Impact of imatinib mesylate on *SLC22A1* gene expression in chronic myeloid leukaemia cell line, K562

By

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DECLARATION

I certify that this dissertation hereby submitted in fulfilment of the Masters in Medical Science (M.Med.Sc) degree in Human Molecular Biology at the University of the Free State is my independent work and that I have not previously submitted the same work for a degree at another University/Faculty. I hereby also waive copyright of the dissertation to the University of the Free State.

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S Sreenivasan

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"I have no strength save what God has given me. My greatest weapon is mute prayer" – Mahatma Gandhi

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LIST OF SCIENTIFIC ABBREVIATIONS AND ACRONYMS

ABL	c-Abelson gene
AP	Accelerated phase
ATP	Adenosine tri-phosphate
α	alpha
aa	Amino acid
ANOVA	Analysis of variance
Bp	Base pairs
β	beta
GUS	β-Glucuronidase gene
BC	Blast crisis
BCR	Breakpoint cluster region gene
CBL	Cellular homologue of Cas Ns-1 oncogene
CML	Chronic myeloid leukaemia
CP	Chronic phase
cDNA	Complementary DNA
CCyR	Complete cytogenetic response
CHR	Complete hematologic response
CMR	Complete molecular response
R^2	Correlation
CRKL	Cr-10 kinase like protein
Da	Dalton
°C	Degree Celsius

Δ	Delta
ΔC_T	Delta threshold cycle (Difference in threshold cycle)
DNA	Deoxyribonucleic acid
DEPC	Diethylpyrocarbonate
et al.	*Et alia* (and others)
EDTA	Ethylenediamine tetra acetic acid
FAM	Fluorescein amidite
FISH	Fluorescence *in situ* hybridisation
g	Gram
HEPES	Hydroxyethyl piperazineethanesulfonic acid
IC_{50}	50% inhibitory concentration
IRIS	International randomized study of Interferon versus STI571
IUR	Intracellular uptake and retention
JAK	Janus-family tyrosine kinase
kb	Kilo-base
l	Litre
MCyR	Major cytogenetic response
MMR	Major molecular response
mRNA	Messenger ribonucleic acid
µg	Micro-gram
µl	Micro-litre
µM	Micro-molar
mg	Milli-gram
ml	Milli-litre
mM	Milli-molar

MYC	Myelocytomatosis oncogene cellular homolog
ng	Nano-gram
nm	Nano-metre
nM	Nano-molar
NCBI	National Centre for Biotechnology Information
NPC	No protein control
NTC	No template control
OCT1	Organic cation transporter 1
%	Percentage
pH	Concentration of hydrogen ions in solution
Ph	Philadelphia
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POU2F1	POU class 2 homeobox 1
P-value	Probability value
RNA	Ribonucleic acid
rpm	Revolutions per minute
STAT	Signal transducer and activator of transcription
SLC22A1	Solute liquid carrier family 22 sub family A member 1
SD	Standard deviation
TAMRA	Tetramethylrhodamine
C_T	Threshold cycle
TE	Tris EDTA
Tris	Tris hydroxymethyl aminomethane
www	World wide web

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PREFACE

Up until 2001, the median survival of patients with chronic myeloid leukaemia (CML) was three years from diagnosis. The introduction of targeted therapy in 2001 using imatinib for treating CML has greatly improved response rates and prolonged survival in the majority of CML patients. However, approximately 25% of CML patients are reported to be at risk of suboptimal response and/or treatment failure.

One of the reasons for suboptimal response has been attributed to decreased uptake of imatinib into target leukemic cells. The membrane protein, SLC22A1, mediates the active cellular influx of imatinib. The activity of SLC22A1 is considered a key determinant of intracellular levels of imatinib achieved within CML cells and hence, patient response. Patients displaying high levels of *SLC22A1* mRNA have been shown to respond favourably to treatment with imatinib compared to patients with low levels. However, there are indications that suggest that imatinib may influence *SLC22A1* expression without necessarily affecting SLC22A1 activity. Given the important role of SLC22A1 in imatinib uptake, the present study was aimed at investigating the effect of imatinib on *SLC22A1* gene expression in an *in vitro* system by treating K562 cells (a CML cell line) with varying doses of imatinib for 24 hours, 48 hours and 72 hours.

This dissertation contains five chapters which include a literature review, followed by three research chapters and a concluding chapter. The literature review provides the background to CML and addresses the important role of

SLC22A1 as a prognostic marker in the treatment of CML with imatinib. The literature review is followed by three research chapters, the first of which investigated the use of ultramers as copy number standards to quantify *SLC22A1* mRNA. This research was necessary due to the lack of commercially available copy number standards for quantifying the copy number of *SLC22A1* mRNA. To this end, we sought to develop a method using ultramer as copy number standard in order to quantify levels of *SLC22A1* gene expression. However, there were gaps in the literature regarding the stability of ultramers and so we undertook an investigation as an additional component which was not part of the original research plan. This chapter was accepted for publication in Gene and has been adapted in this dissertation to include only data relevant to this study. The following two research chapters were aimed at studying the impact of imatinib on the expression of *SLC22A1* mRNA and SLC22A1 protein, respectively. The research chapters in this dissertation have been written in the format of a research article, each with its own introduction and conclusion. Although care has been taken to avoid unnecessary duplication, some repetition of information was necessary to contextualise the arguments in each research chapter. The final chapter draws conclusions across the entire dissertation and highlights the impact of the findings from this study in the context of patients with CML treated with imatinib. Following the concluding chapter is a summary in English and Afrikaans and a reference list. At the end of the dissertation are appendices A, B and C which contain raw data and extra information corresponding to research chapters 2, 3 and 4, respectively. Throughout this dissertation, tables and figures are numbered according to the chapter in which they occur and have been referred to in text where applicable.

The names of genes and encoded protein products are presented in italicised form and normal text respectively, as per convention in literature.

While reading this dissertation, please consider that the focus of this study was not to validate the prognostic role of *SLC22A1* mRNA in the treatment of CML with imatinib, but rather, to specifically investigate the impact of imatinib on *SLC22A1* gene expression.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction to chronic myeloid leukaemia (CML)

Leukaemia describes a group of cancers involving the haematopoietic system and is characterised by clonal proliferation, altered survival and differentiation of affected cells. Chronic myeloid leukaemia (CML) affects the myeloid cell lineage including granulocytes, erythroid and megakaryocytic lineages and leads to uncontrolled proliferation of immature blood cells in peripheral blood (Faderl *et al.*, 1999; Sawyers, 1999). The median age of onset of CML is usually between 45 to 55 years (Faderl *et al.*, 1999) but the disease may occur at all ages. Males are affected more frequently than females in a ratio of 2:1 (Frazer *et al.*, 2007). CML has a global annual incidence of one to two in 100,000 individuals (Faderl *et al.*, 1999). In South Africa, approximately 450 new cases are diagnosed annually (Coetzee *et al.*, 2009). Overall, CML accounts for 15% of all adult and no more than 5% of all paediatric leukaemias (Faderl *et al.*, 1999).

1.2 Clinical course of CML

CML progresses through three phases, an initial chronic phase (CP) followed by an accelerated phase (AP) ultimately ending in the fatal blast crisis (BC). CP lasts for approximately three to four years and is characterised by the accumulation of granulocytes in peripheral blood which retain the ability to differentiate and function normally (Savage and Antman, 2002). Most CML patients remain asymptomatic or display very mild symptoms in this phase and are diagnosed coincidentally when they present with elevated white blood cell counts (Savage *et al.*, 1997; Faderl *et al.*, 1999). As the disease progresses into the AP, the maturation of blood cells is arrested and immature blood cells

or blast cells begin to infiltrate the peripheral blood and bone marrow. The AP lasts for six to 18 months and is followed by the fatal BC (Calabretta and Perroti, 2004; Esfahani *et al.*, 2006; Radich, 2007). Leukemic cells in BC are growth factor independent and often accumulate additional cytogenetic abnormalities (Derderian *et al.*, 1993; Mitelman, 1993). The median survival of patients in this disease phase is three to nine months (Savage *et al.*, 1997; Cortes, 2004). The clinical symptoms of CML include fatigue, loss of appetite, fever and an enlarged spleen (Savage *et al.*, 1997; Sawyers, 1999). CML patients are susceptible to infection as a result of dysfunctional leukemic white blood cells (Savage *et al.*, 1997). In addition to this, the production of red blood cells decreases, resulting in anaemia (Savage *et al.*, 1997).

1.3 Genetics of CML

1.3.1 Philadelphia chromosome

Although rare in occurrence, CML is one of the best understood malignancies in cancer biology. A significant breakthrough in CML research was made in 1960 when Nowell and Hungerford from Philadelphia, discovered the genetic basis of CML, commonly referred to as the Philadelphia (Ph) chromosome (Nowell and Hungerford, 1960; Rowley, 1973). The Ph chromosome, a shortened chromosome 22, is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22 (Figure 1.1). Approximately 95% of CML patients present with the Ph chromosome (Sawyers, 1999). The other 5% of CML patients have variant and complex translocations involving additional chromosomes but experience clinical symptoms identical to Ph positive CML patients (Kaeda *et al.*, 2002; Babicka *et al.*, 2006; Costa *et al.*, 2006). CML was

the first cancer in which an association with a single acquired chromosomal rearrangement was demonstrated.

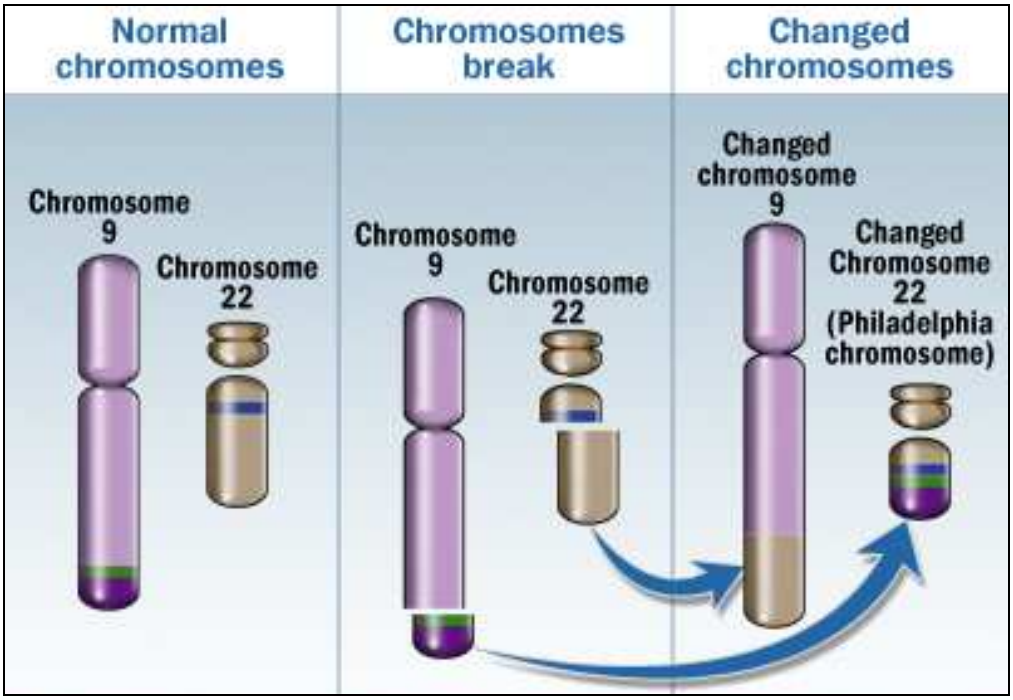


Figure 1.1. Schematic of the Philadelphia chromosome. Schematic representation of the reciprocal translocation between the long arms of chromosome 9 and 22, resulting in the Ph chromosome (Copied from Mayo Foundation for Medical Education and Research: http://www.mayoclinic.com/health/medical/IM03579).

1.3.2 *BCR-ABL* oncogene and BCR-ABL tyrosine kinase

The reciprocal translocation between the breakpoint cluster region (*BCR*) gene on chromosome 22 and the c-Abelson (*ABL)* gene on chromosome 9 gives rise to the *BCR-ABL* fusion oncogene (Faderl *et al.*, 1999). The primary function of *BCR* in normal cells remains unclear and various studies have suggested that it plays a role in intracellular signalling (Malmberg *et al.*, 2004; Oh *et al.*, 2010).

The *ABL* gene normally encodes for a regulated tyrosine kinase that facilitates the transfer of a phosphate group from adenosine tri-phosphate (ATP) to a tyrosine residue on a substrate protein. The phosphorylated substrate activates signal transduction pathways that regulate cell growth, differentiation and apoptosis (Tang *et al.*, 2007). However, the expression of *ABL* in *BCR-ABL* cells is constitutive due to the loss of the regulatory domain of *ABL* as a result of the translocation with *BCR*, resulting in its oncogenic nature (Sawyers, 1999; Deininger *et al.*, 2000). The *BCR-ABL* oncogene encodes for a tyrosine kinase that constitutively phosphorylates several substrate proteins including CRKL (ten Hoeve *et al.*, 1994), p62Dok (Carpino *et al.*, 1997), paxillin (Salgia *et al*., 1995), CBL (de Jong *et al.*, 1995) and RIN (Afar *et al.*, 1997), resulting in the activation of downstream signal transduction pathways involving RAS (Mandanas *et al.*, 1993), RAF (Okuda *et al.*, 1994), phosphatidylinositol-3 kinase (Skorski *et al.*, 1995), JUN kinase (Raitano *et al.*, 1995), MYC (Sawyers et al., 1992) and JAK/STAT (Shuai *et al*., 1996; Clarkson *et al.*, 1997; Steelman *et al*., 2004). The activation of these pathways results in reduced apoptotic response, increased cellular proliferation, disruption of the cell cycle, decreased cellular adherence to bone marrow and overall genomic instability (Figure 1.2) (Sawyers, 1999; Steelman *et al*., 2004).

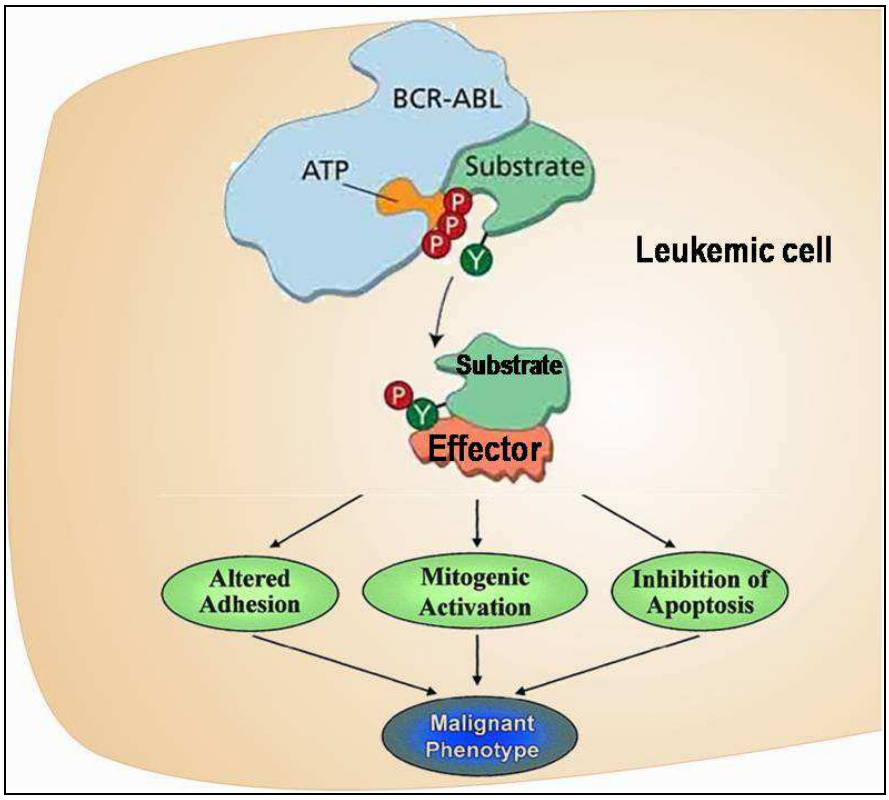


Figure 1.2. Schematic of the function of the BCR-ABL oncoprotein. The relevant substrate is phosphorylated, in the presence of ATP, on a tyrosine residue (Y) and, in its phosphorylated state, interacts with and activates other downstream effector molecules resulting in malignant phenotype (Copied from Deininger *et al.*, 2000; Goldman and Melo, 2001).

