The application of Real-time quantitative PCR in the diagnostics of Chronic Myeloid Leukaemia

By

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May 2009

Submitted in accordance with the requirements for
the degree Magister Scientiae in Medical Science
in Molecular Biology
(M.Med.Sc. Molecular Biology)

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Declaration

I declare that this dissertation hereby submitted by me for the Masters in Medical Science in Molecular Biology (M.Med.Sc. Molecular Biology) degree at the University of the Free State is my own independent work and has not previously been submitted by me at another university. I furthermore cede copyright of the dissertation in favour of the University of the Free State. There is no conflict of interest with regard to the proposed study and principle investigator Mr. J.J. van Deventer. This study was funded by the Department of Haematology and Cell Biology, an NHLS grant and funds generated by contract research. Furthermore, there is no contractual obligation limiting the publication of the results from this study.

J.J. van Deventer

"We shall not cease from exploration. And the end of all our exploring will be to arrive where we started and know the place for the first time."

T. S. Eliot

Met ootmoed en nederigheid dra ek hierdie werk op aan die afgestorwe Jaco Taute en ander leukemie lyers.

"We may yearn for a 'higher' answer - but none exists."
Stephen Jay Gould

Acknowledgements

This research does not belong to one person. Its success is bound by a collaborative group effort by those, who have committed themselves to improve our understanding of CML monitoring. It is for this reason that I am greatly indebted to the following people:

- All the CML patients from the National Hospital in Bloemfontein as well as those receiving treatment at the INR Clinic at the Department of Haematology and Cell Biology, Universitas Hospital, who have participated in this research effort.
- Prof. C.D. Viljoen for sharing valuable thoughts on human molecular biology, frequently engaging in discussion about genetics and promoting ideas that would guide this project and make it a roaring success.
- Prof. V.J. Louw providing critical insight into the disease of CML as well as strategic approaches that gave me as a scientist access to the clinical environment.
- All the registrars, especially Dr. Reinette Weyers, for providing valuable patient information.
- The Department of Haematology and Cell Biology (UFS) for providing facilities and resources.
- The National Health Laboratory Services for providing resources in order to complete this research.
- The GMO testing facility (UFS) for providing facilities and resources to enable this project.
- All the staff from the INR Clinic (UFS) as well as ward 28 from the National Hospital for their assistance with CML patients.
- A special thank you to Sister Elsa du Preez from the INR Clinic (UFS).
- To all my colleagues for their help and support.
- To my parents for their continued support, love and belief in me as a scientist.
- My family and friends for encouragement and support.

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Abbreviations

ABL	Abelson
ALL	Acute lymphoblastic leukaemia
AMV RT	Avian myeloblastoma leukaemia virus reverse transcriptase
Ara-C	Cytarabine
ASO-PCR	Allele specific oligonucleotide PCR
B2M	β-2-Microglobulin
BCR	Breakpoint cluster region
BU	Busulfan
CCR	Complete cytogenetic response
cDNA	Complementary DNA
CR	Cytogenetic response
CHR	Complete hematologic response
CML	Chronic myeloid leukaemia
CMR	Complete molecular response
CNL	Chronic neutrophilic leukaemia
CV	Coefficient of variance
DNA-FCM	DNA flow cytometry
EAC	Europe Against Cancer
EGF	Epidermal growth factor
FDA	Food and Drug Administration
FISH	Fluorescence in situ hybridization
Gab	GRB-2 associated binding protein
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GS	Gene specific
GSc	Gene specific column purification
GUS	β-Glucuronidase
HLA	Human leukocyte antigen
HU	Hydroxyurea
IFN-α	Interferon-α
IRIS	International randomized study of Interferon versus STI-571
Jak	Janus kinase
LOD	Limit of detection
LOQ	Limit of quantification
MAPK	Mitogen activated protein kinase
m-*bcr*	Minor-*bcr*
M-*bcr*	Major-*bcr*
MCyR	Major cytogenetic response
MDR	Multidrug resistance
MMR	Major molecular response
M-MuLV RT	Moloney murine leukaemia virus reverse transcriptase
MRD	Minimal residual disease
PBGD	Porphobilinogen deaminase
PDGF	Platelet derived growth factor
PFS	Progression free survival
Ph	Philadelphia
PI3K	Phosphoinositide 3-kinase
QoL	Quality of life
RAR-α	Retinoic acid receptor

RH	Random hexamer
RHc	Random hexamer column purification
RQ-PCR	Real-time quantitative PCR
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
SS	SuperScript
STAT	Signal transducers and activator of transcription
TBP	TATA binding protein
TK	Tyrosine kinase
TKIs	Tyrosine kinase inhibitors
µ-bcr	Mikro-*bcr*

Preface

Chronic Myeloid Leukaemia (CML) is a myeloproliferative disorder that leads to neoplastic transformation of the hematopoietic system. It is a clonal disease and carries a consistent genetic aberration that is the result of a reciprocal translocation between chromosomes 9 and 22. The result is a shortened chromosome 22, known as the Philadelphia (Ph) chromosome that carries the *BCR-ABL* fusion oncogene. *BCR-ABL* is the causative agent and remains central to the pathogenesis of CML. It encodes a constitutively activated non-receptor tyrosine kinase that affects control of the cell cycle, proliferation, differentiation and adhesive properties of immature leucocytes.

Real-time quantification of *BCR-ABL* mRNA has become the most sensitive molecular technique to monitor patients' response to targeted drug therapy. This technique, also known as RQ-PCR, is used in routine laboratories across the globe to determine the efficacy of imatinib and second-generation tyrosine kinase inhibitors as first line treatment to combat CML. Molecular monitoring through the introduction of Real-time quantification of *BCR-ABL* mRNA has revolutionised, improved our understanding of the dynamics of CML and targeted treatment thereof.

This thesis contains a literature review (Chapter 1), an in-depth analysis of published methods to quantify *BCR-ABL* mRNA using Real-time quantitative PCR (Chapter 2), a research chapter that establishes and validates a method for the quantification of *BCR-ABL* mRNA as well as a discussion and conclusions chapter. The literature review has been written in such a way as to avoid unnecessary duplication. Both subsequent chapters are written in article format and there is some repetition between the respective introductions and the literature review. This is necessary to place each research question within the correct context, as well as to enable the literature review and protocol to exist as separate entities.

Throughout this dissertation, you will find that the tables and figures are numbered consecutively starting from "Table 1" and "Figure 1", and not numerical according to the specific chapter within which it functions. Tables and figures are only included within the text in the literature review. In the subsequent chapters, they are included at the end of each chapter. Furthermore, in some instances you will find a reference to tables and figures from a previous chapter. To facilitate the individual chapters to function as separate entities, each chapter has its own reference list. On a more technical note, you will find that the terms RQ-PCR, Real-time quantification of *BCR-ABL* mRNA, *BCR-ABL* quantification, and Real-time PCR for *BCR-ABL* are used throughout the text to describe the same method.

When reading this thesis, please consider the importance of current efforts in an attempt to standardize Real-time quantification of *BCR-ABL* mRNA.

Chapter 1:
Literature review

1.1 Introduction to chronic myeloid leukaemia

1.1.1 Molecular biology of CML

Chronic Myeloid Leukaemia (CML) was first described in 1845 and is a clonal myeloproliferative disorder that affects one to two individuals in 100,000 (Faderl *et al.* 1999). It accounts for approximately 20% of all Leukaemia (Warmuth *et al.* 1999), and is diagnosed at a median age of 55 years (Hehlmann *et al.* 2007), affecting more males than females at a ratio of 2:1 and it appears to be more common in Caucasians (Frazer *et al.* 2007) than other ethnic groups. CML is a haematological malignancy that results in clonal expansion of primitive hematopoietic progenitor cells (Faderl *et al.* 1999; Frazer *et al.* 2007).

Hematopoietic stem cells differentiate into common myeloid and lymphoid progenitors. In CML, the myeloid lineage is implicated, affecting granulocyte, macrophage and megakaryocyte progenitors downstream in a clonal manner. The result is that the hematopoietic production of granulocytes, macrophages, red blood cells and platelets are adversely affected with an increase in leucocytes (Ren 2005). CML also results in a marked increase in myeloid- and erythroid cells as well as platelets in the peripheral blood and myeloid hyperplasia in the bone marrow (Sawyers 1999). The accumulation of immature and undifferentiated white blood cells, forces the normal and functional cells out of the bone marrow, which leads to the symptoms of anaemia and leukaemia (Calabretta and Perrotti 2004). Furthermore, this excess of undifferentiated white blood cells results in hyper viscosity of the peripheral blood which is also symptomatic of leukaemia (Brunstein and McGlave 2001). The most common symptoms at presentation include fatigue, weight loss, abdominal fullness, bleeding, purpura, splenomegaly, leukocytosis, anaemia, and thrombocytosis (Faderl *et al.* 1999). However, the majority of patients are initially asymptomatic in the benign chronic phase and are

sometimes diagnosed by chance based on results from a routine full blood count (Heaney and Holyoake 2007). After the chronic phase, the disease progresses rapidly to the accelerated phase, and terminates in the blastic phase within three to five years if not treated (Sawyers 1999).

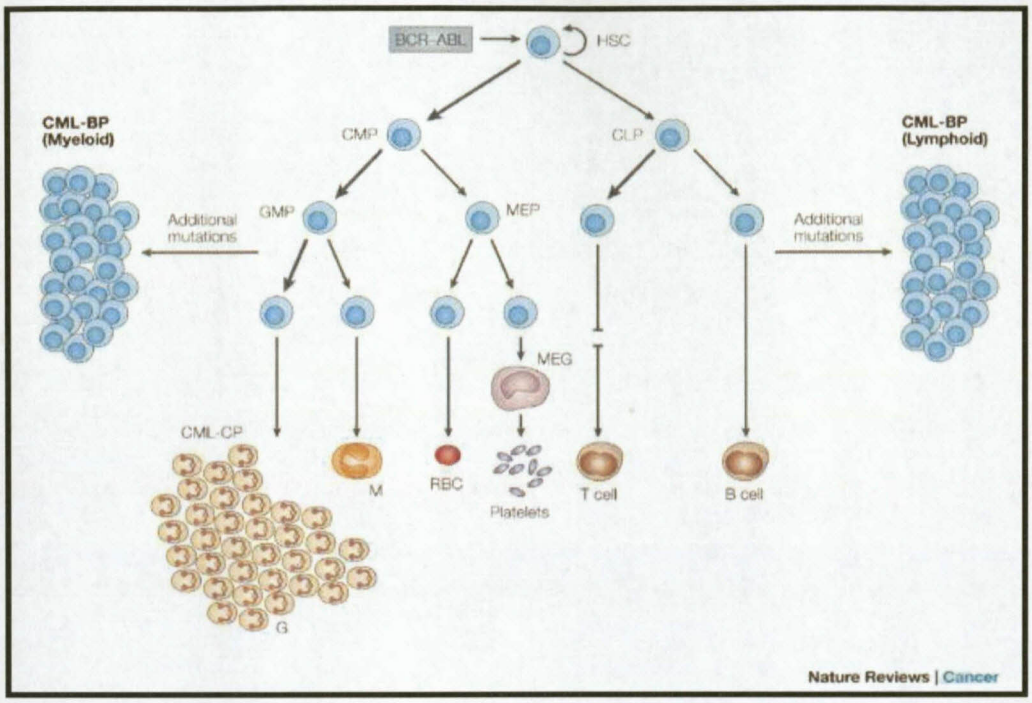

Figure 1: **The development of chronic myelogenous leukaemia (copied from Ren 2005). Self-renewing hematopoietic stem cells differentiate into common myeloid progenitors (CMPs), which then differentiate into granulocyte/macrophage progenitors (GMPs: progenitors of granulocytes (G) and macrophages (M) and megakaryocyte/erythrocyte progenitors. The initial chronic phase of CML is characterized by a massive expansion of the granulocytic cell series.**

CML was the first cancer where leukemogenesis was associated with a consistent chromosomal abnormality (Melo and Barnes 2007). In 1960, Nowell and Hungerford described a minute acrocentric chromosome in cells cultured at their Philadelphia laboratory from CML patients that became known as the "Philadelphia (Ph) chromosome" (Ren 2005). Due to advances in karyotyping,

it was discovered in 1973 that the Ph chromosome was the result of a reciprocal translocation between chromosomes 9 and 22, denoted as t(9;22)(q34:11) (Figure 2) (Sawyers 1999; Geary 2000). It was also discovered that the reciprocal translocation involved the partial displacement and juxtaposition of the *ABL* (Abelson Kinase) and *BCR* (Breakpoint Cluster Region) proto-oncogenes on the long arms of chromosomes 9 and on 22, respectively (Melo and Barnes 2007). The Ph chromosome is present in 90% to 95% of CML patients (Hehlmann *et al.* 2007). CML patients who do not have the Ph chromosome often have other complex genetic aberrations (Babicka *et al.* 2006; Costa *et al.* 2006), usually involving other chromosomes (Kaeda *et al.* 2002).

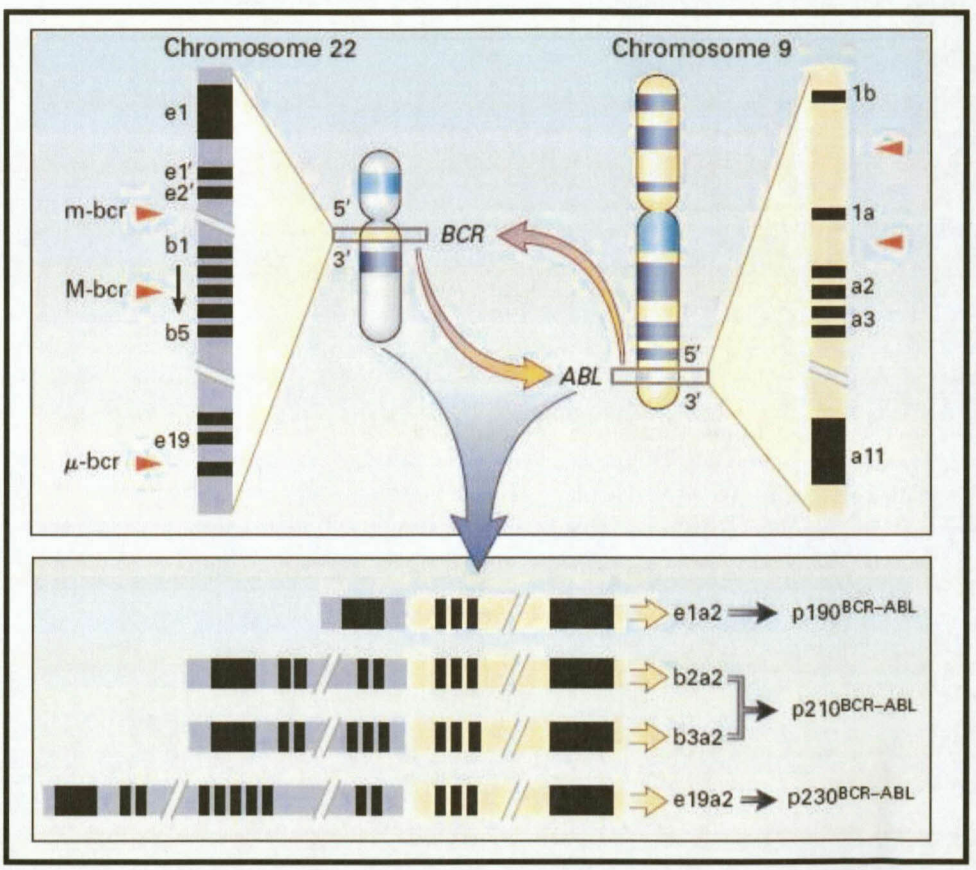

Figure 2: **The reciprocal translocation t(9;22)(q34;q11) in CML (copied from Faderl *et al.* 1999). A segment from the ABL gene on chromosome 9 is fused head to tail (5' to 3') to the BCR gene on chromosome 22.**

The *BCR-ABL* oncogene encodes for an intracellular non-receptor tyrosine kinase (TK) with constitutive activity (Bagg 2002). In normal, BCR-ABL negative cells, the TK (ABL) is encoded by the SH1 domain and controlled by the N-terminal cap region of normal c-ABL (Laneuville 1995; Saglio and Cilloni 2004). BCR-ABL (p210 BCR-ABL) lacks the ABL cap region and the dimerization domain encoded by the first exon of BCR is responsible for the constitutive activation of the ABL SH1 domain (Goldman and Melo 2003). This results in uncontrolled signal transduction and an abnormal cellular phenotype.

Other functional domains in ABL include the SH3 and SH2 regulatory domains, as well as the nuclear-localization signal motif, the nuclear-export signal motif, the DNA-binding domain, and the G-actin and F-actin DNA-binding domains. F- and G-actin are important for the control of cyto-skeletal organization, cell adherence, cell motility, and integrin receptor-mediated signal transduction. BCR is responsible for the oligomerization and autophosphorylation of monomer, inactive BCR-ABL into activated tetramers resulting in dysfunctional cellular activity (Melo *et al.* 2003). The loss of a segment of ABL together with the juxtaposition of the foreign BCR sequence corrupts the regulatory domains of the oncogene resulting in uncontrolled and deregulated tyrosine kinase activity (Melo *et al.* 2003) (Figure 3).

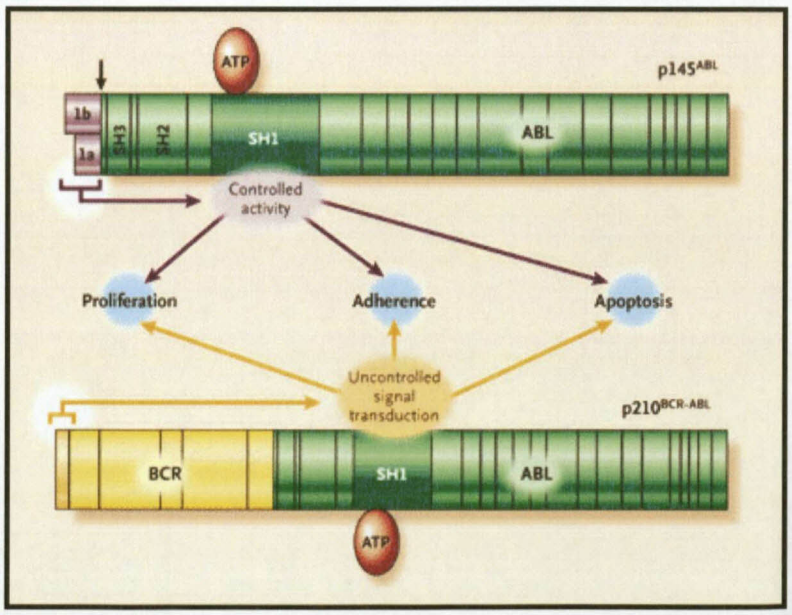

Figure 3: Regulation by the normal ABL protein and deregulation by BCR-ABL of key cellular processes such as proliferation, adherence and apoptosis (copied from Goldman and Melo 2003).

Cells require kinase enzymes to pass phosphate groups between different molecules as a means of internal communication (signal transduction cascades). The constitutive tyrosine kinase activity of BCR-ABL disrupts this process. The pathways that are involved in BCR-ABL downstream signal transduction include Ras/MAPK, Jak/Stat, PI3K and Myc (Figure 4) (Deininger *et al.* 2000; Steelman *et al.* 2004; McCubrey *et al.* 2006). The activation of the phosphorylation cascade in the cell by BCR-ABL initially occurs through the phosphorylation of adapter proteins such as GRB-2, GAB2, DOK and CRKL (Sattler *et al.* 2002).

