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# Evaluation of the signature LTX multiplex RT-PCR assay for the detection of leukaemia-associated translocations

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# EVALUATION OF THE SIGNATURE LTX MULTIPLEX RT-PCR ASSAY FOR THE DETECTION OF LEUKAEMIA-ASSOCIATED TRANSLOCATIONS

A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the branch of Haematology.

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0303533D

# Declaration

I Estée Liezl Benadé declare that this research report comprises my own work. It is being submitted for the degree of Master of Medicine in the branch of Haematology to the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination at any other University.

Estée Liezl Benadé

On this day of the 24th of May 2016

# Dedication

To my husband Neil and our family for their limitless patience, support and encouragement in the completion of this work and to my mother, Maritha whose passion and love for Medicine started it all.

# Abstract

**Introduction**

Genetic abnormalities play an integral role in the pathogenesis, diagnosis, prognosis and treatment of leukaemia. As the technology advances in the field of molecular testing, so will our approach to the diagnosis and treatment of patients presenting with suspected leukaemia. Our aim was to evaluate the Signature® LTx Multiplex RT-PCR assay which combines multiplex RT-PCR (Reverse Transcription Polymerase Chain Reaction) with multiplex fluorescent bead-array detection and to compare results with those of routine diagnostic tests FISH (Fluorescent In Situ Hybridisation) and cytogenetics analysis. The assay has 12 translocations commonly associated with acute lymphoblastic leukaemia (ALL), acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML).

**Material and Methods**

RNA was extracted from bone marrow aspirate and peripheral blood samples from both adult and paediatric patients presenting with ALL, AML and CML and analysed 70 samples using the multiplex assay with four steps of reverse transcription, DNA amplification, hybridisation and detection. Qualitative analysis was performed with a pre-determined mean fluorescence intensity (MFI) cut off level as well as quantitative analysis of signal distributions for positive, negative and endogenous controls (GAPDH: glyceraldehyde 3-phosphate dehydrogenase). Results were then compared to those of cytogenetics and FISH.

**Results**

Eighty six percent of the samples had concordant results and 14% showed discordant results compared to routine diagnostic tests. Of these discordant samples, some results could be explained by suboptimal RNA quantity or GAPDH MFI signals and if these were repeated or

discarded, the number of discordant samples would decrease to four percent. The multiplex detected four translocations which were not identified by FISH or cytogenetics and 6 mutations were missed by the assay, however the majority of these are attributable to suboptimal RNA and internal control signals which would be repeated in the routine use of the assay.

**Conclusion**

Overall, the Signature® LTX Multiplex RT-PCR assay showed good correlation with gold standard test techniques. While the assay provides possible advantages including ease of use and improved turnaround times, drawbacks include problems with RNA quantity after extraction and logistical concerns including the need to batch samples for analysis which will affect the turnaround time. Careful cost analysis and possible modification of the translocations included in the assay would be required before the assay could be incorporated as part of the routine diagnostic work-up of patients presenting with leukaemia.

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## Ethics Clearance

Ethics clearance was obtained from the University of the Witwatersrand Human Research Ethics Committee (M130110) as well as the Faculty of Health Sciences Research Ethics Committee at the University of Pretoria (Protocol 385/2013).

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# Nomenclature

| | |
|---|---|
| ABL1 | Abelson murine leukaemia viral oncogene homolog 1 |
| AF4 | AFFI (AF4/MR2 family, member 1) |
| AF9 | ALL 1 fused gene from chromosome 9 |
| ALL | Acute lymphoblastic Leukaemia |
| AML | Acute myeloid Leukaemia |
| AML1 | Acute myeloid Leukaemia 1 |
| APL | Acute promyelocytic leukaemia |
| ATRA | All-trans retinoic acid |
| BCR | Breakpoint cluster region |
| BM | Bone marrow aspirate |
| CBFA | Core binding factor subunit alpha |
| CBFB | Core binding factor subunit beta |
| cDNA | Complementary DNA |
| CHBAH | Chris Hani Baragwanath Academic Hospital |
| CMJAH | Charlotte Maxeke Johannesburg Academic Hospital |
| CML | Chronic Myeloid Leukaemia |
| DGMC | Donald Gordon Medical Centre |
| DIC | Disseminated Intravascular Coagulation |
| DNMT3A | DNA methyltransferase 3A |
| E2A | E2A immunoglobulin enhancer binding factors E12/E47 |
| EDTA | Ethylenediaminetetra-acetic acid |
| ETV6 | Ets variant 6 |
| FAB | French-American-British classification |
| FLT3 | Fms-related tyrosine kinase 3 |
| FISH | Fluorescent in situ hybridisation |

| | |
|---|---|
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| IDH | Isocitrate dehydrogenase |
| kDa | Kilo-Dalton |
| MFI | Median fluorescent intensity |
| MLL | Mixed lineage leukaemia |
| MLLT1 | Mixed lineage leukaemia; translocated to, 1 |
| MLLT2 | Mixed lineage leukaemia; translocated to, 2 |
| MLLT3 | Mixed lineage leukaemia; translocated to, 3 |
| mRNA | Messenger RNA |
| MYH11 | Myosin heavy chain 11 |
| NCR | National Cancer Registry |
| NOTCH1 | Notch homolog 1 |
| NPM1 | Nucleophosmin 1 |
| PB | Peripheral blood |
| PBX1 | Pre-B-cell leukaemia homeobox |
| PCR | Polymerase Chain Reaction |
| Ph+ | Philadelphia chromosome positive |
| PML | Promyelocytic leukaemia gene |
| RARa | Retinoic acid receptor alpha |
| RUNX1 | Runt-related transcription factor 1 |
| RUNX1T1 | Runt-related transcription factor 1; translocated to, 1 |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| TAT | Turnaround time |
| TCF3 | Transcription factor 3 |
| WHO | World Health Organisation |

# 1) Introduction

## 1.1 Leukaemia

Leukaemia comprises a diverse group of diseases characterised by abnormal proliferation and development of leukocyte precursors and subsequent bone marrow infiltration and failure [1]. The different subtypes include chronic and acute leukaemia affecting both myeloid and lymphoid lineages. Aetiology may be complex with both genetic and environmental contributors including congenital syndromes linked to predisposition to malignancy. Morphology, cytochemistry and immunophenotypic analysis are all crucial in the diagnosis and categorisation of these varied subtypes, but there has been an increasing emphasis on the molecular pathogenesis of the disease, not only to understand the aetiology and prognosis but to tailor treatment according to the underlying genetic lesion. Some categories of acute myeloid leukaemia (AML) for example are designated as an acute leukaemia according to the specific recurrent genetic abnormality, regardless of whether or not the required 20 % blast count is met [2]. As the technology advances in the field of molecular testing, so will our approach to the diagnosis and treatment of patients presenting with suspected leukaemia. For the purposes of this research report, the focus will be on the acute leukaemias (myeloid and lymphoid) as well as chronic myeloid leukaemia (CML).

## 1.2 Leukaemia statistics

According to the South African Paediatric Tumour Registry, leukaemia is the most common childhood cancer, accounting for 25.4% of all cancers. The annual incidence of all tumours in children aged 0-14 years living in South Africa over the period 2003-2007 ranged from 33.4 to 47.2 per million [3]. The National Cancer Registry (NCR) established by the National Health Laboratory Service (NHLS) in 1986 serves an important epidemiological function by keeping a record of all the diagnoses of malignancies made. It is a pathology-based registry and relies on tissue diagnosis and accurate reporting by pathologists. The 2008 annual report states that leukaemia comprised 1.47 % of all cancers in adult males with an age-standardised incidence rate of 1.32 per 100 000 and 1.08 % of all cancers in adult females with age-standardised incidence rate of 2.03 per 100 000 [4].

According to the NCR data collected from 2000-2005 at Chris Hani Baragwanath Academic Hospital (CHBAH) and Charlotte Maxeke Johannesburg Academic Hospital (CMJAH), a total of 203 cases of Acute Lymphoblastic Leukaemia (ALL), 26 cases of acute promyelocytic leukaemia (APL), 96 cases of CML and 179 cases of AML were reported.

In order to confirm and update these figures at our centre, data was collected from the Flow Cytometry Register over the period of 18 months starting January 2012 at CMJAH. Samples from CHBAH, Donald Gordon Medical Centre (DGMC), Helen Joseph Hospital (HJH) and peripheral hospitals are sent to CMJAH for immunophenotypic analysis, therefore the numbers are considered an adequate representation of our referral population, including even more centres than the NCR statistics which is reflected in the increased numbers of all the above malignancies based on the Flow Cytometry data. In this time period, 54 cases of B-ALL and 30 cases of T-ALL were diagnosed. CML comprised 55 of new leukaemia samples with AML and APL accounting for 94 and 32 of all new cases respectively. If an ambiguous lineage leukaemia was suspected or the

sample was inadequate for complete assessment, but the overall blast percentage was greater than 20%, the samples were characterised as Acute Leukaemia (n = 11). These figures are represented in Figure 1 below.

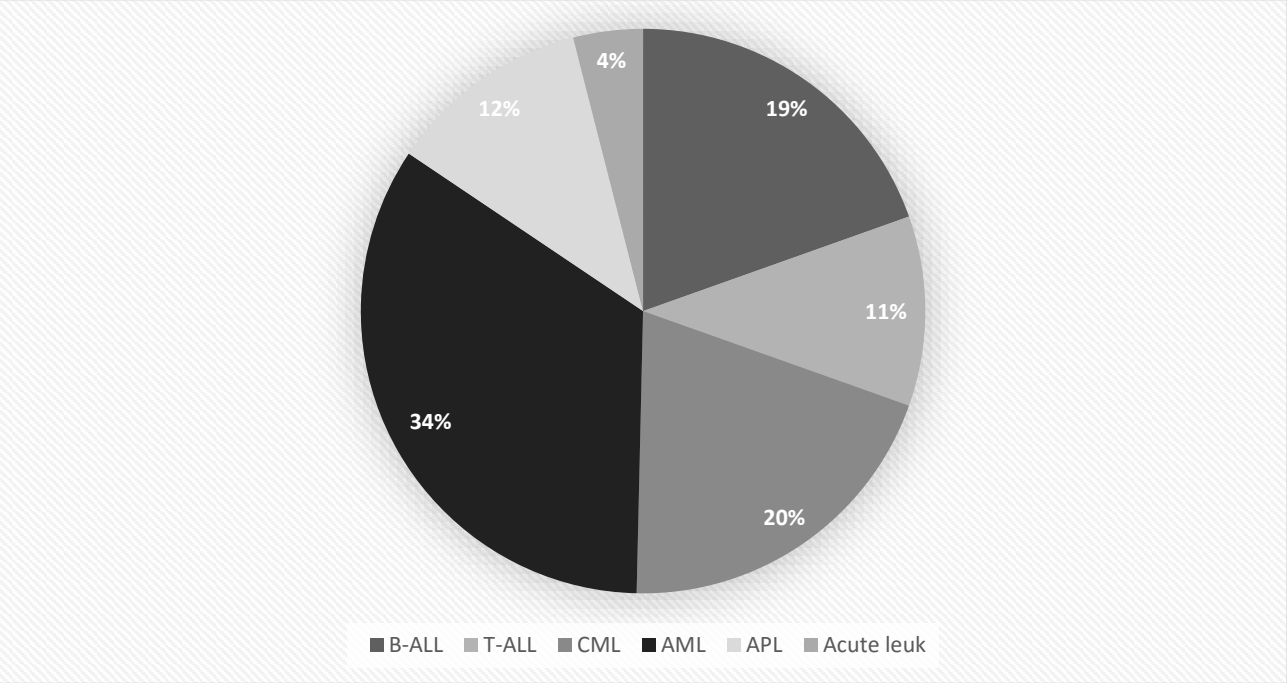



**Figure 1.1: New leukaemia cases according to immunophenotypic data at CMJAH from January 2012 until July 2013.**

## 1.3 Diagnosis

Current World Health Orginisation (WHO) diagnostic criteria in leukaemia require the use of a multifaceted approach. This incorporates the use of morphological, cytochemical, immunophenotypic as well as cytogenetic and molecular analysis [2]. Of the detection methods available, genetic studies provide the most important information with regard to diagnosis, risk stratification and therapeutic strategies. The 2008 WHO classification emphasises the importance of this genetic information by using specific balanced translocations as the basis for a large proportion of its leukaemia classification system, which will be discussed in more detail for the purposes of this study [2].

### 1.3.1 Acute myeloid leukaemia

AML is a defined by clonal expansion of myeloid blasts in the peripheral blood, bone marrow or other tissues [2]. It includes a wide range of clinical, morphological and genetic features. The different categories are shown in Table 1.1. With regards to leukaemogenesis, two groups of mutations have been defined *i.e.* class I and class II mutations. Class I mutations cause proliferation and increased survival of leukaemic cells by activation of signal transduction pathways, whereas class II mutations cause impaired differentiation by affecting components of transcription factors[5]. Class II mutations include the Core Binding Factor leukaemias AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1* and AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*.

**Table 1.1: Categories of AML according to WHO 2008 classification[2]**

| |
|---|
| AML with recurrent genetic abnormalities:<br>AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*<br>AML with inv(16)(p13.1;q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*<br>APL with t(15;17)(q22;q12); *PML-RARa*<br>AML with t(9;22)(p22;g23); *MLLT3-MLL*<br>AML with t(6;9)(p23;q34); *DEK-NUP214*<br>AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*<br>AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*<br>Provisional AML with mutated *NPM1*<br>Provisional AML with mutated *CEBPA* |
| AML with myelodysplasia-related changes |
| Therapy-related myeloid neoplasms |
| AML, not otherwise specified (NOS) |
| Myeloid sarcoma |
| Myeloid proliferations related to Down Syndrome |
| Blastic plasmacytoid dendritic cell neoplasms |

In AML, the core binding factor complex mutations, inv (16)(p13.1q22);*CBFB-MYH11* and t(8;21)(q22:q22);*RUNX1-RUNX1T1*are diagnostic of AML irrespective of blast count and are associated with higher rates of complete remission and a lower risk of relapse [6]. The t(8;21) mutation is found in approximately 5% of AML and inv(16) in 5-8% of cases [2]. These two mutations fall under the category of Acute Myeloid Leukaemia with recurrent genetic abnormalities and both affect subunits of the core binding factor complex, a transcription factor complex essential for regulation of haemopoiesis particularly myeloid differentiation[7]. The t(8;21)(q22:q22);*RUNX1-RUNX1T1*mutation involves the RUNX1 (runt-related transcription factor 1) gene encoding CBFA (core-binding factor subunit alpha) and *RUNX1T1 (ETO)*(runt-related transcription factor 1; translocated to, 1) gene. This results in impaired haemopoietic differentiation since *RUNX1T1 (ETO)* associates with N-Cor (nuclear receptor co-repressor) and blocks transcription of target genes [8]. This translocation is often associated with French American British (FAB) classification AML M2 (acute myeloid leukaemia with differentiation) and is commonly found in younger patients (median 36 years) [2, 9, 10].