1.4 Treatment of CML

Prior to 2001, treatment options for patients with CML were limited to interferon-α and other chemotherapeutic drugs including busulfan and hydroxyurea. However, these therapies only reduce white blood cell counts and do not prolong the overall survival of CML patients (Hehlmann *et al.*, 1994; Silver *et al.*, 1999; Baccarani *et al.*, 2002; Hehlmann *et al.*, 2003). Up to 2001, the median survival of CML patients was less than three years from diagnosis (Whittaker *et al.*, 2000; Irfan and Bhurgri, 2009). A major breakthrough in CML treatment was

made in 2001 with the development of a tyrosine kinase inhibitor, imatinib mesylate, which is the first example of targeted drug therapy for cancer (Druker *et al.*, 2001). Imatinib inhibits the kinase activity of BCR-ABL, thereby arresting the malignant phenotype. Imatinib binds to the ATP binding site in the kinase domain of BCR-ABL and stabilises the protein in an inactive conformation (Schindler *et al.*, 2000). Since ATP cannot bind to the inactive form of BCR-ABL, subsequent phosphorylation and activation of downstream signal transduction pathways is inhibited (Figure 1.3). Inhibition of the BCR-ABL kinase arrests uncontrolled cellular proliferation and induces apoptosis of leukemic cells (Gambacorti-Passerini *et al.*, 2003), thus restoring normal cellular processes.

The efficacy of imatinib for treating CML has been demonstrated in several clinical trials. The first of these was the International Randomized Study of Interferon-α and STI571 (later named imatinib) known as the IRIS trial in 2000 (Druker *et al.*, 2001; O'Brien *et al.*, 2003). The IRIS trial demonstrated the benefits of treating CML with imatinib compared to a combination of interferon-α and cytarabine. Up to 96% of CML patients treated with imatinib displayed disease progression-free survival compared to 91% in patients treated with a combination of interferon-α and cytarabine (Druker *et al.*, 2001; O'Brien *et al.*, 2003). An eight year follow up of the IRIS trial has established that the survival of CML patients on imatinib was 85% and of these, 92% were disease progression free (Deininger *et al.*, 2009). Other subsequent trials with imatinib have shown similar results and currently, imatinib is the first-line for CML

treatment in the world (Homewood *et al.*, 2003; Johnson *et al.*, 2003; Carella *et al.*, 2008; Palandri *et al.*, 2008).

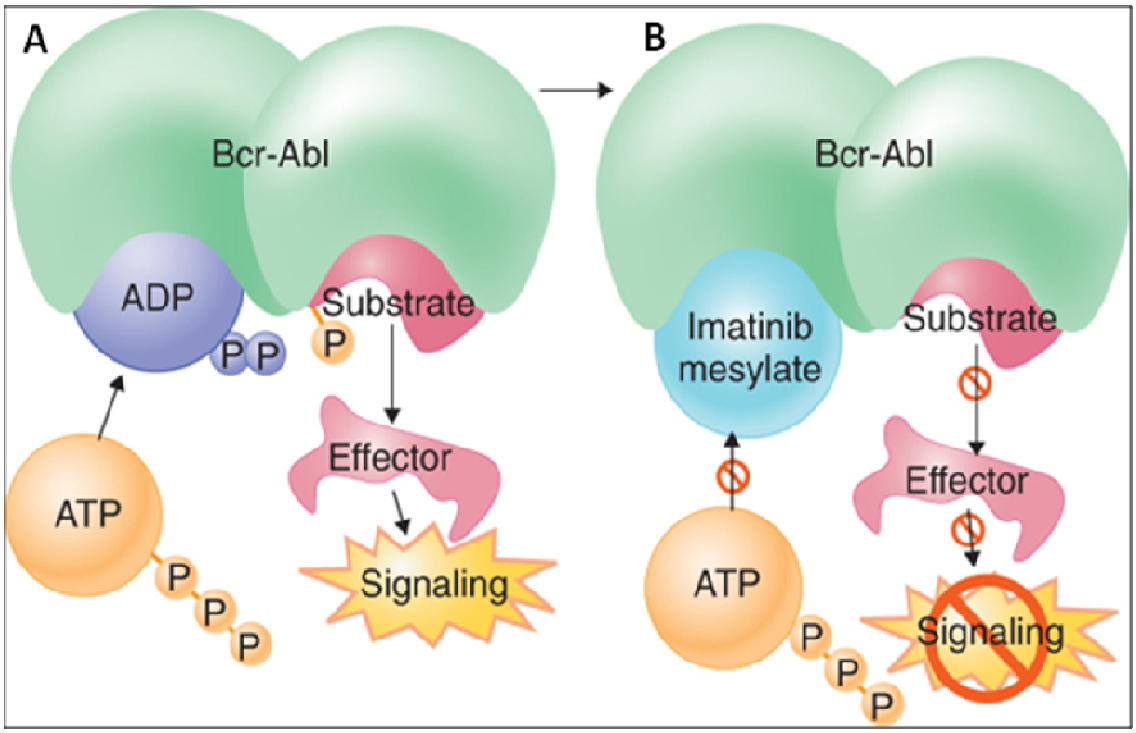


Figure 1.3. Mechanism of action of BCR-ABL in the absence and presence of imatinib. Panel **A** shows the action of BCR-ABL in the presence of ATP, resulting in activation of downstream effector molecules leading to aberrant cell signalling. Panel **B** represents the inhibition of BCR-ABL by imatinib to prevent constitutive kinase activity and restore normal cellular processes (Copied from Rosenbloom *et al.*, 2010).

Since the development of imatinib, more potent tyrosine kinase inhibitors, including dasatinib, nilotinib, bosutinib, and ponatinib, have been developed to treat CML. Although the newer tyrosine kinase inhibitors appear to be more potent than imatinib in clinical trials, (Kantarjian *et al.*, 2010; Saglio *et al.*, 2010; Cortes *et al.*, 2012; Goldman, 2012) they have been approved as second-line

treatment for patients who develop resistance or intolerance to imatinib therapy. In 81 countries including South Africa, Novartis has implemented the Glivec International Patient Assistance Program since 2002 to provide imatinib at no cost to patients who qualify (Lassarat and Jootar, 2006; Capdeville *et al.*, 2008; Au *et al.*, 2009; Louw, 2012).

1.5 Monitoring response to treatment

Monitoring is an integral part of effective treatment and management of CML patients. A number of clinical response criteria to treatment have been implemented throughout the world to determine optimal response, suboptimal response (failure to achieve a pre-defined landmark response to imatinib) and/or treatment failure (Table 1.1) (Druker *et al.*, 2006; Marin *et al.*, 2008; Baccarani *et al.*, 2009; Hughes and Branford, 2009). Responses are categorised as haematological, cytogenetic, and molecular. A complete haematological response is characterized by the normalisation of blood parameters with a reduction in white blood cells to $<10 \times 10^9$/l, a platelet count less than 450×10^9/l and absence of immature cells in peripheral blood. A complete cytogenetic response (CCyR) is defined as the absence of Ph positive bone marrow metaphases using fluorescence *in situ* hybridisation (FISH) (Baccarani *et al.*, 2009; Hughes and Branford, 2009). A molecular response is determined by quantifying the level of *BCR-ABL* mRNA, with a major molecular response (MMR) representing a ≥ 3 log reduction in *BCR-ABL* mRNA from baseline, and a complete molecular response (CMR) is characterised by the absence of detectable *BCR-ABL* mRNA using real-time quantitative polymerase chain reaction (PCR) (Branford and Hughes, 2006). The loss of an achieved

response can either be due to non-compliance (Marin *et al*., 2010) or resistance development and in such cases, mutational screening of the BCR-ABL kinase domain is recommended to detect the presence of mutations that may result in resistance to therapy (Branford, 2007; Hughes and Branford, 2009).

Table 1.1. European Leukaemia Network definition and criteria of haematological, cytogenetic and molecular response and monitoring for CML treated with tyrosine kinase inhibitors (Druker *et al.*, 2006; Marin *et al.*, 2008; Baccarani *et al.*, 2009; Hughes and Branford, 2009)**.**

Response	Definition	Monitoring
Haematological response (complete)	White blood cell count < $10x10^9$/l Platelet count < $450x10^9$/l Differential: without immature granulocytes and with < 5% basophils Non palpable spleen	Check every two weeks until complete response achieved and confirmed, then every three months until otherwise specified
Cytogenetic response	Complete: Ph+ none Partial: Ph+ 1% - 35% Minor: Ph+ 36% - 65% Minimal: Ph+ 66% - 95% None: Ph+ > 95%	Check every six months until complete response achieved and confirmed
Molecular response (ratio of BCR-ABL: control gene according to an international scale)	Complete: *BCR-ABL* transcript non-detectable Major: < 0.1%	Check every three months; mutational analysis only in case of failure, suboptimal response or increased level of transcript

1.6 Prognostic markers of response to treatment with imatinib

Despite the success of imatinib, up to 25% of CML patients experience primary resistance (failure to respond to the drug from the start of treatment) or a suboptimal response (Marin *et al.*, 2008; Engler *et al.*, 2011). A study by White *et al.* (2007) reported that patients who were identified as suboptimal responders benefited from an increase in dose of imatinib from 400 mg/day to 600 mg/day. As a result, several studies have sought to identify key molecular markers that can be used as early indicators of a patient's response to imatinib. Having a strong and reliable predictor of response to imatinib therapy at diagnosis is critical to identify those patients who are likely to have suboptimal responses.

A major determinant of suboptimal response to imatinib therapy in CP CML patients is inadequate inhibition of the BCR-ABL kinase (White *et al.*, 2005). Insufficient inhibition of the BCR-ABL kinase is a result of low intracellular concentrations of imatinib achieved in target leukemic cells (White *et al.*, 2006). Patients with high intracellular levels of imatinib have been shown to respond favourably to treatment since they achieve an adequate concentration of the drug required for the inhibition of BCR-ABL. In contrast, patients displaying low intracellular levels of imatinib are generally associated with a poor response to treatment (White *et al.*, 2005; White *et al.*, 2007).

Various studies have reported that the intracellular concentration of imatinib achieved in leukemic cells is determined by SLC22A1, the influx transporter of imatinib (Thomas *et al.*, 2004; White *et al.*, 2007; Wang *et al.*, 2008). Studies

have found that the influx transporter of imatinib, and not the efflux transporter, is a clinical determinant of levels of imatinib achieved within leukemic cells since differential expression of efflux transporters did not seem to affect imatinib uptake into cells (White *et al.*, 2007; Wang *et al.*, 2008; Kim *et al.*, 2009; Gromicho *et al.*, 2011). Therefore, the activity of SLC22A1 is considered to be of prognostic value in CML patients treated with imatinib (White *et al.*, 2007; Labussiere *et al.*, 2008; Wang *et al.*, 2008; Marin *et al.*, 2010; White *et al.*, 2010; Engler *et al.*, 2011).

1.7 Influx transporter of imatinib, SLC22A1

SLC22A1 is mapped onto the long arm of chromosome 6 and is approximately 39 kb in size and contains 11 exons (Koehler *et al.*, 1997; Koepsell *et al.*, 2003). Four alternative splice variants of *SLC22A1* have been identified in humans, of which, only the longest full length isoform a, comprising 554 amino acids (aa) codes for a functional transporter (Hayer *et al.*, 1999).

While conducting a literature review on SLC22A1, it became evident that the nomenclature of this protein is inconsistent in literature and as a result, confusing. The gene, *SLC22A1* encodes for an organic cation transporter 1 protein which is commonly abbreviated as OCT1. However, the arbitrary use of “OCT1” instead of *SLC22A1* to refer to this gene in literature is misleading and has resulted in errors while researching this transporter.

1.7.1 OCT1 identity crisis[1]

Two different proteins, organic cation transporter 1 (Swiss-Prot:O15245) and octamer binding transcription factor 1, also known as octamer binding protein (Swiss-Prot:P14859) are both referred to as "OCT1" in the literature. Organic cation transporter 1 is encoded by *SLC22A1* (Genbank:AL353625) and is a membrane transport protein, while octamer binding transcription factor 1 is encoded by *POU2F1* (Genbank:AL136984). Both proteins play an important physiological role and have been extensively researched. A search in Google Scholar from 2008 revealed that 3140 articles referred to "OCT1", as either "SLC22A1" or "POU2F1".

The use of "OCT1" for either "SLC22A1" or "POU2F1" is also being used erroneously in commercial product descriptions. Most commercial websites or product data sheets refer synonymously to "SLC22A1" or "POU2F1" as "OCT1" and this is resulting in the erroneous selection of a product or assay. We have identified three recent studies that have used the incorrect gene expression assay for *SLC22A1* as a result of the incorrect use of "OCT1" in commercial literature (Hirayama *et al.*, 2008; Zhang *et al.*, 2008; Xiang *et al.*, 2009). These studies investigated the gene expression of *SLC22A1* by incorrectly using a commercial gene expression kit for *POU2F1* that lists *OCT1* as a gene alias (Hs00231250_m1) (Applied Biosystems). We have also found instances where product descriptions of antibody for POU2F1 and SLC22A1 (listing OCT1 as protein alias) mistakenly interchange the protein function or provide the

[1] This section (1.7.1) has been published as a letter to the editor: Sreenivasan S and Viljoen CD. 2013. OCT1 identity crisis. *Gene* 516:190-191.

incorrect link to SwissProt for these two proteins (Acris Antibodies GmbH, 1998-2012; Creative Biomart, 2011; NovaTeinBio, 2012; Novus Biologicals, 2012). A study by Heise *et al.* (2012) mistakenly used an antibody against POU2F1 while intending to study the protein expression of SLC22A1. Such errors, although unintentional, invalidate published data. The importance of promoting a standard nomenclature for genes and proteins has been emphasized previously (Nature, 1999; White *et al.*, 1999). We suggest that the OCT1 identity crisis be solved by referring to the genes and gene products as "SLC22A1" and "POU2F1", respectively.

1.7.2 Determining SLC22A1 activity for predicting response to imatinib

Currently, there are two ways used to determine SLC22A1 activity. One method measures the level of *SLC22A1* mRNA which gives an indication of the amount of SLC22A1 protein being produced (Wang *et al*., 2008). In a study by Wang *et al.* (2008), it was found that the uptake of imatinib was significantly higher in cells over expressing *SLC22A1* compared to the parental line. This demonstrated that *SLC22A1* mRNA expression can be used as a measure of SLC22A1 function and hence, imatinib uptake. The second method to determine SLC22A1 activity measures the difference in the intracellular uptake and retention (IUR) of radio-labelled imatinib in the presence and absence of prazosin, a potent SLC22A1 inhibitor (White *et al.*, 2006). The difference in IUR is expressed as SLC22A1 activity. A greater difference in IUR is associated with higher SLC22A1 activity. However, in comparison to determining *SLC22A1* mRNA expression using real-time quantitative PCR, the IUR assay

requires the use of cell culturing of individual patient cells and radio-labelled chemistry and is therefore technically demanding and not practical for use in a routine setting.

A number of studies have investigated the relationship between the levels of *SLC22A1* mRNA in CML patients as a prognostic marker to predict treatment response to imatinib. The first study to investigate this was by Crossman *et al.* (2005) who demonstrated that CML patients who had achieved CCyR by 12 months had significantly higher *SLC22A1* mRNA expression compared with those who did not. Wang *et al.* (2008) also found that those patients with high *SLC22A1* mRNA expression had significantly better rates of overall and progression free survival compared to those with low *SLC22A1* mRNA levels. Using univariate analysis, they demonstrated that patients with high levels of *SLC22A1* mRNA had significantly better rates of achieving a CCyR at six months (Wang *et al.*, 2008). Furthermore, Labussiere *et al.* (2008) and more recently, Marin *et al.* (2010) have found a significant correlation between levels of *SLC22A1* mRNA at diagnosis in newly diagnosed CP CML patients and treatment outcome with imatinib. However, these findings are questioned by Zhang *et al.* (2009) who were unable to discern a difference in levels of *SLC22A1* mRNA at diagnosis between patients who did and did not achieve a satisfactory outcome on imatinib therapy. White *et al.* (2007), by grouping patients into high and low IUR groups as measured by the IUR assay using radio-labelled imatinib, demonstrated that those patients with high IUR of imatinib achieved significantly better molecular responses to imatinib by 24 months of therapy than those with low IUR (White *et al.*, 2007). However, White

et al. (2007) report that the same observation did not always hold true for *SLC22A1* mRNA levels as patients with high *SLC22A1* mRNA expression at diagnosis did not always achieve a molecular response compared to those with low *SLC22A1* mRNA expression (White *et al.*, 2007; White *et al.*, 2010). Based on their data, White *et al.* (2010) suggest that the IUR assay is a more reliable predictor of response to imatinib compared to *SLC22A1* mRNA levels.

One of the considerations by White *et al.* (2007) for questioning the use of *SLC22A1* mRNA as a measure of imatinib uptake was the observation that imatinib may affect the expression of *SLC22A1* (White *et al.*, 2007; White *et al.*, 2010). In a study by Crossman *et al.* (2005) involving a small group of 15 CML patients, there was a change (increase or decrease) in expression of *SLC22A1* from baseline in eight patients after imatinib treatment. Similarly, Engler *et al.* (2011) observed that seven out of 16 patients who had achieved CCyR after 12 months of imatinib treatment had increased expression of *SLC22A1* from baseline. Based on the observation by Crossman *et al.* (2005) and Engler *et al.* (2011), White *et al.* (2010) and a few other studies have speculated that the change in *SL22A1* expression from baseline, may be as a result of treatment with imatinib (Engler *et al.*, 2011; Gromicho *et al.*, 2011; Gromicho *et al.*, 2013). While these studies have suggested that imatinib may affect the expression of *SLC22A1*, to date, no conclusive evidence exists on the impact of imatinib on *SLC22A1* gene expression.