BCR-ABL impacts signal transduction pathways via the adapter proteins that are responsible for the activation or repression of gene transcription and apoptosis, cytoskeletal organization and the degradation of inhibitory proteins (Goldman and Melo 2003). Therefore, uncontrolled, constitutive TK activity, because of BCR-ABL, has severe implications for cellular signal transduction and the cell's ability to respond to external stimuli. The inability to respond to stimuli such as growth factors including Interleukin-3 (IL-3), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) and granulocyte/macrophage colony stimulating growth factor (GM-CSF) (Deininger *et al.* 2000) leads to abnormal proliferation of haematopoietic cells.

BCR-ABL has also been implicated in the activation of hematopoietic growth factor signal transduction pathways through activation of PI3K, Raf, Ras and STAT (Sawyers *et al.* 1995; Skorski *et al.* 1995; Carlesso *et al.* 1996; Sattler and Salgia 1997; Skorski *et al.* 1997; Neshat *et al.* 2000). Myeloid progenitor cells lose the ability to respond to growth-regulating factors (cytokines) and thus proliferate uncontrollably resulting in tumour expansion (Pasternak *et al.* 1998).

In CML, myeloid progenitors are released prematurely from the bone marrow into the peripheral blood. This is as a result of decreased adhesion due to the influence that BCR-ABL has on adhesion proteins. BCR-ABL affects focal adhesion proteins like paxillin, tallin and tensin via integrin, which has downstream effects on actin (Deininger *et al.* 2000; Hantschel *et al.* 2005). Therefore, CML progenitor cells have reduced adhesion properties, are less able to adhere to the bone marrow stroma cells and extracellular matrix due to defects in integrin function (Salesse and Verfaillie 2002). Furthermore, proteins responsible for DNA repair (DNA-PKcs), genomic stability (p53) and cell cycle control ($p14^{ARF}$ and $p16^{INK4a}$), either malfunction or are down regulated due to the synergistic and pleiotropic effects of BCR-ABL (Honda *et al.* 2000; Deutsch *et al.* 2001; Cividin *et al.* 2006; Wendel *et al.* 2006). The greatest implication for neoplastic transformation is the anti-apoptotic activity as a result of phosphorylation of the anti-apoptotic protein Bcl-x_L (Canman and Kastan 1995; Cortez *et al* 1995; Fernandez-Luna 2000; Horita *et al.* 2000).

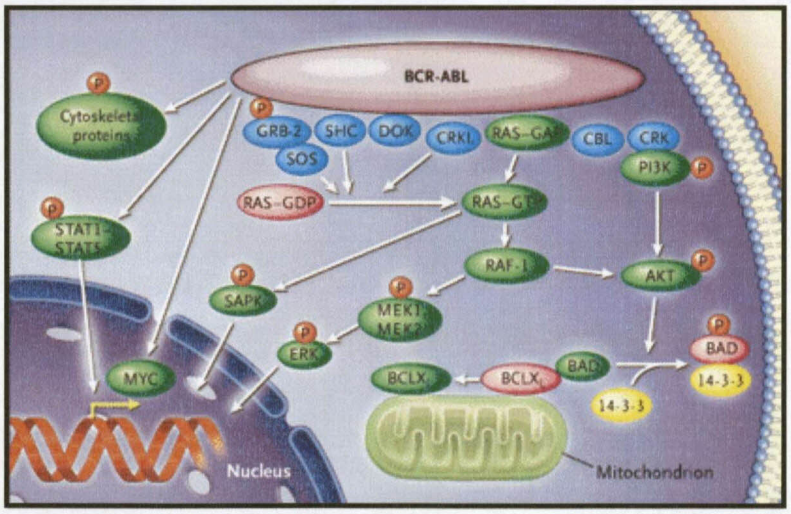

Figure 4: Signal transduction pathways affected by BCR-ABL (copied from Goldman and Melo 2003).

1.1.2 Treatment options for CML with specific reference to imatinib

The *BCR-ABL* hybrid fusion-oncogene encodes one of three different sized protein products, depending on where within *BCR* the break point occurs (Figure 2). The position of the break within *ABL* is consistent and occurs at the 5' end of exon *a2* (Goldman and Melo 2003). There are three different breakpoint regions within *BCR* known as minor-*bcr* (m-*bcr*), major-*bcr* (M-*bcr*) and micro-*bcr* (µ-*bcr*) (Faderl *et al.* 1999). If a break should occur at the m-*bcr* (*e1a2*), a p190 BCR-ABL is encoded. This form of the protein is also common in Ph-positive acute lymphoblastic leukaemia (ALL). The translocation involving the µ-*bcr* (*e19a2*) region encodes a p230 BCR-ABL associated with chronic neutrophilic leukaemia (CNL) and/or thrombocytosis (Pasternak *et al.* 1998) while M-*bcr* is most commonly associated with CML. Depending on whether the break occurs before or after exon *b3* (*e14*), it produces either splice variant *b2a2* or *b3a2* and encodes a p210 BCR-ABL (Bagg 2002).

The treatment options for CML patients include allogeneic stem cell transplantation (Allo-SCT), chemotherapy, interferon-alpha, cytarabine and tyrosine kinase inhibitors like imatinib (Stone 2004). Allogeneic hematopoietic stem cell transplantation is currently the only curative treatment for CML, but is accessible to only about 40% of patients (Stone 2004). Chemotherapeutic agents commonly used to treat patients with CML are hydroxyurea (HU) and busulfan (BU). However, these are considered palliative since they do not prolong overall survival (Hehlmann *et al.* 2007). Hydroxyurea results in the inhibition of ribonucleotide reductase, which controls the concentration of deoxyribonucleotides present in the cell for DNA synthesis. Busulfan is an alkylating agent that slows the growth of cancer cells (Negrin 2004). Busulfan causes serious adverse effects, including myelo-suppression and pulmonary, hepatic and cardiac fibrosis. Hydroxyurea has better efficacy and side-effect profile compared to BU, but still results in side effects such as nausea and other gastrointestinal reactions, myelo-suppression, skin atrophy and drug fever. Long-term side effects include lichenoid dermopathy, lower-extremity skin ulcers, cutaneous squamous cell carcinoma, gangrene of the toes, vasculitis and life-threatening pulmonary reactions (Stone 2004).

Interferon-alpha (IFN-α) is a glycoprotein with antiproliferative properties that down regulates the expression of oncogenes (Faderl *et al.* 1999; Frazer *et al.* 2007). Acute side effects such as flu-like symptoms and chronic reactions, such as fatigue and lethargy, depression, weight loss, myalgias and arthralgias occur in approximately 50% of patients (Stone 2004). The most important persistent side effects are neuropsychiatric resulting in a decreased quality of life (Stone 2004). Cytarabine (Ara-C) is an antimetabolite agent and is rapidly converted to cytosine arabinoside triphosphate, which damages DNA when the cell cycle is in the S-phase. Furthermore, it inhibits DNA- and RNA-polymerase and nucleotide reductase enzymes. Combination therapy of IFN-α with Ara-C is more effective in terms of achieving haematological, cytogenetic and molecular remissions, than either alone, or in conjunction with chemotherapeutic agents, BU and HU (Hughes *et al.* 2003; Henkes *et al.* 2008). Combination therapy of IFN-α and Ara-C produces side effects such as nausea, vomiting, diarrhoea, and thrombocytopenia (Negrin 2004).

In May 2001, the Food and Drug Administration approved imatinib mesylate® (STI571) (Gleevec, Novartis, Basel, Switzerland), a drug that has revolutionized the treatment of CML (Frazer *et al.* 2007). Imatinib was the first example of targeted molecular therapy for CML (Hughes and Branford 2006). Chronic phase CML patients who failed on IFN-α therapy was administered 300 mg (or more) imatinib daily (Kantarjian *et al.* 2002). High response rates were observed during phase II trials of imatinib administered to chronic-phase, accelerated-phase and myeloid-blast-crisis CML patients (Henkes *et al.* 2008). Imatinib produced good responses and improved the overall survival rate and quality of life of CML patients. The IRIS trial, a phase III study, demonstrated the effectiveness of imatinib as mono-therapy at 400 mg daily for newly diagnosed CML patients, compared to the combination of interferon and cytarabine (O'Brien *et al.* 2003). In the IRIS trial groups two comprising 553 individuals participated. Crossover to the alternative group was allowed if stringent criteria for treatment failure and/or intolerability of the drug were met (O'Brien *et al.* 2003). The most common side effects of imatinib treatment were diarrhoea, nausea, oedema, skin rash and de-pigmentation, muscle cramps and myalgia, elevated liver transaminase and myelo-suppression (Guilhot 2004).

Furthermore, imatinib was better tolerated than combination therapy with interferon and cytarabine (O'Brien *et al.* 2003).

Imatinib mesylate (STI571) was the first drug designed to specifically inhibit the tyrosine kinase activity of BCR-ABL (Mauro and Druker 2001; Savage and Antman 2002; Sharifi and Steinman 2002; Manley *et al.* 2005). It was developed by Novartis, Switzerland after 2-phenylaminopyromidine compounds were identified as having tyrosine kinase inhibitory activity (Mauro and Druker 2001). These compounds later become known as tyrphostins. To date, CML is the best studied molecular model of leukaemia (Kantarjian *et al.* 2000) and the first neoplasia where elucidation of the genotype led to the development of rationally designed therapeutics of the phenotype (Hehlmann *et al.* 2005). Gambacorti-Passerini and co-workers have indicated that inhibition of ABL kinase activity blocks the proliferation of BCR-ABL positive leukemic cells and induces apoptosis (Gambacorti-Passerini *et al.* 1997). Targeted treatment affects primarily tumour cells and specifically acts by inhibiting the protein product of the oncogene (Drummond and Holyoake 2001; Sharifi and Steinman 2002; Goldman and Melo 2003; Stone 2004; Manley *et al.* 2005).

Imatinib prevents the binding of ATP to BCR-ABL by stabilizing the enzyme in the inactive state (Gambarcorti-Passerini *et al.* 2003). Thus, targeted treatment affects tumour cells by inhibiting the oncoprotein (Drummond and Holyoake 2001; Sharifi and Steinman 2002; Goldman and Melo 2003; Stone 2004; Manley *et al.* 2005). Imatinib has also been shown to inhibit Abelson (ABL), platelet derived growth-factor receptor (PDGFR) α and β, KIT and Abelson-related gene (ARG) (Melo *et al.* 2003). As c-kit and PDGFR are implicated in other solid tumours, imatinib is also used as treatment in some of these conditions (Drummond and Holyoake 2001). Although the exact cellular mechanism of why tyrosine kinase inhibitors reduce levels of BCR-ABL is unknown, it appears that the inhibition of downstream signal transduction pathways suppresses the clonal proliferation of oncogenic cells.

A follow-up of the IRIS trial at 19 and 60 months, respectively, indicated the highly significant superiority of imatinib with a complete cytogenetic response

rate (CCyR) of 95% and 87%, respectively, compared to the 55% of patients with a CCyR on interferon-alpha at 19 months (Druker *et al.* 2006; Henkes *et al.* 2008). 57% of patients who had a CCyR after 12 months, also indicated a 1000-fold (3 log) decrease in *BCR-ABL* transcript level, as compared to only 24% of patients in the group given interferon plus cytarabine (Hughes *et al.* 2003). There was a 100% chance of progression free survival (PFS) for patients with a CCyR together with a thousand times (3 log) reduction in BCR-ABL transcript levels at 24 months, compared to 85% for patients who were not in CCyR at 12 months (Hughes *et al.* 2003). Furthermore, it was estimated that 39% of all patients treated with imatinib, but only 2% of all those given interferon plus cytarabine had a reduction in BCR-ABL transcript levels of at least 1000-fold (Hughes *et al.* 2003). The estimated overall survival of patients who received imatinib as first-line therapy was 89% at 60 months (O'Brien *et al.* 2008). As a result of its success, imatinib has replaced stem cell transplantation as first line therapy for CML.

1.1.3 Acquired resistance to imatinib in CML patients

Despite its success, resistance to imatinib has been recorded approximately two percent of patients with CML and can be innate or acquired. Resistance to imatinib is defined as a failure to achieve complete haematological remission (CHR) after three months of therapy, and/or failure to achieve at least a cytogenetic response (CyR) after six months of therapy, and/or failure to achieve a major cytogenetic response (MCyR) at 12 months of therapy, and/or loss of an earlier obtained CHR or CyR (Wei *et al.* 2006).

Several studies have indicated that mutations in the BCR-ABL kinase domain are the cause for acquired imatinib resistance, with the T315I mutation inferring absolute resistance towards treatment with all types of tyrosine kinase inhibitor including imatinib, dasatinib, nilotinib and bosutinib (Branford *et al.* 2002; Roche-Lestienne *et al.* 2002; Branford *et al.* 2003; Kantarjian *et al.* 2003; Liu and Makrigiorgos 2003; Sacha *et al.* 2003; Hayette *et al.* 2005; Jabbour *et al.* 2006[a]; Jabbour *et al.* 2006[b]; Nicolini *et al.* 2006). Threonine at position 315

forms a crucial hydrogen bond with imatinib and the absence of an oxygen atom in the substituted isoleucine prevents this bond from forming (Gambacorti-Passerini *et al.* 2003). It was also proposed that the bulkier isoleucine induces a steric clash with imatinib, which led to designating the residue at 315 as the gatekeeper of imatinib (Gambacorti-Passerini *et al.* 2003).

A variety of other mutations within the kinase domain, located at the P-loop (ATP binding site), the catalytic domain and the activation loop bring about different levels of resistance, most of which can be overcome by increasing imatinib dosage (Apperley 2007). These kinase domain mutations may be present at very low frequencies at the onset of treatment and manifest later through the selective pressure invoked upon them by imatinib therapy, or alternatively mutations can be acquired during the course of therapy (Roche-Lestienne *et al.* 2002). The exact mechanism of how mutations are acquired is not known, primarily because the detection of mutations by DNA sequencing is not sensitive enough (Apperley 2007; Roche-Lestienne *et al.* 2002).

The development of mutations in the kinase domain is but one of many mechanisms involved in resistance to imatinib. Other possible explanations for imatinib resistance can be attributed to over-expression of BCR-ABL (Hochhaus *et al.* 2002[a]; Barnes *et al.* 2005) as well as *BCR-ABL* gene duplication (Weisberg and Griffin 2003) and clonal evolution (Apperley 2007). The duplication of the Ph-chromosome has been proposed as a possible mechanism of resistance to imatinib in patients with CML (Ossard-Receveur *et al.* 2005). Furthermore, the concentration of the drug in the target cell can be influenced by increased P-glycoprotein levels as a result of over-expression of the MDR1 (multi-drug resistance) gene (Tauchi and Ohyashiki 2004). Additionally, vast quantities of imatinib are bound to alpha one acid glycoprotein as well as albumin, which prevent the drug from reaching its intracellular target (Henkes *et al.* 2008). The intracellular availability of imatinib can also be influenced by active drug efflux through the ABCB1 and ABCB 2 trans-membrane ATPases (Apperley 2007).

Hochhaus and La Rosee (2004) proposed dose escalation, interruption or cessation of imatinib therapy, upfront combination therapy and second-line combination therapy as strategies to treat resistant patients. Discontinuation of therapy with imatinib has been shown to decrease the prevalence of mutations such as T315I that confer absolute resistance to the drug (Weisberg and Griffin 2003). Most other resistant mutations can be overcome by increasing imatinib dosage (Nicolini *et al.* 2006), or with second-generation tyrosine kinase inhibitors, such as dasatinib and nilotinib, as well as combination therapy with imatinib and decitabine, homoharringtonine and interferon (Jabbour *et al* 2006[c]).

Currently, many novel targeted therapies are also being explored to overcome imatinb resistance in CML (Walz and Sattler 2006). For example, AG957 reverses the effects of multidrug resistance (Yeheskely-Hayon *et al.* 2005); nilotinib selectively inhibits native and mutant BCR-ABL (Golemovic *et al.* 2005; Weisberg *et al.* 2005); dasatinib is a highly potent dual SRC/ABL inhibitor (O'Hare *et al.* 2005); zoledronate is a bisphosphonate that inhibits the oncogenicity of Ras, an important downstream effector of BCR-ABL (Chuah *et al.* 2005); and berbamine selectively induces cell death of imatinib resistance Ph-positive CML cells (Xu *et al.* 2006). SKI-606 is a promising dual SRC/ABL inhibitor that inhibits phosphorylation of cellular proteins, including STAT5, and is currently undergoing clinical trials (Golas *et al.* 2003). VX-680 is the only small molecule inhibitor to show activity against the T315I mutant cultures since it is bound to ABL in a mode that accommodates the substitution at the gatekeeper position and may hold the promise of re-sensitizing these mutant clones to treatment (Young *et al.* 2006). It has also been shown that MK-0457, an aurora kinase inhibitor has remarkable clinical activity against T315I clones (Martinelli *et al.* 2007).

1.2 Molecular methods used in CML diagnostics

Monitoring the outcome of treatment with imatinib in CML patients is necessary to determine the prognosis. Up to 90% of CML patients on imatinib have a positive prognosis and progression free survival for up to 24 months (Hughes *et al.* 2003). Accumulating evidence of first-line imatinib treatment from various

clinical trials, as opposed to conventional chemotherapy, clearly indicates the superior efficacy of this drug. When patients undergo treatment with imatinib, a decrease in *BCR-ABL* mRNA level can be correlated to a haematological, cytogenetic or molecular response (Jabbour *et al.* 2008). These responses are based on monitoring CML patients with different techniques including FISH (fluorescence in situ hybridization), conventional cytogenetic analysis (karyotyping) and BCR-ABL quantification, respectively (Kantarjian *et al.* 2002).

Patients in accelerated or blastic phase responding favourably to treatment with imatinib, can revert to the chronic phase, and eventually achieve remission. A complete haematological response, measured as a platelet count less than $450x10^9$/L, WBC (white blood count) less than $10x10^9$/L, differential without immature granulocytes and with less than 5% basophils together with a non-palpable spleen, is as a result of proliferation being stunted (Baccarani *et al.* 2006). A cytogenetic response is determined according to the amount of Ph-positive metaphases, with a value of 0% indicative of a complete cytogenetic response (Hughes 2006). Molecular response is measured by Real-time *BCR-ABL* mRNA quantification. *BCR-ABL* transcripts are quantified relative to a control gene and results are represented on a logarithmic scale with a three-log reduction from a standardized baseline indicative of a major molecular response (Hughes *et al.* 2006; Branford *et al.* 2008). When the real-time assay is sensitive enough, a measured four (or more) logarithmic decrease in *BCR-ABL* mRNA transcripts are considered a complete molecular response and is also accompanied by a complete cytogenetic response (Martinelli *et al.* 2006[a]).

1.2.1 Karyotyping and FISH

The Ph chromosome was originally detected as an abnormally short G-group chromosome in analysis of bone marrow metaphases from CML patients (Van Etten 2004) which led to the development of the fluorescence in situ hybridization technique. Fluorescent in situ hybridization (FISH) is used to identify the Philadelphia chromosome (Kaeda *et al.* 2002; Madon *et al.* 2003). FISH analysis is performed by hybridization with probes specific for *BCR* and *ABL* on interphase nuclei of patients (Raanani *et al.* 2004). FISH has a false-

positive rate of 1% when using dual labelled probes and therefore results of up to 1% are considered negative (Raanani *et al.* 2004).

The disadvantage of FISH is that it cannot be used to distinguish between the different *BCR-ABL* breakpoints and is not considered quantitative (Van Etten 2004). FISH is a laborious technique and considered approximately a hundred times less sensitive than quantitative Real-time PCR (Tefferi *et al.* 2005). It relies on the objectivity of the investigator and is unsuitable for monitoring minimal residual disease in CML (Tefferi *et al.* 2005). Furthermore, it has been determined that no significant relationship exists between the percentage of positive nuclei by FISH and the *BCR-ABL/ABL* ratio (Kim *et al.* 2002).