The inv (16)(p13.1q22);*CBFB-MYH11*[or *t(16;16)(p13.1;q22)*] mutation fuses the *CBFB* gene (encoding core binding factor beta subunit) at 16q22 to the *MYH11* gene at 16p13.1. *MYH11* which codes for smooth muscle myosin heavy chain. The fusion protein also associates with co-repressor complexes and leads to subsequent differentiation arrest [11, 12]. It is a cryptic translocation and may be missed on conventional cytogenetics therefore Fluorescent In Situ Hybridisation (FISH) analysis or Reverse Transcription Polymerase Chain Reaction (RT-PCR) methods are recommended to detect this mutation [13]. It is commonly identified in AML M4 Eo subtypes with characteristic eosinophil component and can be found in all age groups, but predominantly younger patients (median 41 years) [2, 14].

APL or AML with t(15;17)(q22;q12); *PML-RARα* accounts for 5-8 % of AML cases and can occur at any age, predominantly adults. The translocation involves the fusion of the retinoic acid receptor alpha (*RARα*) gene on 17q12 to a nuclear regulatory gene *PML* (promyelocytic leukaemia) on 15q22, however in the minority of cases (<5%) other translocations involving fusion partners other than the *PML* gene are identified [15]. There are three *PML-RARα* transcript subtypes depending on the location of the breakpoints in the PML region - bcr1, bcr2 and bcr3 (breakpoint cluster region). Bcr3 is known as the short (S) isoform and bcr1 and bcr2 together constitute the long (L) isoform [16].The abnormal *PML-RARα* fusion protein, through various targets of action, results in transcriptional repression of retinoic acid signalling [17]. Identifying this translocation allows for molecularly targeted therapy all-trans retinoic acid (ATRA) and therefore confers a favourable prognosis [2]. APL is considered a haematological emergency and treatment should be started as soon as possible, highlighting the need for a molecular testing technique with a rapid turnaround time (TAT).

### 1.3.2 Chronic myeloid leukaemia

Chronic Myeloid Leukaemia is characterised by t(9;22)(q34;q11.2);*BCR-ABL1,* also known as the Philadelphia chromosome . The translocation fuses *BCR* (breakpoint cluster region) at 22q11.2 and *ABL1* (Abelson murine leukaemia viral oncogene homolog 1), a cytoplasmic tyrosine kinase gene at 9q43. The resultant oncogene encodes a protein with constitutive activity of the kinase [18]. In most patients with CML and in approximately one third of patients with Philadelphia-positive ($Ph^+$) B-cell ALL, the breakpoint within BCR is the major breakpoint cluster region (M-bcr) resulting in a 210-kDa (kilo-Dalton) protein (p210 *BCR-ABL1*) by either b2a2 or b3a2 splicing (refer Figure 1.2). In two thirds of patients with $Ph^+$ B-ALL and rare cases of CML, the minor breakpoint cluster region (m-bcr) is between exons e1 and e2 and a p190 *BCR-ABL* fusion protein is translated from the e1a2 transcript as shown in Figure 1.2 [18]. The detection of t(9; 22) *BCR-*

*ABL1* allows for the selection of specific tyrosine kinase inhibitors *e.g.* Imatinib as treatment which has significantly improved outcomes of patients with CML [19].

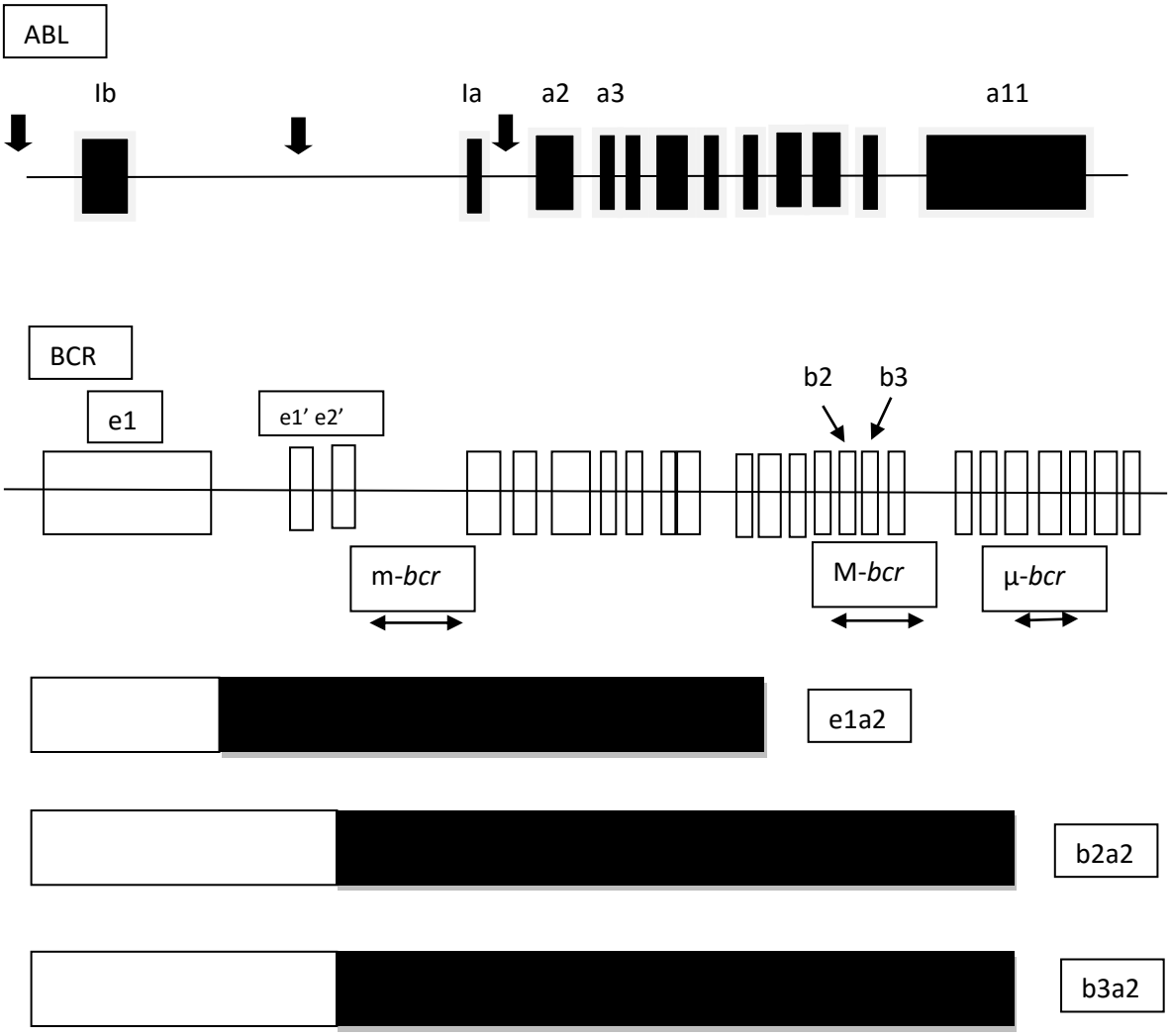



**Figure 1.2 Breakpoint locations in *ABL* and *BCR* genes in chronic myeloid leukaemia (adapted from [20])**

### 1.3.3 Acute lymphoblastic leukaemia

ALL affects mostly children with 75% of cases under the age of six years [2]. ALL is a neoplasm of lymphoblasts either committed to the B-cell or T-cell lineage. B-ALL is more common than T-ALL which accounts for 10-15 % of childhood ALL cases and 25 % of adult cases [21]. Our immunophenotypic data show that 64 % of all ALL cases were of the B-cell lineage.

In the WHO 2008 Classification of Tumours of Haematopoietic and Lymphoid Tissues [2], the group of B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities includes the four translocations t(9;22)(q34;q11.2); *BCR-ABL1* , t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*, t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1),* and t(v;11q23) *MLL* rearranged.

The t(9;22)(q34;q11.2); *BCR-ABL1* ($Ph^+$) mutation in ALL is more common in adults accounting for 25% of cases versus 2-4% of childhood ALL and confers a poor prognosis in both age groups [2, 22]. Its presence in paediatric cases is associated with older age, high white cell count and more frequent involvement of the cerebrospinal fluid (CSF) [23]. The use of imatinib in conjunction with chemotherapy is associated with an improved event-free survival [24].

The t(12;21)(p13;q22);*TEL-AML1 (ETV6-RUNX1)* and t(1;19)(q23;p13.3);*E2A-PBX1 (TCF3-PBX1)* translocations have prognostic significance in B lymphoblastic leukaemia and are associated with a favourable and intermediate prognosis respectively. The t(12;21) mutation is found in 25% of cases of B-ALL and results in a fusion protein that interferes with normal function of RUNX1, a transcription factor[25]. The t(1;19) mutation is identified in 6% of B-ALL cases. This abnormal fusion protein acts as an oncogene which acts as a transcriptional activator and interferes with other transcription factors coded by the two genes involvedE2A (E2A/transcription factor 3) and PBX1 (pre-B-cell leukaemia homeobox 1) [2, 26]. While originally thought to confer a poor

prognosis, more intensive chemotherapy regimens have improved the outcome and it is currently considered to have an intermediate prognosis [27].

The *MLL (*mixed lineage leukaemia) gene on chromosome 11q23 is associated with many fusion partners including *ENL (MLLT1)* (19p13) and *AF9 (MLLT3)*(9p22), however, the most common gene involved is *AF4 (MLLT2)* on chromosome 4q21. MLL rearrangements are identified in 80% of infant B-ALL cases and 10 % of B-ALL in children [28]. Patients with t(4;11);*AF4-MLL*translocation have a poor prognosis, particularly infants less than 6 months of age [2].

In the T lymphoblastic leukaemia/lymphoma group, an abnormal karyotype is identified in 50-70% of cases with a wide spectrum of genetic aberrations involving tumour suppressor genes, cell signalling pathways, cell cycle regulators and proto-oncogenes. Fifty percent of cases have mutations involving the *NOTCH1* (Notch homolog 1) gene causing constitutive activation of this signalling pathway [29]. Translocations involving *MLL* occur in 8 % of cases of T-ALL but t(4;11); *AF4-MLL* has not been reported in this subgroup [30]. The t(9;22)(q34;q11.2); *BCR-ABL1* mutation is identified in <1 % of T-ALL cases and confers a poor prognosis [31].

## 1.4 Established Diagnostic Techniques

The diagnosis of haematological malignancies requires a multidisciplinary approach with several techniques utilised, each with their own advantages and disadvantages. With the rapidly advancing molecular technologies becoming available, it is essential to evaluate these new techniques and assess their potential incorporation in the diagnostic work-up of patients with suspected leukaemia.

### 1.4.1 Non-molecular techniques

Morphology remains an integral part of leukaemia diagnosis. Cytochemical stains are of value in both acute leukaemia and myelodysplastic syndromes *e.g.* in determining granulocytic and monocytic components. Immunohistochemistry detects cells with specific antigen expression by means of monoclonal or polyclonal antibodies. These methods are labour intensive, time-consuming and require skill and experience to interpret. Flow cytometry has the same principle as immunohistochemistry with fluorochrome-labelled monoclonal antibodies detecting specific antigens on cells. Light scatter patterns are analysed in order to classify populations of cells. This method is faster, less labour intensive and analyses large numbers of cells and is particularly useful for assessing the presence of antigen co-expression and is a valuable tool for monitoring minimal residual disease due to its sensitivity [32, 33].

### 1.4.2 Conventional cytogenetics

Conventional cytogenetics remains the gold standard genetic testing method, enabling detection of not only common translocations and complex karyotypes, but also numeric aberrations and rare translocations which may not be detectable with newer molecular methods [34]. Karyotyping is however labour intensive, requires very skilled staff, is time-consuming and dependent on good quality sample and cells in metaphase [35] . Ideally twenty metaphases are analysed depending on the quality and if this is not possible, a result may be reported with the number of metaphases that could be analysed. The turnaround time (TAT) of conventional cytogenetics in our centre is up to 30 days according to the Standard Operating Procedure (SOP).

### 1.4.3 Fluorescence In Situ Hybridisation (FISH)

FISH analysis uses fluorescent labelled probes to detect specific deoxynucleic acid (DNA) sequences of interest. It allows for detection of genetic abnormalities in non-dividing cells, provides improved sensitivity and reliability and enables detection of cryptic translocations which conventional cytogenetics does not detect *e.g.* t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)* [36-38]. Owing to the use of specific primers and probes, abnormalities other than the specific translocation sought will not be detected by FISH analysis. This technique requires experience and skill particularly in interpreting co-localisation or loss of signals [32].

### 1.4.4 Polymerase Chain Reaction (PCR)

PCR is the amplification of a specific DNA target flanked by regions of a specific sequence. Primer design is therefore a crucial step in ensuring accurate diagnosis. There is also a risk of contamination and false negative results, particularly in the case of poor quality DNA. It has a greater sensitivity than FISH and cytogenetics and is less labour-intensive. This technique plays an integral role in molecular diagnostics *e.g.* in investigating clonality (T-cell receptor and immunoglobulin heavy chain (IgH) rearrangement) and detecting gene rearrangements [32].