1.8 Rationale for the study

The activity of SLC22A1 is considered a clinical determinant of how patients with CML respond to treatment with imatinib. Levels of *SLC22A1* mRNA are used as a measure of SLC22A1 activity (Wang *et al.*, 2008; Marin *et al.*, 2010). However, there is a concern that imatinib may impact on expression of *SLC22A1* and as such, levels of *SLC22A1* mRNA may not be a reliable measure of how patients will respond to treatment with imatinib (White *et al.*, 2007; White *et al.*, 2010). Although various studies have suggested that imatinib may affect expression of *SLC22A1* (Crossman *et al.*, 2005; Engler *et al.*, 2011; Gromicho *et al.*, 2011; Gromicho *et al.*, 2013), none of these studies provide conclusive evidence on the effect of imatinib on expression of *SLC22A1*. The aim of this study was to clarify the impact of imatinib on *SLC22A1* gene expression in K562, a CML cell line.

CHAPTER 2

STABILITY OF ULTRAMER AS COPY NUMBER STANDARDS IN REAL-TIME PCR

2.1 Introduction[2,3]

Real-time PCR has become a routine technique to quantify DNA or RNA copy number. However, one of the challenges in real-time PCR is the lack of commercially available copy number standards and/or reference material (Rutledge *et al.*, 2004; Yan *et al.*, 2010). As a result, genomic DNA, cDNA, PCR amplicon, plasmid constructs or synthetic oligonucleotides are often used in serial dilution as standards (Yun *et al.*, 2006; Bustin *et al.*, 2009). Recent studies have investigated the stability of genomic DNA (Yun *et al.*, 2006; Röder *et al.*, 2010; Rossmanith *et al.*, 2011), PCR amplicon (Dhanasekaran *et al.*, 2010) and plasmid constructs (Dhanasekaran *et al.*, 2010; Martinez-Martinez *et al.*, 2011). It has been reported that although genomic DNA was more stable at -20^{o}C than 4^{o}C, degradation resulting in a change in copy number still occurred (Röder *et al.*, 2010; Rossmanith *et al.*, 2011). It has also been found that high concentrations of genomic DNA resulted in inhibition of PCR which can be overcome by additionally shearing the DNA (Yun *et al.*, 2006). In addition, freeze thawing also affects genomic DNA copy number standards adversely. Compared to this, while PCR products have been found to be more stable at -20^{o}C than 4^{o}C, storage still results in variation in copy number (Dhanasekaran *et al.*, 2010). Thus although different sources of nucleic acids are available for use as copy number standards, their stability in terms of storage remains a problem.

[2]This section has been adapted from a short communication that was accepted for publication in Gene to include only the section which is relevant to this dissertation (Viljoen CD, Thompson GG, Sreenivasan S. 2013. Stability of ultramer as copy number standards in real-time PCR. *Gene* 516:143-145).

[3] Although I am not the first author of this publication, I played an integral role in the formulation and execution of this research, and its publication thereof.

Due to advances in oligonucleotide synthesis, several studies have reported using ultramers in a native form as copy number standards for real-time PCR (Williams *et al.*, 2004; Zhang *et al.*, 2004; Vermeulen *et al.*, 2009; Flynn *et al.*, 2011; Ghiselli *et al.*, 2011; Kavlick *et al.*, 2011; O'Callaghan and Fenech, 2011). Ultramers have been used successfully as standards in either single stranded (Williams *et al.*, 2004; Zhang *et al.*, 2004; Flynn *et al.*, 2011; O'Callaghan and Fenech, 2011) or double stranded form (Ghiselli *et al.*, 2011; Kavlick *et al.*, 2011). While some studies have reported on the storage conditions at -80^{o}C in TE (Kavlick *et al.*, 2011), or -20^{o}C in either water (O'Callaghan and Fenech, 2011) or buffer (Ghiselli *et al.*, 2011), very few studies have investigated the effect of storage on ultramers. Kavlick *et al.* (2011) reported that ultramers were stable at -80^{o}C for over seven months. Compared to this, O'Callaghan and Fenech (2011) commented that ultramers were only stable for up to two weeks at 4^{o}C. From current studies it appears that the storage conditions for synthetic oligonucleotides used as standard in real-time PCR may have a considerable effect on copy number determination. Thus the aim of this study was to determine the stability of ultramer as copy number standards when stored at 4^{o}C and -20^{o}C over a 30 day period using different mixing methods.

2.2 Materials and Methods

A TaqMan gene expression kit which amplified only the full length functional isoform of *SLC22A1* was used to determine gene expression of *SLC22A1* (Applied Biosystems). The probe in the kit (Hs 00427555_m1) spanned the junction of exons nine and ten of *SLC22A1* (the shorter isoform of *SLC22A1* lacks exon 9). By adding approximately 95 bases to either side of the

nucleotide which forms the middle of the amplicon (provided in product information), an ultramer of 190 bases was synthesised (Integrated DNA Technologies) which was inclusive of the assay target region (Table 2.1). Copy number was calculated according to Godornes *et al.* (2007) using 330 Da as the average molecular weight of a base (Cheng *et al.*, 2003) and molecular weight of ultramer was determined from the online calculator ((http://www.unc.edu/~cail/biotool/oligo).

$$\text{Number of copies} = \frac{\text{Mass (g) x Avogadro's number (molecule/mole)}}{\text{Average molecular weight of a base (g/mol/base) x Template length (bases)}}$$

Concentration of ultramer = 2.9×10^{-9} mole

Molecular weight of ultramer = 58717.8 Da

Mass of ultramer = Molecular weight (Da) x Concentration (mole)

= 58,717.8 Da x 2.9×10^{-9} mole

= 170281.62×10^{-9} g

$$\text{Number of copies} = \frac{170281.62 \times 10^{-9}\ \text{g} \times 6.023 \times 10^{23}\ \text{molecule/mole}}{330\ \text{g/mol/base} \times 190\ \text{bases}}$$

Table 2.1. Sequence, fragment length and scale of synthesis of ultramer. Assay location designating middle of target sequence (nucleotide 1606) is indicated in red.

Gene	Ultramer sequence (5'-3')	Fragment length	Scale of synthesis
SLC22A1 (NM_003057.2)	GTTCCTCCCTGTGTGACATAGGTGG GATAATCACCCCCTTCATAGTCTTC AGGCTGAGGGAGGTCTGGCAAGCC TTGCCCCTCATTTTGTTTGCG**G**TGT TGGGCCTGCTTGCCGCGGGAGTGA CGCTACTTCTTCCAGAGACCAAGGG GGTCGCTTTGCCAGAGACCATGAA GGACGCCGAGAACCTTGG	190 bases	2.9 nmole

The lyophilised ultramer was suspended in 0.1x TE (10 mM Tris/1 mM EDTA, pH 7.5). The ultramer was serially diluted in 0.1x TE to obtain 10^2, 10^3, 10^4, 10^5 and 10^6 copies/5 µl. The copy number standards were mixed during preparation and prior to use either by vortexing, pipetting or inverting the tubes. The copy number standards were stored at 4^{o}C and -20^{o}C and used in real-time PCR assays at day 0, 7, 15 and 30.

Real-time PCR using the ultramer was performed on the 7500 Fast Real-Time PCR System. The real-time PCR reactions consisted of 2x TaqMan Fast Advanced Mastermix (Applied Biosystems), 5 µl of respective copy number standard, 900 nM forward and reverse primer and 250 nM probe (Hs

00427555_m1) and made up to 20 µl with nuclease-free sterile water. Thermal cycling conditions were 2 minutes at 50^{o}C, 10 minutes at 95^{o}C followed by 45 cycles of 15 seconds at 95^{o}C and 1 minute at 60^{o}C.

2.3 Results and Discussion

In contrast to O'Callaghan and Fenech (2011) who suggested that "working stocks of oligomers should be made fresh", ultramer copy number standards were found to be highly stable whether stored at 4^{o}C or -20^{o}C over a 30 day period in 0.1x TE (Figure 2.1). Less than a 5% variance was observed in threshold cycle (C_T) values for the different copy number standards regardless of storage condition or mixing method over a period of 30 days. Furthermore, the method of mixing to prepare the ultramer and prior to use either by vortexing, pipetting or inverting appeared to make no difference in the reproducibility and linearity of results (Figure 2.2). Thus it was concluded that 4^{o}C is suitable for storage of ultramer copy number standard for up to one month and for longer term storage at -20^{o}C.

The relative cost of using ultramers as copy number standards appears negligible compared to commercial standards (if available). A rough estimation was that at a standard scale of synthesis (4 nmole), sufficient ultramer is synthesised for approximately 7x10^{8} assays, using copy number standards in duplicate, for under $0.01 per assay (Table 2.2). Thus the use of ultramer is a highly cost efficient method of generating copy number standards that are up to 200 bases in size compared to commercially available standards.

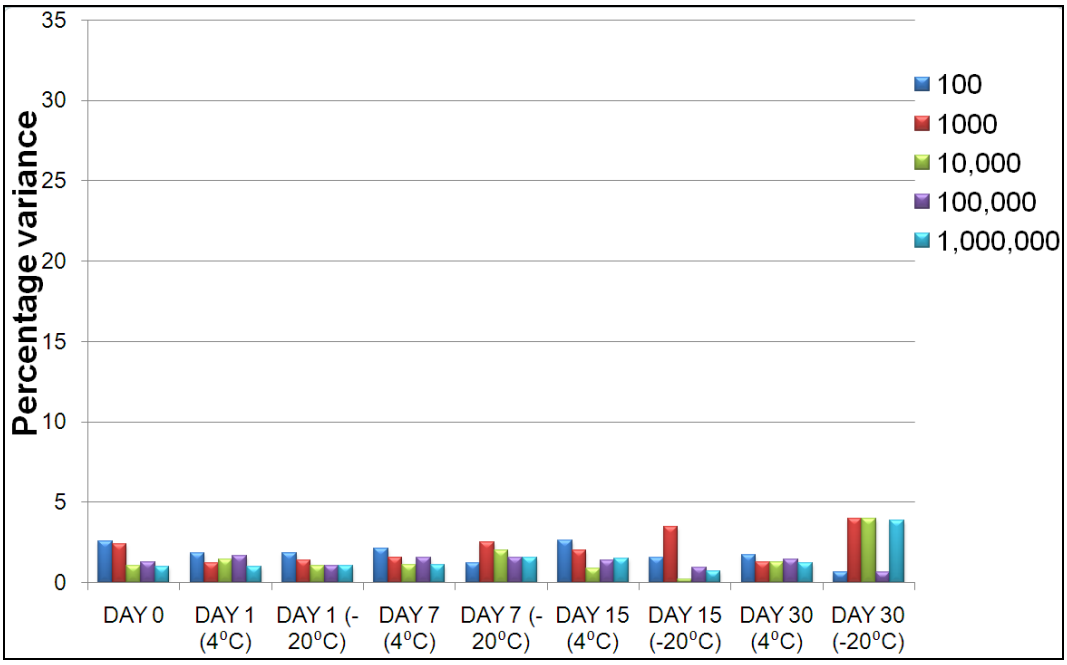


Figure 2.1. Mean percentage of variance in C_T value of *SLC22A1* for ultramer copy number standards (10^2 to 10^6) at 0, 1, 15 and 30 days at 4°C and -20°C analysed using ANOVA. The data for each day represent a combination of mixing treatment by vortex, pipette and hand inversion.

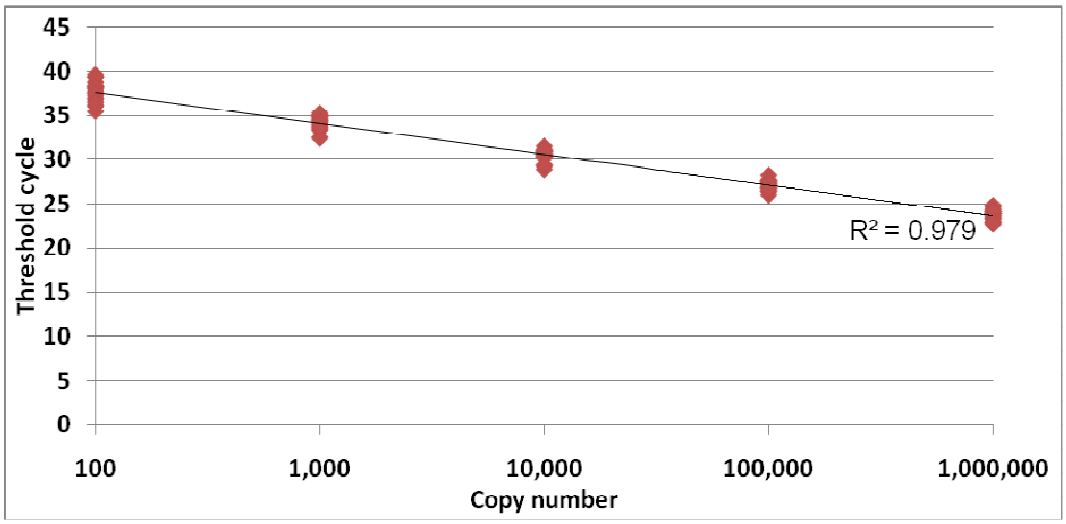


Figure 2.2. The plot represents a compilation of threshold cycle data from *SLC22A1* copy number standards (10^2 to 10^6) prepared using all three mixing methods, for 24 assays at 0, 1, 15 and 30 days at 4°C and -20°C.

Table 2.2. Cost efficiency of using ultramer as copy number standards.

Gene	Calculated copies of ultramer[1]	Theoretical number of assays[2]	Estimated cost per assay ($)
SLC22A1	$1.64x10^{15}$	$7x10^{8}$	<0.01

[1]At a standard scale of synthesis (4 nmole).

[2]Assuming that copy number standards are used in duplicate.

2.4 Conclusion

The use of ultramer as copy number standards has several advantages over other sources of DNA for copy number standards: 1. Since the ultramer is synthetic, it is free of any biological contamination that could affect overall stability and reproducibility; 2. Up to 200 bases of ultramer can be synthesized without additional preparation including cloning or extraction; 3. Ultramer is highly stable compared to genomic DNA or PCR amplicon; 4. Preparations of different batches of ultramer appear to provide reproducible results (Kavlick *et al.* 2011). Based on these results, ultramer is stable, convenient and cost effective for use as copy number standards in real-time PCR.

CHAPTER 3
DETERMINATION OF *SLC22A1* mRNA EXPRESSION

3.1 Introduction

The treatment of chronic myeloid leukaemia (CML) with imatinib is one of the best examples of targeted therapy for cancer. The clinical use of imatinib has resulted in favourable response rates in up to 85% of CML patients (Deininger *et al.*, 2009). However, approximately 25% of CML patients are reported to display a suboptimal response to treatment with imatinib (Marin *et al.*, 2008; Engler *et al.*, 2011). White *et al.* (2006) suggested that a suboptimal response in patients can be attributed to inadequate BCR-ABL inhibition in target leukemic cells as a result of decreased intracellular accumulation of imatinib. The intracellular uptake of imatinib in leukemic cells is mediated by SLC22A1, and so, its activity is considered an important clinical determinant of how patients will respond to therapy (White *et al.*, 2007; Wang *et al.*, 2008).

There are two methods described in literature to determine SLC22A1 activity. The first method uses levels of *SLC22A1* mRNA as measured by real-time quantitative PCR, as a measure of SLC22A1 activity. In a study by Wang *et al.* (2008), it was demonstrated that the uptake of imatinib was significantly higher in cells over expressing *SLC22A1* mRNA, and so, Wang *et al.* (2008) suggested that levels of *SLC22A1* mRNA can be used a measure of imatinib uptake, and hence, SLC22A1 activity. The second method measures the difference in the intracellular uptake and retention (IUR) of imatinib in the absence and presence of prazosin, a potent inhibitor of SLC22A1. In this method, patient cells are incubated with radio-labelled imatinib in the presence and absence of prazosin. The difference in IUR of radio-labelled imatinib in the presence and absence of prazosin is measured using a Scintillation counter and is termed as SLC22A1

activity. A greater difference in IUR is associated with increased SLC22A1 activity. Although there is no general consensus in literature as to which method is a more reliable measure of SLC22A1 activity, the use of radio-labelled chemistry poses a considerable problem in implementing the IUR assay in a routine setting, due to the safety and availability of reagents, equipment and expertise required. In comparison, determining levels of *SLC22A1* mRNA using real time quantitative PCR is more amenable as most CML diagnostic laboratories have access to real-time PCR analysis.