Karyotyping and FISH is also essential for the detection of novel secondary chromosomal abnormalities that may develop as complex Philadelphia translocations during CML and ALL pathogenesis (Costa *et al.* 2006). Trisomy 8 and 19, iso-chromosome 17q and a double Philadelphia chromosome are some of the most common secondary cytogenetic phenomena associated with CML (Calabretta and Perrotti 2004). These cytogenetic aberrations involve gene clusters responsible for DNA break-repair, telomere maintenance, cell cycle control, oncogene expression and apoptosis (Radich 2007). Such secondary chromosomal changes are often an indication of disease progression (Gordon *et al.* 1999).

1.2.2 Flow cytometry

The use of flow cytometric techniques in the detection and monitoring of CML has remained limited. The technique has been used to identify abnormal populations of cells in CML patients with monoclonal antibodies, being either HLA-DR-positive or HLA-DR-negative (Ligler *et al.* 1985). HLA-DR-positive populations underwent no clonal evolution, but progressed to a lymphoid blastic crisis whereas HLA-DR-negative populations exhibited chromosomal abnormalities in addition to the Philadelphia chromosome and progressed to a myeloblastic acute phase (Ligler *et al.* 1985). Furthermore, it has been determined by DNA-flow cytometry (DNA-FCM) that bone marrow cell

proliferation in CML patients at diagnosis and during apparent remission is not essentially different from normal. However, during malignant metamorphosis changes occur in ploidy level and proliferative activity can be detected by DNA-FCM in an early phase (Holdrinet *et al.* 1983). Recent work has focused on the investigation of flow cytometry together with in situ polymerase chain reaction (Preudhomme *et al.* 1999[a]) using labelled primers in an attempt to extend the analytic power of flow cytometry into the molecular arena (Jennings and Foon 1997).

1.2.3 PCR based techniques

The latest PCR-based techniques to detect ABL kinase domain mutations, include denaturing high-performance liquid chromatography (Deininger *et al.* 2004), ASO-PCR (Iqbal *et al.* 2004; Kang *et al.* 2006) and high resolution melting curve analysis (Gutierrez *et al.* 2005; Polakovà *et al.* 2008). These techniques in combination with Real-time quantification of *BCR-ABL* mRNA, have become increasingly important for the treatment of CML (Branford *et al.* 2004). It is for this reason that molecular diagnostic laboratories committed to *BCR-ABL* mRNA quantification have realized the vital role of RNA stabilization and preservation during the development of a successful, accurate and highly sensitive Real-time quantification assay (Muller *et al.* 2002; Thörn *et al.* 2005; Fleige and Pfaffl 2006). Real-time quantification of *BCR-ABL* mRNA is currently the most sensitive method for disease monitoring in CML, especially for minimal residual disease (MRD) (Tefferi *et al.* 2005) and has been shown to predict the likelihood of relapse (Kantarjian *et al.* 2003), allowing potentially beneficial treatment adjustments for patients at high risk (Lange *et al.* 2004; Shüler and Dölken 2006). Perhaps the biggest disadvantage and limit to Real-time quantification of *BCR-ABL* mRNA is the lack of international method standardization as the robustness of the technique has led to countless laboratories developing in-house assays (Muller *et al.* 2007).

1.2.4 Real-time PCR quantification: The gold standard

The amount of BCR-ABL mRNA (and subsequently its cDNA) is directly correlated to the disease-load and stage (Elmaagacli *et al.* 2000), hence real-time quantification was quickly established as the most effective and sensitive technique to monitor patient response to imatinib treatment (Gabert *et al.* 2003; Hughes and Branford 2006). Real-time quantification of BCR-ABL expression gives a clear indication of disease progression and prognosis (Martinelli *et al.* 2006[b]). The level of BCR-ABL is directly correlated with pathogenesis and the higher the level of BCR-ABL mRNA, the poorer the prognosis (Moravcová *et al.* 2004; Michor *et al.* 2005; Prejzner 2002). In this state more of the oncoprotein is present within the cytoplasm to interfere with signal transduction cascades. Malfunctioning cell cycle regulation and a loss of equilibrium facilitates neoplastic transformation. It would seem that by inhibiting the BCR-ABL tyrosine kinase itself, the amount being transcribed also decreases (Elmaagacli *et al.* 2000). By reverse transcribing the mRNA into complementary DNA (cDNA), which is subjected to real-time PCR, BCR-ABL is quantified relative to a control gene (Beillard *et al.* 2003).

1.2.5 Prognostic markers in CML

Cytogenetic analysis remains central to monitoring CML (Jha *et al.* 2006). For FISH, minimal cytogenetic response, minor cytogenetic response and partial cytogenetic response are defined as 65 to 95%, 35 to 65%, and 1 to 35% Ph^+ metaphases, respectively (Baccarani *et al.* 2006). *BCR-ABL* quantification results are represented on a logarithmic scale (Figure 5). Patients who have a percentage *BCR-ABL* decrease of more than three-log, to 0.1% from the baseline value (determined at the start of therapy) are considered to have had a major molecular response (MMR) (Hughes and Branford 2006). Since percentage *BCR-ABL* expression levels were first compared with FISH, a CCyR is equivalent to a two-log (100-fold) reduction of the initial *BCR-ABL* level. A complete molecular response (CMR) corresponds to undetectable *BCR-ABL* mRNA (Frazer *et al.* 2007). Hence, Real-time quantification of *BCR-ABL* mRNA

is a more sensitive technique and offers a far greater level of resolution in terms of molecular, instead of cytogenetic, monitoring of CML treatment.

Patients with a MMR and/or CMR are monitored for minimal residual disease (MRD) and have undetectable levels of *BCR-ABL* mRNA (Hochhaus *et al.* 2000). A 10-fold (logarithmic) increase in *BCR-ABL* indicates a loss of response and possible development of resistance to TK inhibitors due to genetic mutation (Barnes *et al.* 2005; Hughes and Branford 2006; Wang *et al.* 2006). If a patient being treated with imatinib achieves a MMR by 12 months this is indicative of a good prognosis with a 98% possibility of PFS (Hughes and Branford 2006). Decreasing levels of BCR-ABL mRNA indicate overall improvement of prognosis (Kantarjian *et al.* 2002). Thus CML disease progression and regression is determined according increasing and decreasing *BCR-ABL* mRNA levels and is essential to monitor how patients respond to treatment (Elmaagacli *et al.* 2000; Hochhaus 2002). How patients respond to treatment provides a platform to predict future response and whether a patient will achieve event-free survival (Faderl *et al.* 2004; Colombat *et al.* 2006; Hughes 2006; Martinelli *et al.* 2006[b] Piazza *et al.* 2006; Gupta and Prasad 2007; Jabbour *et al.* 2008).

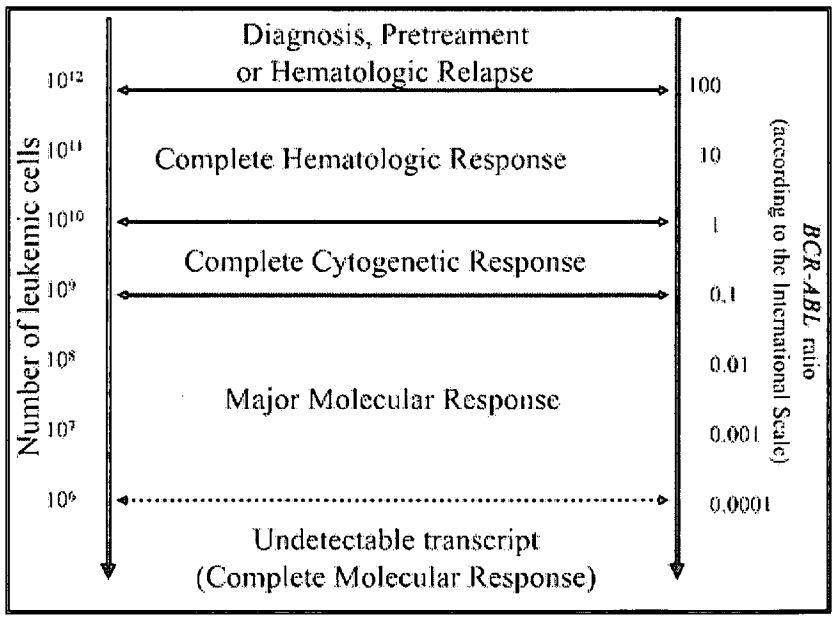

Figure 5: The international scale for *BCR-ABL* log reduction (proposed by Hughes *et al.* 2006 and copied from Baccarani *et al.* 2006). This scale depicts logarithmic decrease in *BCR-ABL* transcript copy number, relative to a control gene, as a percentage. A Major Molecular Response is shown as a three-log decrease from a standardized baseline of 100% and is measured at a level of 0.1% *BCR-ABL*.

Compared to this, patients that have not achieved a one-log reduction in *BCR-ABL* mRNA by six months are unlikely to attain a sustained molecular response (Hughes and Branford 2006). A three-log reduction by 12 months is indicative of a very good prognosis (Faderl *et al.* 2004; Jabbour *et al.* 2008). However, there are patients that require a year or more to achieve a MMR and still have a good prognosis (European LeukaemiaNet).

According to results from the IRIS trial (International Randomized study of Interferon versus STI-571), approximately 39% of all first line imatinib treated patients achieved a greater than or equal to three log reduction (MMR) of *BCR-ABL* by 12 months of therapy (Table 1) (Hughes and Branford 2006). Patients, who achieved a CCyR, but not a three-log reduction by 12 months, had an 8% risk of progression, whereas patients who did not achieve a CCyR had a 20% risk of progression (Hughes and Branford 2006). According to the five-year follow-up study of CML patients receiving imatinib, those who had a reduction of *BCR-ABL* levels of at least three log after 18 months of treatment, had an estimated rate of progression-free survival of 100% (Druker *et al.* 2006).

It has been suggested that patients with rising levels of BCR-ABL transcripts should be screened for kinase domain mutations, especially those in the advanced phase of the disease (Branford *et al.* 2004). For chronic phase patients who started treatment with tyrosine kinase inhibitors, mutation screening is recommended if there is an inadequate response or any loss of response. This includes patients who have failed to achieve a complete haematological response at three months, minimal cytogenetic response at six months, or a major cytogenetic response at 12 months (Hughes *et al.* 2006).

Loss of response is defined provisionally as haematological relapse, relapse from complete cytogenetic response to Ph positive, or an increase in percentage *BCR-ABL* of one-log or greater (Hughes *et al.* 2006). Misinterpretation of results may have serious effects on the treatment and prognosis of the patient (Hughes *et al.* 2006).

For patients in molecular remission, monitoring of minimal residual disease by Real-time quantification of *BCR-ABL* mRNA is necessary to determine whether CMR is being maintained (Ginzinger 2002; Cazzaniga *et al.* 2006). Monitoring allows the detection of changes or shifts in *BCR-ABL* mRNA levels, which is indicative of a loss of MRD (Radich 2000; Oehler and Radich 2003). It is recommended that if the level of *BCR-ABL* is increasing in a patient, they be monitored every three months (Table 2) (Baccarani *et al.* 2006; Hughes *et al.* 2006; Laneuville *et al.* 2006; Martinelli *et al.* 2006[a]). For patients in remission, it is recommended that the levels of *BCR-ABL* mRNA be monitored once every six months (Hughes *et al.* 2006). Treatment with Imatinib is made effective through the use of Real-time PCR quantification to monitor response to treatment and help patients achieve progression-free survival with a better quality of life (QoL). Thus Real-time quantification of *BCR-ABL* mRNA gives the most accurate and precise indication of patient response to treatment as well as disease stage for monitoring and prognosis (Shüler and Dölken 2006).

Table 1: Proposed response criteria for chronic phase CML patients treated with 400 mg imatinib (from European LeukaemiaNet and copied from Baccarani *et al.* 2006).

Time	Failure	Suboptimal response	Warnings	Optimal response
Diagnosis	N/A	N/A	High risk, del9q+, ACAs in Ph+ cells	N/A
3 months after diagnosis	No HR (stable disease or disease progression)	Less than CHR	N/A	CHR
6 months after diagnosis	Less than CHR, no CgR (Ph+ > 95%)	Less than PCgR (Ph+ > 35%)	N/A	At least PCgR (Ph+ ≤ 35%)
12 months after diagnosis	Less than PCgR (Ph+ > 35%)	Less than CCgR	Less than MMolR	CCgR
18 months after diagnosis	Less than CCgR	Less than MMolR	N/A	MMolR
Any time	Loss of CHR* Loss of CCgR† Mutation‡	ACA in Ph+ cells§ Loss of MMolR§ Mutation#	Any rise in transcript level Other chromosomal abnormalities in Ph– cells	N/A

N/A Not applicable

ACA Additional chromosomal abnormalities

HR Haematological response

CCyR Complete cytogenetic response

PCgR Partial cytogenetic response

* To be confirmed on two occasions unless associated with progression to accelerated phase/blast crisis

† To be confirmed on two occasions unless associated with CHR loss or progression to accelerated phase/blast crisis

‡ High level of insensitivity to imatinib

§ To be confirmed on two occasions unless associated with CHR or CCyR loss

\# Low level of insensitivity to imatinib

Table 2: Definitions of responses to CML therapy and recommended monitoring (from European LeukaemiaNet and copied from Druker *et al.* 2006).

	Definition	Monitoring
Hematologic response (complete)	Platelet count < 450 x 10^9/L WBC count < 10 x 10^9/L Differential: without immature granulocytes and with < 5% basophils Non-palpable spleen	Check every 2 weeks until complete response achieved and confirmed, then every 3 months unless otherwise specified
Cytogenetic response	Complete: Ph+ none Partial: Ph+ 1–35% Minor: Ph+ 36–65% Minimal: Ph+ 66–95% None: Ph+ > 95%	Check every 6 months until complete response achieved and confirmed
Molecular response (BCR-ABL: control gene ratio according to an international scale)	'Complete': transcript non-detectable Major: ≤ 0,1 %	Check every 3 months; mutational analysis only in case of failure, suboptimal response or increased level of transcript

1.3 Real-time PCR quantification of *BCR-ABL*

1.3.1 Requirement for standardization

To date, approximately 33 papers on the use of Real-time quantification of *BCR-ABL* have been published (Table 3). Although these publications discuss different variations on the same theme, there are significant differences in RNA extraction, cDNA synthesis, Real-time PCR including the use of reference gene, quantification standards and interpretation of results. Only one method has been regionally standardized through an initiative known as the Europe Against Cancer (EAC) program (Gabert *et al.* 2003). However, many other efforts have been made regarding method standardization for BCR-ABL quantification (Hochhaus 2003; Branford *et al.* 2006; Hughes *et al.* 2006).

It has been suggested that assay standardization would allow for better correlation of results between different institutions (Fossey *et al.* 2005). Fossey and co-workers (2005) concluded that standardization should focus on the maintenance of RNA integrity and the use of appropriate calibration controls. Skern *et al.* (2005) recommended that RQ-PCR results should be analyzed with

caution, preferably by using two or more analytical approaches to validate conclusions and emphasized that an effort should be made to standardize methods.

In a comparative study by Zhang *et al.* (2007), it was found that primers, enzymes, different PCR kits, and reagents did not affect the reported log results for *BCR-ABL* quantification. Furthermore, they found that the use of diluted RNA, cDNA, plasmid DNA or cell lines for standard curves did not affect the reporting of results. In contrast to this, Curry *et al.* (2002[a]) and Branford *et al.* (2008) stated that minor alterations in an analytical system such as primer concentration, the type of reverse transcriptase and the selection of reference gene could have a significant impact on the measurement of *BCR-ABL* transcripts.

To elucidate the potential problems in the quantification of BCR-ABL using Real-time PCR without a standardized approach, Yamada *et al.* (2008) established a collaborative effort with four diagnostic laboratories in Japan, comparing quantitative reverse transcription PCR (RT- PCR) based detection for minimal residual disease in leukaemia. It was found that 38.4% of samples displayed a more than 10-fold inter-laboratory difference in quantitative results. The greatest differences between laboratories were evident in methods for RNA extraction and resulted in the variability of PCR results. From this it was concluded that the RNA extraction and PCR steps are most crucial for method standardization. Müller *et al.* (2007; 2008) suggested that standardization of quantitative PCR is possible and independent of the specific equipment platform being used. Müller *et al.* (2007) concluded that the comparability of RQ-PCR data depends upon using the same method of analysis between laboratories.

Most methods to quantify BCR-ABL mRNA are developed in-house and cannot easily be implemented without access to proprietary standards. Furthermore, the variability in the use of control gene to normalize *BCR-ABL* mRNA copy number can result in misinterpretation of data (Jabbour *et al.* 2000; Branford *et al.* 2008). Thus the greatest limitation in implementing a method for the

quantification of *BCR-ABL* is the lack of readily available copy number standards, especially for *BCR*, as well as reference controls for *BCR-ABL*.

1.4 Conclusions

CML is a stem cell disorder that leads to neoplastic transformation of blood cells. CML is presented in a benign chronic phase, but the disease rapidly progresses to an accelerated and blastic phase, within three to five years if left untreated (Sawyers 1999). BCR-ABL encodes a non-receptor tyrosine kinase with constitutive activity. BCR-ABL affects signalling pathways that inhibit apoptosis, cell cycle control, differentiation and cellular adhesion. The combined effect of malfunctioning signal transduction is neoplastic transformation and malignancy.

Imatinib prevents the binding of ATP to BCR-ABL by stabilizing the enzyme in the inactive conformation (Gambarcorti-Passerini *et al.* 2003). The effective management of CML treatment requires the quantification of *BCR-ABL* transcripts by RQ-PCR since this is correlated to disease stage and prognosis (Elmaagacli *et al.* 2000). However, more than thirty methods for the quantification of *BCR-ABL* have been published. Considering the variety of techniques and of standards and reference material, it is difficult to compare results from different assays (Branford *et al.* 2008). Thus there is a need to standardize methods for *BCR-ABL* quantification considering the impact of these on the management of patient treatment.

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Chapter 2:

A comparison of published methods for Real-time PCR quantification of *BCR-ABL*

2.1 Introduction

In 2001, imatinib mesylate® (Novartis, Switzerland) was first introduced as targeted molecular therapy for chronic myeloid leukaemia (CML) (Savage and Antman 2002). The functioning of the drug is to block the ATP binding pocket of the BCR-ABL kinase and thus stabilize it in the inactive state (Gambacorti-Passerini *et al.* 2003). With the tyrosine kinase inhibited, it cannot phosphorylate downstream effector molecules and subsequent cellular events are abrogated (Mauro and Druker 2001). A five-year follow-up study has determined that patients receiving imatinib from the start of treatment have an 89% survival rate compared to 68% for those receiving interferon plus cytarabine (Druker *et al.* 2006).

An important part of successfully treating CML patients with imatinib is to monitor *BCR-ABL* mRNA transcript levels. Real-time quantitative PCR is sensitive and robust, and has been implemented in various laboratories to monitor *BCR-ABL* mRNA transcript levels in CML patients (Saffroy *et al.* 2000). It was used with great success during the IRIS trial, (International Randomized Study of Interferon vs. STI 571 (imatinib)), a phase III clinical trial which compared the use of imatinib and interferon for the treatment of CML and monitor patients' response to imatinib therapy (Hughes and Branford 2006). The IRIS trial demonstrated that there is a correlation between levels of *BCR-ABL* mRNA and disease progression (Hughes and Branford 2006). Hughes and Branford (2006) also determined that the decrease of *BCR-ABL* mRNA in CML patients being treated with imatinib could be used as a prognostic marker to determine treatment success.