For RT-PCR, messenger RNA (mRNA) is reverse transcribed to complementary DNA (cDNA) followed by amplification. In this way, only exons are targeted for analysis. Multiplex assays have been developed to detect several translocations in a single PCR reaction in combination with various detection methods including gel-based techniques, capillary electrophoresis, bead array or micro-array [39].

### 1.4.5 Other molecular techniques

For the purposes of this study, the focus will be on the techniques used for routine investigation of patients with leukaemia, however many more techniques are already established *e.g.* comparative genomic hybridisation, microarrays and sequencing. Next generation sequencing or massively parallel sequencing has been used to sequence entire genomes and has been particularly enlightening in the field of AML by identifying more mutations with prognostic and treatment implications *e.g.* Isocitrate dehydrogenase 1 and 2 (IDH 1 and IDH2) and DNA methyltransferase 3A (DNMT3A) [40-42].Challenges include its cost and need for sophisticated bioinformatics systems.

There is therefore a need to optimise existing diagnostic techniques to improve detection of leukaemia-associated genetic abnormalities in a cost-effective way while providing an efficient service with a rapid turnaround time.

## 1.5 Multiplex RT-PCR Assays

Multiplex RT-PCR assays offer several advantages. Multiple translocations can be detected in one assay which is often more cost-effective and less labour-intensive [43]. Since small amounts of RNA are required, these assays can be used on limited diagnostic specimens to screen for several fusion transcripts [34, 39] with the potential for a rapid TAT, being as little as five hours in one study [43]. Cryptic translocations not detectable by conventional cytogenetics can be identified by the multiplex assays. There are several commercial assays available currently with varied performance characteristics for the classification of leukaemias. These include the Signature® LTx v2.0 assay (LTx; Asuragen, Austen, TX) and the HemaVision® 7Q (DNA Technoglogy A/S, Aurhus, Denmark) assays which will be discussed below.

### 1.5.1 Signature® LTx v2.0 assay

In the Signature® LTx v2.0 assay, multiplex RT-PCR is combined with multiplex fluorescent bead-array detection. Ribose nucleic acid (RNA) is reverse transcribed to cDNA and amplified by multiplex PCR. The PCR product is then hybridised to beads bearing oligonucleotide probes specific for each translocation and a reporter molecule is introduced with the fluorescence then measured by flow cytometry (Table 1.2). The median fluorescence intensity (MFI) is calculated for each and a qualitative result obtained [44]. Chromosome regions tested are the same as those targeted by FISH probes at our centre [45] and are represented in Table 1.2. Probes used in FISH analysis are the LSI (Locus Specific Identifier) BCR/ABL ES (Extra Signal) dual Colour translocation Probe (Vysis, Abbott, Illinois, USA), LSI PML/RARA Dual Colour Translocation Probe (Vysis), LSI TEL/AML1 ES Dual Colour Translocation Probe (Vysis), LSI MLL Dual Colour Probe (Vysis), LSI TCF3/PBX1 dual colour, dual fusion DNA probe and the LSI CBFB inv(16) dual colour probe.

The Signature® LTx assay was selected for evaluation since the translocations included correlate with the FISH analysis probes used at our centre in the diagnostic investigations of patients presenting with suspected leukaemia *e.g.* a new paediatric patient diagnosed with B-cell ALL will be tested for t(12;21)(p13;q22) *TEL-AML1 (ETV6-RUNX1)*, t(1;19)(q23;p13.3) *E2A-PBX1 (TCF3-PBX1),* t(4;11) *AF4-MLL*and t(9;22)(q34;q11.2) *BCR-ABL.* The turnaround time for FISH analysis in our centre is 3-10 days for smears and for conventional cytogenetics, 30 days are acceptable according to the SOP. Testing for those four translocations in one multiplex assay could potentially improve turnaround times as well as total cost considering the price per FISH probe. These tests could potentially be performed by staff with a more readily available skill set.

**Table 1.2: Signature® LTx Leukaemia Translocation Panel v2.0***

| Classification | Translocation | Fusion transcript and Specific variant |
|---|---|---|
| **CML** | t(9;22)(q34;q11) | BCR/ABL1 (b2a2)<br>BCR/ABL1 (b3a2) |
| **ALL** | t(9;22)(q34;q11) | BCR/ABL1 (e1a2) |
| | t(12;21)(p13;q22) | TEL/AML1 (e5/e2) |
| | t(1;19)(q23;p13) | E2A/PBX1 (e13/e2) |
| | t(4;11)(q21;q23) | MLL/AF4 (e9/e5)<br>MLL/AF4 (e10/e4) |
| **APL** | t(15;17)(q24;q21) | PML/RARα (L form, bcr1)<br>PML/RARα (S form, bcr3) |
| **AML** | Inv(16)(p13;q22) | CBFB/MYH11 (A type, e5e12)<br>CBFB/MYH11 (D type, e5e8) |
| | t(8;21)(q22;q22) | AML1/ETO (e5/e12) |

### 1.5.2 HemaVision® 7Q

The HemaVision® 7Q is an RT-PCR based multiplex assay for qualitative screening of 7 frequently identified translocations involved in CML and Acute Leukaemia as shown in Table 1.3 below. RNA is extracted from whole blood or bone marrow followed by cDNA synthesis and qualitative PCR analysis. An Internal Amplification Control is included in the assay, as well as a reference gene Abelson (ABL1) as a control. If fluorescence exceeds the threshold level before cycle 35 (Ct<35), the sample is considered positive for the translocation. The primers in this assay are designed to detect multiple breakpoints and splice variants [46].

This assay has been evaluated and demonstrated to be cost-effective and showed good correlation with routine diagnostic test results, in some cases detecting translocations which were not identified by other laboratory assays. These translocations were subsequently confirmed using conventional cytogenetics [47].

The HemaVision ®7Q was initially included in our study protocol, but owing to funding limitations, only one multiplex assay was chosen for evaluation at our centre. Both assays are comparable, but the Signature® LTx was chosen due to cost per assay.

**Table 1.3: Translocations included in the HemaVision® 7Q assay (adapted from HemaVision® 7Q package insert [46])**

| Translocations |
|---|
| t(1;19)(q23;p13)(TCF3-PBX1) |
| t(4;11)(q21;q23)(MLL-AFF1) |
| t(8;21)(q22;q22)(RUNX1-RUNX1T1) |
| t(9;22)(q34;q11)(BCR-ABL1) – p210, p190 and p230 isoforms |
| t(12;21)(p13;q22)(ETV6-RUNX1) |
| t(15;17)(q24;q21)(PML-RARA) – L and S isoforms |
| inv(16)(p13;q22)(CBFB-MYH11) |

## 1.6 Aim and Objectives

The aim of the study was to compare the multiplex RT-PCR platform Signature® LTx with FISH analysis and conventional cytogenetics in the diagnosis of common fusion genes implicated in leukaemia. The hypothesis was that this assay is not inferior to gold standard diagnostic methods at Charlotte Maxeke Johannesburg Academic Hospital and offers additional advantages of improved turnaround time and cost-efficiency.

The objectives were

1. To test samples of patients being investigated for acute leukaemia or CML using the commercially available Multiplex RT-PCR assay Signature® LTx. The results will be compared to results obtained using FISH and conventional cytogenetics analysis.
2. To assess the accuracy and usefulness of multiplex RT-PCR methods for the routine molecular detection of common leukaemia-associated translocations in our setting and thereby determine the feasibility of incorporating the Signature® LTx assay with FISH analysis in the diagnostic work-up of patients with leukaemia.
3. To compare the TAT and cost-effectiveness of the Signature® LTx panel and FISH analysis at our centre.

# 2) Materials and Methods

## 2.1 Patient samples

This was a prospective study and sample collection was commenced in June 2013 at CHBAH CMJAH, DGMC, from private Clinical Haematologists in Johannesburg as well as Steve Biko Academic Hospital (SBAH) as part of our collaboration with the University of Pretoria. A total of 112 samples were collected with the query of having the diagnosis of leukaemia. Funding was available for four assays, each analysing 21 samples therefore a total of 84 samples could be tested. Since some samples had to be discarded throughout the stages of analysis, sample collection was extended in order to complete four assays. Reasons for discarding samples will be discussed below.

The bone marrow or peripheral blood samples were taken by the attending paediatricians/oncologists after obtaining informed consent during the routine investigation of new patients presenting with suspected ALL, AML, APL and CML.

Exclusion criteria for sample collection were

- No informed consent obtainable from the patient or parent/legal guardian
- Difficult aspirate with limited material for routine diagnostic techniques including flow cytometry, FISH and cytogenetics analysis

Patient demographics and results were obtained from the laboratory information system (LIS) and included gender, age, sample type (peripheral blood, bone marrow aspirate), cytogenetics and FISH analysis results as well as the final diagnosis. Peripheral blood or bone marrow aspirate samples were collected in PAXgene RNA tubes (PreAnalytix, Qiagen) and transported to the PCR laboratory at CMJAH for storage at -20°C.

PAXgene tubes require 2.5 mL of bone marrow aspirate or peripheral blood sample and can be stored for three days at room temperature (15-25$^{o}$C), up to five days at 2-8$^{o}$C and for 8 years at -20$^{o}$C or -70$^{o}$C according to the manufacturer's specifications.

## 2.2 RNA Extraction and quantification

Initially RNA was extracted from peripheral blood or bone marrow specimens using the NucliSENS® easyMAG® (bioMerieux,Lyon, France) as per the standard of practice for our laboratory (HAEM1564). Specimens were added to NucliSENS® Lysis Buffer containing guanidine thiocyanate and Triton X-100 which inactivates RNases and DNases in the specimen. Magnetic silica added to the lysate initiates the isolation process. Nucleic acids present in NucliSENS® Lysis Buffer bind the magnetic silica dioxide particles under high salt conditions. The magnetic silica is then washed and nucleic acids are eluted form the solid phase making them available for use in downstream applications. This technique yielded suboptimal RNA quantities from three test samples and an alternative manual extraction technique using the PAXgene Blood RNA Kit (PreAnalytix, Qiagen, Limburg, Netherlands) was used for the remainder of the samples.

The samples were equilibrated and stored for at least 2 hours at room temperature to ensure blood cell lysis via the cell lysis buffer already in the tube. All necessary buffers were reconstituted and kept at the prescribed temperatures.

PAXgene tubes (PreAnalytix, Qiagen, Limburg, Netherlands) were centrifuged for 10 minutes at 3000-5000 x g (times gravity) using a swing-out rotor (Digicen 21R, Ortoalresa, Madrid, Spain). The supernatant was removed and 4 mL (millilitres) RNase-free water added to the pellet. The tubes were vortexed until the pellet was visibly dissolved and centrifuged again for 10 minutes at 3000-5000 x g. The supernatant was discarded again and 350 µl (microliters) resuspension buffer was added, followed by vortexing.

The samples were then transferred to 1.5mL microcentrifuge tubes and 300 µl binding buffer and 40 µl proteinase K added. The samples were vortexed and incubated for 10 minutes at 55°C using a shaker-incubator at 400-1400 rpm (revolutions per minute) (FMH instruments, B&M Scientific cc, Helderberg, South Africa). The lysate was then transferred into a PAXgene Shredder spin column and centrifuged for 3 minutes at maximum speed (not exceeding 20 000 xg) (Eppendorf, Hamburg, Germany). The supernatant was transferred to a fresh microcentrifuge tube without disturbing the pellet. Three hundred and fifty microliters of ethanol (100 % purity grade p.a. pro analysi) was added to the tube, followed by vortex and brief centrifuge (1-2 seconds at 500-1000 x g). A portion of this sample (700 µl) was pipetted into a PAXgene RNA spin column and centrifuged for 1 minute at 8000-20 000 xg.

The spin column was placed in a new 2mL processing tube and the old processing tube containing flow-through was discarded. The remaining sample was pipette into the PAXgene RNA spin column and centrifuged again for 1 minute at 8000-20 000 xg. A new processing tube was used again and the old one discarded. Wash buffer 1 (350 µl) was added to the RNA spin column and centrifuged for 1 minute at 8000-20 000 xg. The spin column was placed in a new processing tube and the old one discarded.

DNase I stock solution (10 µl per sample) and DNA digestion buffer (70 µl per sample) were added to a 1.5 mL microcentrifuge tube and gently mixed. Eighty microliters of the incubation mix was placed onto the PAXgene RNA spin column membrane and left at room temperature for 15 minutes.

This was followed by a series of wash and centrifuge steps as demonstrated in Figure 2.1 below:

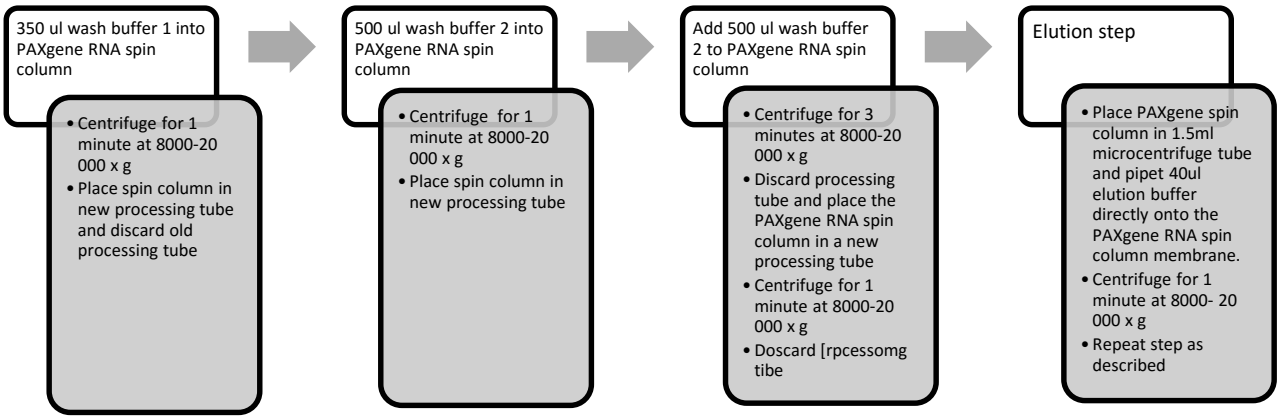



**Figure 2.1: Wash and centrifuge steps of RNA extraction using the PAXgene Blood RNA Kit (PreAnalytix, Qiagen) manual method (adapted with permission from Asuragen package insert [44])**

The eluate was incubated for 5 minutes at 65°C (FMH instruments, B&M Scientific cc, Helderberg, South Africa) and the samples were transferred to ice and either stored at -20°C or taken to the laboratory for RNA quantitation and subsequent RT-PCR steps as shown in Figure 2.2.