Several studies have demonstrated a correlation between *SLC22A1* expression and patient response to imatinib (Crossman *et al.*, 2005; Labussiere *et al.*, 2008; Wang *et al.*, 2008; Marin *et al.*, 2010). One of the first such studies was by Crossman *et al.* (2005) who reported that patients who had eight times higher levels of *SLC22A1* mRNA were associated with a favourable treatment outcome compared to those with low levels of *SLC22A1* mRNA. The latter group did not achieve CCyR after 12 months of imatinib treatment. Since then other studies have also demonstrated that patients who achieved a favourable treatment outcome with imatinib had significantly higher levels of *SLC22A1* mRNA compared to non-responders (Labussiere *et al.*, 2008; Wang *et al.*, 2008; Marin *et al.*, 2010). However, in contradiction to this, White *et al.* (2007) reported that grouping patients into high and low *SLC22A1* mRNA did not always predict the response to treatment with imatinib and rather stratifying patients into high and low IUR groups, was a more reliable predictor of response. Furthermore, Crossman *et al.* (2005) observed that 12 out of 15 patients showed a change (increase or decrease) in *SLC22A1* expression 12

months after imatinib therapy. In addition, Engler *et al.* (2011) observed that there was an increase, although not significant, in *SLC22A1* mRNA expression in seven out of 16 patients who had achieved CCyR after 12 months of treatment with imatinib. Based on this and the data from Crossman *et al.* (2005), White *et al.* (2010) suggested that levels of *SLC22A1* mRNA may not be a reliable measure of imatinib uptake since imatinib may impact on levels of *SLC22A1* mRNA, without a consequent change in imatinib uptake. While it is generally accepted in literature that imatinib may influence expression of *SLC22A1* (White *et al.*, 2010; Engler *et al.*, 2011; Gromicho *et al.*, 2011; Gromicho *et al.*, 2013), this has not been proven conclusively. Thus, the aim of this study was to determine whether imatinib has any regulatory effect on *SLC22A1* mRNA in a K562 CML cell line using real-time quantitative PCR.

3.2 Materials and Methods

3.2.1 Study design

This was a case-controlled study to investigate the effect of imatinib on *SLC22A1* gene expression in a K562 CML cell line. The CML cell line, K562, was chosen in this study to minimize experimental differences as a result of external factors and inter-individual variation. Levels of *SLC22A1* mRNA were determined using real-time PCR in control cells and cells treated with 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2 µM, 5 µM and 10 µM of imatinib for 24 hours, 48 hours and 72 hours.

3.2.2 Cell culture

The K562 CML cell line was obtained from the European Collection of Cell Cultures (Sigma-Aldrich). K562 cells are non-adherent, suspension cells which are highly undifferentiated granulocytes. The cells were maintained in RPMI-1640 (Roswell Park Memorial Institute-1640) medium containing 2 mM L-glutamine and 25 mM HEPES (hydroxyethyl piperazineethanesulfonic acid) (HyClone). The medium was supplemented with 10% foetal bovine serum (GIBCO), 5 µg/ml penicillin-streptomycin solution (Sigma-Aldrich) and 5 µg/ml plasmocin (Invivogen). Cells were cultured in T75 flasks (Corning) at 37°C in a humidified atmosphere of 5% carbon dioxide. K562 cells in exponential growth phase were counted using the TC10 Automated Cell Counter (BIO-RAD) to determine cell viability by the addition of a mixture of 10 µl of cell suspension and 10 µl of 0.4% trypan blue (BIO-RAD) onto a TC10 counting slide. Subculturing of cells was performed once every two days by centrifugation of the cell suspension at 2,500 rpm for 5 minutes. The cell pellet was then re-suspended in fresh growth medium to a concentration of 2×10^6 cells/ml.

3.2.3 Treating cells with imatinib

Imatinib was kindly provided by Novartis for this study (Novartis Pharmaceuticals). A stock solution of 10 mM of imatinib was made in nuclease-free sterile water and kept at -70ºC until used. Cells were incubated with 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2 µM, 5 µM and 10 µM of imatinib for 24 hours, 48 hours and 72 hours. The experimental period was limited to 72 hours since the doubling period of K562 cells is 24 hours and the majority of cells undergo apoptosis by 72 hours. At the end of each time interval, control and

experimental cells were counted using the TC10 Automated Cell Counter (BIO-RAD), diluted to a concentration of 2 x 10^6 cells/ml and divided into two equal parts, for RNA extraction and the Taqman protein assay (refer to chapter 4, section 4.3.4.1).

3.2.4 RNA extraction and concentration determination

RNA extraction was performed in a dedicated area where all equipment, reagents, glassware and plasticware used for RNA extraction was treated with DEPC (Diethyl pyrocarbonate) and RNase-ZAP to prevent RNAase contamination. Total RNA was extracted from K562 cells using TRI Reagent (Sigma Aldrich). Approximately 2 to 5 x 10^6 cells were pelleted by centrifugation at 2,500 rpm for 5 minutes. The cell pellet was homogenized with 1 ml TRI Reagent (Sigma Aldrich) to which 15 µl of 20 mg/ml Proteinase K (Roche) was added and incubated at 65°C for 20 minutes. This was followed by the addition of 350 µl chloroform (Merck), vortexing for 15 seconds and incubation on ice for 3 minutes. Thereafter, the sample was centrifuged at 10,000 rpm for 15 minutes to allow phase separation. The upper aqueous phase was removed from which RNA was precipitated by the addition of equal volumes of ice-cold iso-propanol (Merck) and incubated for 10 minutes at room temperature followed by centrifugation at 10,000 rpm for 10 minutes. The pellet of total extracted RNA was washed twice in 1 ml of 75% ethanol and re-dissolved in 40 µl nuclease-free sterile water aided by incubation at 55°C for 15 minutes. The concentration of extracted RNA was determined using the Quant-iT RNA Assay Kit according to manufacturer's instructions (Invitrogen). Calibration standards were prepared by the addition of 10 µl of each Quant-iT RNA standard 1 (0

ng/ml RNA) and Quant-iT RNA standard 2 (10 ng/ml RNA) provided in the kit to 189 µl of Quant-iT RNA buffer and 1 µl of Quant-iT RNA Reagent also provided in the kit. The concentration of extracted RNA was determined by the addition of 1 µl of extracted RNA to 198 µl of Quant-iT RNA buffer and 1 µl of Quant-iT RNA Reagent. The mixture was vortexed and briefly centrifuged, followed by incubation at room temperature for 3 minutes. Concentration measurements were performed on the Qubit fluorometer (Invitrogen). Extracted RNA was stored at -70°C until used.

3.2.5 cDNA (complementary DNA) synthesis

A standard amount of 2 µg of extracted RNA was reverse transcribed to cDNA. All cDNA synthesis reactions were performed in duplicate using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). The cDNA synthesis cocktail contained (per reaction) 10 µl of 2x RT Buffer (provided in the kit), 1 µl of 20x Enzyme Mix (provided in the kit), 2 µg of extracted RNA (to a maximum volume of 9 µl) made up to a total volume of 20 µl with nuclease-free sterile water. The cDNA synthesis cocktail was incubated in a GeneAmp 9700 (Applied Biosystems) thermal cycler at 37°C for 60 minutes, followed by 95°C for 5 minutes and stored at 4°C until used.

3.2.6 Real-time quantitative PCR

SLC22A1 copies of mRNA were quantified by real-time PCR reactions consisting of 10 µl TaqMan Fast Advanced Mastermix (Applied Biosystems), the equivalent of 500 ng of RNA converted to cDNA, 900 nM forward and reverse *SLC22A1* primer respectively, 250 nM *SLC22A1* probe (Hs

00427555_m1) and nuclease-free sterile water made up to a final reaction volume of 20 µl. *GUS* was used as the reference gene for quantification and similar reaction mixtures were prepared which consisted of 250 nM forward and reverse *GUS* primer respectively, and 125 nM *GUS* probe instead of *SLC22A1* primer and probe. The real-time PCR reactions were performed in duplicate using a 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal cycling conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute. The primer and probe sequences for *GUS* were obtained from Beillard *et al.* (2003) and synthesized by Applied Biosystems (Table 3.1). A commercially available TaqMan Gene Expression Assay Kit was used to perform gene expression of *SLC22A1* (Hs 00427555_m1) (Applied Biosystems). The TaqMan Gene Expression Kit for *SLC22A1* contains proprietary primers and probe sequences designed to span the junction of exons nine and ten of *SLC22A1*. Commercial copy number standards (10^3, 10^4 and 10^5 copies) were used to quantify copies of *GUS* mRNA (Ipsogen) and serial dilutions (10^2, 10^3, 10^4, 10^5, 10^6 copies) of an ultramer oligonucleotide synthesized by Integrated DNA Technologies were used to quantify copies of *SLC22A1* mRNA (refer to chapter 2). Standard curves for both *GUS* and *SLC22A1* were generated by plotting mean threshold cycle (C_T) values against template copy number. No-template controls were included to serve as contamination controls.

3.2.7 Real-time data analysis

Real-time analysis was performed with a fluorescence threshold setting of 0.1. Only standard curves with $R^2 \geq 0.98$ and slope between -3.1 and -3.6 were used

for quantification of both reference and target gene copy number. Results were expressed as the mean ratio of *SLC22A1* to *GUS* in order to correct for sampling variation and inherent biological sample variability (Lee *et al.*, 2006). PCR efficiency was calculated according to the formula: Efficiency = $[10^{(-1/slope)}]$ – 1 (Peters *et al.*, 2004). The statistical significance ($p < 0.05$) for differences in expression of *SLC22A1* between control and imatinib treated cells at 24 hours, 48 hours and 72 hours were calculated using ANOVA (analysis of variance) and Student's t-test using Microsoft Excel, with a 95% confidence interval.

Table 3.1. Primers and probe sequences from Beillard *et al. (*2003) used for real-time PCR of *GUS*. **F** - Forward primer; **R** - Reverse primer; **P** – Probe labelled with FAM and TAMRA.

Gene	Sequence (5' to 3')
GUS	**F:** GAAAATATGTGGTTGGAGAGCTCAT **R:** CCCGAGTGAAGATCCCCTTTTTA **P:** FAM CCAGCACTCTCGTCGGTGACTGTTCA TAMRA

3.3 Results and Discussion

3.3.1 Impact of imatinib on *SLC22A1* mRNA expression

The results from this study indicate that there is a non-linear correlation between *SLC22A1* expression and imatinib concentration at 24 hours, 48 hours and 72 hours. Interestingly, it was observed that, although not always significant, there appeared to be an increase in *SLC22A1* expression compared to control (untreated cells), at all concentrations of imatinib. Overall, there was

an increase in *SLC22A1* expression from 0.1 µM to 0.5 µM imatinib, followed by a decline between 0.5 µM to 1 µM imatinib. At 24 hours, 48 hours and 72 hours, there was a significant increase in expression from 2 µM to 5 µM, ultimately reaching peak expression at 10 µM imatinib which resulted in up to 8-fold ($p = 0.01$), 22- fold ($p = 0.00$) and 62- fold ($p = 0.00$) increase in expression of *SLC22A1*, respectively, from baseline (Figure 3.1). The clinical importance of imatinib-induced gene expression observed at 10 µM imatinib is questionable as the maximum plasma concentration of imatinib achieved in CML patients irrespective of therapeutic dose, has been reported to be 3910 ng/ml which is equivalent to only 6 µM imatinib (Larson *et al*., 2008). Interestingly, it was observed that there was a non-linear relationship between *SLC22A1* expression and imatinib concentration at 24 hours, 48 hours and 72 hours.

A striking observation was that the level of *SLC22A1* expression at a particular concentration was not statistically significant over time. Whereas, expression of *SLC22A1* was dependent on the concentration of imatinib, *SLC22A1* expression did not change significantly over time of exposure to imatinib (Table 3.2 and Table 3.3). Thus, the findings from this study suggest that while *SLC22A1* expression changes due to exposure to imatinib, this change is evident at 24 hours, after which no further significant increase in expression due to imatinib exposure was observed for up to 72 hours.

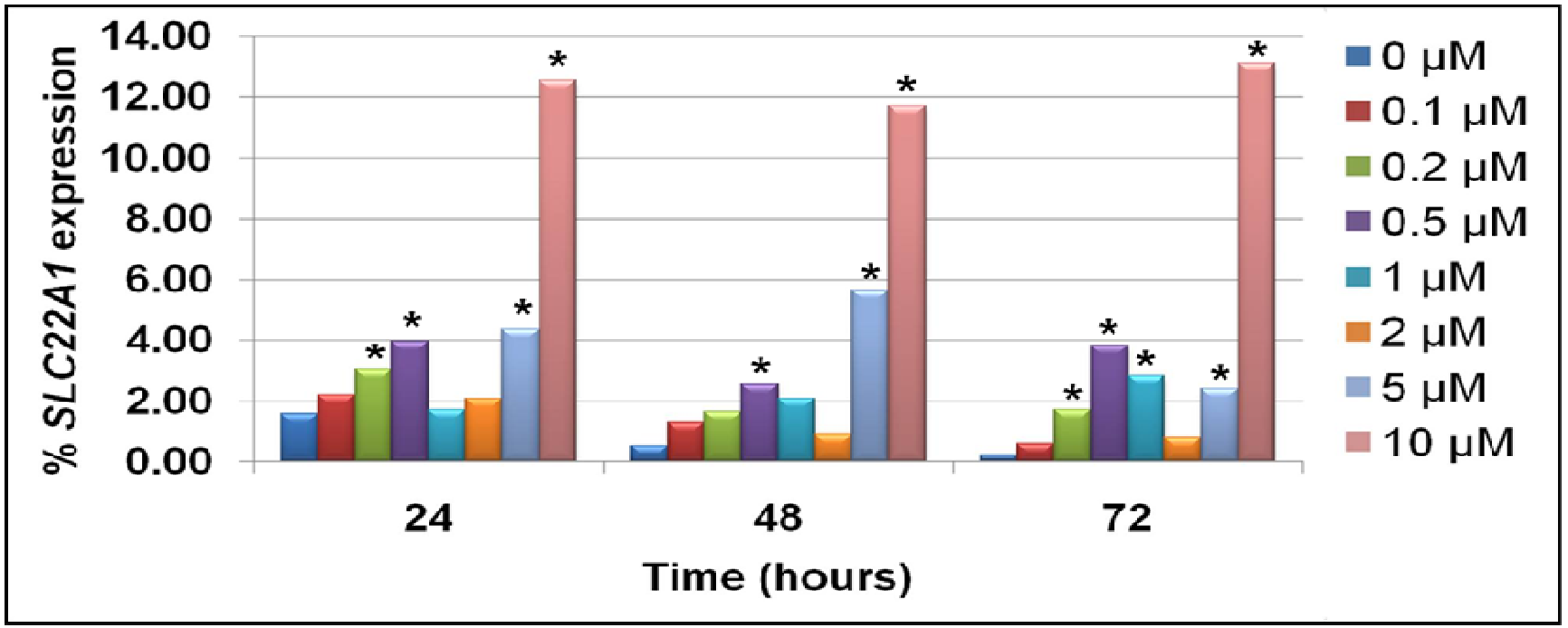


Figure 3.1. Change in *SLC22A1* expression in K562 cells treated with 0.1 µM to 10 µM imatinib compared to the control. Statistically significant differences ($p < 0.05$) between control and treated cells determined using ANOVA and Student's t-test are indicated with asterisk (*).

Table 3.2. Significant differences in *SLC22A1* expression between K562 control cells and imatinib treated cells over time for 24 hours, 48 hours and 72 hours. Significance was calculated using ANOVA at a 95% confidence interval. Cells have been crossed out to avoid repetition of p-values.

Time	24 hours	48 hours	72 hours
24 hours	-	p = 0.6195	p = 0.5889
48 hours	-	-	p = 0.9416

Table 3.3. Significant differences in *SLC22A1* expression between cells treated with varying doses of imatinib. Significance was calculated using ANOVA at a 95% confidence interval. Significant differences in *SLC22A1* expression ($p < 0.05$) between K562 cells treated with 0.1 µM to 10 µM imatinib are indicated with asterisk (*). Cells have been crossed out to avoid repetition of p-values.

Imatinib	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM
0.1 µM	-	0.28	0.002*	0.11	0.61	0.003*	1.34×10^{-8}*
0.2 µM	-	-	0.01*	0.65	0.11	0.01*	2.41×10^{-8}*
0.5 µM	-	-	-	0.02*	0.0006*	0.33	1.12×10^{-7}*
1 µM	-	-	-	-	0.03*	0.014*	1.82×10^{-8}*
2 µM	-	-	-	-	-	0.001*	7.69×10^{-9}*
5 µM	-	-	-	-	-	-	1.00×10^{-6}*
10 µM	-	-	-	-	-	-	-

The results obtained from this study may explain the heterogeneous expression of *SLC22A1* reported by other studies on CML patients treated with imatinib

(Crossman *et al.*, 2005; Engler *et al.*, 2011; Gromicho *et al.*, 2013). These studies found a change (increase or decrease) in *SLC22A1* expression in response to imatinib, although not always statistically significant. It has been reported in literature that there is a large inter-patient variability in plasma levels of imatinib achieved between patients on the same dose of the drug. A pharmacokinetic analysis of the IRIS trial revealed that patients given 400 mg/day of imatinib exhibited trough plasma levels ranging from as little as 153 ng/ml to as high as 3910 ng/ml after 24 hours (Larson *et al.*, 2008). Thus, a combination of variable individual trough levels of imatinib and the non-linear correlation to *SLC22A1* expression may explain the seemingly inconsistent expression of *SLC22A1* in CML patients on imatinib. Furthermore, a significant change in *SLC22A1* expression from baseline was observed only at concentrations of 0.5 µM, 5 µM and 10 µM imatinib (Figure 3.1). This may explain the observations by Engler *et al.* (2011) that while there was an increase in *SLC22A1* expression in patients treated with imatinib, it was not always statistically significant.