BCR-ABL mRNA transcript levels were correlated to disease stage and progression (Elmaagacli *et al.* 2000), is predictive of a cytogenetic response (Branford *et al.* 2003) and strongly correlated to prognosis (Merx *et al.* 2002; Wang *et al.* 2002). Furthermore BCR-ABL transcript levels are used to predict the duration of a complete cytogenetic response and prolonged response (Press *et al.* 2006; Piazza *et al.* 2006). Therefore, Real-time quantification of *BCR-ABL* mRNA provides a reliable monitoring alternative to bone marrow cytogenetics (Branford *et al.* 1999). Furthermore, RQ-PCR is the current standard for PCR quantification in CML (Faderl *et al.* 2004) and it is considered to be the most sensitive method to monitor patients and to predict relapse (Elmaagacli *et al.* 2001; Branford *et al.* 2004; Crossman and O'Brien 2004; Wang *et al.* 2006). Although RQ-PCR may be very effective, it requires careful design and validation (Hughes and Branford 2006).

Approximately 33 papers have been published describing methods for Real-time quantification of *BCR-ABL* mRNA. These publications differ with regard to RNA extraction, cDNA synthesis, primers and probes as well as the use of standards and reference material. There have been attempts to standardize methodology to facilitate international cooperation between diagnostic laboratories (Burmeister *et al.* 2000; Hochhaus 2003; Skern *et al.* 2005; Branford *et al.* 2006; Hughes *et al.* 2006; Jobbagy *et al.* 2007; Müller *et al.* 2007; Müller *et al.* 2008). However, these attempts have been frustrated by the availability of commercial controls and standards, the use of different types of RT-enzyme, primer and probe sets and especially the choice of control gene (Fossey *et al.* 2005; Rulcová *et al.* 2007; Zhang *et al.* 2007; Yamada *et al.* 2008; Branford *et al.* 2008; Cross *et al.* 2008). The lack of standardization makes implementation of a method for Real-time quantification of *BCR-ABL* mRNA in a laboratory difficult, especially in terms of knowing which variables are important.

The Europe Against Cancer (EAC) program (Gabert *et al.* 2003; Beillard *et al.* 2003) was aimed at regionally standardizing a method to quantify BCR-ABL through the use of controls and standards that are available commercially. Hughes *et al.* (2006), Branford *et al.* (2008) and Martinelli *et al.* (2006) have

argued for the need to standardize methods through the use of laboratory specific conversion factors whereby a major molecular response is internationally represented as a three-log reduction in *BCR-ABL* transcript level from a predetermined baseline value of 100% to 0.1%. This would allow laboratories to continue to use their existing assays and express results according to an international scale (Cross *et al.* 2008). Despite this, a laboratory will find it difficult to implement a method for Real-time quantification of *BCR-ABL* mRNA since there are a myriad of different approaches available in current literature. The aim of this study was to make a detailed analysis of published methods for Real-time quantification of *BCR-ABL* mRNA. The synthesis of common practices for Real-time quantification of *BCR-ABL* mRNA would provide guidance on what could be used as best practice. This was done with emphasis on the methods published by Branford *et al.* (1999) and Gabert *et al.* (2003) for the Real-time quantification of *BCR-ABL* mRNA since these are the only two published methods that have attempted standardization.

2.2 Materials and Methods

A total of 33 publications, describing the quantification of *BCR-ABL* mRNA were analyzed and compared in terms of the following main areas of methodology: RNA extraction, purification, quantification and storage; reverse transcription (RT-enzyme and priming method for cDNA synthesis); the selection of a control gene for copy number normalization and relative expression of levels of *BCR-ABL* mRNA; the use of splice variant discrimination and copy number standards for absolute quantification (Table 3).

2.3 Results and Discussion

Considerable differences exist in the published methods for BCR-ABL quantification from sample processing to Real-time PCR and result analysis. It is interesting to note that the majority of methods were developed in-house and do not make use of commercial kits for either RNA extraction or Real-time quantification of BCR-ABL. The most considerable differences between the published methods are in RNA extraction, cDNA synthesis, the selection of

BCR-ABL primers and probes, the choice of control gene and the use of standards and reference material.

2.3.1 RNA extraction for quantification of *BCR-ABL*

One of the most crucial stages during Real-time quantification is RNA extraction and its preservation (Schmittgen *et al.* 2000; Müller *et al.* 2002; Thörn *et al.* 2005; Fleige and Pfaffl 2006). Different methods for RNA extraction range from conventional guanidinium-thiocyanate acid-phenol-chloroform to gradient centrifugation (Table 4). The most commonly used extraction method is cell homogenization, phase separation and RNA precipitation. From the literature it was evident that Trizol is most commonly used for RNA extraction (Table 4). Most laboratories may prefer to use Trizol compared to commercial kits since the former produces superior yields of RNA. In addition, Trizol is also a suitable reagent for storing homogenized leukocytes. RNA can be preserved for much longer in the un-extracted form in Trizol at -80℃. Furthermore, this technique allows for optimal use of patient samples since DNA can also be extracted from the Trizol homogenate. Trizol also has RNase inhibiting properties, resulting in optimal RNA yield whilst providing the opportunity to repeat extractions as well as easy storage of sample material.

Most published techniques require 5 to 10 ml of peripheral blood for RNA extraction. During the process of sample preparation, Trizol is used to homogenize total isolated leukocytes from the blood sample (Branford *et al.* 1999). It is essential that the entire sample of white blood cells be dissolved in Trizol for optimal RNA yield. Some laboratories still use conventional guanidinium-thiocyanate extraction techniques, which are based upon the same principal as that of Trizol (Mensink *et al.* 1998). RNA yields can range from 200 ng/µl to 4500 ng/µl of which between 1000 ng and 2000 ng of total extracted RNA is used as template for cDNA synthesis.

2.3.2 cDNA synthesis for quantification of *BCR-ABL*

For cDNA synthesis for Real-time quantification *of BCR-ABL* mRNA, most publications use random hexamer to prime the reverse transcription together with MMLV reverse transcriptase (RT). Some publications refer to the use of AMV-RT for reverse transcription (Wittor *et al.* 2000; Elmaagacli *et al.* 2001; Press *et al.* 2006). However, Wong *et al.* (1998) determined that the efficacy of MMLV-RT superseded that of AMV-RT and suggested that MMLV-RT should be used in cDNA synthesis above AMV-RT (Wong *et al.* 1998) (Table 4).

All publications for Real-time quantification of *BCR-ABL* mRNA use random hexamer to prime cDNA synthesis. However, Zhang and Byrne (1999) determined that the use of random hexamer could lead to an overestimation of mRNA copy numbers, while Nolan *et al.* (2006) found that random hexamer had varying efficiencies between targets. In comparison, gene specific priming is considered more efficient than random hexamer, especially for low copy numbers of mRNA (Wacker and Godard 2005) and has a greater range of linearity (Deprez *et al.* 2002; Bustin and Nolan 2004; Bustin *et al.* 2005). However, it has been postulated that primer dimers, when using gene specific priming, could result in a lower detection of copy number (Peters *et al.* 2004). In contrast to this, Freeman *et al.* (1999) found that the use of gene-specific priming enhanced copy number detection, especially for rare mRNA species, with decreased background noise associated with other types of priming. Additionally, Deprez *et al.* (2002) also found that gene specific priming for cDNA synthesis is more efficient than random hexamer for standard curves constructed from serial diluted template. One disadvantage of gene specific priming is that it only targets a specific RNA species and does not produce a representative pool of cDNA (Nolan *et al.* 2006). Thus random hexamer priming is considered to be the least reliable method, but if applied correctly, it would still yield reproducible results since it introduces the least bias in the resulting cDNA (Bustin and Nolan 2004). Nolan *et al.* (2006) concluded that it is important to use the same reverse transcriptase enzyme, priming strategy and experimental conditions if result is to be comparable between laboratories. This

is in agreement with findings from Branford *et al.* (2008) that stipulated that the type of RT-enzyme as well as primer concentration could have a significant impact on the measurement of BCR-ABL transcripts even though Zhang *et al.* (2007) found the impact from these variables to be negligible.

The quantity and quality of cDNA is considered one of the most critical variables in RQ-PCR analysis (Cross *et al.* 2008). For this reason, the use of minimal control gene copy number has been suggested by both Gabert *et al.* (2003) as well as Branford *et al.* (1999). According to Branford *et al.* (1999) when using BCR, a minimum of 40 000 copies per microgram of RNA is a prerequisite for quantification and samples generating control gene copy numbers below this level are considered degraded. In the same instance, Gabert *et al.* (2003) suggest a cut-off level of 1300 copies per microgram of RNA for ABL. However, there does not appear to be any specific justification for using these levels.

2.3.3 Primers and probes for quantification of *BCR-ABL*

Different primer and probe designs make it possible to either detect different forms of *BCR-ABL* without discriminating between them, whereas others have the ability to differentiate between p190 BCR-ABL, p210 BCR-ABL and p230 BCR-ABL, as well as the two splice variants for p210 BCR-ABL (Table 5 and 6). According to published literature, about 10% of methods discriminate between p210 BCR-ABL splice variants, whereas the rest either detect all possible p210 BCR-ABL forms, or a combination of p210 BCR-ABL and p190 BCR-ABL. However, most publications do not discriminate between p210 BCR-ABL variants. Notable exceptions are Branford *et al.* (1999), Eder *et al.* (1999) and de Lemos *et al.* (2005) that distinguish between the p210 BCR-ABL splice variants *b2a2* and *b3a2*. However, it is not certain if the use of splice variant discrimination offers any prognostic value above the added cost. De Lemos *et al.* (2005) suggest that patients with the *b2a2* splice variant may respond better to treatment with imatinib than those that carry *b3a2*. However, there is no substantial data to support this finding.

All primers for *BCR-ABL* are designed to span intron/exon boundaries to ensure that only cDNA and not DNA is amplified (Table 5). Reference to amplicon size is very seldom made and only sequence information is provided. However, from an analysis of the binding positions of primers, it is evident that most amplicons are 100 bp or less in size.

Considering all available primers and probes, great discrepancies could arise as reported by Branford *et al.* (2008). Quantification results from assays that did not use the same primers may not be comparable, especially when used in combination with different standards (Branford *et al.* 2008). Furthermore, the use of primers to discriminate between splice variants makes the assay unnecessarily expensive.

2.3.4 Selection of control gene for quantification of *BCR-ABL*

During quantification, levels of *BCR-ABL* are normalized against a reference or control gene. The RNA of a suitable control gene should have degradation kinetics similar to that of *BCR-ABL* in order to maintain the ratio between target and control gene (Wang *et al.* 2006). In addition, the expression of the control gene should remain unaffected by the treatment of the patient (Rulcová *et al.* 2007).

Different control genes are used for copy number normalization including *ABL*, *BCR*, *GUS*, to name a few (Lee *et al.* 2006). Beillard *et al.* (2003) suggested that ABL is the most suitable control gene for RQ-PCR based diagnosis and MRD monitoring since its expression profile did not differ significantly between normal and leukemic samples at diagnosis. However, recent research has shown that *GUS* expression levels were found to be the least affected by imatinib treatment compared to *ABL* (Lee *et al.* 2006). Furthermore, it was found that *ABL* expression is not the same in CML cells compared to other hematopoietic cells (Wang *et al.* 2006).

Irrespective of the arguments for or against, *ABL* is the most commonly used control gene for normalization and Real-time quantification of *BCR-ABL* mRNA (Table 5). It is also the control gene used and recommended by the Europe Against Cancer initiative (Gabert *et al.* 2003). However, recent publications from Lee *et al.* (2006), Wang *et al.* (2006) and Rulcová (2007) provide evidence that *GUS* is a better control gene for *BCR-ABL* quantification compared to *ABL*. They concluded that since *ABL* is part of *BCR-ABL*, this undermines *ABL's* fundamental role as control gene during copy number normalization, especially at high levels of *BCR-ABL* expression. In c-amplification, normal and fusion *ABL* can lead to an increase in total *ABL* transcript level resulting in an underestimation of disease load (Rulcová *et al.* 2007). Lee *et al.* (2006) and Wang *et al.* (2006) also found that *ABL* did not degrade at the same rate as *BCR-ABL* and that its expression is influenced by treatment with imatinib. Furthermore, it was recommended by members of the Bethesda CML group (2005) that *GUS*, compared to *ABL* and *BCR*, should be used as a control gene (Cross *et al.* 2008).

Currently only one method makes use of *BCR* as a control gene for the Real-time quantification of *BCR-ABL* mRNA (Hughes *et al.* 2006). There is no specific evidence that *BCR* is unsuitable as control gene. However, since *BCR* is also part of *BCR-ABL*, similar to *ABL*, it may result the same underestimation of *BCR-ABL* at high levels.

2.3.5 Standards and reference material for quantification of *BCR-ABL*

During Real-time PCR quantification, standards and reference material are necessary in order to accurately and reliably determine the level of BCR-ABL mRNA (Branford *et al.* 1999; Jones *et al.* 2003). Without the availability of standards and reference material, it becomes almost impossible to implement a method in a local laboratory that has been developed elsewhere. This may be the single biggest contributing factor to frustrating efforts for method standardization.

Currently, mRNA extracted from cell lines K562 and HL60 are used as positive and negative controls, respectively (Table 7). Unfortunately, the requirement for cell culturing adds a further complexity and technical difficulty to standardization. It would be very helpful if commercial reference cDNA were available instead of having to use cell cultures.

Most published methods for Real-time quantification of *BCR-ABL* mRNA use plasmid standards that are developed in-house (Table 7). This makes implementing a method for Real-time quantification of *BCR-ABL* mRNA quite challenging since it requires each laboratory to develop their own standards or obtain them from another laboratory. To standardize the use of standards, the EAC uses commercial standards available from Ipsogen. Ipsogen produce copy number standards for *BCR-ABL* as well as *ABL*, *BCR* and *GUS*.

2.3.6 Analysis of results for quantification of *BCR-ABL*

Publications indicate little if anything about data analysis, and results are expressed as a ratio percentage of *BCR-ABL* to control gene. Some laboratories also express results as copy number per microgram of RNA, although this is not common (Hughes *et al.* 2006). The recommended approach is to report the results on a log scale from a standardized baseline for untreated patients (Hughes *et al.* 2006). On this scale, a MMR is considered a three log reduction from the baseline level of expression from 100% to 0.1% (Hughes *et al.* 2006). This approach allows laboratories around the world to express their results on an international scale through the use of conversion factors, determined by Branford *et al.* (2008).

In order to facilitate international co-operation and method standardization, results should be expressed on an international scale. This scale is defined by 2 values, the standardized baseline as established in the IRIS trial (100%) and a three-log reduction from it (MMR), fixed at 0.1% (Hughes *et al.* 2006; Branford *et al.* 2008). In order to standardize this method of reporting results, a conversion factor for each participating laboratory, must be determined

according to a verified set of samples of known value (Branford *et al.* 2008). Subsequently, percentage BCR-ABL values are multiplied by the conversion factor and expressed on the international scale. The conversion factor is derived by dividing the value for a MMR established in the IRIS trial (0.1) by the value for a MMR according to the independent laboratory participating (Hughes *et al.* 2006; Branford *et al.* 2008).

Published methods do not indicate parameters that are often unique to each Real-time instrument. Despite this, Silvy *et al.* (2005) determined that the EAC method for Real-time quantification of *BCR-ABL* could be transferred to different instruments without any modification. However no reference is made to setting threshold levels of fluorescence. Although Silvy *et al.* (2005) used Ct values to determine variance. Hughes *et al.* (2006) oppose this approach since Ct values are logarithmic units and may result in a misrepresentation of reproducibility.

One of the most important considerations for quality control is the inclusion of high and low positive controls to monitor the performance of the assay including stability of standards and linearity and the success of the RT and quantitative PCR steps. Unfortunately, such reference material is not commercially available. Furthermore, published methods all agree on the use of 0.98 as minimum correlation coefficient for standard curves.

2.4 Summary and conclusions

This analysis has not only identified the differences in methods used for Real-time quantification of *BCR-ABL* mRNA, but has also attempted to identify common practices. Although the common practice is not necessarily best practice, it can be used as a guide when establishing a new method in the laboratory. From the synthesis of the literature, the following approaches are common or supported by the majority of publications:

a) RNA extraction: Trizol is used by most laboratories for blood stabilization and RNA extraction. It has the advantage of high RNA yield as well providing optimal extraction and storage conditions through its RNase

inhibitory functions. Trizol allows for multiple RNA extractions from one blood sample while preserving the sample intact at -80℃.

b) cDNA synthesis: Most methods use random hexamer and MMLV-RT for cDNA synthesis. None of the cDNA synthesis methods for Real-time quantification of BCR-*ABL* mRNA use gene specific priming. There is no obvious explanation for this except that gene specific priming limits the cDNA to target genes only.

c) Primers and probes for *BCR-ABL*: Most methods use BCR-ABL primers that do not distinguish between different splice variants. Furthermore, the availability of BCR-*ABL* commercial standards is also a limiting factor – since there are currently no commercial standards available for Real-time quantification of the different splice variants. Furthermore, there is not evidence that monitoring different splice variants has any prognostic value.

d) Control gene: *ABL* is the most commonly used control gene for normalization and Real-time quantification of *BCR-ABL* mRNA. However, given the inherent problems with native *ABL* being part of *BCR-ABL*, not degrading at the same rate as *BCR-ABL* as well as having its expression influenced by the treatment of imatinib, *GUS* is considered to be a more appropriate candidate.

e) Standards and Reference material: Most methods use in-house developed standards and reference material. However, for a laboratory introducing the Real-time quantification of *BCR-ABL* mRNA it is more appropriate to use the commercially available standards from Ipsogen, especially given the additional technical considerations to produce such plasmid standards.

f) Analysis: Apart from the use of a 0.98 correlation coefficient for standard curves, there are very little recommendations on result analysis. From a synthesis of the literature it would appear that using a predetermined minimum copy number for the control gene is useful to ensure that the quality and quantity of RNA is sufficient for Real-time quantification of *BCR-ABL*. There is no commercial reference material available for

quality control with the exception of using the cell line K562, which apart from its use as positive control has no other value in terms of sample quantification.

In conclusion, this comparative analysis of published approaches to quantify *BCR-ABL* has identified common practice that can be used as guidelines for best practice. This study also highlights the importance of making standards and reference materials commercially available.

Table 3: List of publication authors for the quantification of *BCR-ABL* used in subsequent tables.

No	Author
1	Mensink *et al.* 1998
2	Branford *et al.* 1999
3	Eder *et al.* 1999
4	Emig *et al.* 1999
5	Kreuzer *et al.* 1999
6	Preudhomme *et al.* 1999[b]
7	Barbany *et al.* 2000
8	Bolufer *et al.* 2000
9	Elmaagacli *et al.* 2000
10	Kreuzer *et al.* 2000
11	Saffroy *et al.* 2000
12	Wittor *et al.* 2000
13	Amabile *et al.* 2001
14	Elmaagacli *et al.* 2001
15	Stentoft *et al.* 2001
16	Kim *et al.* 2002
17	Lee *et al.* 2002
18	Schoch *et al.* 2002
19	Wang *et al.* 2002
20	Gabert *et al.* 2003
21	Jones *et al.* 2003
22	Kantarjian *et al.* 2003
23	Neuman *et al.* 2003
24	Raanani *et al.* 2004
25	Cortes *et al.* 2005
26	de Lemos *et al.* 2005
27	Fossey *et al.* 2005
28	Gutièrrez *et al.* 2005
29	Jabbour *et al.* 2006
30	Martinelli *et al.* 2006a
31	Press *et al.* 2006
32	Stock *et al.* 2006
33	Ishikawa *et al.* 2006

Table 4: Summary of variables for the Real-time quantification of *BCR-ABL* mRNA.