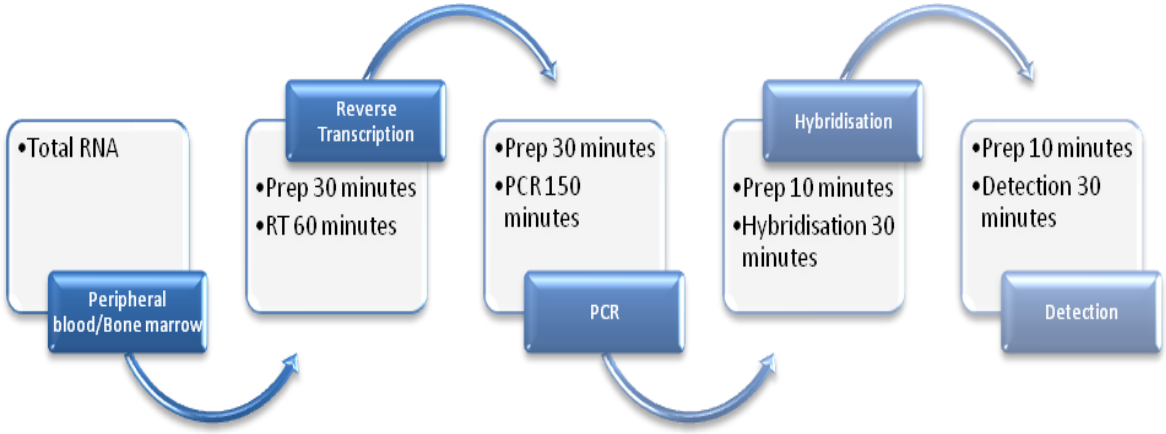



**Figure 2.2: Signature® LTx workflow adapted from Asuragen package insert [44].**

(RT – Reverse Transcription; Prep – Preparation; PCR – Polymerase Chain Reaction; Total time estimate ~6 hours)

Multiplexed translocation analysis testing for the fusion transcripts and an endogenous control transcript glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was performed using the four steps: reverse transcription, DNA amplification, hybridisation and detection (Figure 2.3).

RNA quantitation was performed using the Nanodrop spectrophotometer (Thermo Scientific, Massachussetts, USA). According to the PAXgene RNA Kit handbook using the manual protocol, $A_{260}/A_{280}$ ratio of sample absorbance values are recommended to fall between 1.8 and 2.2. The recommended RNA concentration is 70 ng/µl [48] but for the purposes of the study, samples were only discarded if the concentration was below 20 ng/µl with a note made of the suboptimal quantity and compared to the results of the gold standard reference techniques.

## 2.3 RT-PCR, DNA amplification and hybridisation

Once RNA concentration had been determined, we performed reverse transcription of 500ng of RNA to cDNA and amplification by multiplex PCR using the GeneAMP PCR System 9700 Thermocycler (Applied Biosystems, Invitrogen, USA). A more detailed description of the steps involved is given in Figure 2.3.

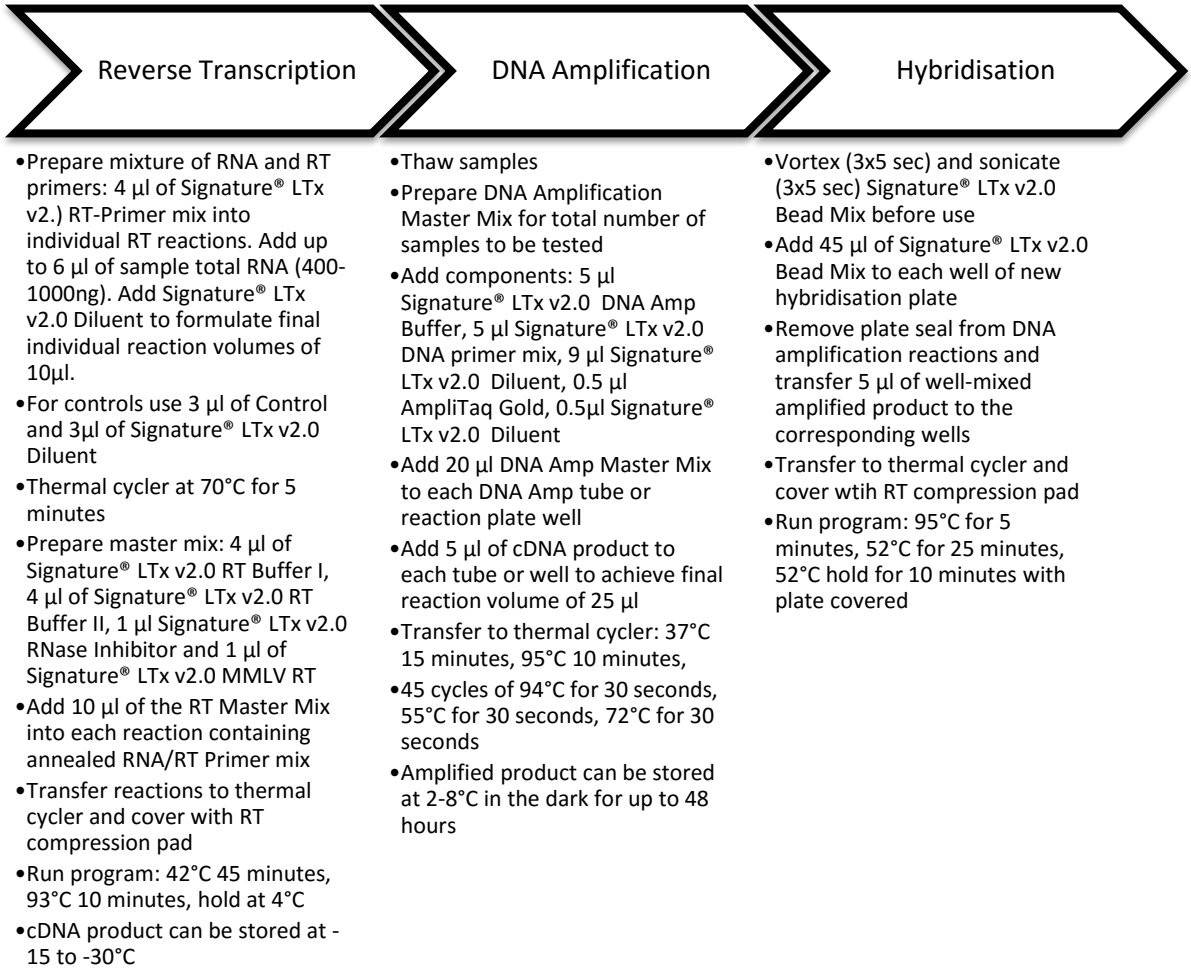



**Figure 2.3: Detailed Workflow for steps 1-3: Reverse transcription, DNA Amplification and Hybridisation (Adapted from Asuragen package insert [44]).**

(MMLV RT – Moloney murine leukaemia virus reverse transcriptase; AMP – amplification)

A total of 5µl PCR product was hybridised to a mixture of 12 probes conjugated to 12 unique carboxylated xMAP beads (Luminex Corp., Austin, TX, USA). This was done using the Bio-Rad Bio-Plex™ 200 instrument and Bio-Plex Manager™ software (Luminex, Texas, USA) for analysis. A minimum of 50 beads for each fusion transcript and the endogenous control GAPDH was analysed to determine the MFI signal for each probe. The positive, negative and no RNA controls included in the assay was included in every run. The Signature® LTx negative control contains only the GAPDH transcript and the positive control is an RNA sample containing the GAPDH transcript and all fusion transcripts detected by the assay [44].

After completion, output files were converted to Excel spreadsheet and results analysed based on MFI cut off as per the manufacturer's instructions [44]

## 2.4 Analysis of Signature® LTx results

Samples were designated as negative or positive for a translocation relevant to the Signature® LTx multiplex RT-PCR assay as determined by cytogenetics and FISH analysis *e.g.* a sample showing MLL rearrangement with t(10;11) on cytogenetic analysis was still classified as negative as the multiplex assay can only detect the t(4;11) *AF4-MLL* fusion partner. The results of the FISH analysis and cytogenetics results were then compared to the transcripts identified by our multiplex assay with an MFI of 350 as the cut off signal for the presence of a specific fusion transcript as per the manufacturer's specifications and confirmed by other multicentre validations of the assay [48]. Mean MFIs for all positive and negative transcripts were also calculated.

According to the manufacturer's protocol, an invalid control result (Table 2.1) would include a positive control with any fusion transcript <350 MFI or GAPDH <1000 MFI; a negative control with any fusion transcript >350MFI or GAPDH <1000 MFI or a bead count for any bead <50 [44]. These results would require re-run of the plate from the reverse transcription step or the hybridisation step in the case of a low bead count. For research purposes however, we could not repeat testing owing to limited assays and 'invalid' control results were still accepted for analysis with description of the problem encountered. Valid control results would include a positive control with fusion transcripts >350MFI and GAPDH >1000 MFI and a negative control with fusion transcripts <350MFI and GAPDH of >1000 MFI.

**Table 2.1: Control results - Description of invalid and valid control results as per manufacturer's recommendation adapted from Asuragen package insert [44]).**

| Run | Sample | Result | Re-test |
|---|---|---|---|
| **Invalid (One or more controls are invalid)** | Positive control | Fusion transcript <350 MFI or GAPDH < 1000 MFI | Re-run from reverse transcription step* |
| | Negative control | Fusion transcript >350 or GAPDH < 1000 MFI | Re-run from reverse transcription step |
| | Any LTx control | Low bead count for any bead | Re-run from hybridisation step |
| **Valid control result** | Positive control | All fusion transcripts >350 MFI and GAPDH > 1000 MFI | N/A |
| | Negative control | All fusion transcripts <350 MFI and GAPDH > 1000 MFI | N/A |

(* This was not done for the purposes of this evaluation and all results were included for analysis, for reasons provided above.)

For diagnosis runs would be deemed invalid if there is a low bead count for any bead or all fusion transcripts of <350MFI and GAPDH <1000 MFI (Table 2.2). This would require a re-run from the hybridisation step in the case of a low bead count; or a re-run from the reverse transcription step or re-extraction of RNA from the same sample in the case of GAPDH <1000 MFI. For our purposes, a GAPDH signal of <1000 MFI was still accepted and the result was compared with those of cytogenetics and FISH analysis for any discrepancies. A tested sample would be called positive for a fusion transcript if a MFI of greater than 350 MFI is generated and negative if all fusion transcripts are <350 MFI and GAPDH >1000 MFI [44].

Genetic mutations other than those included in the Signature® LTx Multiplex RT-PCR assay were also documented in order to recommend alterations or additions to the assay.

**Table 2.2: Sample results - Description of valid and invalid patient sample results as per manufacturer's recommendation as adapted from Asuragen package insert [44].**

| Run | Sample | Result | Interpretation | Re-test |
|---|---|---|---|---|
| **Valid (Bead count for every control bead >50 and all control results valid)** | Tested sample | All fusion transcripts <350 MFI and GAPDH < 1000 | Invalid samples result | Re-run from reverse transcription or re-extract RNA from same/new specimen |
| | | Low bead count for any bead | Invalid sample result | Re-run from hybridisation step |
| | | Any fusion transcript >350 MFI | Fusion transcript detected | N/A |
| | | All fusion transcripts <350 MFI and GAPDH > 1000 MFI | Fusion transcripts not detected | N/A |

## 2.5 Fluorescence in situ hybridisation

FISH was performed by the staff of the Somatic Cell Genetics Unit in the NHLS, CMJAH as per standard operating procedure (SOP HAE0234).

### 2.5.1 Sample preparation and pre-treatment

Peripheral blood or bone marrow collected in an ethylenediaminetetra-acetic acid (EDTA) or heparin tube is the ideal material, alternatively unstained smears can be used. The sample should be processed as soon as possible, preferably within 2 days. The sample is centrifuged for 10 minutes at 1500 rpm. With a glass Pasteur pipette, a drop of buffy coat about 2 mm in diameter is placed 10 mm from the frosted area and spread with a spreader at $30^{o}$ angle. The smear dries for 10 minutes at room temperature.

The slides are placed in Coplin jars filled with methanol for 30 minutes at $-20^{o}C$ followed by 2 hours in a fixative filled Coplin jar. The slides are dehydrated in a series of ethanol solutions graduating from 70 %, 90 % and 100 % for 5 minutes each at room temperature. Slides are placed in protease solution for 10 minutes at $37^{o}C$, washed and fixed in 1 % formaldehyde for 5 minutes. After another wash, the slides are dehydrated again in 70, 90 and 100 % ethanol for 1 minute each.

### 2.5.2 Hybridisation

Slides are dried and warmed at 60$^{o}$C for 5 minutes. They are then placed in a denaturing solution for 5 minutes in a water bath set at 76$^{o}$C. The slides are then transferred into ice cold ethanol (70 %, 90 % and 100 %) for 5 minutes each on a shaker and dried in an upright position. Probes are prepared just before the denaturing step and denatured at 76$^{o}$C for 5 minutes while the slides are in 90% ethanol on the shaker. Ten microlitres of probe mixture is added onto the slide with a pipette and a coverslip is applied followed by sealing with rubber cement. Slides are then incubated at 37$^{o}$C overnight.

### 2.5.3 Washing

After incubation, the rubber cement is removed and the slides are treated with several washing steps as well as addition of 50 µl DAPI (4', 6-Diamidino-2-Phenylindole double stranded DNA staining). The slides are kept in the dark and analysed with a fluorescent microscope with various filters and objectives for analysis.

### 2.5.4 Analysis and reporting results

One to two hundred cells are analysed by a trained technologist/scientist and checked by a senior member of staff. Findings are recorded on an analysis sheet and reported as a percentage. Nomenclature is used according to the (SICN) 2013 International System for Human Cytogenetic Nomenclature.