Engler (2011) also found that the expression of *SLC22A1* in the HL-60 CML cell line treated with 2 µM imatinib was not significantly different from untreated cells at 24 hours and seven days. Based on this, Engler (2011) suggested that short term exposure to imatinib does not affect *SLC22A1* expression. Compared to this, in the current study, there was also no significant difference in expression of *SLC22A1* between control cells and K562 cells treated with 2 µM imatinib at 24 hours ($p = 0.2178$), 48 hours ($p = 0.0668$) or 72 hours ($p = 0.0693$). It appears that due to the non linear correlation between *SLC22A1* expression

and imatinib concentration, 2 µM imatinib does not appear to significantly impact *SLC22A1* expression. Thus, in contradiction to the assumption by Engler (2011) that short term imatinib exposure may not impact on expression of *SLC22A1*, the results from the current study show that expression of *SLC22A1* is not time dependent but rather that significant changes in *SLC22A1* expression is dependent on the intracellular levels of imatinib.

3.3.2 Quantification of *SLC22A1*

Table 3.4. **Comparison of mean and standard deviation (SD) of correlation (R^2), slope and efficiency of five standard curves for *GUS* and *SLC22A1* respectively.**

Gene	*GUS*		*SLC22A1*	
	Mean	**SD**	**Mean**	**SD**
R^2	0.999	0.0009	0.995	0.004
Slope	-3.50	0.04	-3.53	0.04
Efficiency	92.91%	1.47	92.83%	1.92

In this study, serial dilutions of a synthesised ultramer (Integrated DNA Technologies) were used as copy number standards to successfully quantify copies of *SLC22A1*. The slope and efficiency of the ultramer standard for *SLC22A1* was similar to that of a commercially available standard for the reference gene, *GUS* (Table 3.4). This is the first study in which quantification of *SLC22A1* copy number has been performed in comparison to previous studies that have expressed relative fold change in expression of *SLC22A1* to

that of a control gene using the delta C_T method (Crossman *et al.*, 2005; Wang *et al.*, 2008; Marin *et al.*, 2010; Gromicho *et al.*, 2011).

3.4 Conclusion

Several studies have reported that levels of *SLC22A1* mRNA expression can be used as a measure of SLC22A1 activity (Wang *et al.*, 2008; Marin *et al.*, 2010; Gromicho *et al.*, 2013). However, some studies have reported that CML patients treated with imatinib displayed heterogeneous expression of *SLC22A1* (Crossman *et al.*, 2005; Engler *et al.*, 2011). Whereas patients in Crossman *et al.* (2005) showed either an increase or decrease after treatment with imatinib, patients in the study by Engler *et al.* (2011) showed an increase in expression after 12 months of treatment. These observations have led researchers to suggest that imatinib may impact *SLC22A1* expression (White *et al.*, 2010; Engler *et al.*, 2011; Gromicho *et al.*, 2013) and question the use of *SLC22A1* as a prognostic marker for treatment response with imatinib. The results from this study demonstrate that there is a non-linear concentration dependent correlation between *SLC22A1* expression and imatinib. Furthermore, the relationship between *SLC22A1* expression and imatinib concentration does not appear to be time dependent after 24 hours. Thus, it is the degree of exposure to imatinib that influences expression of *SLC22A1* and not the overall time of exposure to imatinib.

It may have been of added value to determine whether changes in *SLC22A1* mRNA expression correlate to imatinib uptake. Unfortunately the challenge in obtaining radio-labelled imatinib and the need for specialized equipment to

perform the IUR assay were limiting factors. Thus, while this study does not exclude the use of expression levels of *SLC22A1* mRNA as a prognostic marker for treatment outcome, results from this study validate the suggestion by White *et al*. (2007) that levels of *SLC22A1* mRNA can only be used reliably in newly diagnosed imatinib naive or previously untreated CML patients since imatinib induces the expression of *SLC22A1*.

CHAPTER 4

DETERMINATION OF SLC22A1 PROTEIN EXPRESSION

4.1 Introduction

The cellular uptake of imatinib is mediated by the membrane protein, SLC22A1 (Thomas *et al.*, 2004), and its activity is considered a key determinant of patient response to imatinib treatment (White *et al.*, 2007; Wang *et al.*, 2008). Studies have reported that CML patients with significantly higher levels of *SLC22A1* mRNA achieved a CCyR at 12 months after treatment with imatinib in comparison to patients with low *SLC22A1* expression who did not respond favourably. Thus, it has been suggested that levels of *SLC22A1* mRNA can be used as a prognostic marker to treatment outcome with imatinib (Crossman *et al.*, 2005; Wang *et al.*, 2008; Marin *et al.*, 2010). Interestingly, Crossman *et al.* (2005) observed a change in expression of *SLC22A1* from baseline, in some CML patients after treatment with imatinib. On the other hand, in a study by White *et al.* (2007), grouping patients in high and low IUR groups as measured by the IUR assay was found to be indicative of treatment outcome. The same observation did not always hold true for levels of *SLC22A1* mRNA since patients with high levels of *SLC22A1* mRNA did not always respond significantly better than patients with low levels of *SLC22A1* mRNA. Based on this, and the data from Crossman *et al.* (2005) study, which found a change in expression of *SLC22A1* in patients after imatinib therapy, White *et al.* (2010) and other studies have suggested that levels of *SLC22A1* mRNA may not be a reliable measure of imatinib uptake as treatment with imatinib may affect the expression of *SLC22A1* (Engler *et al.*, 2011; Gromicho *et al.*, 2011; Gromicho *et al.*, 2013).

Based on the results from the current study (presented in chapter 3) there is conclusive evidence to substantiate the suggestion that imatinib does affect *SLC22A1* mRNA expression. However, the increase in *SLC22A1* expression, due to exposure to imatinib, does not appear to result in increased imatinib uptake (White *et al*., 2007; White *et al.*, 2010). It is therefore unknown whether increased levels of *SLC22A1* mRNA result in increased SLC22A1 protein or whether other factors are involved in determining the uptake of imatinib. Thus far, there have been no studies that have investigated the correlation in gene expression between *SLC22A1* mRNA and SLC22A1 protein.

Currently, different methods including western blotting and immunohistochemistry are used to determine relative protein expression (Lipman *et al.*, 2005). However, these techniques are cumbersome and laborious as they require extensive protein extraction and purification. Recently, a novel technique to quantify a specific protein was described. The Taqman protein assay enables the relative quantification of a protein present in crude cell lysates using real-time PCR. The Taqman protein assay has several advantages to other protein detection methods since it enables an integrated real-time PCR approach for measuring relative changes in mRNA and protein expression from the same sample on a single analytical platform and does not require extensive protein extraction and purification (Swartzman *et al.*, 2010).

The Taqman protein assay is an antibody based ligation assay for the detection of proteins (Fredriksson *et al.*, 2002; Gullberg *et al.*, 2004). The Taqman protein assay uses two biotinylated antibodies to the target protein, to which

streptavadin linked oligonucleotides have been attached (Figure 4.1a). The Taqman protein assay can be applied to crude cell lysates exposing the epitopes of the target protein for antibody recognition (Figure 4.1b). When the antibodies bind to the target protein at a distance of approximately 21 nm (Figure 4.1c), the oligonucleotides are ligated together forming the template for real-time PCR (Figure 4.1d). The extent of real-time amplification is therefore relative to the amount of ligated oligonucleotide and hence, the target protein (Figure 4.1e-f). A key determinant of the success of the Taqman protein assay is the selection of an optimal antibody pair to detect the target protein. Antibodies used in the Taqman assay have to comply with certain criteria which include antibody binding characteristics (relating to the specificity and affinity of antibody to target epitope sites) and the solution in which the antibody is stored (Table 4.1).

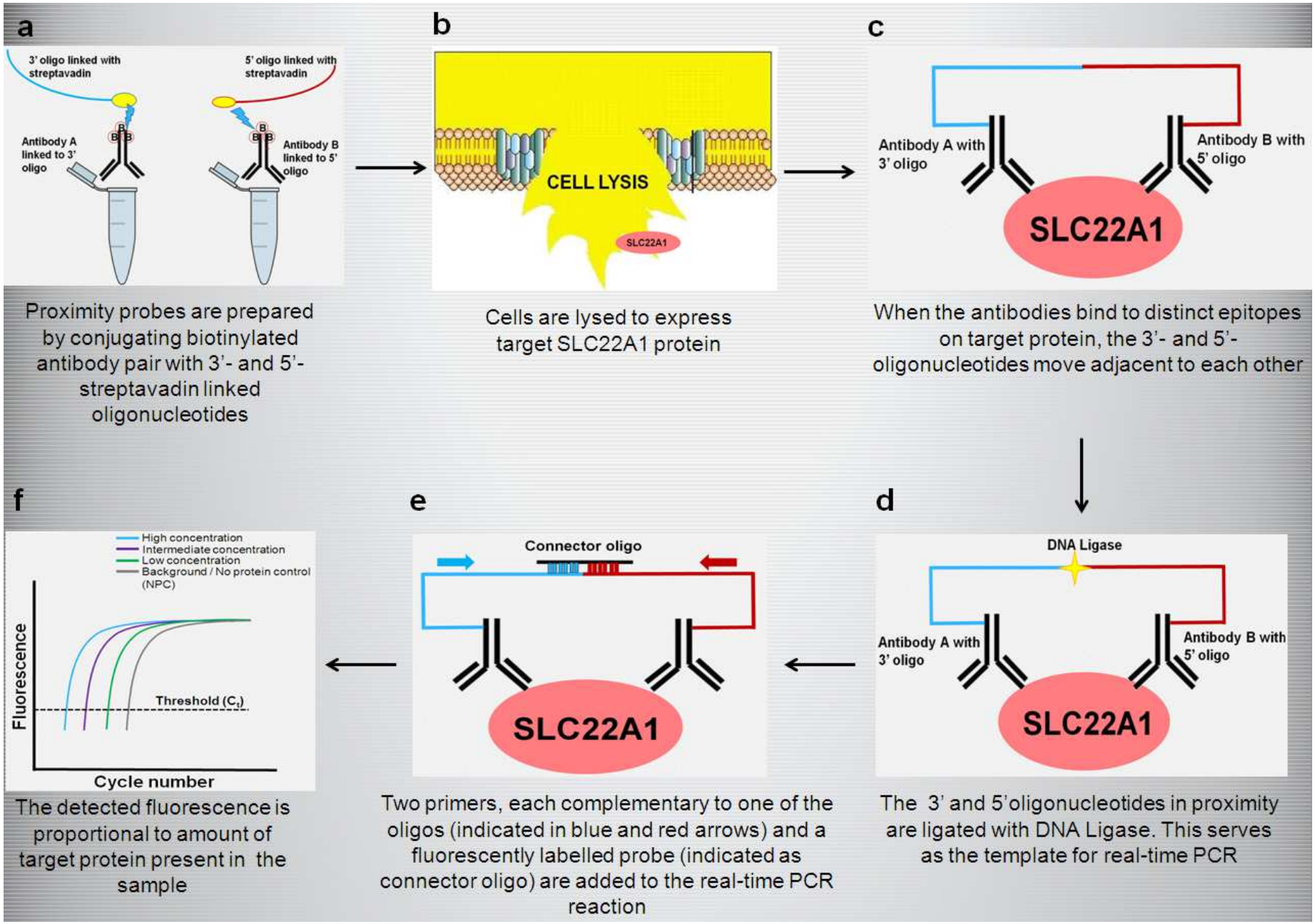


Figure 4.1. Overview of the Taqman protein assay principle.

Table 4.1. Criteria determining antibody suitability for use in the Taqman protein assay.

Criteria	Reason for set criteria
Polyclonal antibody should be raised against the full length or near-full length of the antigen (target protein).	Small antigenic sequences do not illicit immune reactions that yield specific and concentrated antibody. In addition, a polyclonal antibody pair against a small epitope region may not be successful since the antibodies may only bind to a single epitope.
The solution in which antibody is stored should not contain carrier protein or amine-containing buffers including >0.1% gelatin, >0.1% azide.	If an antibody solution contains carrier proteins and >0.1% of amine-containing buffers, these will also get biotinylated and may result in under biotinylation of the antibody.
Biotinylated antibody solution must not contain free biotin.	Based on unpublished data from the Taqman protein assays probe development protocol (Applied Biosystems), as little as 80 nM of free biotin in the antibody preparation has been reported to diminish assay performance.
The polyclonal antibodies must be affinity purified.	Non-affinity purified antibodies usually contain significant amounts of non-specific antibodies and their presence will impact on the sensitivity and specificity of the assay.

4.2 Aim of the study

The aim of this study was to determine the effect of imatinib on the expression of SLC22A1 protein in K562 cells treated with 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2 µM, 5 µM and 10 µM of imatinib compared to control cells, for 24 hours, 48 hours and 72 hours, using the Taqman protein assay.

4.3 Materials and Methods

4.3.1 Cytotoxicity assay

The K562 CML cell line was maintained as explained in chapter 3 (section 3.2.2). K562 cells in exponential growth phase were diluted to a concentration of 5 x 10^4 cells per 100 µl, plated in triplicate, and incubated in 100 µl of RPMI-1640 medium containing 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, 2 µM, 5 µM and 10 µM of imatinib for 24 hours at 37°C in an incubator with 5% carbon dioxide. After 24 hours, 10 µl of Alamar Blue (Invitrogen) was added to the cells and incubated for 6 hours. Alamar Blue or resazurin (blue and non-fluorescent) is reduced to resorufin (pink and fluorescent) by living cells. The resulting change in absorbance is used as a measure of cell viability. Blank controls consisted of media and Alamar Blue. Absorbance was measured at 570 nm and 600 nm using a plate reader (Synergy^{TM} HT, BioTek Instruments). The concentration of imatinib required to inhibit cell growth by 50% (IC_{50}) was calculated using the percentage difference in reduction of Alamar Blue between treated and control cells (Xie *et al*., 2008). The IC_{50} was reported as mean value of two independent experiments, performed in triplicate for the seven concentrations tested.

$$\text{Cell viability (\% of control)} = \frac{(\varepsilon_{OX(600)} \times T_{(570)}) - (\varepsilon_{OX(570)} \times T_{(600)})}{(\varepsilon_{RED(570)} \times C_{(600)}) - (\varepsilon_{RED(600)} \times C_{(570)})} \times 100\%$$

Where;

$\varepsilon_{OX(570)}$=Molar extinction coefficient of oxidized Alamar Blue at 570nm= 80,586

$\varepsilon_{OX(600)}$=Molar extinction coefficient of oxidized Alamar Blue at 600 nm= 117,216

$\varepsilon_{RED(570)}$=Molar extinction coefficient of reduced Alamar Blue at 570nm= 155,677

$\varepsilon_{RED(600)}$=Molar extinction coefficient of reduced Alamar Blue at 600 nm= 14,652

$T_{(570)}$=Absorbance for test cells at 570 nm

$T_{(600)}$=Absorbance for test cells at 600 nm

$C_{(570)}$=Absorbance for control cells at 570nm

$C_{(600)}$=Absorbance for control cells at 600nm

4.3.2 Antibody screening, selection and biotinylation

A total of 56 commercially available antibodies against human SLC22A1 were compared in terms of their suitability for use in the Taqman protein assay (Table 4.1). The Easy Link Biotin (type A) kit (Abcam) was used to biotinylate the selected SLC22A1 antibodies. In order to biotinylate 20 µg of antibody, either 10 µl or 4 µl of EL-Modifier reagent (supplied with the kit) was added to 100 µl or 40 µl of antibody, respectively, depending on the initial concentration of the antibody (0.2 mg/ml or 0.5 mg/ml respectively). This mixture was added to the lyophilised EL-Biotin (type A) mix (supplied with the kit) and incubated in the dark for 3 hours at room temperature. Following incubation, 10 µl or 4 µl of EL-Quencher reagent was added to either 0.2 mg/ml or 0.5 mg/ml antibodies, respectively, and incubated at room temperature for 30 minutes. The biotinylated antibodies were stored at -70 °C.

