98% between PCR-CE and NGS as well as a sensitivity of 96% and 95% for NGS and PCR-CE clonality testing. The results of this study therefor showed that NGS and PCR-C are comparable in their ability to detect clonality with a slightly better sensitivity for NGS testing.(20)

The overall consensus of almost all the in Data Group A described studies can be summarized that NGS based clonality testing performs comparable to slightly better in regards to sensitivity and specificity than classic PCR-CE methodology and that it's main advantage lies in the detailed sequence description which can be used for consecutive minimal residual disease testing and is more difficult to do so via PCR-CE.

6.3 Minimal Residual Disease

Minimal residual disease testing via detection of the clonal sequence plays a major role of planning the therapy regiment in acute lymphoblastic leukemia as well as in multiple myeloma. Up to now flow cytometry and allele-specific oligonucleotide PCR (ASO-PCR) are the main methods for detecting residual clonal lymphocytes and relapse.(32,33,38)

As mentioned before in the study of Arcila et al PCR-CE usually shows a Limit of detection between 5 to 10% of clonal DNA in a polyclonal background which is not enough regarding the assessment of minimal residual disease. The subjectivity in the interpretation of the fragment analysis especially if few clonal cells are included are another reason why PCR-CE is not appropriate to use for MRD assessment.(15,38)

ASO-PCR is a sensitive method suitable for the detection of minimal residual disease. Usually a clonality analysis using PCR-CE and BIOMED-2/ Euroclonality recommended primer is done for identification of the patient specific rearrangement.

Subsequent patient specific primer are designed for real-time quantitative PCR detection of the malignancy over the course of the treatment. This technique is highly specific but unfortunately also quite time consuming and complex. Additionally not all rearrangements are equally eligible for Real Time Quantitative Polymerase-Chain-Reaction (RQ-PCR) primer design.(33)

Eight studies were included in Data Group C evaluating and discussing the use of Next-Generation-Sequencing for clonality and subsequent minimal residual disease testing. Seven of these studies used an Illumina platform for their testing. Several different self-designed primers and assays were used. Only four of all included studies stated which DNA input amount they used which ranged between 50 ng (usually at first diagnosis) up to 650 ng for subsequent testing.

In their study Ladetto et al compared NGS and ASO-PCR for the use of minimal residual disease testing in 378 samples of patients with Acute Lymphoblastic Leukemia, Mantle Cell Lymphoma and Multiple Myeloma. In their study NGS showed a high concordance to ASO-PCR/ RQ-PCR and therefor a comparable sensitivity with the advantage of not having to design patient specific primer.(33)

Theunissen et al showed in their study evaluating 42 paired samples (bone marrow or peripheral blood of children with ALL) another advantage of the usage of NGS for minimal residual disease testing in hematooncological diseases. In their examined patient collective only 27% of clonal rearrangements proofed to be the same in initial diagnosis as well as in relapse. Regarding the usage of ASO-PCR and patient specific primer this would lead to false negative results whereas in NGS the there is a possibility to detect emerging new subclones.(35)

Brüggemann et al also state that in their study NGS was able to detect 4% more clonal rearrangements compared to PCR-CE with subsequent primer design and ASO-PCR. They also concluded that NGS shows a better performance in the presence of a broad polyclonal background for minimal residual disease detection.(36)

In their study on 236 paired samples of patients with B- and T-ALL Mai et al came to a similar conclusion with minimal residual disease detection rates via NGS of 57,5% (B-ALL) and 80% (T-ALL) after initial treatment which were higher than in conventional ASO-PCR or flow cytometry.(37)

6.4 Conclusion and outlook

Summarizing the literature which was systematically reviewed in this thesis Next-Generation-Sequencing, especially when using Euroclonality approved primer sets, is a highly sensitive and specific method for the detection of B- and T-cell clonality and therefor can help to distinguish lymphoid malignancy from reactive disorders. Advantages of this method, compared to the gold standard method of PCR-CE, include the specific detection and calling of the predominant sequences which can be used for following specific clones for minimal residual disease determination. The usage of both Illumina as well as IonTorrent platforms could be found in the literature with studies concluding their applicability. Invivoscribe® assays with the usage of the LymphoTrack® software were the most commonly used methods. Some studies like Arcilla et al and Schumacher et al stated that the interpretation algorithms have to be assessed further and are crucial for an objective assessment of the data. Nollet et al highlighted that the overall cost of NGS based clonality testing represent a major downside of this method compared to PCR-CE.

In conclusion, especially for minimal residual disease testing and in difficult cases with multiple clonal rearrangements, NGS seems to show promising results. As PCR-CE is a well-studied and comparatively low-priced technique for clonality testing, the decision which method to prefer is still difficult and advantages as well as disadvantages for implementation of NGS based clonality testing have to be evaluated in each laboratory individually considering the given resources and main clinical areas of application of clonality testing.

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