## 2.6 Conventional Cytogenetics

Testing and analysis of all samples were performed by the Somatic Cell Genetics Unit in the NHLS, CMJAH as per standard operating procedure (SOP HAE0221 and HAE0224).

### 2.6.1 Initiation of culture

One to five millilitres of peripheral blood collected in a sodium heparin vacutainer or 1.0-2.0mL of fresh bone marrow obtained from the initial aspirate are used for testing. Cultures were performed within 24 hours of bone marrow collection. The specimen is centrifuged in transport media tube at 1500 rpm for 10 minutes at room temperature followed by removal of most of the supernatant. The remaining pellet is re-suspended with the remaining supernatant. Fifteen to thirty drops (0.25-0.50 mL) of suspension is added to sterile 50 mL culture flasks containing 3-5 mL of supplemented medium. The flasks are incubated at 37$^{o}$C overnight.

### 2.6.2 Harvesting

For harvesting, the culture is centrifuged at 1500 rpm for 8-10 minutes and the supernatant removed. Colcemid (0.1 mL) and warm potassium chloride (KCl) (6.0 mL) are added followed by mixing and incubation for 25 minutes. Three drops of cold 3:1 methanol-ascetic acid (fixative) is added followed by mixing and centrifugation. After the supernatant has been removed, the pellet is re-suspended and 5mL of cold 3:1 fixative is added followed by thorough mixing. Second and third fix and centrifuge steps follow and the tubes can be stored at -20$^{o}$C until slide making is required.

### 2.6.3 Slide preparation

Slide preparations involved dropping harvested cell suspensions on a slide flooding with fixative and air-drying. The slide is flooded with fresh 3:1 methanol-acetic acid fixative followed by 3 drops of cell suspension. The slide is steamed and aged in the oven at 90$^{o}$C for 45 minutes to 2 hours. Slides are dipped in room temperature trypsin for 5-60 seconds and rinsed briefly in Coplin

jars containing 0.9% NaCl. Trypsin interacts with the protein-DNA complex and creates a staining differential between condensed and less condensed sections. Finally, the slide is stained for 1-3 minutes with Wright's stain-buffer solution (0.1 mL stain and 4.0 mL buffer) followed by rinsing and blot drying with tissue paper. Wright's stain creating a pattern of light and dark bands. Each chromosome has a characteristic pattern for identification.

Adequately prepared slides should yield well spread mitotic figures for G0banding and chromosome analysis.

### 2.6.4 Analysis and reporting of results

Slides are examined for metaphases and if the metaphase spread is suitable, a more detailed examination is performed. For photography and karyotyping, a semi-automated chromoscan system (Cytovision, Leica Biosystems, IL, USA) and the karyotype is reported with tabulation of cells analysed, chromosome counts and karyotyping according to the International System for Human Cytogenetic Nomenclature (ISCN 2005). Twenty metaphases are analysed ideally, if fewer are obtained this is stated in the report.

## 2.7 Statistical analysis

Percent agreements were calculated using the GraphPad Prism version 4.00 for Windows (GraphPad Softward, San Diego, CA), comparing the qualitative results with the gold standard cytogenetics and FISH analysis. Quantitative analysis of the signal distributions for each type of signal; positive, negative and endogenous control was done using box plots. Analysis of variance (ANOVA) testing was used to calculate significant difference in means of the positive, negative and internal controls and a *post hoc* analysis was done to determine between which means there is a difference in order to ensure correct cut off values in terms of mean fluorescence intensity and determine the robustness of the assay with regards to the GAPDH as internal control. The mean MFI signals for all positive and all negative transcripts were also calculated.

# 3) Results

## 3.1. Patient samples

A total of 112 samples were collected with the suspected diagnosis of leukaemia. Of these, 6 were discarded once a diagnosis other than ALL, AML, CML and APL was confirmed as shown in Figure 3.1. The remaining 106 peripheral blood and bone marrow aspirate samples were stored for analysis in batches. The details of all collected samples are shown in the Table 3.1 below. Thirty five (34.31%) peripheral blood and 67 (65.69 %) bone marrow aspirate samples were collected.

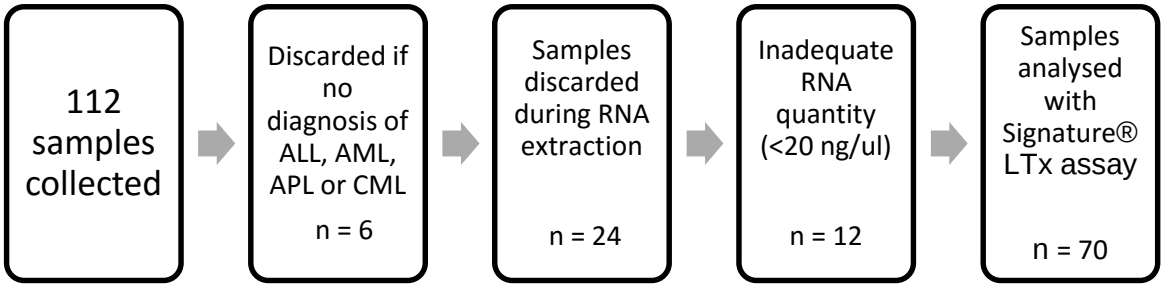



**Figure 3.1: Flow diagram of all samples collected and analysed.**

A total of 112 samples were collected, six discarded with diagnoses other than ALL, AML, APL or CML. A further 24 samples were discarded during RNA extraction and 12 were considered of inadequate RNA quantity. A final total of 70 samples could yield results for comparison to FISH and cytogenetics results.

Fifty three (51.96 %) male patients and 49 (48.04 %) female patients agreed to participate in the study (Table 3.1). Of the samples, 55 (53.92 %) were from paediatric oncology units (0-18 years) and 47 (46.08 %) from the adult oncology patients (18 years and older). The final diagnoses after initial investigations were 9 CML cases (8.82 %), 30 B-cell ALL cases (29.41 %), 12 T-cell ALL cases (11.76 %), 2 cases of ambiguous lineage leukaemia (1.96 %), 11 cases of APL (10.78 %) and 38 cases of AML (37.25 %) as shown in Table 3.2.

These numbers correlated with those collected from the flow cytometry register in the case of APL, AML and T-ALL, however our sample group had more cases of B-ALL and fewer cases of CML.

**Table 3.1: Details of all collected samples (n = 106)**

| **Gender** | **Number (%)** |
|---|---|
| Male | 57 (53.77) |
| Female | 49 (46.23) |
| **Age, n (%)** | |
| Paediatric (0-18 yrs) | 55 (51.89) |
| Adult (>18 yrs) | 51 (48.11) |
| **Sample type** | |
| Peripheral blood (PB) | 35 (33.02) |
| Bone marrow aspirate (BM) | 71 (66.98 |

All patients had either cytogenetics or FISH analysis results on the laboratory information system (Figure 3.2). Cytogenetics results were available for 64 of the 70 patients. In two cases, no cytogenetics analysis was requested and in four cases, there were no adequate metaphases for analysis. All six cases had FISH analysis results, with a translocation or deletion detected in four patient samples. Of all the cytogenetics results, less than 20 metaphases were analysed in 35 cases. FISH analysis was not requested or had been performed at the referral institute before transfer of the patient in four cases and cytogenetics results were available for all these patients.

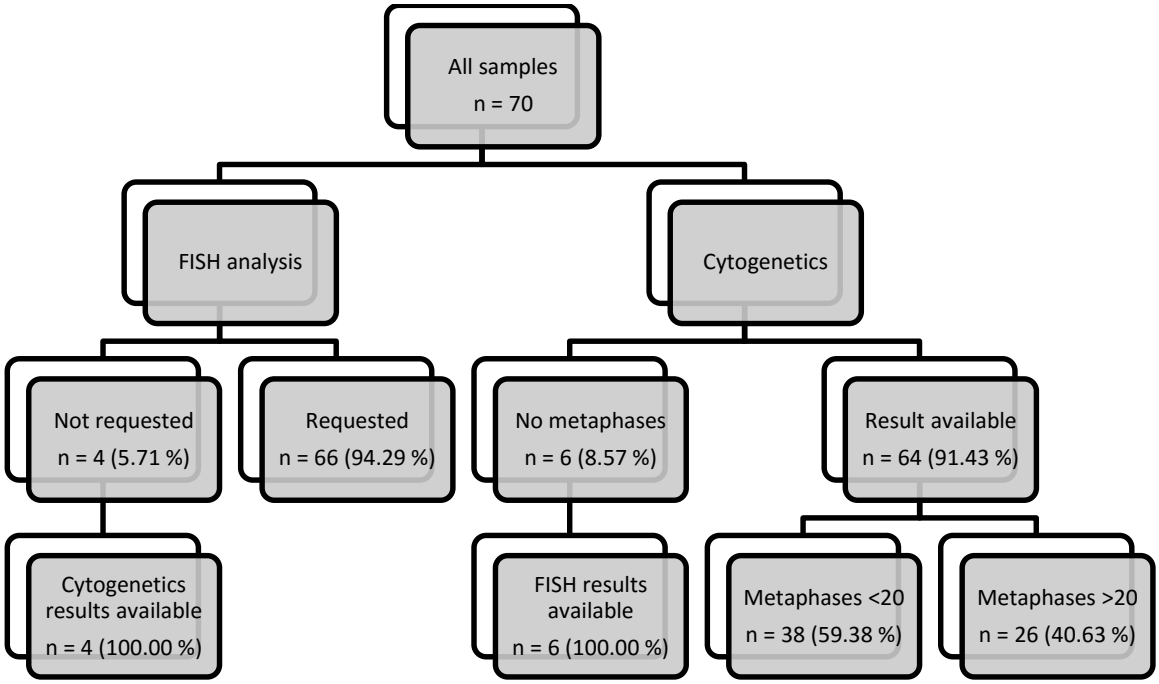



**Figure 3.2: Cytogenetics and FISH analysis availability for all samples analysed using the multiplex assay**

Of all the samples, FISH analysis was available for 66 cases and in the four where FISH was not requested, a cytogenetics report was issued. In the 70 samples analysed, cytogenetics results were available for 64, of which 38 had fewer than the ideal twenty metaphases.

For the translocations included in the Signature® LTx multiplex RT-PCR assay, a total of 51 cases were positive by FISH or cytogenetic analysis including 10 cases of t(8;21)(q22:q22); *RUNX1-RUNX1T1*, 3 cases of inv (16)(p13.1q22); *CBFB-MYH11* and 11 cases of t(15;17)(q24;q21); *PML-RARa*. In the ALL category, 9 cases of t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)*, 3 cases of t(9;22)(q34;q11.2); *BCR-ABL1*and five cases of t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)* were identified. In keeping with the nine diagnoses of CML, nine t(9;22)(q34;q11.2); *BCR-ABL1* mutations were positive using FISH or cytogenetic analysis (Table 3.2).

The samples were both extracted and run in batches from June to end of July 2014. Since samples had to be discarded during and after the extraction process, an additional assay remained and sample collection was extended until December 2014 with the final analysis completed in the same month.

**Table 3.2 Details of patient diagnosis, conventional cytogenetics and FISH analysis results (relevant to Signature® LT Multiplex RT-PCR assay) of all collected samples (n = 102)**

| Diagnosis Total number, n (%) | Chromosomal target | Fusion transcript | Number (%) |
|---|---|---|---|
| **Chronic myeloid leukaemia, 9 (8.82)** | t(9;22)(q34;q11) | *BCR-ABL1* | 9 (8.82) |
| **Acute promyelocytic leukaemia, 11 (10.78)** | t(15;17)(q24;q21) | *PML-RARα* | 11 (10.78) |
| **Acute myeloid leukaemia, 38 (37.25)** | t(8;21)(q22;q22) | *RUNX1-RUNX1T1* | 10 (9.80) |
| | inv(16)(p13;q22) | *CBFB-MYH11* | 3 (2.94) |
| | Other mutations | | 25 (24.51) |
| **Acute lymphoblastic leukaemia,* 42 (41.18)** | t(9;22)(q34;q11) | *BCR-ABL1* | 3 (2.94) |
| | t(12;21)(p13;q22) | *ETV6-RUNX1 (TEL-AML1)* | 5 (4.90) |
| | t(1;19)(q23;p13) | *TCF3-PBX1 (E2A-PBX1)* | 9 (8.82) |
| | Other mutations | | 25 (24.51) |
| **Ambiguous lineage acute leukaemia, 2 (1.96)** | | | 2 (1.96) |

[* B-cell Acute Lymphoblastic Leukaemia n=30 (29.41), T-cell Acute Lymphoblastic Leukaemia n = 12 (11.76)]

## 3.2 RNA extraction and quantification

Total RNA was initially isolated from peripheral blood or bone marrow specimen using the NucliSENS® easyMAG® (bioMerieux,Lyon, France). This however yielded suboptimal RNA quality and quantity despite troubleshooting and a manual extraction kit PAXgene Blood RNA Kit (PreAnalytix, Qiagen) was ordered. The manual extraction yielded better RNA concentration (ng/µl) and quality ($A_{260}/A_{280}$ ratio of sample absorbance). During RNA extraction however, 24 of the 102 samples (23.53%) were discarded as they became viscous after the incubation step at 55°C (Figure 3.1). Furthermore, an additional 12 out of 102 samples (11.76 %) could not be used in the RT-PCR step as the RNA concentration was below 20 ng/µl (Figure 5). Although the manufacturer's instructions recommend a target of 70 ng/µl, samples between 20 and 70 ng/µl were included for analysis. All the samples met the criterion of absorbance value $A_{260}/A_{280}$ ratio

between 1.8 and 2.2. This resulted in 70 samples meeting the required criteria for analysis. Of note was that samples which had been stored for less than six months yielded better RNA concentration and were less likely to be discarded during the extraction process (Table 3.3).