4.3.3 Forced proximity probe test

A forced proximity probe test was performed according to manufacturer's instructions to determine whether antibody was adequately biotinylated. The biotinylated antibodies were diluted to 200 nM by the addition of either 42.5 µl or 47 µl of Antibody Dilution Buffer (supplied in the TaqMan Protein Assay Buffer Kit) to either 7.5 µl (for 0.2 mg/ml antibody) or 3 µl (for 0.5 mg/ml) antibody, respectively. The diluted 200 nM biotinylated antibody (2 µl) was added to 2 µl of a mixture containing equal amounts of 200 nM 3′ Prox-Oligo (3' streptavadin linked oligonucleotide) and 200 nM 5′ Prox-Oligo (5' streptavadin linked oligonucleotide) (both supplied in the TaqMan Protein Assays Oligo Probe Kit) to prepare proximity probes. A no protein control (NPC) was included which contained 2 µl of Antibody Dilution Buffer (supplied in the TaqMan Protein Assay Buffer Kit) instead of 200 nM of biotinylated antibody (Table 4.2).

Table 4.2. Reagents and reaction volumes used for forced proximity probe test.

Reaction components	Forced proximity probe test	NPC
200 nM biotinylated antibody	2 µl	-
200 nM oligo mix	2 µl	2 µl
Antibody Dilution Buffer	-	2 µl

The forced proximity probe assay including NPC was incubated at room temperature for 60 minutes to conjugate the biotinylated antibody to the

streptavadin linked oligonucleotides. To this mixture, 396 µl of Assay Probe Dilution Buffer (supplied in TaqMan Protein Assay Buffer Kit) was added and incubated at room temperature for 30 minutes. Following incubation, 2 µl each of the forced proximity probe test and NPC were aliquoted in quadruplicate to 2 µl of Lysate Dilution Buffer (supplied in TaqMan Protein Assay Buffer Kit). To this mixture, 96 µl of ligation solution which consisted of 2 µl of 500x DNA Ligase (diluted 1:200 in Ligase Dilution Buffer supplied in the TaqMan Protein Expression Assays Core Reagents Base Kit), 50 µl of 20x Ligation Reaction Buffer (supplied in the TaqMan Protein Expression Assays Core Reagents Base Kit) and 908 µl of nuclease free water was added to get a total reaction volume of 100 µl. The solution was incubated in a GeneAmp 9700 (Applied Biosystems) thermal cycler at 37°C for 10 minutes followed by the addition of 2 µl of 100x Protease (diluted 1:100 in PBS supplied in the TaqMan Protein Expression Assays Core Reagents Base Kit), and incubated in a GeneAmp 9700 (Applied Biosystems) thermal cycler at 37°C for 10 minutes to terminate the ligation reaction and thereafter, at 95°C for 5 minutes to inactivate the protease.

The protease-treated ligation product was used as template in a real-time PCR assay to confirm that the oligonucleotide linked to the antibody was able to undergo ligation for real-time PCR amplification. To 9 µl of the protease-treated ligation product, 10 µl of 2x Taqman Protein Expression Fast Master Mix (Applied Biosystems) and 1 µl of 20x Universal PCR Assay (supplied in the TaqMan Protein Expression Assays Core Reagents Base Kit) were added. The Universal PCR Assay mix contains the primers and the fluorescently labelled

connector oligonucleotide necessary to facilitate real-time amplification of the template in the reaction. Real-time PCR was performed using the 7500 Fast Real-Time PCR System at 95°C for 20 seconds followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. The real-time PCR data was analysed using a threshold setting of 0.1 and automatic baseline. The difference in the mean of threshold cycle (C_T) values obtained for NPC and forced proximity probe was used to calculate delta C_T (mean $C_{T(NPC)}$ – mean $C_{T(\text{forced proximity probe})}$ = ΔC_T). The ΔC_T value determines whether the antibody is adequately biotinylated and suitable for use in the Taqman protein assay. According to the manufacturer, a ΔC_T value ≥ 8.5 indicates that the antibody is biotinylated sufficiently and is suitable for use in the Taqman protein assay.

4.3.4 Taqman protein assay for quantification of SLC22A1 protein

4.3.4.1 Cell lysate preparation

Cell lysates were freshly prepared from K562 cells at a concentration of 2 x 10^6 cells/ml (refer to chapter 3, section 3.2.3). K562 cells were washed twice with 1x phosphate buffered saline (PBS) (GIBCO) to remove residual media by centrifugation at 2,000 rpm for 5 minutes. The supernatant was discarded. Cells were lysed to release target protein by the addition of 1 ml of Sample Lysis Buffer (provided in the TaqMan Protein Quant Sample Lysis Kit), 10 µl of 1x Calbiochem Protease Inhibitor Cocktail Set I (Merck) and 10 µl of 1x Calbiochem Phosphatase Inhibitor Cocktail Set II (Merck). The cell lysates were stored at -70ºC at a concentration of 2,500 cells/µl in sample lysis solution. For stability reasons, cell lysates were serially diluted to 1:10, 1:20, 1:40, 1:80,

1:160, 1:320 and 1:640 in Lysate Dilution Buffer (supplied in the TaqMan Protein Assays Buffer Kit),.immediately prior to performing the Taqman protein assay.

4.3.4.2 Performing the Taqman protein assay

Proximity probes were freshly prepared by conjugating the biotinylated antibodies to the streptavadin-linked oligonucleotides. To 5 µl of 200 nM biotinylated antibody, 5 µl of either 200 nM of 3' Prox-Oligo or 5 µl 200 nM of 5' Prox-Oligo (supplied in the TaqMan Protein Assays Oligo Probe Kit) was added to get 3' proximity probe and 5' proximity probe, respectively. The 3' and 5' proximity probes were incubated at room temperature for 60 minutes followed by the addition of 90 µl of Assay Probe Storage Buffer (supplied in the TaqMan Protein Assays Buffer Kit), and incubated for a further 20 minutes. A mixture containing both proximity probes was prepared by adding 12 µl of each 3' and 5' proximity probes to 216 µl of Assay Probe Dilution Buffer (supplied in the TaqMan Protein Assays Buffer Kit). To 2 µl of this mixture in triplicate, 2 µl of serially diluted cell lysates (1:10, 1:20, 1:40, 1:80, 1:160, 1:320 and 1:640) were added in triplicate. A NPC that contained only 18 µl of Lysate Dilution Buffer (supplied in the TaqMan Protein Assays Buffer Kit) was also included. The mixture containing cell lysate and proximity probe was incubated in a GeneAmp 9700 (Applied Biosystems) thermal cycler at 37°C for 60 minutes to allow antibodies to bind to target protein. To the incubated mixture, 96 µl of ligation solution consisting of 0.1 µl of 500x DNA Ligase (diluted 1:200 in Ligase Dilution Buffer supplied in the TaqMan Protein Expression Assays Core Reagents Base Kit), 5 µl of 20x Ligation Reaction Buffer (supplied in the

TaqMan Protein Expression Assays Core Reagents Base Kit) and 90.9 µl of nuclease free water was added to get a total reaction volume of 100 µl. The solution was incubated in a GeneAmp 9700 (Applied Biosystems) thermal cycler at 37°C for 10 minutes, followed by the addition of 2 µl of 100x Protease (diluted 1:100 in PBS supplied in the TaqMan Protein Expression Assays Core Reagents Base Kit) and incubated in a GeneAmp 9700 (Applied Biosystems) thermal cycler at 37°C for 10 minutes and 95°C for 5 minutes.

The protease treated mixture serves as a template for real-time PCR. To 9 µl of the protease treated mixture, 10 µl of 2x TaqMan Protein Expression Fast Master Mix (Applied Biosystems) and 1 µl of Universal PCR Assay (supplied with the TaqMan Protein Expression Assays Core Reagents Base Kit) were added. Real-time PCR was performed on the 7500 Fast Real-Time PCR system (Applied Biosystems) using fast thermal cycling conditions: 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds. The real-time PCR data was analysed using a threshold setting of 0.1 and automatic baseline setting. NPC allowed for the correction of background ligation. This corrected value is represented as ΔC_T. The ProteinAssist software Version 1.0 (Applied Biosystems) was used to analyse the real-time data obtained, in order to determine the relative SLC22A1 protein expression in K562 cells treated with imatinib compared to control.

4.4 Results and Discussion

4.4.1 Cytotoxicity assay

The average percentage reduction of Alamar Blue in treated cells compared to control was calculated and used to generate equation of the trend line which was then used to calculate IC_{50} imatinib (Moharamzadeh *et al.*, 2007). The IC_{50} of imatinib for the K562 CML cell line was calculated to be 0.50 ± 0.03 µM (Figure 4.2). This data is comparable to the IC_{50} values for the K562 CML cell line determined by other studies (Druker *et al.*, 1996; Deininger *et al.*, 1997; Deguchi *et al.*, 2008; Assef *et al.*, 2009). The equivalent therapeutic threshold dose for 0.5 µM imatinib is 200 mg/day (Peng *et al.*, 2004) and since at this concentration cell proliferation is inhibited by 50%, pharmacokinetic studies have determined the standard dose for effective inhibition of BCR-ABL positive leukemic cells as 400 mg/day of imatinib which represents 2 µM imatinib used in this study (Peng *et al.*, 2004). During optimisation of the TaqMan protein assay for SLC22A1 protein determination, experiments were performed using cells treated with 0.5 µM imatinib and compared to the control.

4.4.2 SLC22A1 antibody selection

Out of the 55 antibodies screened for suitability for use in the Taqman protein assay to quantify SLC22A1 protein, only three antibodies fulfilled the necessary criteria (Table 4.3). The selected SLC22A1 antibodies, one each from Santa Cruz Biotechnology, Abcam and Sigma-Aldrich, respectively, were polyclonal, affinity purified and recognised the correct isoform of *SLC22A1*. The remaining 52 SLC22A1 antibodies did not comply with the requirements for use in the Taqman protein assay (Appendix C).

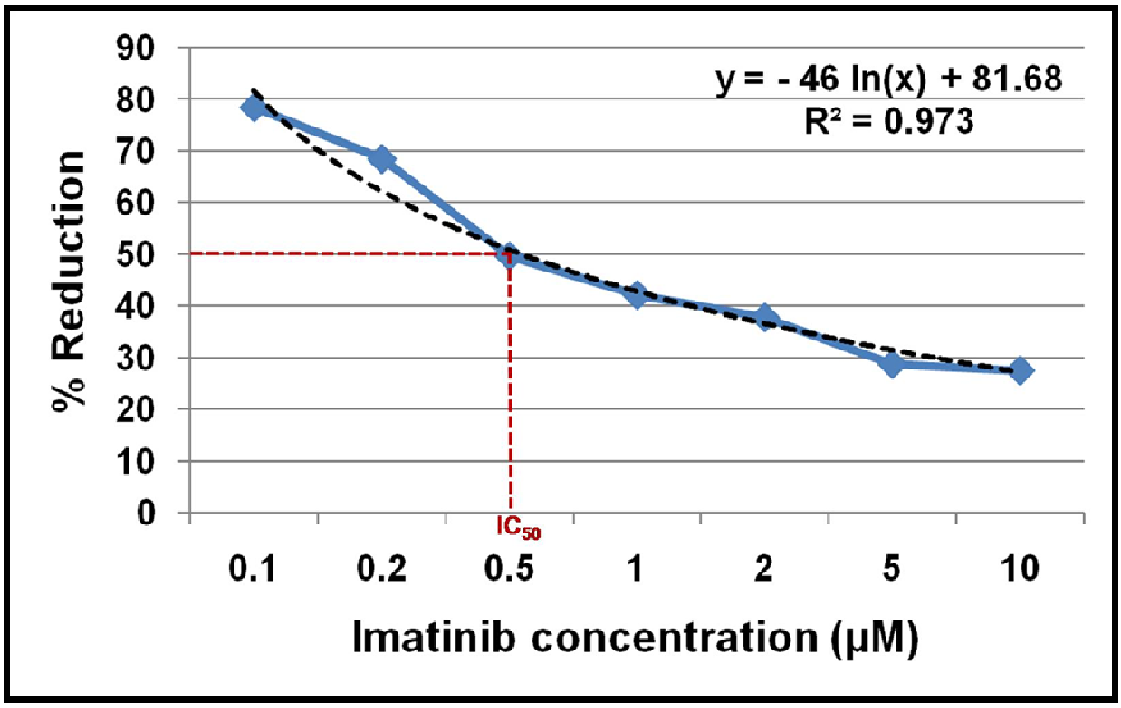


Figure 4.2. IC_{50} imatinib for K562 cells after 24 hours of exposure to imatinib. Data points represent the mean percentage reduction in treated cells relative to untreated control ± SD of two independent experiments performed in triplicate. The equation of the curve is derived from the trend-line (black dotted line).

Table 4.3. SLC22A1 antibodies used in the study.

Manufacturer of SLC22A1 antibody	Santa Cruz Biotechnology	Abcam	Sigma Aldrich
Catalogue number of antibody	sc-19309	ab93491	HPA029846-100UL-EA
Concentration of antibody in solution	0.2 mg/ml	0.5 mg/ml	0.5 mg/ml
Antibody storage solution	0.1% gelatine and <0.1% azide	0.01% azide, 50% glycerol and PBS	0.02% azide, 40% glycerol and PBS

4.4.3 Forced proximity probe test

The forced proximity probe test was performed to determine the suitability of a biotinylated antibody for use in the Taqman protein assay. All three SLC22A1 antibodies tested passed the forced proximity probe test with a $\Delta C_T \geq 8.5$ indicating that they were adequately biotinylated, that there was no excess biotin in antibody solution and thus suitable for use in the Taqman protein assay (Table 4.4).

Table 4.4. ΔC_T values for antibodies tested using forced proximity probe test.

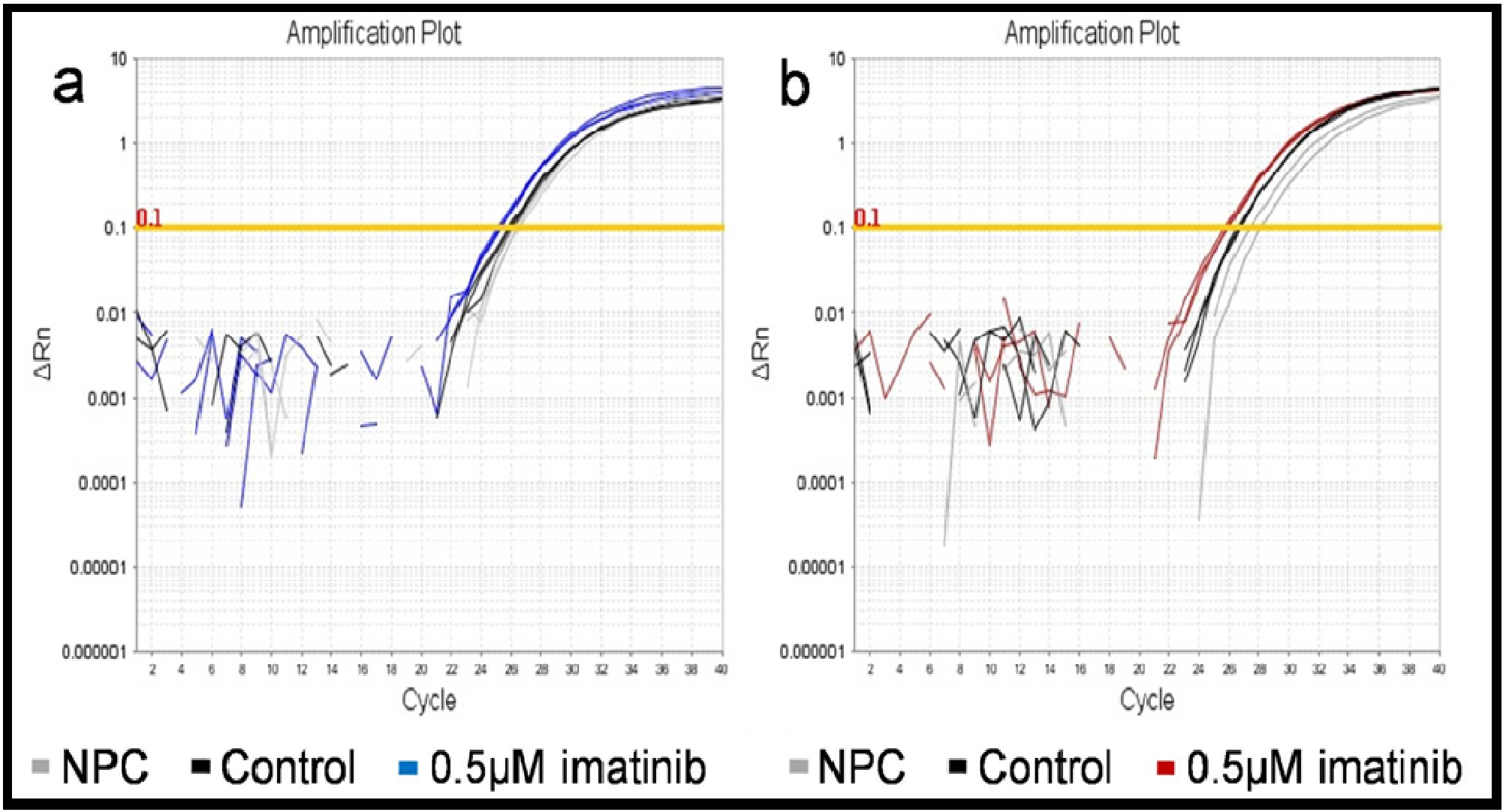
Sample Name	C_T	Mean C_T	ΔC_T = (Mean C_T NPC – Mean C_T antibody)
Abcam NPC	33.65	33.36	13.13
	33.22		
	33.29		
	33.26		
Abcam antibody	20.36	20.23	
	20.24		
	20.11		
	20.21		
Santa Cruz NPC	33.10	33.47	12.10
	33.51		
	33.33		
	33.94		
Santa Cruz antibody	21.50	21.37	
	21.29		
	21.30		
	21.37		
Sigma NPC	25.60	25.57	11.86
	25.54		
	25.43		
	25.72		
Sigma antibody	13.83	13.71	
	13.77		
	13.74		
	13.51		