Method summary	
Method	**Authors**
RNA Extraction	
Guanidinium thiocyanate	1, 5, 8, 9, 10, 11, 33
Trizol	2, 3, 6, 15, 17, 21, 22, 25, 26, 29, 27
Commercial kits	12, 14, 18, 19, 23, 24, 31
Other[1]	4, 7, 13, 16, 28, 30
RT Enzyme	
MMLV	1, 3, 4, 6, 7, 8, 9, 13, 15, 20, 21, 22, 23, 29, 30
SS	2, 17, 18, 19, 20, 25, 27, 28, 30, 32
AMV	16, 23, 31
Multiscribe	11
Control Gene	
BCR	2, 27
ABL	4, 6, 8, 11, 13, 15, 16, 18, 19, 20, 22, 24, 25, 26, 29, 32, 33
GAPDH	3, 7, 9, 14, 21, 28
GUS	15
B2M	15, 30
G6PDH	4, 12, 23, 31
RAR-a	17
B-actin	10
PBGD	1
PCR Platform	
ABI	1, 2, 3, 6, 7, 10, 11, 15, 17, 20, 21, 22, 24, 25, 26, 27, 29, 30, 33
Lightcycler	4, 5, 8, 9, 12, 14, 18, 19, 23, 28, 31, 32
Mircoamp	13
PCR Detection	
TaqMan probes	1, 2, 3, 5, 6, 7, 8, 9, 10, 11, 13, 15, 16, 17, 20, 21, 22, 24, 25, 26, 27, 29, 30, 33
Hybridization probes	4, 12, 14, 18, 19, 23, 31, 32
SybrGreen	28

[1] Other approaches include CsCl gradient, Ficoll-Hypaque density gradient, Histopaque density gradient and manifold assisted RNA extraction.

Table 5: List of primer and probe sequences for Real-time *BCR-ABL* mRNA quantification.

Author	Forward Primer	Reverse Primer	Probe
1	(b2) 5'-CGGGAGCAGCAGAAGAAGTGT-3'	(a2) 5'-AAAGGTTGGGGTCATTTTCAC-3'	(a2) 5'-TET-TCAGCGGCCAGTAGCATCTGACTT-(TAMRA)-3'
2	(b2) 5'-ACTCGTGGAGCTGCAGATG-3' (b3) 5'-GGGCTCTATGGGTTTCTGAATG-3'	(a2) 5'-CGCTGAAGGGCTTCTTCCTT-3' (a2) 5'-CGCTGAAGGGCTTTTGAACT-3'	(b2) 5'-CCAACTCGTGTGTGAAACTCCAGACTGTCC-3' (b3) 5'-CATCGTCCACTCAGCCACTGGATTTAAGC-3'
3	(b2) 5'-TGTGAAACTCCAGACTGTCCACA-3' (b3) 5'-TCCACTCAGCCACTGGATTTAA	(a2) 5'-AAAGTCAGATGCTACTGGCCG-3' (a2) 5'-TGAGGCTCAAAGTCAGATGCTACT-3'	(b2) 5'-TGACCATCAATAAGGAAGAAGCCCTTCAGC-3' (b3) 5'-CAGAGTTCAAAAGCCCTTCAGCGGC-3'
4	(b2) 5'-TTCAGAAGCTTCTCCCTGACAT-3'	(a2) 5'-CCCAACCTTTTCGTTGCACTGT-3'	(a3) 5'-LC Red 640-AATGGGGAATGGTGTGAAGCCCAAA-P
	(e1) 5'-CAGATCTGGCCCAACGATGG-3'		(a3) 5'-TGAAAAGCTCCGGGTCTTAGGCTATAATCA-F
5	(e1) 5'-AGATCTGGCCCAACGATGG (b2) 5'-AGCATTCCGCTGACCATCA	(a2) 5'-AGCGGCTTCACTCAGACCC (a3) 5'-GCGTGATGTAGTTGCTTGGGAC	(e1) 5'-AGGCTCAAAGTCAGATGCTACTGGCCG (a3) 5'-TTTGGGCTTCACACCATTCCCCATTG
6	(b3) 5'-CGTGTGTGAAACTCCAGACTGTCCA-3'	(a2) 5'-CTTCAGCGGCCAGTAGCATCT-3'	(a2) 5'-TTCACCTTTAGTTATGCTTAGAGTGTTATCTCCAC-3'
7	(b2) 5'- GCATTCCGCTGACCATCAATA-3' (b3) 5'- CCACTGGATTTAAGCAGAGTTCAA-3'	(a2) 5'- TCCAACGAGCGGCTTCAC-3'	(a2) FAM-CAGCGGCCAGTAGCATCTGACTTTGA-TAMRA
8	(b2) 5'-CGGGAGCAGCAGAAGAAGTGT-3'	(a2) 5'-CGAAAAGGTTGGGGTCATTTTC-3'	(a2) 5'-CGGCCAGTAGCATCTGACTTTGAGC-F (a2) 5'-LC Red640-TCAGGGTCTGAGTGAAGCCGCTC-P
9	5'-GAGCGTGCAGAGTGGAGGGAGAACA-3' (b2,2nd) 5'-CGGGAGCAGCAGAAGAAGTGT-3'	(a3) 5'-GGTACCAGGAGTGTTTCTCCAGACTG-3' (a2,2nd) 5'-AAAGGTTGGGGTCATTTTCAC-3'	Probe a2 5'-FAM-TCAGCGGCCAGTAGCATCTGACTT-TAMRA-3'
12	Roche-Kit	Roche-Kit	Roche-Kit
13	(e1) 5'-CGCAAGACCGGGCAGAT-3' (b2) 5'-GCATTCCGCTGACCATCAAT-3' (b3) 5'-TCCACTCAGCCACTGGATTTAA-3'	(a2) 5'-TGGGTCCAGCGAGAAGGTT-3'	(a2) 5'-FAM-CCAGTAGCATCTGACTTTGAGCCTCAGGG-TAMRA-3'
15	(b2) 5'-CATTCCGCTGACCATCAATAAG-3'	(a2) 5'-CAACGAGCGGCTTCACTCA-3'	(a2) Fam-TCAGATGCTACTGGCCGCTGAAGG-Tamra
16	(b2) 5'-GATGCTGACCAACTCGTGTG-3'	(a2) 5'-AACGAAAAGGTTGGGGTCAT-3'	(a2) 5'-AGACCCTGAGGCTCAAAGTCAGATGCTACT-3'
17	(e1) 5'-ACCGCATGTGGGACAAAA-3' (b2) 5'-ACAGCATTCCGCTGACCATCAATAAG-3'	(a2) 5'-TGTTGACTGGCGTGATGTAGTTGCTTG-3'	(a2) 6-FAM-AAGAAATTCAGAGGCCAGTAGC-TAMRA
20	(b2) 5'-GATGCTGACCAACTCGTGTG-3'	(a2) 5'-ACCGAAAAGGTTGGGGTCAT-3'	(a2) 5'-AGACCCTGAGGCTCAAAGTCAGATGCTACT-3'
21	(b2) 5'-TGCTGACCAACTCGTGTGTG-3' (e1) 5'-AACTCGCAACAGTCCTTCGAC-3'	(a2) 5'-CCATTCCCCATTGTGATTATAGC-3'	(a2) 5'-FAM-AAGACCCGGAGCTTTTCACCTTTAGTTATGC-TAMRA
22	(b3) 5'-CGTCCACTCAGCCACAT-3' (b2) 5'-TGCAGATGCTGACCAACTCG-3'	(a2) 5'-TCCAACGAGCGGCTTCAC	5'-CAGTAGCATCTGACTTTGAGCCTCAGGGTCT-3'
23	e1 Forward Roche Kit b2 Forward Roche Kit	a4 Reverse Roche Kit	Hybridization Probes, a3
24	(b2) 5'-CTGGCCCAACGATGGCGA-3'	(a2) 5'-CACTCAGACCCTGAGGCTCAA-3'	(a2) 5'-CCCTTCAGCGGCCAGTAGCATCTGA-3'
25	(e1) 5'-GCAAGACCGGGCAGATCT-3' (b2) 5'-ACAGCATTCCGCTGACCAT-3'	(a2) NED 5'-CGAGCGGCTTCACTCAGA-3'	(a2) 5'-6FAMR-CCTGAGGCTCAAAGTCAGATGCTACTGG-TAMRA-3'
26	(b2) 5'-TGACCAACTCGTGTGTGAAACTC-3' (b3) 5'-CCACTGGATTTAAGCAGAGTTCAA-3'	(a2) 5'-TCAGACCCTGAGGCTCAAAGTC-3'	(a2) 5'-VIC-AGCCCTTCAGCGGCCAGTAGCATC-TAMRA-3'
28	(b2) 5'- TCAGAAGCTTCTCCCTGACATCCGT-3' (e1) 5'- ACCTCACCTCCAGCGAGGAGGACTT-3'	(a2) 5'-TCCACTGGCCACAAAATCATACAGT-3'	SYBR Green I
32	(e1) 5'-GCAGATCTGGCCCAACGAT-3'	(a2) 5'-TCAGACCCTGA-GGCTCAAAGTC	6-FAM-CATGGAGACGCAGAAGCCCTTCAGC-TAMRA

Table 6: Summary of *BCR-ABL* break point discrimination in different publications for the quantification of *BCR-ABL*.

Original methods		
Author	**Detects**	**Primers discriminate between splice variants**
1	*b2a2, b3a2*	NO
2	*b2a2, b3a2*	YES
3	*b2a2, b3a2*	YES
4	*b2a2, b3a2, b2a3, b3a3, e1a2*	NO
5	*b2a2, b3a2, b2a3, b3a3, e1a2*	NO
6	*b2a2, b3a2, b2a3, b3a3*	NO
7	*b2a2, b3a2*	YES
8	*b2a2, b3a2*	YES
9	*b2a2, b3a2*	NO
12	*b2a2, b3a2*	NO
13	*b2a2, b3a2, e1a2*	NO
15	*b2a2, b3a2*	NO
16	*b2a2, b3a2*	NO
17	*b2a2, b3a2, e1a2*	NO
20	*b2a2, b3a2*	NO
21	*b2a2, b3a2, e1a2*	NO
22	*b2a2, b3a2*	NO
23	*b2a2, b3a2, b2a3, b3a3, e1a2, e19a2*	NO
24	*b2a2, b3a2*	NO
25	*b2a2, b3a2, e1a2*	NO
26	*b2a2, b3a2*	YES
28	*b2a2, b3a2, e1a2*	YES
32	*e1a2*	NO

[1] Splice variants for P210 BCR-ABL (*b2a2 and b3a2*) as well as P190 BCR-ABL (*e1a2*) and P230 BCR-ABL (*e19a2*).

Table 7: Summary of controls and standards for *BCR-ABL* quantification

Authors	**Control / Standard**
1-17, 20, 22, 23, 25-29	K562
All except 7, 11, 21, 22, 27, 28	HL60 negative control
20, 24, 33	Ipsogen standards
1-19, 21-23, 25-33	In-house plasmid standards

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Chapter 3:
Real-time quantitative PCR for CML diagnostics and method validation

3.1 Introduction

The reciprocal t(9;22)(q34;q11) translocation known as the Philadelphia chromosome, defines the cytogenetic basis of CML (Melo and Deininger 2004). The consequence of this is the *BCR-ABL* fusion oncogene that encodes a tyrosine kinase (TK) with constitutive activity. There are three breakpoint regions within *BCR* and depending on which one is involved in the translocation, either a p190 BCR-ABL, p210 BCR-ABL or p230 BCR-ABL can be encoded. BCR-ABL disrupts signal transduction in the cell and results in malignant cell growth (Melo and Deininger 2004). The progression of the disease can be determined by monitoring levels of *BCR-ABL* mRNA through Real-time PCR (Elmaagacli *et al.* 2000; Hardling *et al.* 2004; Baccarani *et al.* 2006; Jabbour *et al.* 2008). This allows for effective treatment response monitoring (Crossman and O'Brien 2004; Hughes and Branford 2006).

Although several methods for quantifying *BCR-ABL* have been published, there has been little attempt to standardize methodology. The Europe Against Cancer initiative was aimed at regional standardization of RQ-PCR in leukaemia, including CML. (Gabert *et al.* 2003). Recommendations have also been made to introduce a laboratory conversion factor to achieve the international standardization of *BCR-ABL* results on the same scale (Branford *et al.* 2006; Hughes *et al.* 2006). However, despite this, there are considerable differences in methodology to quantify *BCR-ABL* mRNA and these can affect results (Branford *et al.* 2008). It was found that even though several different laboratories use the same primers, probes and control gene combination, their conversion factors differed (Branford *et al.* 2008).

When required to implement a method for the Real-time quantification of *BCR-ABL* mRNA, our laboratory was faced with the daunting task of carefully analyzing and comparing the methods in the literature to quantify BCR-ABL mRNA for protocol development. After a thorough literature survey, it became evident that the Branford *et al.* (1999) and Gabert *et al.* (2003) methods were on the forefront of Real-time *BCR-ABL* quantification method development and standardization. It was decided to evaluate these two methods and to implement the one that was most suitable to our laboratory requirements. GUS was used as control gene in comparing the Branford *et al.* (1999) and Gabert *et al.* (2003) methods due to the lack of commercially available *BCR* copy number standards at the time of investigation. In addition, *ABL* was not considered a suitable control gene for quantification due to recommendations from available literature. Furthermore, splice variant specificity (Branford *et al.* 1999) was compared to the non-discriminatory application of Gabert *et al.* (2003). Other subtle differences were not considered important and the main motivation for using gene specific priming was to improve copy number detection. The aim of this study was to develop a standardized molecular diagnostic to quantify levels of *BCR-ABL* mRNA in CML patients using, the Branford *et al.* (1999) and Gabert *et al.* (2003) methods as basis. In addition, it was also decided to optimize the cDNA synthesis step in order to achieve maximum copy number detection.

3.2 Materials and Methods

3.2.1 Design of the study

The papers by Branford *et al.* (1999) and Gabert *et al.* (2003) were used as basis to develop and validate a method to quantify *BCR-ABL* mRNA in CML patients. Total RNA was extracted from peripheral blood and used to synthesize cDNA that was used to determine *BCR-ABL* and *GUS* copy numbers using Real-time PCR. Different experimental approaches were tested during cDNA synthesis to enhance copy number detection.

3.2.2 Study population

Blood samples from 40 CML patients were analyzed after informed consent was obtained. Samples were obtained from CML patients that routinely visit the Haematology Clinic, in the Department of Haematology and Cell Biology at the Universitas Hospital, for treatment and consultation from January 2007 to December 2008. Available CML related patient data was also obtained including disease stage, white blood cell count and FISH results, together with the treatment regimen. A fresh peripheral blood sample was collected from each patient at consecutive periods during treatment and stabilized in Trizol solution within two hours to prevent RNA degradation. Short term cDNA storage was at 4℃ and medium to long term was at - 20℃. Ethics approval for this study was obtained from the Ethics Committee of the University of the Free State (ETOVS 32/07).

3.2.3 RNA extraction

Peripheral blood (5 to 15 ml) was treated to a final volume of 50 ml with lysis buffer (containing 25 ml of 0.144 M NH_4Cl and 25 ml of 0.01 M NH_4HCO_3) within 2 h of sampling (Branford *et al.* 1999). The mixture was incubated at room temperature for 10 min and centrifuged at 3500 rpm for 15 min. The supernatant was discarded and the pellet was re-dissolved in lysis buffer to a volume of 25 ml. After incubation for 5 min, the mixture was centrifuged at 3500 rpm for 10 min. The supernatant was discarded and the isolated leucocytes were used for RNA extraction. Total RNA was extracted by the addition of 3.2 ml Trizol (Invitrogen, USA) with the addition of 0.35 ml of chloroform per 1.6 ml of Trizol homogenate. After homogenization, RNA was extracted by phase separation and precipitation. Total extracted RNA was washed with 75% ethanol and dissolved in 40 µl of RNAse-free water at 55℃ for 15 min and subsequently quantified using the Quant-iT Assay and Qubit fluorometer. Extracted RNA was either immediately used for cDNA synthesis or stored at -80℃. RNA degradation was checked by copy number d etection during Real-time quantification.

3.2.4 cDNA synthesis

Reverse transcription was performed using MMLV reverse transcriptase (RT) and priming with either random hexamer and/or gene specific primer. *BCR-ABL* and *GUS* antisense primers were used simultaneously for gene specific cDNA synthesis. A standard amount of 2000 ng of RNA was used per cDNA synthesis reaction. The RNA/primer mix consisted of 2000 ng of RNA together with one µl 10 mM random hexamer and/or gene specific antisense primers for *BCR-ABL* and *GUS*. The RNA/primer mixture was incubated at 70℃ for 5 min. Thereafter, 12.5 µl of cDNA synthesis cocktail was added to the RNA/primer mixture and incubated at 42℃ for 65 min. The cDNA synthesis cocktail contained (per reaction) 200U MMLV RT (200U/µl, Fermentas Life Sciences), 20 U RiboLock ribonuclease inhibitor (40 U/µl, Fermentas Life Sciences), 2 µl dNTP mix (10 mM, Fermentas Life Sciences), 4 µl $MgCl_2$ (25 mM, Roche), 1 µl DTT (0.1 M, Invitrogen) and 4 µl 5X RT-buffer (Fermentas Life Sciences). The final reaction volume for cDNA synthesis was 25 µl. Thereafter, the cDNA was treated with 10 U of Ribonuclease H (Takara Bio INC., Japan) and stored at 4℃ for future use.

The effect of removing cDNA fragments less than 100 bp in size was investigated through column purification using the GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare). Capture buffer (500 µl) was added to 25 µl of cDNA, mixed and loaded onto the GFX MicroSpin column. The column was centrifuged at 16,000 rpm for 30 sec and the elute removed. The column was washed twice by addition of 500 µl of wash buffer followed by centrifugation at 16,000 rpm for 30 sec. The cDNA was eluted by the addition of 20 µl of elution buffer and incubation at room temperature for 1 min. followed by centrifugation at 16,000 rpm for 1 min.

For all further Real-time PCR assays, the concentration of the RNA was calculated according to the final cDNA synthesis reaction volume of 25 µl. To determine the effect of different priming methods on the linearity of the assays, a serial dilution spanning four log concentrations was performed. The cDNA was diluted serially based on the final concentration of RNA used, from 100 ng

RNA (100%) to 0.05 ng RNA (0.05%) in increments of 100 ng, 80 ng, 50 ng, 30 ng, 10 ng, 5 ng, 1 ng, 0.5 ng, 0.1 ng and 0.05 ng RNA.

3.2.5 Real-time PCR

Real-time PCR of *BCR-ABL* was performed using primer and probe sets from Branford *et al.* (1999) and Gabert *et al.* (2003), respectively (Table 9). Lyophilised primer and probe were reconstituted and diluted in 0.1X TE (1 mM Tris, pH 7.4 and 0.1 mM EDTA). The reaction consisted of the following: 12.5 µl TaqMan Universal PCR Mastermix (Applied Biosystems), containing Taq polymerase, dNTPs and buffer, 0.2 µM forward and reverse primers, 0.1 µM probe, 5 µl cDNA (equivalent to 400 ng of RNA) and PCR grade water to a final reaction volume of 20 µl. The following cycling conditions were used: 1 cycle at 95℃ for 10 min followed by 50 cycles at 95℃ for 1 5 sec and 60℃ for 1 min.

Standard curves were constructed for both control and target genes, using Ipsogen Cancer Profiler copy number standards for *GUS* (CGRS-03) and *BCR-ABL* (FGRS10). The copy number standards for *GUS* were 1000, 10,000 and 100,000 copies, while those for *BCR-ABL* were 10, 100, 1000, 100,000 and 1,000,000 copies, respectively. The Copy number standards used by Branford *et al.* (1999) were not commercially available and the Ipsogen standards were used for the *b3a2* splice variant.