**Table 3.3: Number of samples discarded after 1 year of storage (June-July 2014) and after 6 months of storage (December 2014)**

| | Analysis (June – July 2014) | Analysis (December 2014) |
|---|---|---|
| **Number of samples discarded (% of total samples collected)** | 33 (32.35) | 3 (2.94) |
| **Median RNA concentration (ng/µl)** | 107.3 | 205.2 |

## 3.3 RT-PCR, DNA amplification and hybridisation

Samples were run and analysed and the results compared with those obtained by FISH and cytogenetics analysis. Forty nine samples (70.00 %) were bone marrow aspirate and 21 (30.00 %) were peripheral blood (Table 3.3). The samples analysed were collected form 40 males and 30 females. Thirty nine paediatric samples could be included in the run and 31 adult samples with 4 cases of CML, 23 cases of B-cell ALL, 5 cases of T-cell ALL, 1 case of ambiguous lineage acute leukaemia, 6 APL cases and 27 AML cases (Table 3.4).

Despite having to discard samples, a good representation of all included translocations was still achieved including 8 samples with t(8;21)(q22:q22); *RUNX1-RUNX1T1*, 2 cases of inv (16)(p13.1q22); *CBFB-MYH11*, 6 cases of t(15;17)(q24;q21;) *PML-RARα*, 8 cases of t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)*, 1 case of t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)*, 2 cases of t(9;22)(q34;q11.2); *BCR-ABL1*in the setting of ALL and 4 cases of t(9;22)(q34;q11.2); *BCR-ABL1*with a diagnosis of CML (Table 3.5).

**Table 3.4: Details of all analysed samples (n = 70)**

| | |
|---|---|
| **Gender, n (%)** | |
| Male | 40 (57.14) |
| Female | 30 (42.86) |
| **Age, n (%)** | |
| Paediatric (0-18 yrs) | 39 (55.71) |
| Adult (>18 yrs) | 31 (44.29) |
| **Sample type** | |
| Peripheral blood (PB) | 21 (30.00) |
| Bone marrow aspirate (BM) | 49 (70.00) |

**Table 3.5 Details of patient diagnosis, conventional cytogenetics and FISH analysis results (relevant to Signature® LT Multiplex RT-PCR assay) of all analysed samples (n = 70)**

| Diagnosis Total number, n (%) | Chromosomal target | Fusion transcript | Number (%) |
|---|---|---|---|
| **Chronic Myeloid Leukaemia, 4 (5.71)** | t(9;22)(q34;q11) | *BCR-ABL1* | 4 (5.71) |
| **Acute Promyelocytic Leukaemia, 6 (8.57)** | t(15;17)(q24;q21) | *PML-RARα* | 6 (8.57) |
| **Acute Myeloid Leukaemia, 31 (44.29)** | t(8;21)(q22;q22) | *RUNX1-RUNX1T1* | 8 (11.43) |
| | inv(16)(p13;q22) | *CBFB-MYH11* | 2 (2.86) |
| | Other mutations | | 21 (30.00) |
| **Acute Lymphoblastic Leukaemia,* 28 (40.00)** | t(9;22)(q34;q11) | *BCR-ABL1* | 2 (2.86) |
| | t(12;21)(p13;q22) | *ETV6-RUNx1 (TEL-AML1)* | 1 (1.43) |
| | t(1;19)(q23;p13) | *TCF3-PBX1 (E2A-PBX1)* | 8 (11.43) |
| | Other mutations | | 17 (24.29) |
| **Ambiguous lineage acute leukaemia, 1 (1.43)** | | | 1 (1.43) |

[* B-cell Acute Lymphoblastic Leukaemia n=23 (32.86), T-cell Acute Lymphoblastic Leukaemia n = 5 (7.14)]

## 3.4 Analysis of Signature® LTx results

After testing was completed, Excel spreadsheets were generated and data captured with regards to controls and transcripts. The GAPDH signals of less than 1000 MFI were still accepted for analysis and a cut-off of 350 MFI was used to determine positive transcripts. These results were then compared to cytogenetics and FISH analysis results. Of the 70 samples suitable for analysis, FISH/cytogenetics detected 31 translocations corresponding to those included in the Signature® LTx Multiplex RT-PCR assay (Figure 3.3). Of these, the result was confirmed using the Signature®

LTx Multiplex RT-PCR assay in 25 cases (81.00 %) with discordant results in 6 samples (19.00 %). In the 39 cases of no detection of one of the 12 multiplex assay translocations by FISH/cytogenetics analysis, 35 (90.00 %) were also negative using the Signature® LTx Multiplex RT-PCR assay where it detected 4 translocations not demonstrated with gold standard genetic testing techniques.

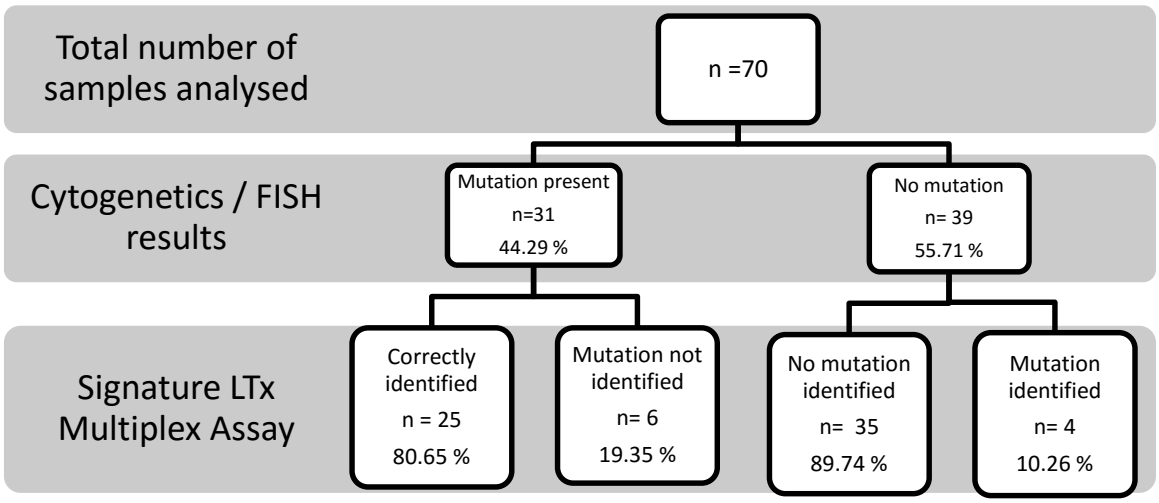



**Figure 3.3: Flow chart of samples evaluated. A mutation was considered to be present if it was identified on FISH/cytogenetics and it was included in the multiplex PCR kit as shown in Table 1.2.**

Tables 3.6-3.7 show a summary of all concordant (n = 60) results and Table 3.8 all discordant (n = 10) results including information on the RNA quantity, quality and GAPDH signal. In three of the four runs all the controls passed, however in the third analysis the positive control GAPDH was less than 1000 MFI (please refer to notes in Table 2.1). In this same run, 14 of the 21 samples had GAPDH levels <1000, however of these, only two results were discrepant (BP12 and GA18 in Table 3.8) with those reported on FISH or cytogenetics analysis. In some runs, samples (n = 10) were included despite not meeting the RNA concentration limit of 70 ng/µl. Of these samples, results correlated with gold standard methods result in seven cases.

A total of 10 cases as described in the flow diagram above did not correlate with each other as shown in Table 3.8 - mutation not identified by the Signature® LTx Multiplex RT-PCR assay (n=6) and mutation identified by the Signature® LTx Multiplex RT-PCR assay which had not been detected by FISH or cytogenetics (n=4).Missed mutations included inv(16) (p13.1q22) *CBFB-MYH11*, t(15;17)(q22;q12); *PML-RARA* and t(9;22)(q34;q11.2) *BCR-ABL1* and three t(1;19)(q23;p13.3) *E2A-PBX1 (TCF3-PBX1)* mutations.

A borderline of MFI (365) was detected in one case for mutation t(8;21)(q22:q22) *RUNX1-RUNX1T1* while the other routine results (FISH analysis and cytogenetics)for this sample showed no translocations (See Table 3.8). Another t(8;21)(q22:q22) *RUNX1-RUNX1T1* (MFI 417.5) was identified in a paediatric patient with B-ALL showing aberrant myeloid marker expression. The third discrepant t(8;21)(q22:q22) *RUNX1-RUNX1T1* was also detected in an adult patient, a mutation which was not detected using cytogenetics and no FISH was requested for the patient. In this case the MFI was strongly positive at 5961 MFI. Finally, BP2 had FISH results showing rearrangement of the *MLL* gene and cytogenetic studies identified an unknown translocation partner t(11;?). Subsequently, a t(4;11) *MLL/AF4* was identified using our assay, but it could not be confirmed.

Table 3.9 shows the further inspection of discrepant results with regards to specific categories of RNA quantity (<70mg/µl), GAPDH signal (<1000 MFI), borderline MFI signals and suboptimal quality of the gold standard testing technique.

**Table 3.6: Summary of all concordant results (positive for translocations relative to the Signature® LTx Multiplex RT-PCR assay)**

| Patient code | RNA ng/ul | $A_{260}$:$A_{280}$ | GAPDH MFI | Fusion transcript MFI | Signature results | FISH/cytogenetics results |
|---|---|---|---|---|---|---|
| | **t(8;21)(q22;q22) AML-ETO** | | | | | |
| **BA8** | 146.5 | 2.07 | 5353 | 8190 | t(8;21) | t(8;21) |
| **DP9** | 211.2 | 2.05 | 6239 | 8937 | t(8;21) | t(8;21) |
| **GA7** | 66.6* | 1.98 | 6511 | 9213 | t(8;21) | t(8;21) |
| **GA15** | 979.6 | 2.10 | 5585 | 8680 | t(8;21) | t(8;21) |
| **BA20** | 266.5 | 2.07 | 46* | 3638 | t(8;21) | t(8;21) |
| **BA10** | 140.1 | 2.14 | 2565 | 7273 | t(8;21) | t(8;21) |
| **GA22** | 111.1 | 2.14 | 3540 | 8256 | t(8;21) | t(8;21) |
| **BA12** | 273.4 | 2.08 | 223* | 3456 | t(8;21) | t(8;21) |
| | **inv(16)(p13;q22) CBFB-MYH11** | | | | | |
| **GP4** | 85.0 | 1.97 | 6700 | 8366; 2902 | inv(16) | Inv(16) |
| | **t(15;17)(q24;q21) PML-RARα** | | | | | |
| **BP15** | 58.9* | 2.07 | 5413 | 8163 | t(15;17) L | t(15;17) |
| **GA1** | 11.4* | 2.44 | 3563 | 4371 | t(15;17) L | t(15;17) |
| **GA5** | 464.4 | 2.02 | 6438 | 7831; 1874 | t(15;17) L&S | t(15;17) |
| **BP8** | 951.0 | 2.10 | 2609 | 5027 | t(15;17) L | t(15;17) |
| **BA9** | 363.4 | 2.07 | 2076 | 5157 | t(15;17) L | t(15;17) |
| | **t(9;22)(q34;q11) BCR-ABL1 (b2a2)** | | | | | |
| **GA10** | 78.4 | 2.01 | 3032 | 6864 | t(9;22) | t(9;22) |
| **BP1** | 131.7 | 2.11 | 2375 | 5972 | t(9;22) | t(9;22) |
| **GA9** | 417.1 | 2.01 | 1233 | 1353 | t(9;22) | t(9;22) |
| | **t(9;22)(q34;q11) BCR-ABL1 (b3a2)** | | | | | |
| **BA13** | 229.4 | 2.12 | 1265 | 2131 | t(9;22) | t(9;22) |
| | **t(9;22)(q34;q11) BCR-ABL1 (e1a2)** | | | | | |
| **DP7** | 188.2 | 2.08 | 959* | 5004 | t(9;22) | t(9;22) |
| | **t(1;19)(q23;p13) E2A-PBX1** | | | | | |
| **BP13** | 93.1 | 2.07 | 49* | 2658 | t(1;19) | t(1;19) |
| **BP20** | 569.8 | 1.94 | 608* | 1100 | t(1;19) | t(1;19) |
| **GP5** | 109.9 | 2.06 | 91* | 2163 | t(1;19) | t(1;19) |
| **DP18** | 114.9 | 2.10 | 3969 | 7064 | t(1;19) | t(1;19) |
| **DP17** | 52.5* | 2.09 | 2385 | 6894 | t(1;19) | t(1;19) |
| | **t(12;21)(p13;q22) TEL-AML1** | | | | | |
| **BP4** | 214.4 | 2.09 | 5522 | 2171 | t(12;21) | t(12;21) |

(* Indicates suboptimal RNA quantity or GAPDH MFI signal)

**Table 3.7: Summary of all concordant results (negative) for translocations relative to the Signature® LTx Multiplex RT-PCR assay**

| Patient code | RNA ng/µl | $A_{260}$:$A_{280}$ | GAPDH MFI | Signature results | FISH/cytogenetics results |
|---|---|---|---|---|---|
| **Negative** | | | | | |
| **SP1** | 988.7 | 2.10 | 7306 | Neg | Neg |
| **GP8** | 230.1 | 2.10 | 5963 | Neg | Neg |
| **DP1** | 38.9* | 1.97 | 2953 | Neg | Neg |
| **GA13** | 17.9* | 2.18 | 4237 | Neg | Neg |
| **BA6** | 332.0 | 2.07 | 7081 | Neg | Neg |
| **GP1** | 73.6 | 2.09 | 4376 | Neg | Neg |
| **DP3** | 77.8 | 2.06 | 1867 | Neg | Neg |
| **BP7** | 182.9 | 2.05 | 7321 | Neg | Neg |
| **BP5** | 89.2 | 2.02 | 866 | Neg | Neg |
| **GA12** | 355.5 | 2.03 | 7497 | Neg | Neg |
| **GP3** | 65.8* | 2.01 | 6493 | Neg | Neg |
| **BP14** | 83.1 | 1.96 | 202* | Neg | Neg |
| **GP6** | 274.0 | 2.08 | 2362 | Neg | Neg |
| **BP3** | 133.9 | 1.99 | 1626 | Neg | Neg |
| **BA5** | 61.3* | 2.01 | 196* | Neg | Neg |
| **BP6** | 58.6* | 2.05 | 29* | Neg | Neg |
| **DP11** | 824.0 | 2.12 | 629* | Neg | Neg |
| **DP13** | 255.3 | 2.05 | 2375 | Neg | Neg |
| **BP22** | 376.3 | 2.02 | 6200 | Neg | Neg |
| **BA21** | 192.8 | 2.08 | 4478 | Neg | Neg |
| **GP11** | 118.2 | 2.10 | 5277 | Neg | Neg |
| **GA23** | 216.8 | 2.01 | 6131 | Neg | Neg |
| **BP26** | 100.8 | 2.07 | 5243 | Neg | Neg |
| **BP25** | 139.7 | 2.09 | 6170 | Neg | Neg |
| **GA20** | 160.6 | 2.10 | 6774 | Neg | Neg |
| **DP14** | 108.3 | 1.97 | 5620 | Neg | Neg |
| **GA21** | 40.5* | 2.18 | 6483 | Neg | Neg |
| **BP23** | 176.7 | 1.94 | 5773 | Neg | Neg |
| **SB1** | 145.7 | 1.96 | 6449 | Neg | Neg |
| **SB2** | 130.5 | 1.98 | 3471 | Neg | Neg |
| **SB3** | 191.4 | 1.96 | 7127 | Neg | Neg |
| **SB4** | 137.0 | 2.05 | 5832 | Neg | Neg** |
| **SB5** | 155.0 | 1.90 | 5138 | Neg | Neg |
| **GA4** | 49.0* | 2.12 | 4272 | Neg | Neg |
| **DP4** | 122.6 | 2.07 | 6362 | Neg | Neg |