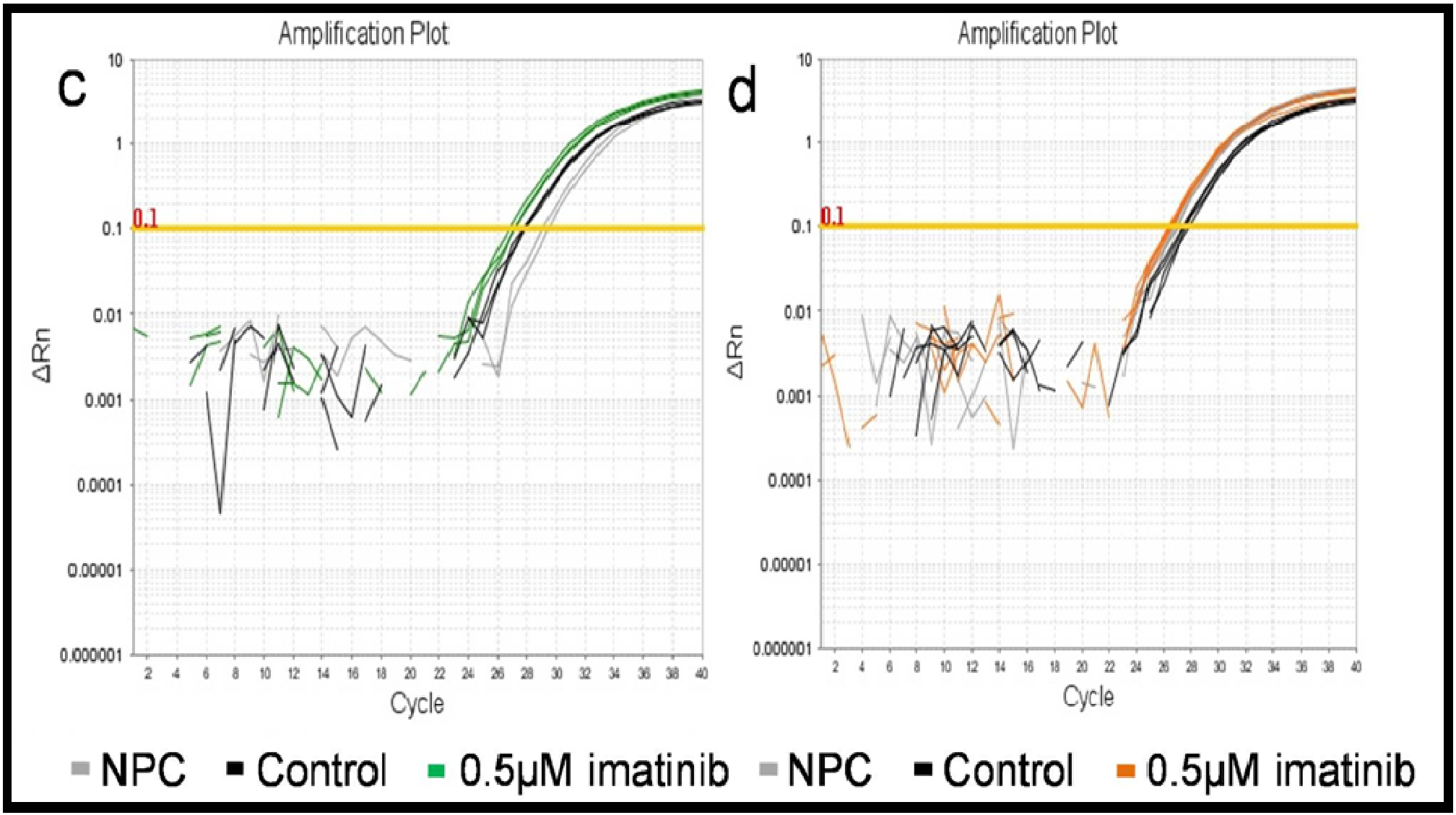
4.4.4 Screening for the most suitable proximity probe pair

Due to the lack of information on the epitope recognition and binding affinity of selected SLC22A1 antibodies, it was not possible to predict which antibodies would function optimally as a proximity probe pair. Since a polyclonal antibody binds to different epitopes on the same target protein, an antibody pair could either be formed by the same polyclonal antibody attached to 3' and 5' oligonucleotides, or with two different polyclonal antibodies attached to a 3' and a 5' oligonucleotide, respectively. Thus, each antibody was tested on its own and in combination with the other antibodies, to determine the pair best suited for the detection of SLC22A1 protein on K562 cell lysates obtained from K562 cells treated with 0.5 µM imatinib for 24 hours against control cells.

Successful amplification was obtained for assays performed using the Santa Cruz antibody on its own and the Sigma antibody on its own. The Abcam antibody on its own and other antibody combinations tested did not result in successful real-time PCR amplification as C_T values for antibody binding were similar to C_T values for NPC (Figures 4.3 a-f). Further analysis of real-time PCR amplification data using the ProteinAssist software version 1.0 (Applied Biosystems) identified the linear range of amplification of each antibody on its own or in combination (Figures 4.4 a-f). All the antibodies produced a narrow linear range of amplification over only two dilution points with the exception of the Sigma antibody which yielded a linear range of amplification over three dilution points when used on its own. As a result of this, further optimisation of the assay using the Sigma antibody was undertaken by investigating whether

the concentration of antibody or an insufficient amount of target protein were limiting factors.


a
Amplification Plot
ΔRn
Cycle
0.1
NPC
Control
0.5µM imatinib
b
Amplification Plot
ΔRn
Cycle
0.1
NPC
Control
0.5µM imatinib


c
Amplification Plot
ΔRn
Cycle
0.1
d
Amplification Plot
ΔRn
Cycle
0.1
NPC
Control
0.5μM imatinib
NPC
Control
0.5μM imatinib

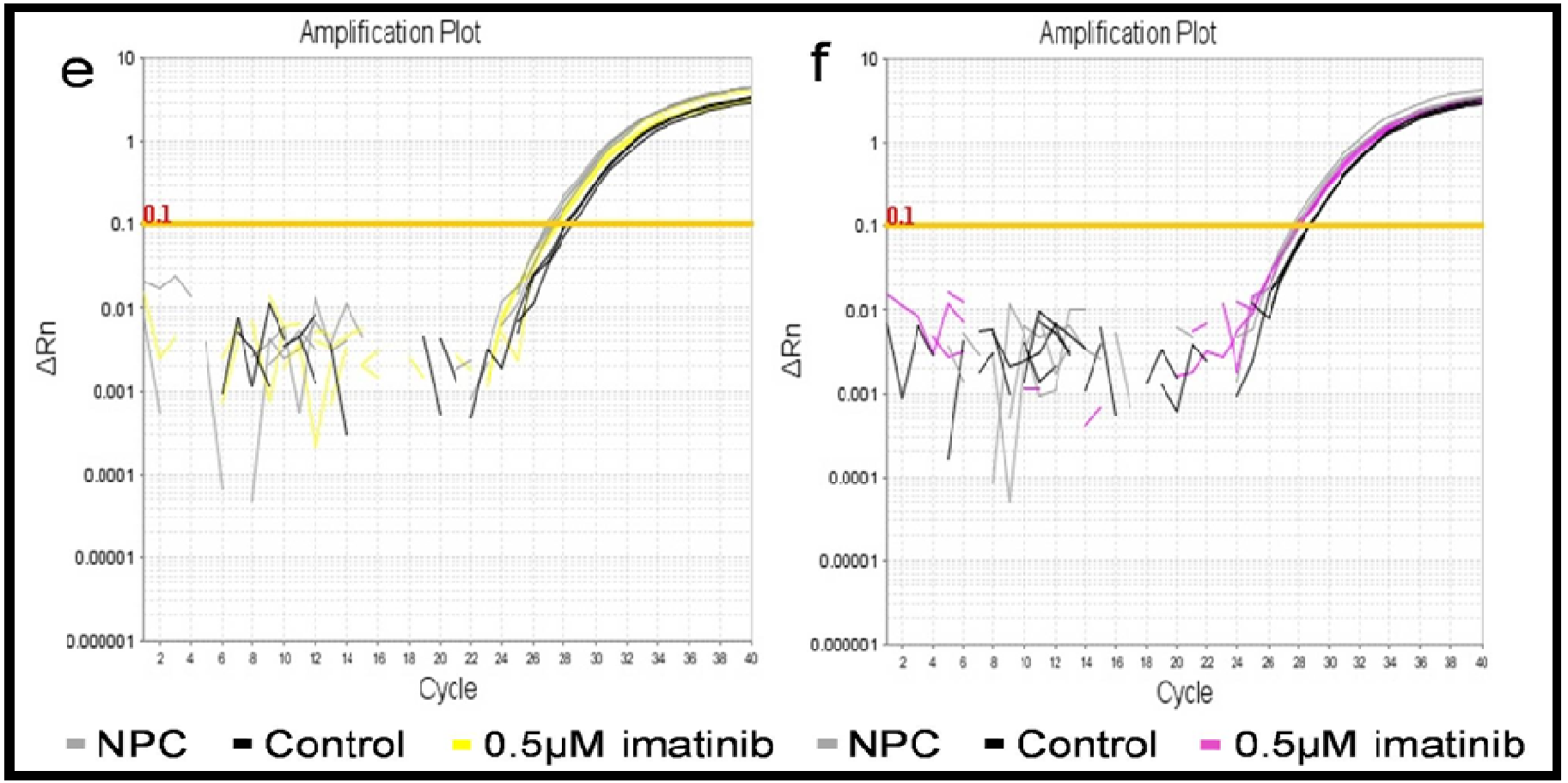
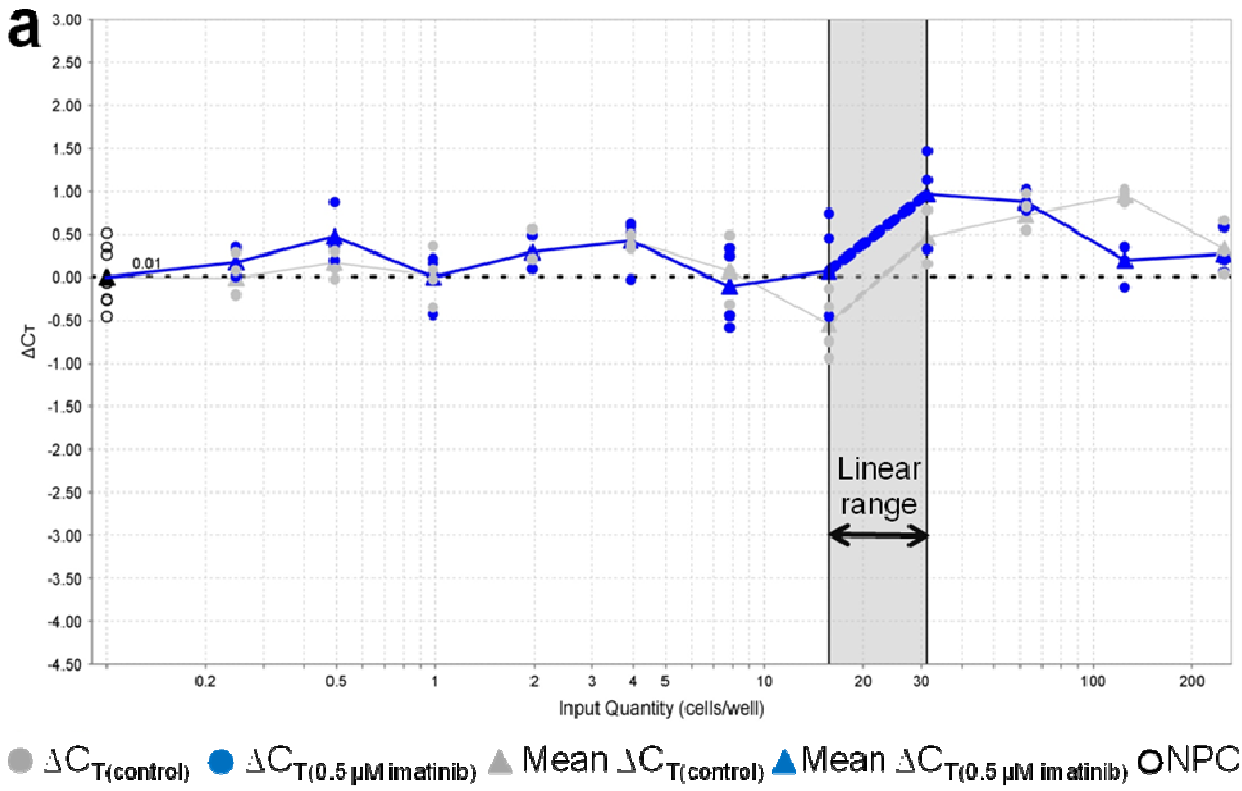
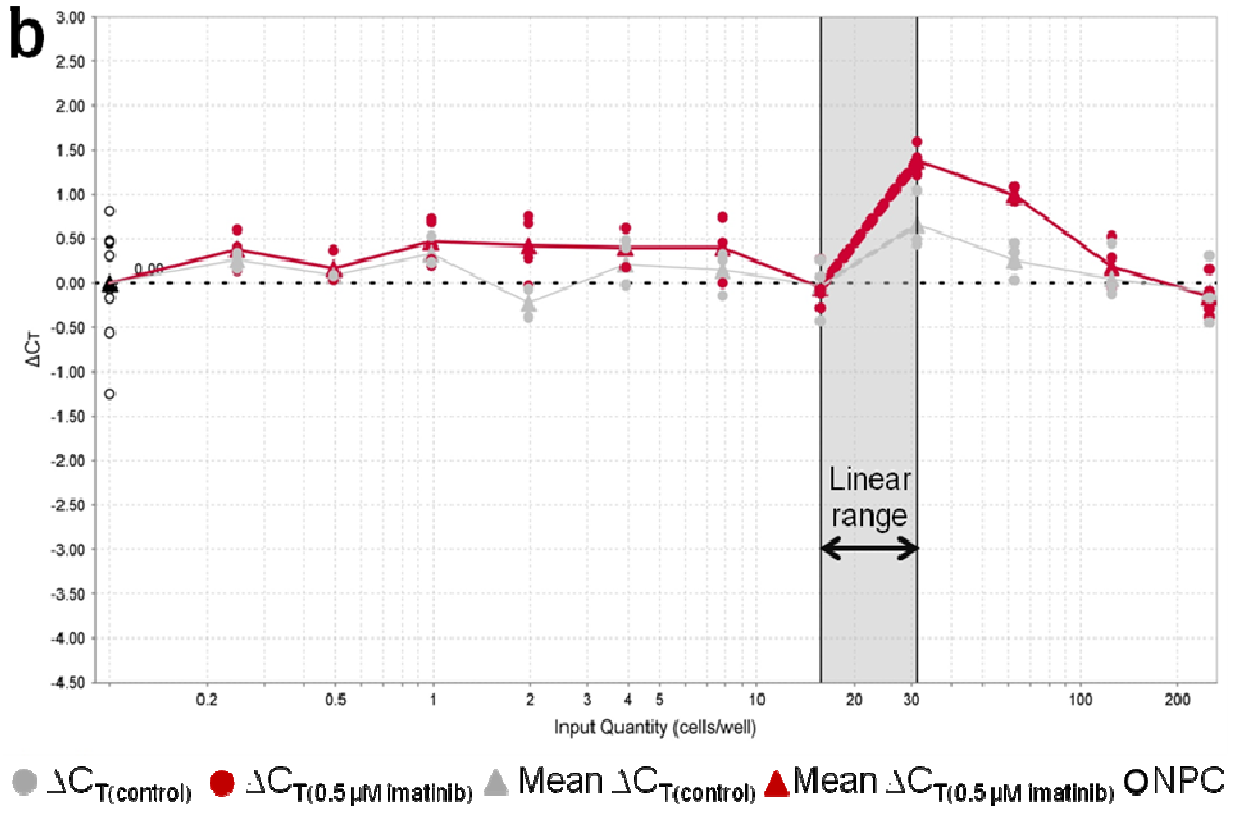
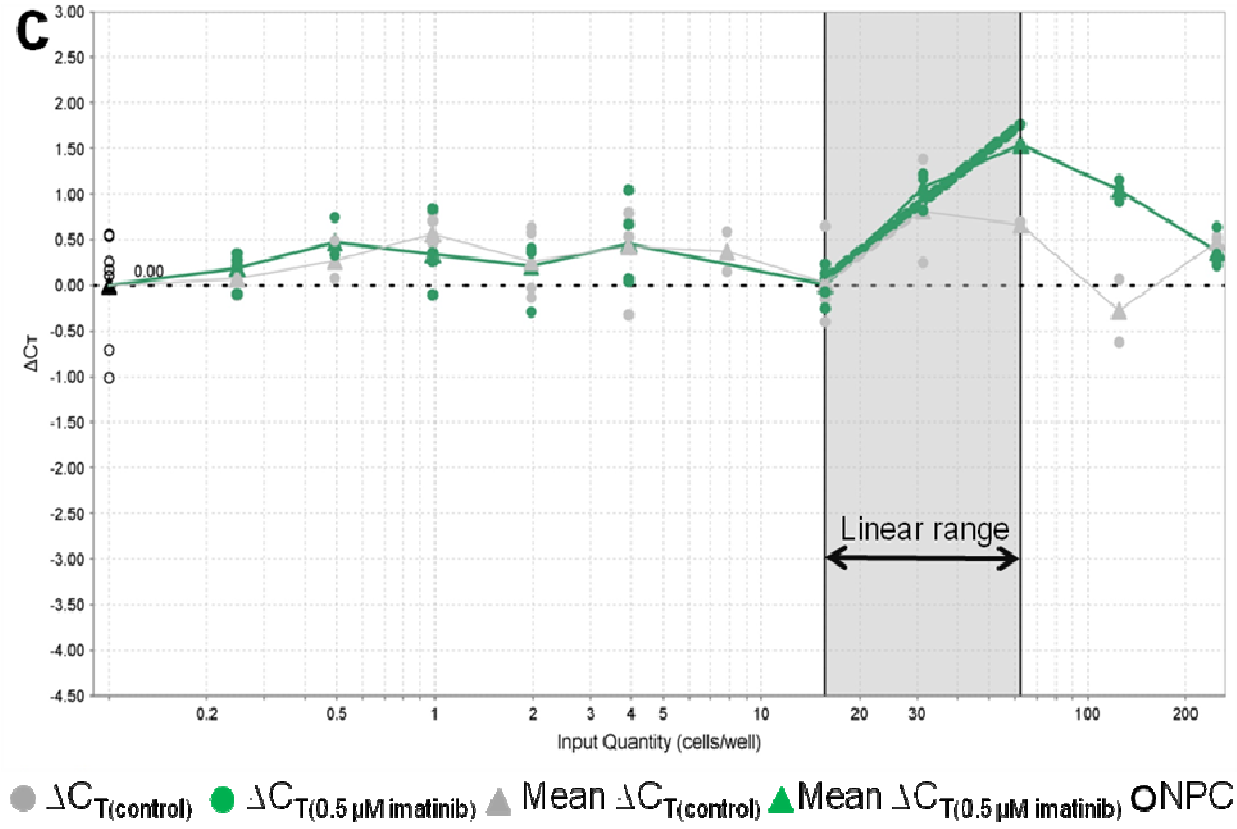
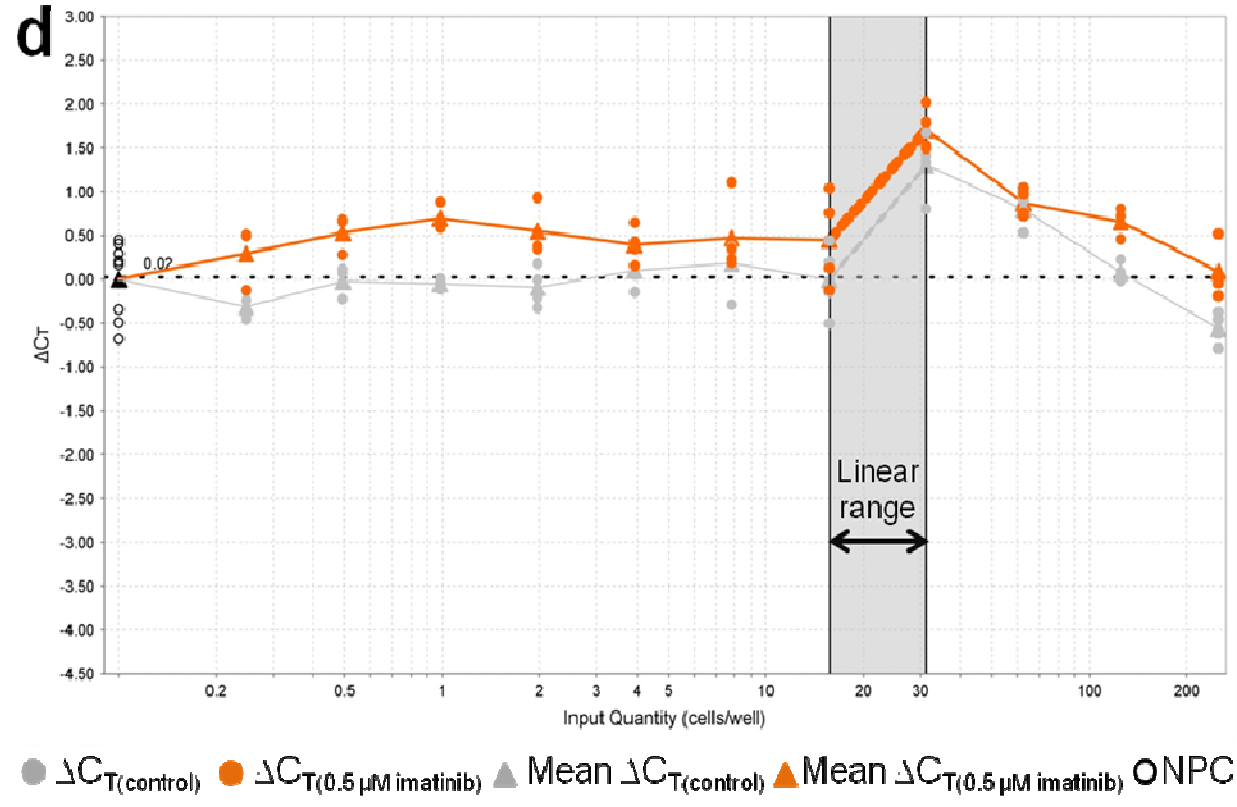