3.2.6 Data analysis

All Real-time assays were performed in duplicate on the ABI 7500 system (Foster City, CA, USA) and repeats were done at the reverse transcription step as recommended by Hughes *et al.* (2006). Real-time PCR product was not sequenced since sequence specific probes were used in the TaqMan assay, which confirmed that the correct template was amplified. No sensitivity controls were used and the copy number standards served as positive controls. The lowest copy number standard for *BCR-ABL* ($1X10^1$) was used as cut-off level for positive samples. Rox served as the passive reference and the fluorescence threshold was set at 0.1. Standard curves with $R^2 \geq 0.98$ were used for absolute quantification of both control and target gene copy number

and PCR efficiency (E) was calculated according to the formula from Peters *et al.* (2004):

$$\text{Efficiency} = [10^{(-1/\text{slope})}] - 1$$

Results were expressed as a percentage ratio of *BCR-ABL/GUS*. The cut-off level for control gene copy number were set at 40 000 copies per microgram RNA. All copy numbers were calculated according to 100 ng of RNA. Samples that did not yield sufficient control gene copy number were considered degraded and not suitable for quantification. Where necessary, means and standard deviations were calculated. The t-test and ANOVA was used to determine whether results from different priming methods were statistically significant. One-way ANOVA and the t-test statistical analysis was performed using Microsoft Excel. For ANOVA, P values were adjusted for multiple testing by the method of Bonferroni-Holm with a significance level of 0.05. The F test was used to compare the variances and t-test their means. For all analyses a P value of <0.05 was considered statistically significant.

3.3 Results and Discussion

Previously, Deprez *et al.* (2002), Bustin and Nolan (2004) and Bustin *et al.* (2005) reported that the linearity of different cDNA priming methods is an important consideration in cDNA synthesis. To investigate the effect of the different priming methods on the linearity of cDNA, the correlation, slope and efficiency of a serial dilution of cDNA was determined (Table 10 and 11). Furthermore, to test the possible effect of incomplete cDNA synthesis on the different priming methods it was decided to apply an additional cleanup step using PCR purification columns to remove cDNA fragments smaller than 100 bp. There was not a considerable difference in the slope or efficiency of the cDNA dilutions for the different treatments (Table 10 and 11). A comparison was also made of the reproducibility of the standard curves for *BCR-ABL* and *GUS* (Figure 6 and 7). A total of 20 standard curves were compared and the mean correlation for BCR-ABL and *GUS* was 0.996 (SD 0.004) and 0.997 (SD 0.003), respectively (Table 12). However, GUS copy numbers determined from

gene specific cDNA synthesis had the best linearity in a dilution series with a correlation of 0.99 (Figure 9). The correlation of all the data from the different treatments combined was 0.99, indicating that apart from producing higher target copy numbers, no bias in linearity was introduced as a result of the different treatments (Figure 10). A further comparison of the percentage *BCR-ABL/GUS* ratio for the different dilutions over the different treatments produced the same statistical result (Figure 11). It was notable that the priming using random hexamer followed by additional column purification produced a greater range in results over the dilution series. A possible reason for this is that the *GUS* copy number was limiting at the lower end of the dilution series.

A true comparison of the Gabert *et al.* (2003) and Branford *et al.* (1999) methods was not possible since standards and reference material for *BCR* is not readily available and the use of *ABL* is discourage in the literature. The Branford *et al.* (1999) method requires its own unique standards for *b2a2* and *b3a2*, respectively, as well as the *BCR* control gene, all of which are not commercially available. However, the Branford *et al.* (1999) primers for the *b3a2* splice variant could be used with the Ipsogen commercial standards for *BCR-ABL*. After comparison of the Branford *et al.* (1999) and Gabert *et al.* (2003) methods using *GUS* as control gene, instead of *BCR* or *ABL*, it was evident that the results from both modified methods was not statistically significantly different ($P > 0.05$ by t-test, $P > 0.05$ by F-test, $P > 0.05$ by ANOVA) (Table 13).

Since none of the published methods for Real-time quantification of BCR-ABL mRNA use gene specific priming during cDNA synthesis assays, it was also decided to evaluate the effect of random hexamer and gene specific priming as well as a combination of both, on both the Branford *et al.* (1999) and Gabert *et al.* (2003) methods. Gene specific priming on its own or combined with random hexamer gave a significantly different *BCR-ABL* result compared to the use of random hexamer (Table 14). However, the low copies of *GUS* from cDNA primed with random hexamer may have resulted in the overestimation of *BCR-ABL* disease load. Based on the results from this experiment it was decided to

investigate the use of gene specific priming using only the Gabert *et al.* (2003) method.

To investigate the effect of different priming methods, three samples were subjected to random hexamer, gene specific priming and a combination of random hexamer and gene specific priming, respectively. The percentage BCR-ABL calculated for the different treatments was not statistically significantly different ($P > 0.05$ by ANOVA) (Table 15) However, the GUS copy number for random hexamer priming was statistically significantly lower than gene specific and a combination of random hexamer and gene specific priming ($P < 0.05$ by ANOVA) (Table 15). Thus the advantage of gene specific priming is that it increases copy number detection without biasing the percentage *BCR-ABL*. Thus gene specific priming on its own or in combination with random hexamer is more effective than random hexamer for cDNA synthesis, especially for samples with partially degraded RNA without biasing the result.

To test the reproducibility of gene specific priming on the percentage *BCR-ABL/GUS* ratio, a sample was tested on eight consecutive days using different cDNA preparations. The mean result was similar with an acceptable standard deviation (Table 16). Furthermore, the copy number detection for *GUS* and *BCR-ABL* using gene specific priming, are comparable to published data (Table 17).

For method validation, 15 samples were tested in duplicate using both random hexamer and gene specific cDNA priming (Table 18). Gene specific cDNA synthesis did not increase overall copy number detection in un-degraded samples, it did allow for an increase in copy number detection of slightly degraded samples with low copy number compared to the use of random hexamer. There were no statistical significant differences between *GUS* copy numbers for random hexamer or gene specific methods ($P > 0.05$ by ANOVA). The differences in percentage *BCR-ABL* for both random hexamer and gene specific methods were also not significant ($P > 0.05$ by ANOVA). Thus, when evaluating the results using the international scale suggested by Branford *et al.*

(1999), the different cDNA priming methods produced statistically similar results (Figures 12 to 16).

Unfortunately it was not possible to monitor CML patients from before the start of treatment. An analysis of patient results revealed that patient 9 failed to respond on 800 mg imatinib and did not achieve a major molecular response (MMR). The white blood counts remained consistent compared to the percentage *BCR-ABL*. The high FISH value also corresponds to a high percentage BCR-ABL. The patient is currently being treated with hydroxyurea. Patient 10 did not respond to 800 mg imatinib and progressed to the accelerated phase. High FISH values (62% and 56%) are in agreement with high percentage *BCR-ABL/GUS*. Patient 12 was responsive to 200 mg imatinib and had, according to the assumption that the baseline was 100% *BCR-ABL* on the international scale, a two-log reduction in BCR-ABL expression over a period of 10 months. According to the last FISH results, this patient had a complete cytogenetic response (CCyR). Patient 18 responded to 400 mg imatinib and experienced a CCyR as well as a major molecular response (MMR) over the course of 12 months. Patient 22 is receiving 200 mg imatinib due to adverse drug reactions. Assuming that the pre-treatment levels of *BCR-ABL* were 100%, this patient has achieved a one log reduction in *BCR-ABL* expression level.

3.4 Conclusions

Sensitive, accurate and reliable copy number detection is the most important variable during Real-time *BCR-ABL* quantification. The data from this study indicated that optimal copy number detection plays a significant role in terms of the accuracy and reproducibility of quantification. The data presented demonstrates that the use of priming method can have a considerable difference in copy number detection, although this did not necessarily result in a different percentage *BCR-ABL*. Column purification did not improve the linearity (correlation) of the cDNA although it did result in higher PCR efficiency. Thus the cost of the additional cDNA purification does not justify its use.

Based on this research, we have implemented and validated a modification of the Gabert *et al.* (2003) method. The most important modification we have made is the use of *GUS* as control gene and gene specific priming during cDNA synthesis (Table 19). Repeat sample testing has shown this approach to be reproducible and robust with the added advantage of improving copy number detection, which is important for accuracy. The final stage of this research will be to request Branford *et al.* (2008) to determine a conversion factor to enable the results to be represented on the international scale for CML disease monitoring.

Table 8: **Comparison of methods for *BCR-ABL* mRNA quantification according to Branford *et al.* (1999) and Gabert *et al.* (2003).**

Method	***Branford et al.* (1999)**	***Gabert et al.* (2003)**
RNA extraction	Trizol ®	Trizol ®
cDNA synthesis	Random Hexamer	Random Hexamer
Control Gene	*BCR*	*ABL*
Standards	In-House	Ipsogen ®
Discrimination	b2a2 and b3a2 BCR-ABL	p210 BCR-ABL
Lower Limit of Quantification	10 copies	10 copies
Sensitivity	$1{:}1x10^5 - 1x10^6$ copies	10 copies
Min. Control Gene copy number	BCR: 4000/100ng RNA	ABL: 1300/100ng RNA

Table 9: Primer and probe sequences from Beillard *et al.* (2003), Gabert *et al.* (2003) as well as Branford *et al.* (1999).

Method	Gene	Sequence
Beillard *et al.* (2003)	*GUS*	F gaaaatatgtggttggagagctcatt R cccgagtgaagatccccttttta P ccagcactctcgtcggtgactgttca
Branford *et al.* (1999)	*b2a2*	F atccgtggagctgcagatg R cgctgaagggcttcttcctt P ccaactcgtgtgtgaaactcagactgtcc
	b3a2	F gggctctatgggtttctgaatg R cgctgaagggcttttgaact P catcgtccactcagccactggatttaagc
Gabert *et al.* (2003)	*BCR-ABL* (M-*bcr*)	F tccgctgaccatcaayaaga R cactcagaccctgaggctcaa P cccttcagcggccagtagcatctga

Table 10. **Comparison of the correlation, slope and efficiency of a standard curve for *GUS* using random hexamer, gene specific and a combination of both for cDNA priming. Corresponding values for the GUS copy number standard curve are included for comparison.**

	GUS Dilution Series				*GUS* Standard Curve
cDNA synthesis	**Random Hexamer**	**Random Hexamer (c)**	**Gene Specific**	**Gene Specific (c)**	
Correlation	0.92	0.96	0.99	0.98	0.99
Slope	-3.65	-3.61	-3.91	-3.55	-3.53
Efficiency	0.88	0.89	0.80	0.91	0.92

The additional use of column cleanup is denoted with c.

Table 11. **Comparison of the correlation, slope and efficiency of a standard curve for *BCR-ABL* using random hexamer, gene specific and a combination of both for cDNA priming. Corresponding values for the *BCR-ABL* copy number standard curve are included for comparison.**

	BCR-ABL Dilution Series				*BCR-ABL* Standard Curve
cDNA synthesis	**Random Hexamer**	**Random Hexamer (c)**	**Gene Specific**	**Gene Specific (c)**	
Correlation	0.99	0.98	0.99	0.99	0.99
Slope	-3.71	-3.63	-4.01	-3.60	-3.52
Efficiency	0.86	0.88	0.76	0.90	0.93

The additional use of column cleanup is denoted with c.

Table 12. **Comparison of the correlation, mean and standard deviation of 20 standard curves for *GUS* and *BCR-ABL*, respectively.**

R^2 values	*BCR-ABL*	*GUS*
Mean	0.996	0.997
SD	0.004	0.003

Table 13: **Comparison of *BCR-ABL* quantification between Branford *et al.* (1999) (*b3a2*) and Gabert *et al.* (2003) systems, using *GUS* as control gene.**

Sample	GUS copy number/100ng RNA	% *BCR-ABL*[1] Gabert *et al.* (2003)	% *BCR-ABL*[1] Branford *et al.* (1999)	Standard[2] Deviation
2(1)	4,426	91.27	86.12	3.64
5(1)	30,696	99.39	81.07	12.95
7(1)	51,646	4.36	9.49	3.63
11(1)	23,303	219.11	190.03	20.56
12(1)	26,194	98.28	95.68	1.84
12(2)	57,421	80.76	60.79	14.12
12(6)	16,747	10.89	11.27	0.27
16(1)	146,750	30.24	15.88	10.15
16(2)	61,782	93.76	54.36	27.86
16(3)	30,583	20.88	6.29	10.32
18(2)	34,458	93.39	57.07	25.68
18(3)	18,668	1.34	2.08	0.52
20(1)	107,667	0.12	0.24	0.08
20(2)	69,206	0.57	0.29	0.20
22(1)	30,713	17.01	7.73	6.56
36(1)	32,476	35.41	24.88	7.45
38(1)	39,875	26.05	35.41	6.62

[1] Percentage *BCR-ABL* using the Gabert *et al.* (2003) and Branford *et al.* (1999) methods was not statistically significantly different ($P > 0.05$ by t-test, $P > 0.05$ by F-test, $P > 0.05$ by ANOVA).

[2] SD for duplicate values.

Table 14: **Comparison of Branford *et al.* (1999) (*b3a2*) and Gabert *et al.* (2003) methods using random hexamer, gene specific primer or a combination of both for priming cDNA synthesis. *GUS* copy number is expressed per 100 ng RNA. Copy numbers for the reference gene below 4000 per 100 ng RNA are indicated for comparative purposes.**

Priming method	*GUS* copy number	*BCR-ABL* copy number Gabert *et al.* (2003)	*BCR-ABL* copy number Branford *et al.* (1999)	% *BCR-ABL* Gabert *et al.* 2003	% *BCR-ABL* Branford *et al.* 1999	Standard[1] Deviation
Random hexamer	2,328	4,990	4,874	214*	209*	4
Random hexamer and gene specific	29,396	42,083	26,403	143	90	38
Gene specific	26,194	32,017	25,287	122	97	18

* The percentage *BCR-ABL* for cDNA primed with random hexamer was significantly different to the corresponding values obtained from gene specific priming and a combination of random hexamer and gene specific priming ($P < 0.05$ by ANOVA).

[1] SD for duplicate values.

Table 15: Comparison of different cDNA priming approaches, random hexamer, gene specific primer and a combination of both, using the Gabert *et al.* (2003) primers and probe for *BCR-ABL* quantification. Copy numbers for the reference gene below 4000 per 100 ng RNA are indicated for comparative purposes.

Sample	Random Hexamer Priming			Gene Specific Priming			RH and GS		
	% *BCR-ABL/GUS*[2]	*BCR-ABL*	*GUS*[1]	% *BCR-ABL/GUS*[2]	*BCR-ABL*	*GUS*[1]	% *BCR-ABL/GUS*[2]	*BCR-ABL*	*GUS*[1]
22(10)	1	2	175	2	258	14,662	1	214	16,802
22(10)	2	2	124	3	621	18,399	2	459	19,632
16(4)	8	1,401	17,696	6	2,380	41,123	6	935	16,802
16(4)	9	1,671	18,044	8	3,222	40,593	1	158	25,119
10(1)	102	2,954,758	2,903,056	98	11,101,815	11,294,151	105	12,766,259	12,136,384
10(1)	97	3,049,912	3,145,201	122	12,766,259	10,446,288	105	14,195,891	13,469,179
Mean			1,014,049			3,642,536			4,280,653

[1] The *GUS* copy number for cDNA from random hexamer was statistically significantly different to that using gene specific primer or a combination of both ($P < 0.05$ by ANOVA).

[2] The percentage *BCR-ABL* for the different priming methods was not statistically significantly different ($P > 0.05$ by ANOVA).

Table 16: **Percentage *BCR-ABL* for sample 22-5 repeated eight times to determine the reproducibility and variability using the Gabert *et al.* (2003) method with modification based on the current study.**

Sample 22-5 (%)	
20.5	
20.7	
22.6	
21.1	
22.1	
17.5	
24.8	
21.7	
Mean	21.4
SD	2.1

Table 17. **Summary of *GUS* and *BCR-ABL* copy number reported in published literature compared to results obtained in the current study (van Deventer). Copy number is expressed per 100 ng RNA. The mean and range for van Deventer is based on 300 values.**

[1]*GUS* mean and range	
20,000 (4,500 – 67,000)	Rulcovà *et al.* 2007
41,000 (2,800 – 320,000)	Beillard *et al.* 2003
28,713 (4,000 – 410,000)	van Deventer
[1]*BCR-ABL* mean and range	
2,700 (1 – 100,000)	Rulcovà *et al.* 2007
3,890 (1 – 630,000)	van Deventer *et al.*

[1] Values are not correlated to disease stage.

Table 18. Validation of *BCR-ABL* quantification using gene specific priming for cDNA synthesis compared to the use of random hexamer. Where available, the white blood counts (WBCs) and FISH data are included for comparison. Copy numbers for the reference gene below 4000 per 100 ng RNA are indicated for comparative purposes. All patients were in the chronic phase of CML.

Sample	Date	WBC	FISH	*GUS*[1] CPN GS	*GUS*[1] CPN RH	*BCR-ABL* CPN GS	*BCR-ABL* CPN RH	% *BCR-ABL* GS[2]	% *BCR-ABL* RH[2]	SD	Treatment
9(1)	19-03-2007	31.40	74%	10,065.07	5,489.06	3,868.45	2,248.41	38.43	40.96	1.79	HU
9(4)	23-07-2007	36.70	N/A	10,670.11	5,568.03	3,388.38	2,007.02	31.76	36.04	3.03	
9(6)	29-10-2007	37.00	N/A	10,563.24	5,784.08	2,893.91	2,135.41	27.39	36.91	6.73	
10(1)	26-03-2007	6.02	62%	26,802.11	18,161.63	56,870.17	22,352.56	212.19	123.08	63.01	800 mg Imatinib
10(4)	13-08-2007	26.00	N/A	27,685.57	21,083.55	50,127.89	27,526.79	181.06	130.56	35.71	
10(5)	10-10-2007	10.00	56%	29,160.08	34,516.61	60,193.11	36,565.11	206.42	105.93	71.06	
12(3)	28-05-2007	4.54	86%	16,477.88	17,468.42	11,307.85	4,947.74	68.62	28.32	25.50	200 mg Imatinib
12(5)	03-09-2007	4.65	16%	10,809.43	9,014.25	4,794.09	2,007.02	44.35	22.26	15.62	
12(7)	07-04-2008	6.60	N/A	4,200.31	4,220.39	22.63	12.65	0.56	0.30	0.18	
18(1)	26-04-2007	358	70%	54,351.46	4,562.76	24,263.30	3,568.21	44.64	78.2	23.73	400 mg Imatinib
18(3)	08-10-2007	5.50	N/A	4,031.72	4,012.12	36.20	35.30	0.89	0.88	0.01	
18(5)	14-04-2008	12.00	1%	20,947.23	5,687.60	10.87	1.28	0.05	0.02	0.02	
22(2)	28-05-2007	24.50	80	19,504.70	8,754.35	5,473.32	5,561.43	28.06	63.52	25.07	200 mg Imatinib
22(6)	06-08-2007	86.46	5	26,115.68	9,784.54	22,352.55	2,658.37	85.59	27.2	41.29	
22(9)	21-04-2008	4.47	Neg.	13,741.31	13,216.81	971.45	282.05	7.07	2.13	3.49	

[1] *GUS* copy number (CPN) for random hexamer (RH) compared to gene specific (GS) priming are not statistically significantly different ($P > 0.05$ by ANOVA).

[2] Percentage *BCR-ABL* using random hexamer (RH) compared to gene specific (GS) priming are not statistically significantly different ($P > 0.05$ by ANOVA.