(* Indicates suboptimal RNA quantity or GAPDH MFI signal; ** Unusual signal pattern with probe results despite rearrangement of chromosome 16)

**Table 3.8: Summary of all discordant results (n = 10)**

| Diagnosis | Patient code | RNA ng/µl | $A_{260}$: $A_{280}$ | GAPDH MFI | Fusion transcript MFI | Signature results | FISH/cytogenetics results |
|---|---|---|---|---|---|---|---|
| **AML** | inv(16)(p13;q22) CBFB-MYH11 | | | | | | |
| | DP2 | 27.6* | 2.49 | 2324 | 100; 73 | Neg | inv(16) |
| | | | | | | | |
| **APL** | t(15;17)(q24;q21) PML-RARα | | | | | | |
| | DP15 | 164.2 | 2.09 | 5830 | 38; 64 | Neg | t(15;17) |
| | | | | | | | |
| **ALL** | t(9;22)(q34;q11) BCR-ABL1 (e1a2) | | | | | | |
| | DP5 | 71.5 | 2.03 | 93* | 92 | Neg | t(9;22) |
| | | | | | | | |
| **ALL** | t(1;19)(q23;p13) E2A-PBX1 | | | | | | |
| | BA1 | 50.5* | 2.11 | 4524 | 113 | Neg | t(1;19) |
| | BP12 | 627.7 | 2.12 | 17* | 113 | Neg | t(1;19) |
| | GA18 | 299.9 | 2.09 | 194* | 41 | Neg | t(1;19) |
| | | | | | | | |
| **Mixed cases** | Negative | | | | | | |
| **ALL** | BP2 | 106.7 | 2.09 | 192* | 4316 | t(4;11) | MLL rearrangement |
| **AML** | GA6 | 82.9 | 2.00 | 640* | 5961 | t(8;21) | Neg |
| **AML** | GA8 | 22.1* | 2.12 | 4360 | 365 | t(8;21) | Neg |
| **ALL** | GP9 | 262.5 | 2.09 | 3608 | 418 | t(8;21) | Neg |

* Indicates suboptimal RNA quantity or GAPDH MFI signal

**Table 3.9: Deviations of optimal criteria noted in discrepant samples**

| Patient code | RNA concentration (<70ng/µl) | GAPDH signal (<1000 MFI) | Borderline MFI* | Suboptimal quality gold standard (<20 metaphases) |
|---|---|---|---|---|
| **DP2** | x (27.6) | | | |
| **DP15** | | | | X (13) |
| **DP5** | | x (198.0) | | |
| **BA1** | x (50.5) | | | |
| **BP12** | | x (865.5) | | |
| **GA18** | | x (194.0) | | x (10) |
| **BP2** | | x (192.0) | | x (6) |
| **GA6** | | x (640.0) | | x (2) |
| **GA8** | x (22.1) | | X (356) | x (10) |
| **GP9** | | | X (418) | |

According to the manufacturer's specifications, the required RNA concentration is > 70ng/µl and a GAPDH signal of <1000 MFI would require re-run from the reverse transcription step. For the purposes of our study we did not repeat steps or repeat extraction. If we strictly adhered to these recommendations, the following numbers of concordant and discordant results would be obtained (Figure 3.4).

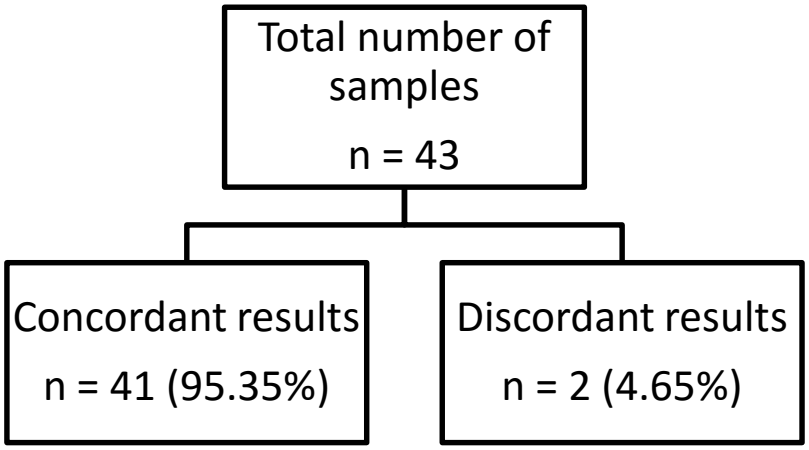



**Figure 3.4: Summary of concordant and discordant results with strict adherence to RNA quantity and GAPDH signal requirements.**

The box plot in Figure 3.5 shows quantitative analysis of signal output with the GAPDH MFI for the positive, negative and blank controls (G POS, G NEG, G BLANK) as well as the mean MFI of the positive, negative and blank transcript controls of all four runs (T POS, T NEG, T BLANK). The boxes represent the $25^{th}$, $50^{th}$ (median) and $75^{th}$ percentiles of the signal distributions. The tails of the distributions are the whiskers – 1.5 times the interquartile range).

The cut off values for a positive signal (350 MFI) and GAPDH signal (1000 MFI) are shown as dash lines. As expected, the GAPDH MFI of the blank control was well below the 1000 MFI cut-off recommended by the manufacturer while the negative control GAPDH was always greater than 1000. In a single run, the positive control GAPDH signal was below 1000, but these results were included in the analysis. The negative and blank transcript controls were consistently less than 350 MFI as required for acceptable analysis according to the manufacturer's recommendations.

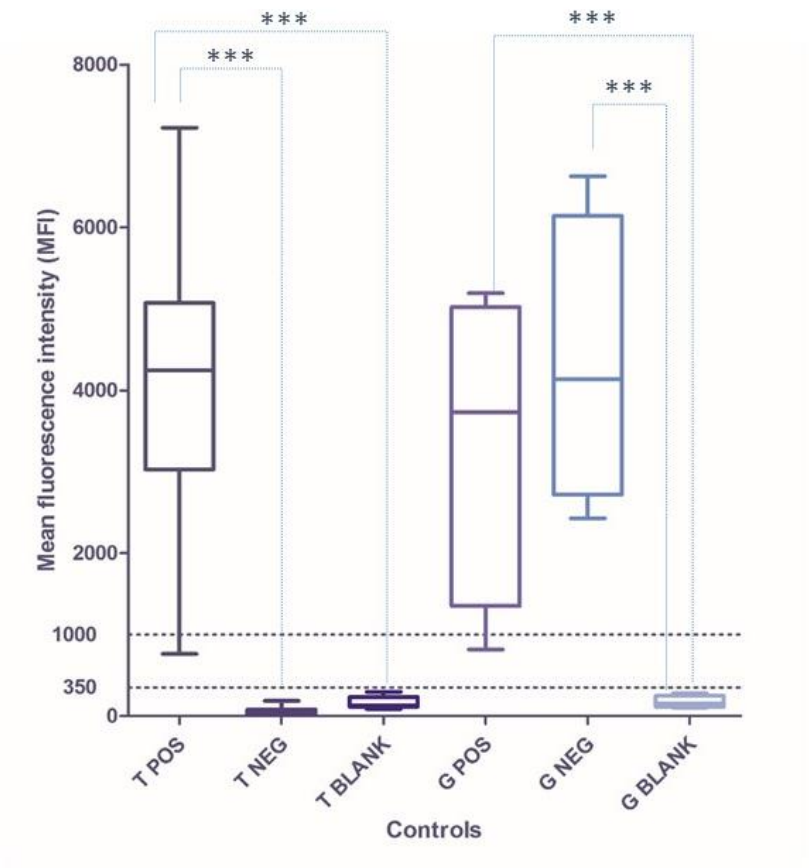



**Figure 3.5: Distribution of transcript positive, negative, blank and GAPDH endogenous control signals (MFI).**

(T POS – transcript positive control; T NEG – transcript negative control; T BLANK – transcript blank control; G POS – GAPDH positive control; G NEG – GAPDH negative control; G BLANK – GAPDH blank control; *** = p value < 0.001).

Analysis of variance (ANOVA) testing showed a mean difference of 4041 MFI between the transcript positive MFI and transcript negative signal ($P < 0.05$) and 3928 MFI between the transcript positive control and transcript blank control signals ($P < 0.05$). No significant difference was shown between the transcript negative and transcript blank controls. A mean difference of 3195 MFI ($P < 0.05$) and 4158 ($P < 0.05$) was shown between the GAPDH of the blank control and those of the positive and negative control GAPDH signals respectively (See Appendix 1).

The MFI signal for positive translocation results were > 1000 in all cases but two, which have already been described above as some of the discrepant results where the multiplex assay detected mutations which were not identified by FISH or cytogenetics. The median MFI for all positive transcripts was 3656. The median negative transcript MFI was 157.

Figures 3.6 and 3.7 show all other mutations using cytogenetics or FISH in samples which were categorised as 'Negative' according to the translocations included in the Signature® LTx multiplex RT-PCR assay. The majority had normal karyotypes (35%), complex karyotypes (10%), partial deletions (13%) and hyperploidy (10%). FISH analysis also detected two cases of deletions involving chromosome five and two samples with deletions of chromosome seven (refer Figure 3.6).

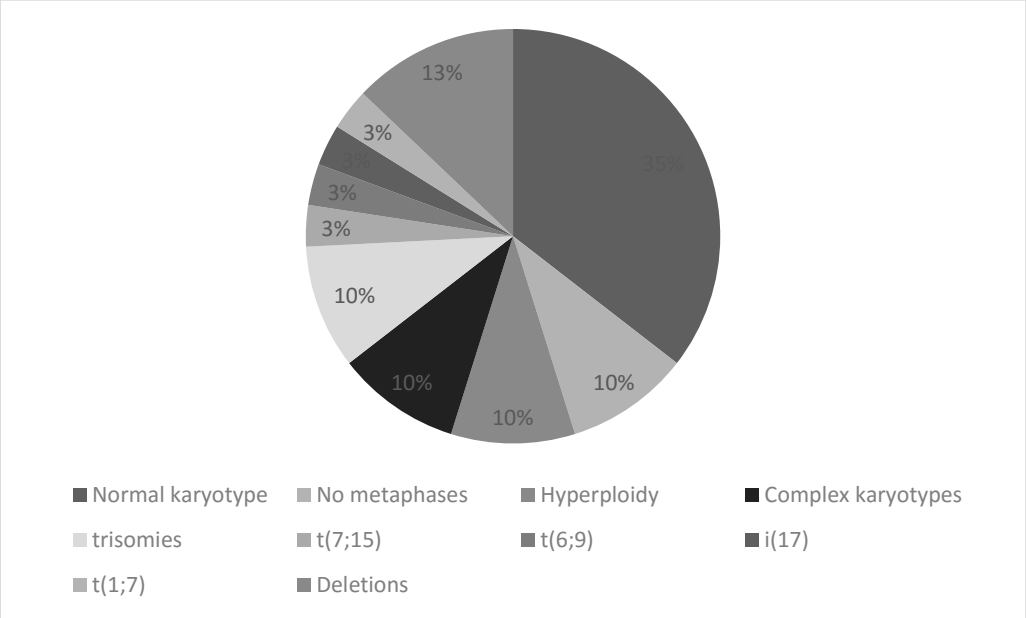



**Figure 3.6: Summary of mutations detected by cytogenetics which are not included in the multiplex assay**

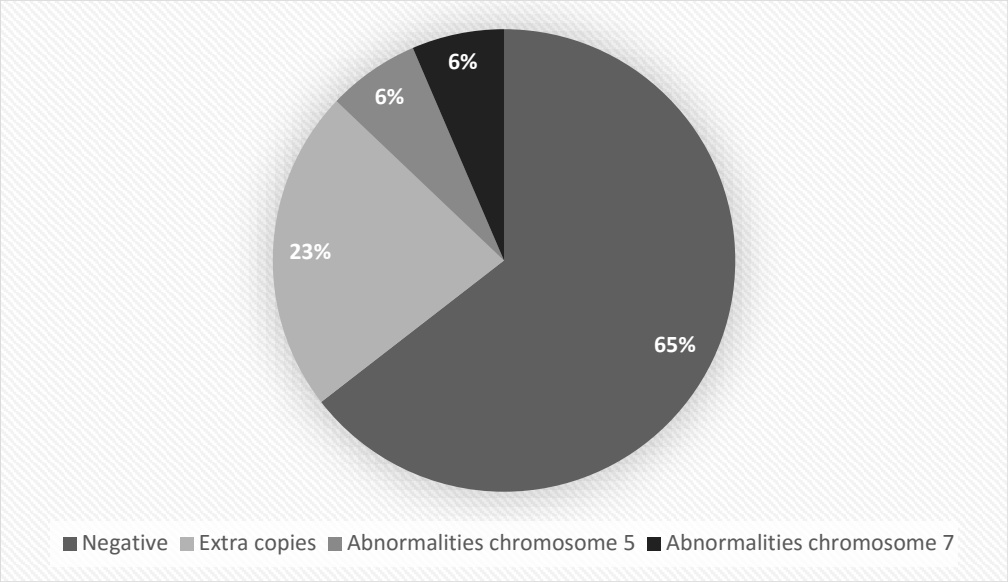



**Figure 3.7: Summary of mutations detected by FISH analysis which are not included in the multiplex assay**

# 4) Discussion

As mentioned, leukaemia is a complex, heterogeneous disorder with numerous genetic abnormalities already identified as well as new potential aberrations which impact on diagnosis, treatment and prognostication. As technology advances and the intricacies of this disease are further elucidated, the haematology laboratory should take advantage of these new methods in order to make the most accurate diagnosis as well as providing information to the treating oncologists within an acceptable time frame. This will not only be useful for prognostication purposes but also for potential targets for therapy. Although cytogenetic karyotyping remains the gold standard for diagnosis of acute leukaemias, other technologies especially those providing molecular diagnoses should be embraced and used in conjunction with more traditional methods in order to make an accurate diagnosis, determine prognosis and tailor therapy to a specific molecular target.