Table 19: Overall comparison of variables between the Branford *et al.* (1999), Gabert *et al.* (2003) and van Deventer for *BCR-ABL* mRNA quantification using Real-time PCR.

Method	Gabert method	Branford method	Van Deventer modification
RNA extraction	Trizol	Trizol	Trizol
cDNA synthesis	Random hexamer	Random hexamer	Gene specific priming
Control Gene	*ABL*	*BCR*	*GUS*
Standards	Ipsogen M-BCR-ABL	*b3a2* and *b2a2* specific standards not commercially available	Ipsogen M-*BCR-ABL*
Assay and Platform	TaqMan ABI 7700	TaqMan ABI 7700	TaqMan ABI 7500

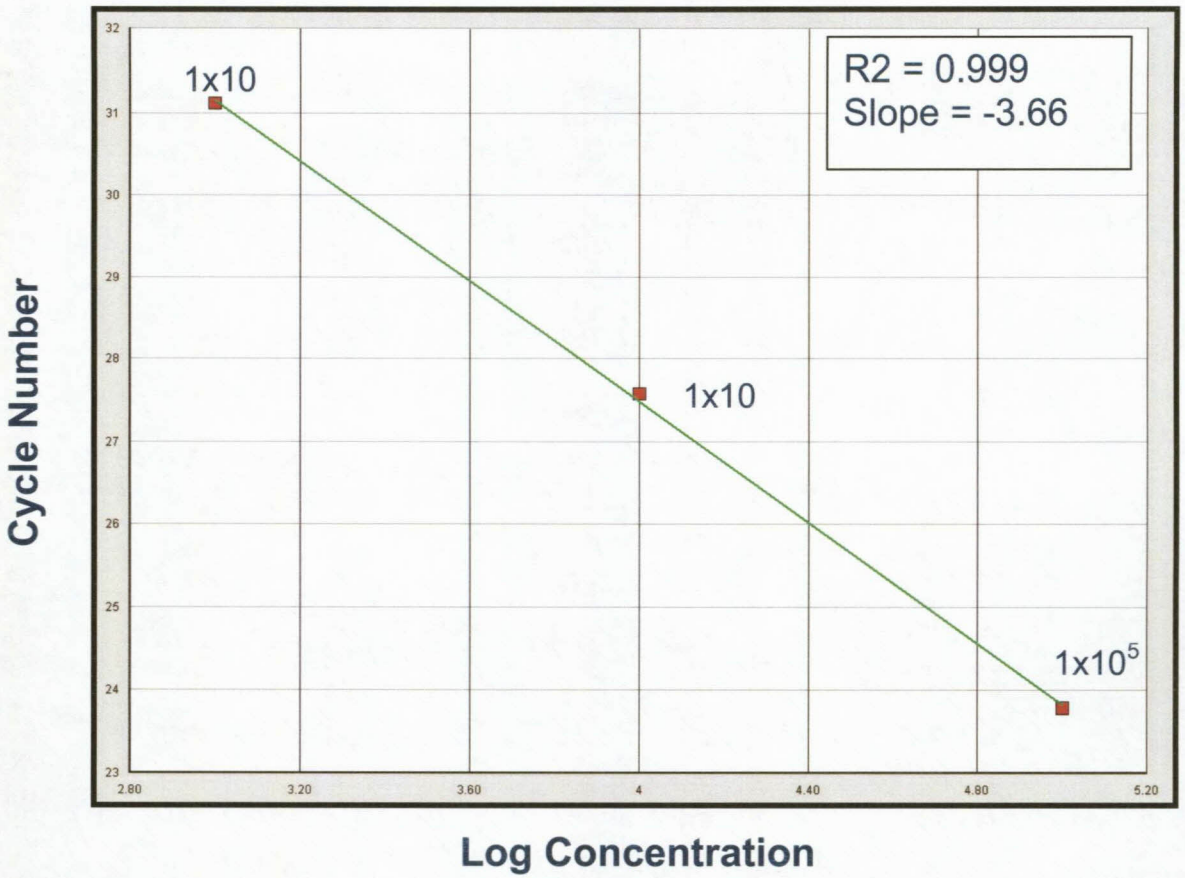

Figure 6: **An example of a copy number standard curve for *GUS* using the Ipsogen standards.**

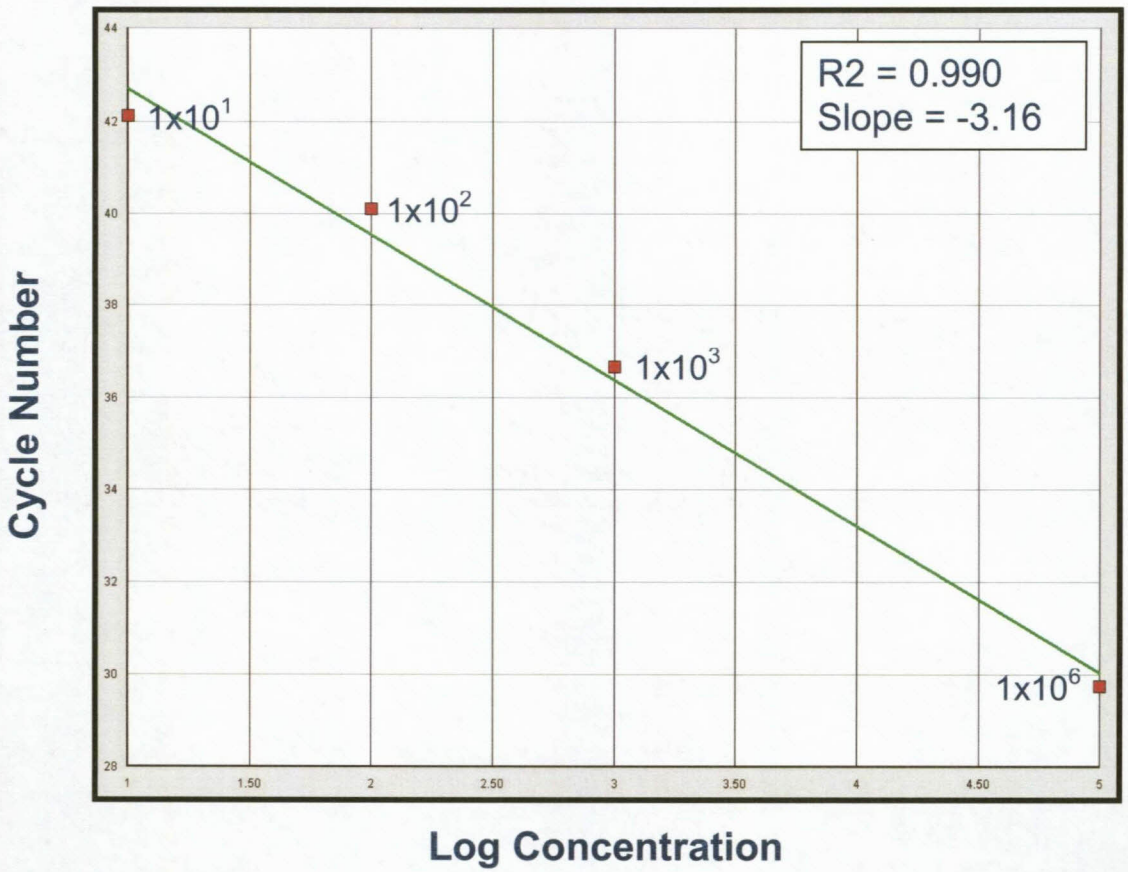

Figure 7: **An example of a copy number standard curve for *BCR-ABL* using the Ipsogen standards.**

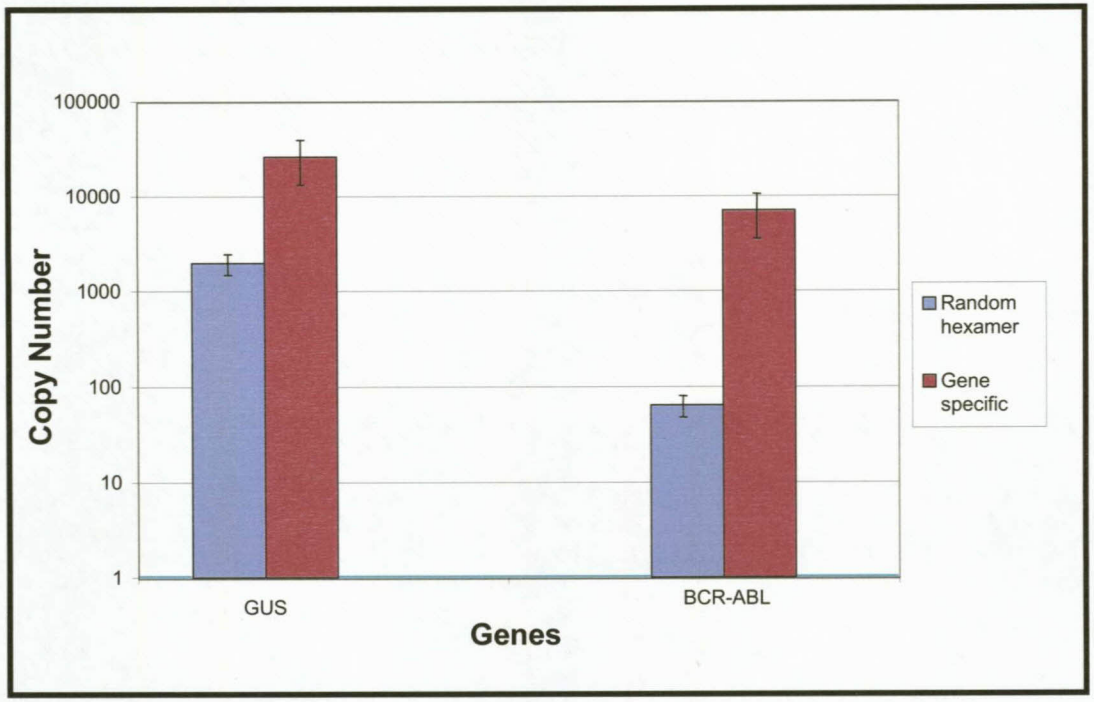

Figure 8: **Comparison of copy number detection for *GUS* and *BCR-ABL* using random hexamer and gene specific priming for cDNA synthesis, respectively.**

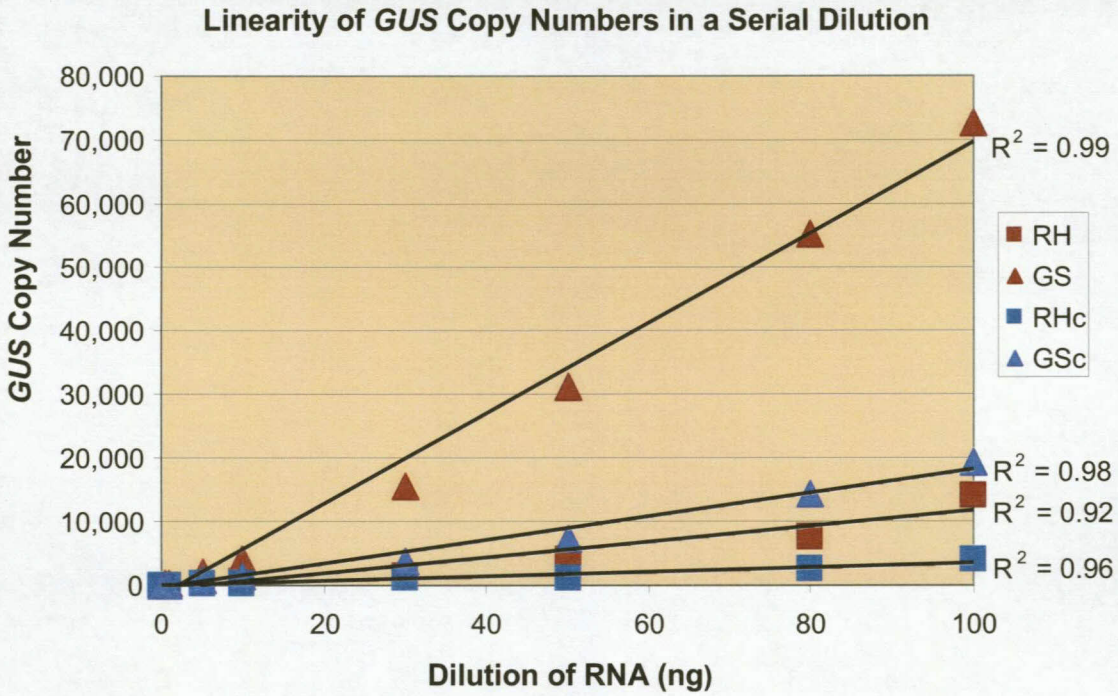

Figure 9: **A comparison of the linearity of different cDNA priming approaches using random hexamer (RH) and gene specific primers (GS) in a serial dilution, respectively. The effect of column purification is also evaluated for random hexamer (RHc) and gene specific (GSc) priming.**

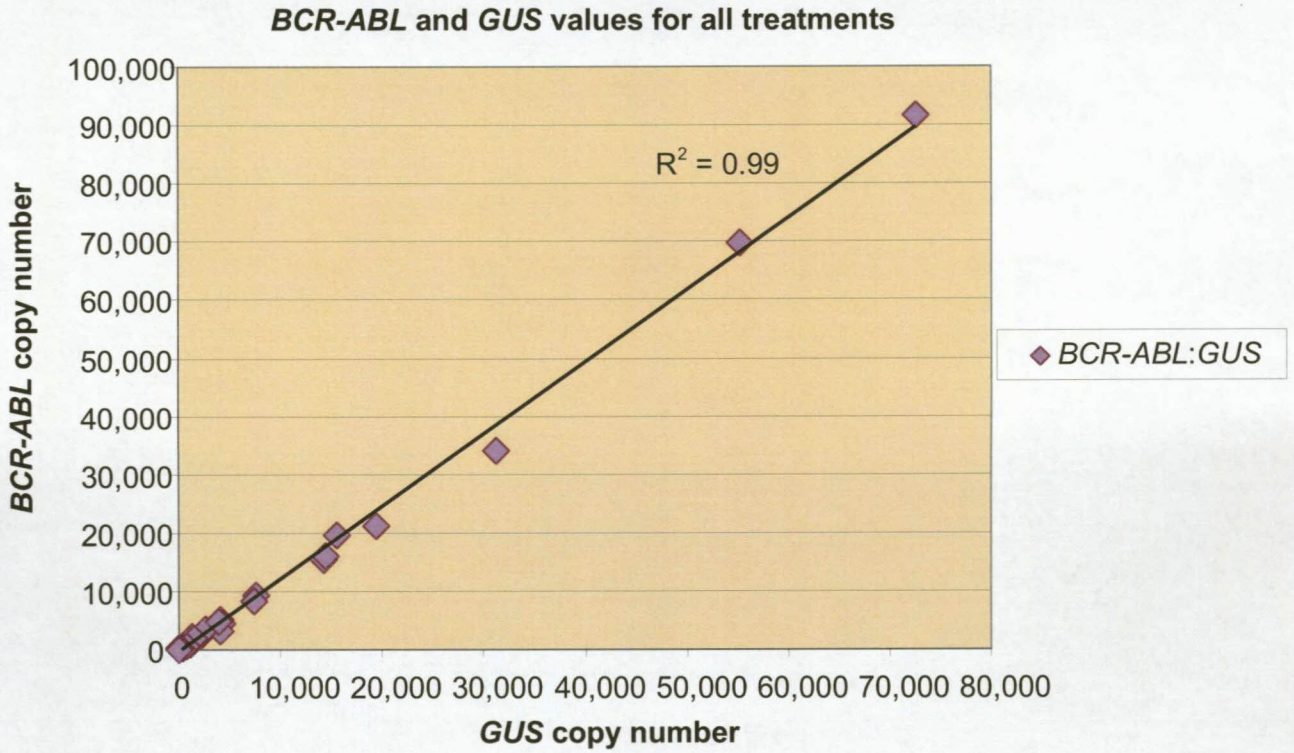

Figure 10: **The combined correlation of different priming methods, random hexamer, gene specific priming and a combination of both, with or without column purification of the cDNA.**

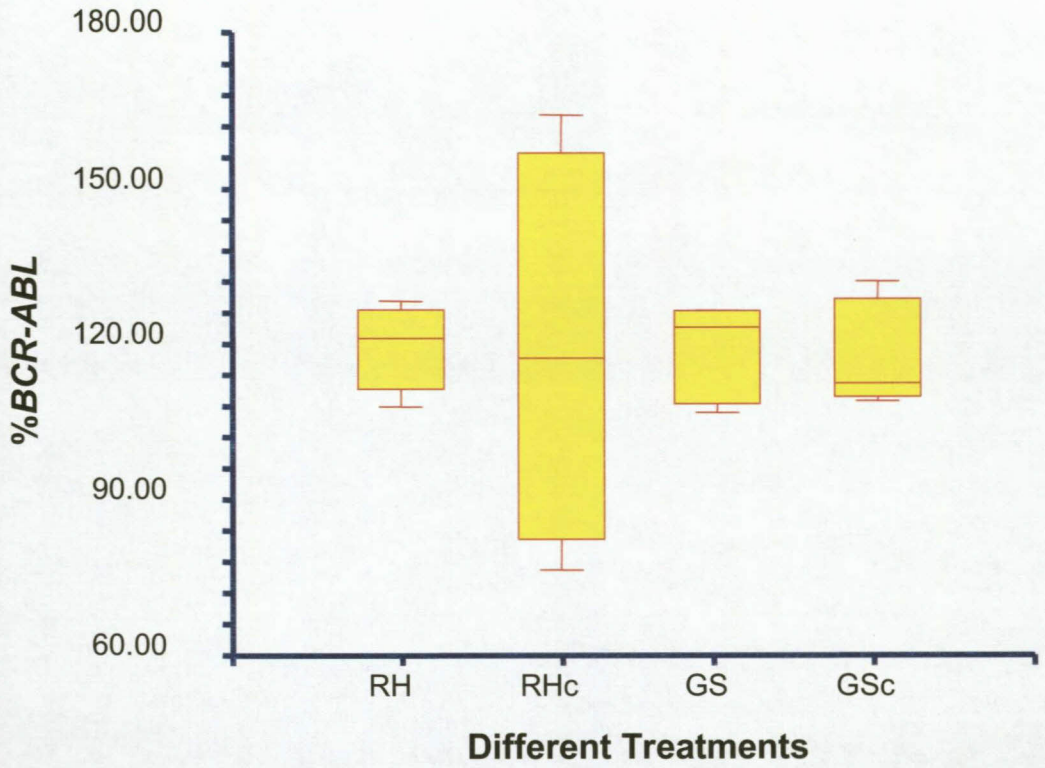

Figure 11: **A graphic representation of the percentage *BCR-ABL* for the different serial dilutions of cDNA generated by random hexamer (RH) and gene specific (GS) priming, respectively. The purified cDNA using random hexamer or gene specific priming is indicated as RHc and GSc, respectively.**