Several studies have investigated multiplex RT-PCR assays in the detection of leukaemia associated translocations and shown that it is a simple, rapid and sensitive testing method and possibly a valuable addition to the routine diagnostic testing of patients presenting with leukaemia [47, 49-52].

The aim of our study was to evaluate the Signature® LTx Multiplex RT-PCR assay in the detection of leukaemia-associated translocations. We compared the results obtained using this assay with those of FISH and cytogenetics analysis which are part of the routine diagnostic work-up of patients presenting with leukaemia.

The study sample set demographics were determined by patient willingness to participate in the study, whether patients were able to consent and whether adequate diagnostic sample material could be collected at the initial bone marrow aspirate biopsy. However, these are all general exclusions and would not have affected one particular group of patients more than another e.g. children vs. adults. We collected fewer CML cases than those reflected in the NCR and CMJAH flow cytometry registries since these patients were often discharged soon after diagnosis and we were unable to collect peripheral blood if a sample was not taken at the time of the initial bone marrow aspirate biopsy. Sample collection was also influenced by the rapid turnover of interns and registrars in the Oncology units who were not always aware of the study and sample collection requirements.

There were several limitations to our study including costing implications, the need for batching of samples, manual extraction problems and evaluation of the assay's assessment of minimal residual disease.

In terms of sample quality, it is noted that in the last run, all samples were less than 6 months old and a consistently improved RNA quality and concentration was observed (Table 3.3). Pre-analytical factors could affect the quantity and quality of RNA. A limitation of this study was that we did not have any measures in place to assess the handling of PAXgene tubes *e.g.* volume of sample and temperature until they were received in the lab for processing and storage. If the Signature® LTx assay is to be implemented as part of the routine diagnostic investigation, there needs to be an emphasis on pre-analytical variables *e.g.* collection of only 2.5 mL of bone marrow aspirate/peripheral blood at the time of collection as well as maintaining appropriate temperature of PAXgene tubes during transport and storage.

Total RNA was initially isolated from peripheral blood or bone marrow specimens using the NucliSENS® easyMAG® (bioMerieux, Lyon, France). This however yielded suboptimal RNA quality and quantity and a manual extraction kit PAXgene Blood RNA Kit (PreAnalytix, Qiagen) was tested as an alternative. The manual extraction yielded better RNA concentrations (70 ng/µl) and qualities (1.8-2.2 $A_{260}/A_{280}$ ratio of sample absorbance), however some samples became viscous after the incubation step at 55°C. This was noted in samples with a wide range of white cell counts and dates of collection with no obvious underlying cause. One of the greatest challenges faced in this study was discarding samples (n = 36) during the RNA extraction process as well as during RNA quantification owing to suboptimal quantity. Since our samples were batched before extraction, we could not assess whether this problem could be averted by extracting the samples upon arrival at the PCR laboratory. The $A_{260}$:$A_{280}$ ratio is not reflective of the integrity of the RNA. AS RNA is highly susceptible to degradation, it may influence the results and explain the low MFI values despite an adequate ratio. Troubleshooting would include comparing different RNA extraction methods in order to minimise discarding of samples or the need for more sample material to be sent by the requesting physician. Manual extraction also adds to the total time to obtain a result with an additional 4-5 hours required.

The possible benefit of faster results and turnaround time may be hampered by the need to batch samples both for the assay and RNA extraction. Batching is required since all three controls need to be included, with only 24 samples per assay. If testing is done on fewer samples at a time *e.g.* as samples are received with immediate extraction and analysis, the full complement of 21 samples will not be analysed per assay which will increase the cost per test and may not necessarily be more cost effective than individual FISH probes in order to achieve the improved turnaround times. An example of how more frequent testing will impact on the cost per test is shown in Appendix 2.

In some runs, samples (n=10) were included despite not meeting the >70 ng/µl RNA concentration requirement. Of these ten samples, results correlated with gold standard methods results in 70% (7/10) of cases. The GAPDH signal was a problem in some cases, particularly in the third batch where the positive control GAPDH signal was less than 1000 MFI. According to the manufacturer's recommendations, this result indicates that a re-run from the RT-PCR step is indicated, however for our purposes, these results were included in the analysis. Two thirds (14/21) of the samples in this run had GAPDH levels <1000 MFI, however of these, ten samples were concordant with the results reported by FISH and cytogenetics.

In our study, all the concordant positive signals of the multiplex assay had MFI values of>1000 MFI with a median signal intensity of 3656. There were two samples that had an MFI of <500. These samples (GA8, GP9) had mutations present which were not detected by either FISH or cytogenetics, and were therefore discordant. Statistically significant differences ($P < 0.05$) in the level of MFI readings were shown between transcript positive controls (T POS) and both transcript negative (T NEG) and blank controls (T BLANK) (Figure 3.5). No statistically significant difference was found between the transcript negative (T NEG) and transcript blank controls (T BLANK). Both of these categories were well below the 350 MFI cut-off signal recommended by the manufacturer. These findings are as expected, as neither the transcript negative or blank controls contain any of the targeted mutations. The GAPDH signals were acceptable throughout all four batches for the positive (>1000 MFI), negative (>1000 MFI) and blank controls (<1000 MFI), with the exception of one batch where, the positive control GAPDH signal was <1000 MFI. The GAPDH signals were not statistically different in the positive and negative controls as expected. In the blank control, the GAPDH signal was consistently <1000 MFI with significantly lower MFI signals as compared to the positive (G POS) and negative (N POS) signals.

In a multicentre validation study of 280 patients, 95 % of true-positive signals (concordant positive results compared to FISH and cytogenetics) were double the 350 MFI cut-off and negative signals were significantly below the same cut-off value [39]. Calculations from the same study showed that 99.99% of probe signals would be below 343 MFI, equating to one potential false-positive signal for every 100 million reactions performed using true-negative samples (concordant negative results compared to FISH and cytogenetics) [39].The recommended MFI signal was therefore found to be an appropriate, even conservative cut-off value.

The Signature® LTx multiplex assay showed good correlation with the gold standard diagnostic technique in our study with 10 (14.29 %) discordant results compared to 60 (85.71 %) concordant results. In these ten cases, there were some possible contributing factors identified *e.g.* RNA quantity, GAPDH MFI and fusion transcript MFI signal (Table 3.8).

In the case of GA6, a patient with known AML with t(8;21)(q22:q22) *RUNX1-RUNX1T1* who was receiving treatment had a repeat bone marrow biopsy, this translocation was identified using the Signature® LTx multiplex assay with a signal of 5814.0 MFI, however no FISH analysis was requested and the metaphases for cytogenetics were of poor quality. The GAPDH signal was less than 1000 MFI. The mutation could not be confirmed by another method and the patient showed evidence of morphological and molecular remission in a bone marrow biopsy performed six months later.

For the sample BP2, FISH analysis showed rearrangement of the *MLL* gene whereas cytogenetics identified a translocation involving chromosome eleven with an unknown fusion partner t(11;?). Therefore our multiplex assay result of t(4;11)(q21;q23) is not necessarily a discordant result, but could not be confirmed using routine diagnostic tests. Nine of the ten discrepant results had

identifiable contributing factors and repeat testing may have yielded concordant results. However, in patient DP15, a diagnosis of APL was made at a referral hospital and the t(15;17)(q22;q12); *PML-RARa* was confirmed by cytogenetics at our centre. Despite adequate RNA quantity and GAPDH signal, the mutation was missed by the Signature® LTx assay with an MFI below 100 for both isoforms.

If extraction were to be repeated with RNA concentration results <70ng/µl and PCR steps were repeated following a GAPDH results of < 1000 MFI, a total of 43 samples could be analysed, of which 95.35 % would be concordant and 4.65 % discordant compared to the FISH and cytogenetics reports.

When discrepant results were identified by comparison to cytogenetics and FISH departments, we did not repeat analysis of these samples due to limited sample. Should this assay be implemented as part of the routine diagnostic investigations of patients presenting with leukaemia, appropriate flow diagrams need to be designed in the case of conflicting results. The role in monitoring of minimal residual disease would form part of an ongoing assessment

Alternative splicing isoforms or rare variants will not be detected with the Signature® LTx multiplex RT-PCR assay with a need for further specific primers to be added to the design; however the same challenge is faced in FISH analysis [39]. Multiplex assays are also not able to detect a complex karyotype, therefore they cannot be used in isolation and conventional cytogenetics remains the gold standard in investigation of patients with acute leukaemia.

We would recommended incorporation of more genetic abnormalities with prognostic significance including *NPM1* (Nucleophosmin 1) and *FLT3* (fms-related tyrosine kinase 3) in AML which both have significant prognostic implications [53]. The mutations 7q- and 5q- would be of value as well, particularly in the case of myelodysplasia as four of the cases in our patient set showed these abnormalities. In ALL, possible additions could include *PAX5* (paired box protein 5) and *IKZF1* (Ikaros family zinc finger protein 1) [34]. The addition of these mutations would be valuable because *PAX5* mutation occurs in a third of B-cell ALL cases and *IKZF1* mutations are identified in 15% of all paediatric B-ALL cases, more than 70% of *BCR-ABL1* lymphoid leukaemia and in 10-15% of *BCR*-AB negative ALL [54]. It is associated with a poor prognosis [55]. Inclusion of both myeloid and lymphoid lineage translocations in one assay may be unnecessary in most cases following morphological, cytochemical or flow cytometry analysis, thereby necessitating development of disease-focused panels [39].

The multiplex panels were expanded in one multicentre validation study [39]. Two additional prototype panels were designed and tested, one focusing on t(9;22), t(12;21), t(4;11) and t(1;19) *i.e.* ALL-associated translocations. This assay could detect rare t(12;21)(p13;q22); *TEL-AML1 (ETV6-RUNX1)* e5e3 and t(1;19)(q23;p13.3); *E2A-PBX1 (TCF3-PBX1)* e13e2i27 variants as well as four additional *MLL-AF1* variants. The second assay focused on AML-associated translocations and included added beads to detect *CBFB-MYH11* type E and *PML-RARα* bcr2 variants as well as three most common *NPM1* transcripts. These extended assays were found to have excellent correlation with cytogenetics and other molecular methods [39].

# 5) Conclusion

In our study, the Signature® LTx Multiplex RT-PCR assay showed good correlation with the gold standard of conventional cytogenetics as well as FISH analysis. In order to utilise the potential improved cost and turnaround time benefits of the assay, careful attention needs to be paid to pre-analytical variables, optimising the RNA extraction methods, possibly modifying the translocations included in the assay and having diagnostic algorithms in place particularly with regards to discordant results in order to incorporate its use within the routine diagnostic work-up of patients presenting with leukaemia.

# Appendix

## Appendix 1: ANOVA testing of transcript positive, negative, blank and GAPDH endogenous control signals (MFI)

| Tukey's Multiple Comparison Test | Mean Difference | q | Significant? P < 0.05? | Summary | 95% CI of difference |
|---|---|---|---|---|---|
| **T POS *vs.* T NEG** | 4041 | 30,20 | Yes | *** | 3493 to 4589 |
| **T POS vs T BLANK** | 3928 | 29,36 | Yes | *** | 3380 to 4475 |
| **T NEG vs T BLANK** | -113,0 | 0,8449 | No | ns | -661.0 to 434.9 |
| **G POS vs G NEG** | -963,5 | 2,171 | No | ns | -2781 to 853.9 |
| **G POS vs G BLANK** | 3195 | 7,199 | Yes | *** | 1377 to 5012 |
| **G NEG vs G BLANK** | 4158 | 9,370 | Yes | *** | 2341 to 5975 |

(T POS – transcript positive control; T NEG – transcript negative control; T BLANK – transcript blank control; G POS – GAPDH positive control; G NEG – GAPDH negative control; G BLANK – GAPDH blank control; *** = p value < 0.001; ns – not significant).

## Appendix 2: Cost breakdown per test

| Reagents | Cost/test (one batch per assay) | Cost/test (two batches per assay) | Cost/test (four batches per assay) |
|---|---|---|---|
| **PAXgene tubes** | R 110.00 | R110.00 | R110.00 |
| **Extraction Cost** | R 252.04 | R252.04 | 252.04 |
| **Consumables:** | | | |
| **Pipette tips** | R 5.83 | R 5.83 | R5.83 |
| **Gloves** | R 1.54 | R 1.54 | 1.54 |
| **Eppendorf tubes** | R 10.45 | R10.45 | R10.45 |
| **Signature LTx translocation Kit (including controls)** | R1795.50 | 2094.75 | R 3142.13 |
| **Co-star 96 well Plate** | R 26.32 | R26.32 | R26.32 |
| **Sheath fluid** | R 18.73 | R18.73 | R18.73 |
| **Costar Thermowell seals** | R 7.88 | R7.88 | R7.88 |
| **TOTAL:** | **R2228.29** | **R2527.54** | **R3574.92** |

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