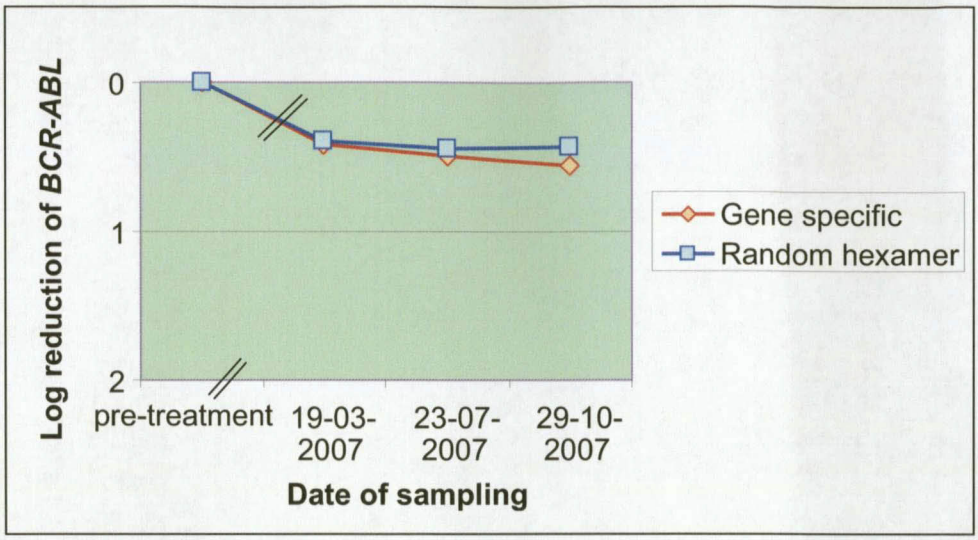

Figure 12: **Log reduction of *BCR-ABL* for patient 9. The pre-treatment levels of *BCR-ABL* are unknown and assumed to be 100% for graphical purposes. The results for *BCR-ABL* quantification are indicated for cDNA synthesis using random hexamer and gene specific priming, respectively.**

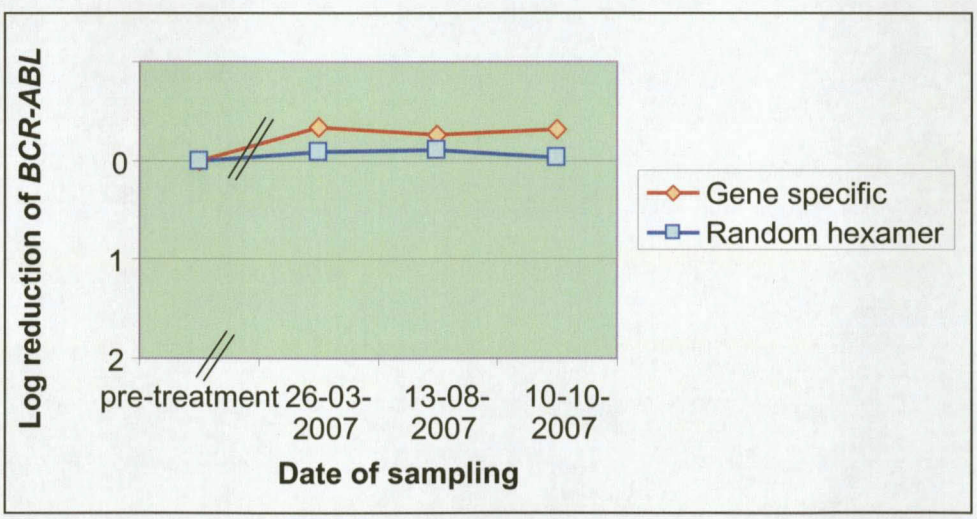

Figure 13: **Log reduction of *BCR-ABL* for patient 10. The pre-treatment levels of *BCR-ABL* are unknown and assumed to be 100% for graphical purposes. The results for *BCR-ABL* quantification are indicated for cDNA synthesis using random hexamer and gene specific priming, respectively.**

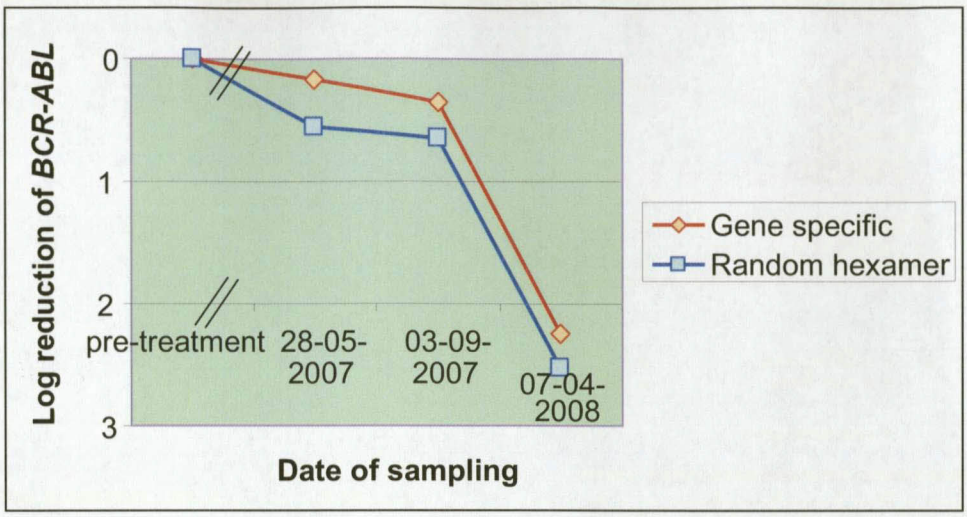

Figure 14: **Log reduction of *BCR-ABL* for patient 12. The pre-treatment levels of *BCR-ABL* are unknown and assumed to be 100% for graphical purposes. The results for *BCR-ABL* quantification are indicated for cDNA synthesis using random hexamer and gene specific priming, respectively.**

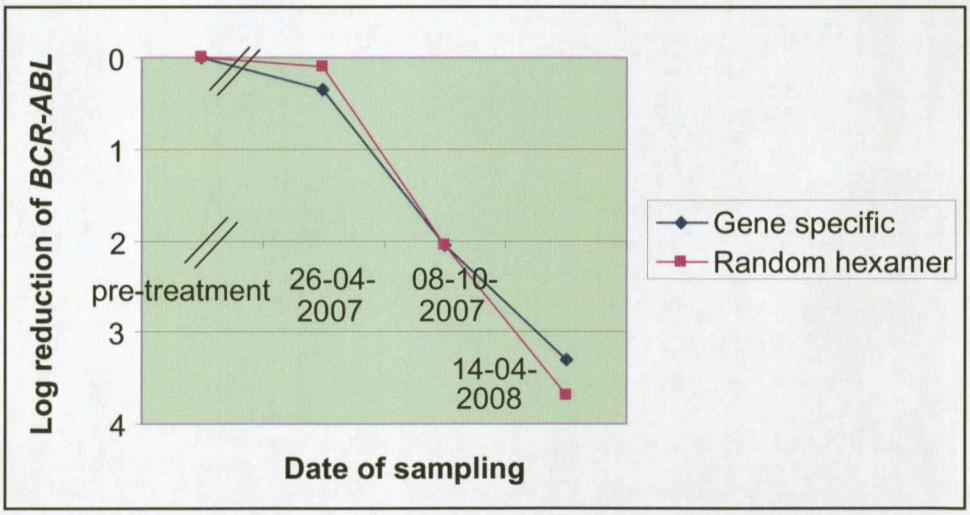

Figure 15: **Log reduction of *BCR-ABL* for patient 18. The pre-treatment levels of *BCR-ABL* are unknown and assumed to be 100% for graphical purposes. The results for *BCR-ABL* quantification are indicated for cDNA synthesis using random hexamer and gene specific priming, respectively.**

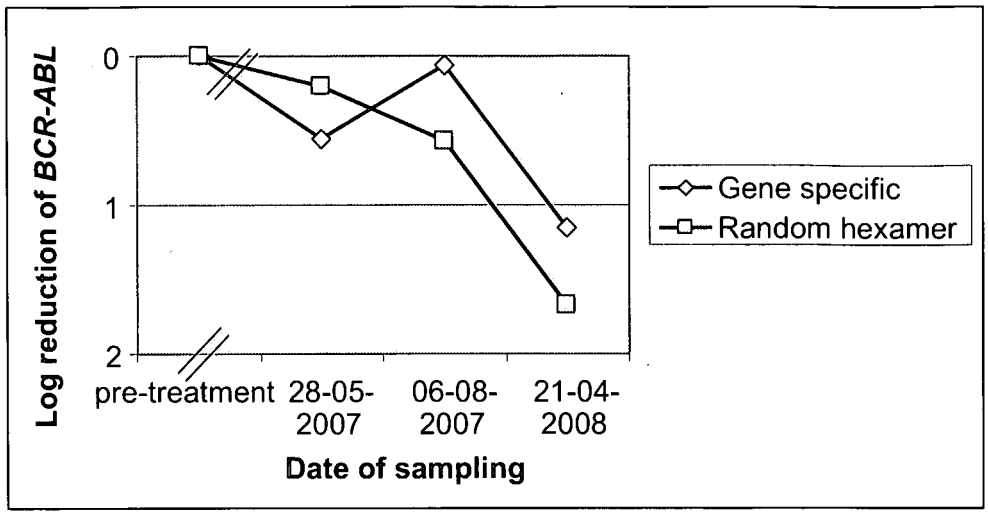

Figure 16: **Log reduction of *BCR-ABL* for patient 22. The pre-treatment levels of *BCR-ABL* are unknown and assumed to be 100% for graphical purposes. The results for *BCR-ABL* quantification are indicated for cDNA synthesis using random hexamer and gene specific priming, respectively.**

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Chapter 4:
Discussion and Conclusions

Initially the idea of setting up a laboratory method to monitor levels of *BCR-ABL* mRNA in CML patients using Real-time PCR quantification seemed easy enough, given the plethora of published methods available. However, a more detailed desktop comparison of the different methods for *BCR-ABL* mRNA quantification revealed that most laboratories use in-house methods each with variations in RNA extraction, cDNA synthesis, the use of control gene, the use of standards and controls and approaches to data analysis. Notable differences in methods related to splice variant detection, the use of control gene and the use of standards.

Most methods do not use PCR primers that distinguish between splice variants for p210 BCR-ABL. Notable exceptions are that of Branford *et al.* (1999), Eder *et al.* (1999) and de Lemos *et al.* (2005). Although no reasons are provided it is possible that the added cost of differentiating between splice variants is not justified in terms of prognostic value.

While the majority of methods advocate the use of *ABL* as control gene, this appears to be historical rather than scientific. Recently Lee *et al.* (2006), Wang *et al.* (2006) and Rulcová (2007) suggested that *GUS* is a more appropriate control gene compared to *ABL* based on empirical data. Although *BCR* has also been suggested as control gene, it is also part of *BCR-ABL*, which was one of the criticisms against the use of *ABL*. Thus, for the purpose of this study neither *ABL* nor *BCR* was considered as suitable control gene for quantification. Furthermore, there was no commercial reference material available for *BCR*.

One of the technically limiting factors in quantifying levels of *BCR-ABL* mRNA is the availability of copy number standards. Most methods use in-house developed plasmid standards. However, preparing these requires additional technical expertise and cost and it is not a ready option for smaller laboratories,

especially in 3rd world countries. To address this problem Gabert *et al.* (2003) based their method on the use of commercial standards for *BCR-ABL* and control gene.

Another problem that is not sufficiently addressed in the literature is that of quality control and data analysis. While most methods include either a positive sample or high/low sample in the analysis, the lack of a commercial reference makes evaluating the quality of data difficult. As a result Hughes *et al.* (2006) and Branford *et al.* (2006) have suggested the use of a conversion factor to allow different laboratories to represent their data on a single international scale. Despite the differences between methods for Real-time quantification of BCR-ABL mRNA, there were also similarities. Most methods use Trizol to extract RNA, random hexamer to prime cDNA synthesis with MMLV reverse transcriptase and Real-time PCR with the TaqMan assay. Thus the approaches in common were used as basis for best practice.

Based on the analysis of different methods for Real-time quantification of *BCR-ABL* mRNA, it was decided to use the Gabert *et al.* (2003) and Branford *et al.* (1999) methods with the following modifications:

a) *GUS* was used as control gene instead of *ABL* (Gabert *et al.* 2003)

b) Only the *b3a2* splice variant primers were used from Branford *et al.* (1999) since these could be used with the only commercially available *BCR-ABL* copy number standards.

c) Gene specific priming for cDNA synthesis was compared to the use of random hexamer.

There were no differences in the limited comparison of methods from Gabert *et al.* (2003) and Branford *et al.* (1999) in terms of the *BCR-ABL* result. However, the different priming approaches had a significant difference in copy number detection with both methods without changing the *BCR-ABL/GUS* ratio. From further investigation of priming methods, it was apparent that the gene specific approach on its own or in conjunction with random hexamer resulted in higher copy number detection than random hexamer on its own, without biasing the

BCR-ABL/GUS ratio. There was no significant effect of gene specific priming compared to the use of random hexamer on the linearity of the assay.

To test the reproducibility of gene specific priming, a single sample was tested repeatedly with no significant differences in result. There were also no significant differences in the percentage *BCR-ABL* for 15 samples tested using both gene specific and random hexamer priming during cDNA synthesis. However, the copy number detection for *GUS* was significantly higher for gene specific priming compared to using random hexamer.

This is the first method that uses dual gene specific cDNA synthesis for Real-time quantification of *BCR-ABL* mRNA in CML monitoring. This method is reproducible, robust and only makes use of commercially available copy number standards. None of the modifications resulted in a significant change in percentage *BCR-ABL*. However, copy number detection was significantly improved that will improve the sensitivity of MRD detection.

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Summary

CML is a cancer of the white blood cells and it effects on average one individual in every 100,000. Since it was first described in 1845 by John Hughes Bennett and the subsequent discovery of the Philadelphia chromosome by Nowell and Hungerford in 1960, this hematopoietic malignancy has received much attention in terms of scientific study. Elucidating the pathogenic pathway has lead to the development of targeted therapy. In 2001 imatinib mesylate was introduced as first line therapy for CML. The success of imatinb was illustrated during the IRIS trial by Real-time quantification of *BCR-ABL* mRNA.

BCR-ABL expression levels are correlated to disease stage and progression. BCR-ABL mRNA quantification is therefore the most accurate and sensitive prognostic marker to monitor CML patients. Hence, Real-time PCR for *BCR-ABL* has been introduced in many international laboratories to allow for accurate and reliable monitoring to improve and manage patient treatment. Standardization became problematic due to the ease of method development and robustness for Real-time quantification of *BCR-ABL* mRNA by different laboratories. As a result a plethora of methods for Real-time quantification of *BCR-ABL* mRNA have been published. This is especially problematic for laboratories with limited means undertaking to develop and implement such a method. Since there are no standardized guidelines, in-house development is required. Furthermore, availability of commercial copy number standards for control and target genes makes it difficult to implement any one method from the literature especially since there is criticism for the genes where standards are commercially available.

From a thorough analysis of the literature, problem areas considering RNA extraction, the choice of priming for cDNA synthesis, primers and probes for Real-time PCR as well as a specific control gene together with copy number standards and reference material were clearly defined. Based on this information, best laboratory practice regarding common methodology from literature was established. Only recently through an initiative known as Europe

Against Cancer (EAC) has there been a concerted effort to facilitate regional standardization of Real-time quantification of *BCR-ABL* mRNA.

During this study a modified EAC method for Real-time quantification of *BCR-ABL* mRNA was developed and validated with the emphasis to improve reproducibility. Instead of *ABL* or *BCR*, *GUS* was used as control gene based on recommendations from literature. Based on statistical analysis it was concluded that the modifications did not bias the percentage *BCR-ABL* result.

It cannot be emphasised enough that standardization for Real-time monitoring of *BCR-ABL* is most crucial as it will ultimately facilitate molecular laboratories to develop this diagnostic with much greater ease. In order for standardization to be realized, copy number standards as well as reference material for quality control purposes needs to become more readily available. In addition to that, specific guidelines for assay criteria such as appropriate Ct values and analysis of data must also be developed. By streamlining Real-time quantification of *BCR-ABL* the treatment and monitoring of CML patients can be improved on a global scale.

Opsomming

CML is 'n maligniteit van die witbloedselle en dit affekteer gemiddeld een individu per 100,000. Vandat dit die eerste keer in 1845 beskryf is deur John Hughes Bennett en die daaropvolgende ontdekking van die Philadelphia chromosoom deur Nowell en Hungerford in 1960, geniet hierdie hematopoïetiese maligniteit veel aandag in terme van wetenskaplike studie. Ontbloting van die patogeniese weg het gelei tot die onwikkeling van teikengerigte terapie. In 2001 is imatinib mesylaat bekendgestel as die nuwe eerste linie vir die bestryding van CML. Die sukses van imatinib was duidelik gedurende die IRIS proewe toe *BCR-ABL* mRNA vlakke met behulp van kwantitatiewe PKR gekwantifiseer is.

BCR-ABL uitdrukkingsvlakke word gekorreleer met siekte toestand en ontwikkeling. *BCR-ABL* mRNA kwantifisering is die akkuraatste en sensitiefste prognostiese merker vir die monitering van CML pasiënte. Gevolglik is "Real-time PCR" vir *BCR-ABL* kwantifisering deur vele internasionale laboratoriums bekend gestel vir akkurate en betroubare monitering wat sal help met die verbetering en bestuur van behandeling vir pasiënte. Standaardisering het problematies geword a.g.v. die gemak waarmee die "Real-time" tegniek deur verskillend laboratoriums ontwikkel kon word. Die uiteinde hiervan was dat 'n aansienlike hoeveelheid metodes vir die kwantifisering van *BCR-ABL* uitdrukkingsvlakke gepubliseer is. Dit het juis 'n probleem geword vir laboratoriums met beperkte hulpbronne om hierdie metode te onwikkel en te implementer aangesien daar geen standaard riglyne beskikbaar is nie. Dit het gelei tot menige intern-ontwikkelde metodes aangesien 'n tekort aan kommersieel verkrygbare kopiegetal standaarde vir kontrole- sowel as teiken geen dit onmoontlik maak om 'n spesifieke metode, soos gepubliseer in die literatuur, te implementer.

'n Deeglike literatuurstudie het probleem areas soos RNA ekstraksie, die keuse van peilstukke vir cDNA sintese, peilstukke en peilers vir "Real-time PCR" sowel

as 'n spesifieke kontrole geen tesame met kopie getal standaarde en verwysingsmateriaal, duidelik gedefinieer. Na aanleiding van hierdie inligting is die beste laboratorium gebruik n.a.v. algemene metodiek van die literatuur vasgestel. Daar is onlangs 'n inisiatief aangewend bekend as "Europe Against Cancer (EAC)" om streeks standaardisasie vir "Real-time" kwantifisering van *BCR-ABL* mRNA te fasiliteer. 'n Tekort aan kommersiële kopie getal standaarde en verwysingsmateriaal staan steeds sentraal tot die frustrasie van ander pogings om die kwessie van metodiek standaardisering op te los. Gedurende hierdie studie is 'n gemodifiseerde EAC metode vir "Real-time" kwantifisering van *BCR-ABL* mRNA ontwikkel en geverifieer met beklemtoning om reprodusering te verbeter. Eerder as om *ABL* of *BCR* as kontrole gene te gebruik, is *GUS* vir hierdie doeleinde geïmplementeer met verwysing na aanbevelings van verskeie publikasies. Statistiese analise het getoon dat hierdie veranderinge nie enige resultate vir *BCR-ABL* kwantifisering onregmatig beïnvloed het nie.

Die belangrikheid van metodiek standaardisering vir "Real-time" kwantifisering van *BCR-ABL* mRNA kan nie genoeg benadruk word nie aangesien dit uiteindelik die implementering van die metode deur verskeie molekulêre laboratoriums sal vergemaklik. Hiervoor sal kopie getal standaarde asook verwysingsmateriaal vir kwaliteitskontrole meer geredelik kommersieel beskikbaar moet wees. Verder sal spesifieke riglyne vir reaksie kriteria soos aanvaarbare Ct waardes en data analise ook ontwikkel moet word. Deur "Real-time" kwantifisering van *BCR-ABL* mRNA te standaardiseer, sal die behandeling en monitering van CML pasiënte op 'n globale skaal verbeter kan word.