Figure 4.3. Real-time amplification plots as presented by the ABI 7500 SDS program for Taqman protein assays performed using different proximity probe pairs. Assays were performed using a) Abcam antibody, b) Santa Cruz antibody, c) Sigma antibody, d) combination of Abcam and Sigma antibodies, e) combination of Abcam and Santa Cruz antibodies and f) combination of Santa Cruz and Sigma antibodies. Amplification plots represented are for one set of dilutions in triplicate.


a
ΔCT
Linear range
Input Quantity (cells/well)
ΔCT(control) ΔCT(0.5 µM imatinib) Mean ΔCT(control) Mean ΔCT(0.5 µM imatinib) NPC


b
ΔCT
Linear range
Input Quantity (cells/well)
ΔCT(control) ΔCT(0.5 µM imatinib) Mean ΔCT(control) Mean ΔCT(0.5 µM imatinib) NPC


c
ΔCT
Input Quantity (cells/well)
Linear range
ΔCT(control)
ΔCT(0.5 µM imatinib)
Mean ΔCT(control)
Mean ΔCT(0.5 µM imatinib)
NPC


d
ΔCT
Input Quantity (cells/well)
Linear range
ΔCT(control)
ΔCT(0.5 µM imatinib)
Mean ΔCT(control)
Mean ΔCT(0.5 µM imatinib)
NPC

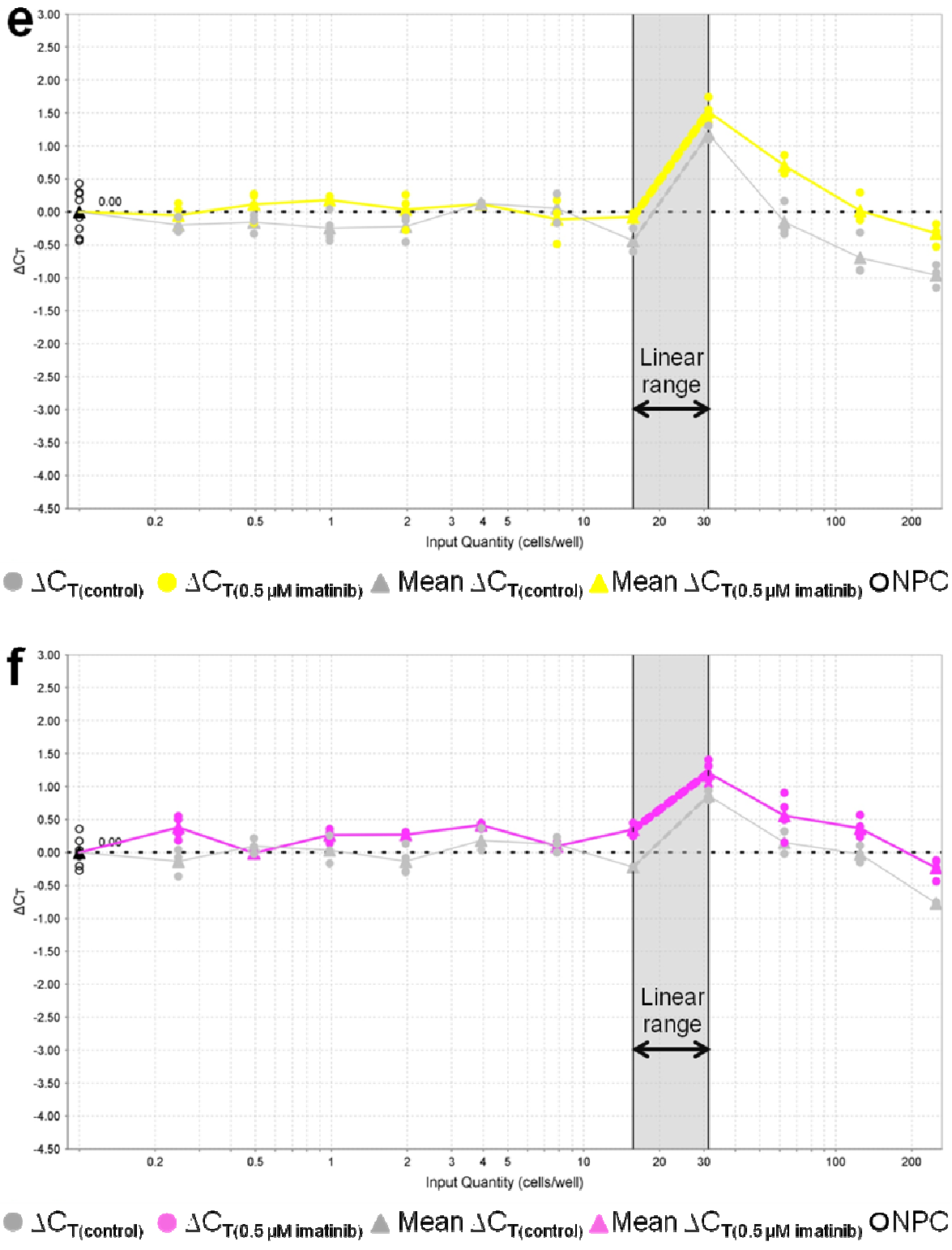


Figure 4.4. Screening proximity probe pairs with the Taqman protein assay. Assays were performed using a) Abcam antibody, b) Santa Cruz antibody, c) Sigma antibody, d) combination of Abcam and Sigma antibodies, e) combination of Abcam and Santa Cruz antibodies and f) combination of Santa Cruz and Sigma antibodies. Region shaded in grey represents linear range of assay.

4.4.5 Optimisation of the Taqman protein assay

Since the antibodies used in the assay were polyclonal and their epitope recognition sites unknown, it was uncertain exactly how many antibodies would bind redundantly to the target protein and not within the correct spatial distance to facilitate the formation of the antibody-oligo complex. Too much redundant antibody binding could result in the antibody becoming limiting. To investigate this potential problem, the assay was performed with 200 nM, 600 nM and 1000 nM of antibody. Although an increase in antibody concentration resulted in a greater ΔC_T, it did not increase the linear range of amplification (Figure 4.5a). A further consideration was that the amount of target protein could be limiting due to the low levels of *SLC22A1* expression based on the results obtained from *SLC22A1* expression analysis in chapter 3 (section 3.3). As a result of this, the concentration of cell lysate was increased from 500 cells/well to 60,000 cells/well which unfortunately resulted in a higher noise to signal ratio (Figure 4.5b). Finally, no improvement in the linear range of amplification was obtained even when the probe concentration was decreased, in order to reduce the noise to signal ratio, which included incubating the antibody with oligonucleotide (proximity probe) and cell lysate overnight at 4°C (Figure 4.5c) to maximise antibody binding to target protein, as recommended by the manufacturer (Applied Biosystems Real-time PCR systems Taqman protein assay chemistry guide).

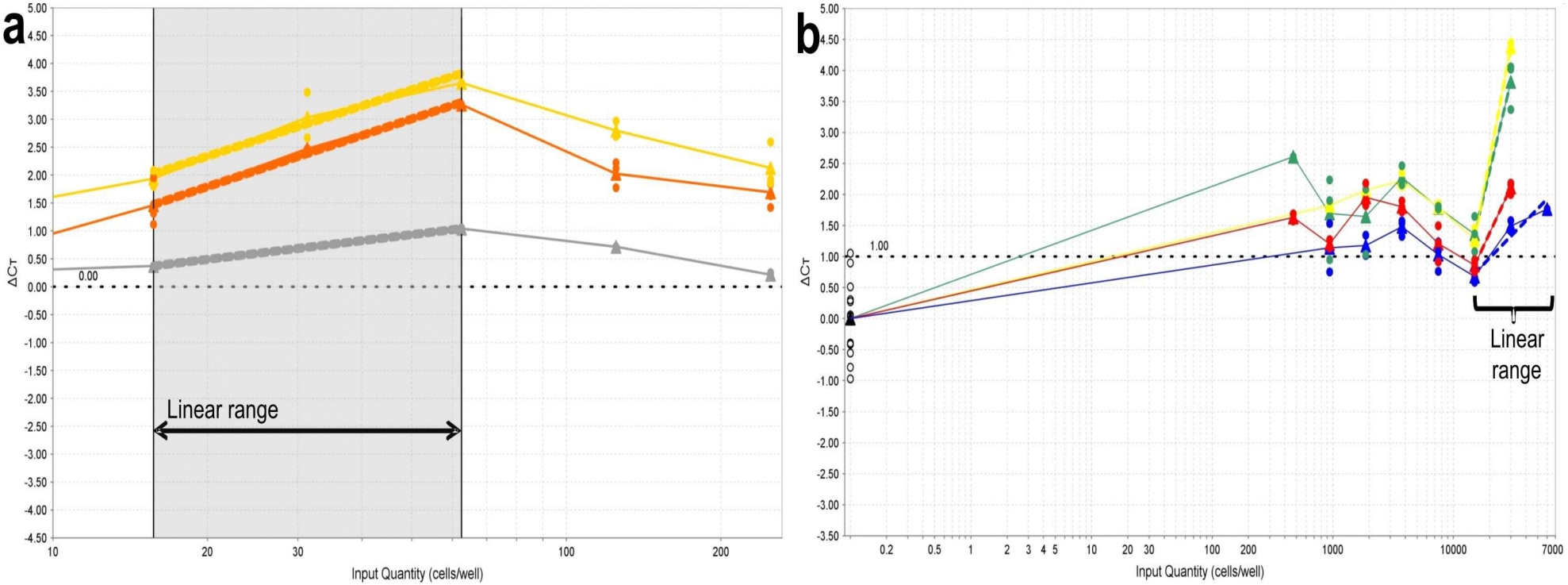


Figure 4.5. Optimisation of the Taqman protein assay. ΔCT plot obtained from ProteinAssist software showing Taqman protein assay comparing **a)** effect of increasing antibody concentrations of Sigma antibody on assay performance - Grey line represents ΔCT plot for the recommended 200 nM of antibody, Orange line represents ΔCT plot for 600 nM of antibody and yellow line represents ΔCT plot for 1000 nM of antibody; **b)** effect of increasing initial concentrations of cell lysates on assay performance using Sigma antibody - Blue and red lines represent ΔCT plot for starting quantity of 60,000 cells/well and 30,000 cells/well for control cell lysates, Yellow and green lines represent ΔCT plot for starting quantity of 60,000 cells/well and 30,000 cells/well for cell lysates of K562 cells treated with 0.5 µM imatinib for 24 hours.

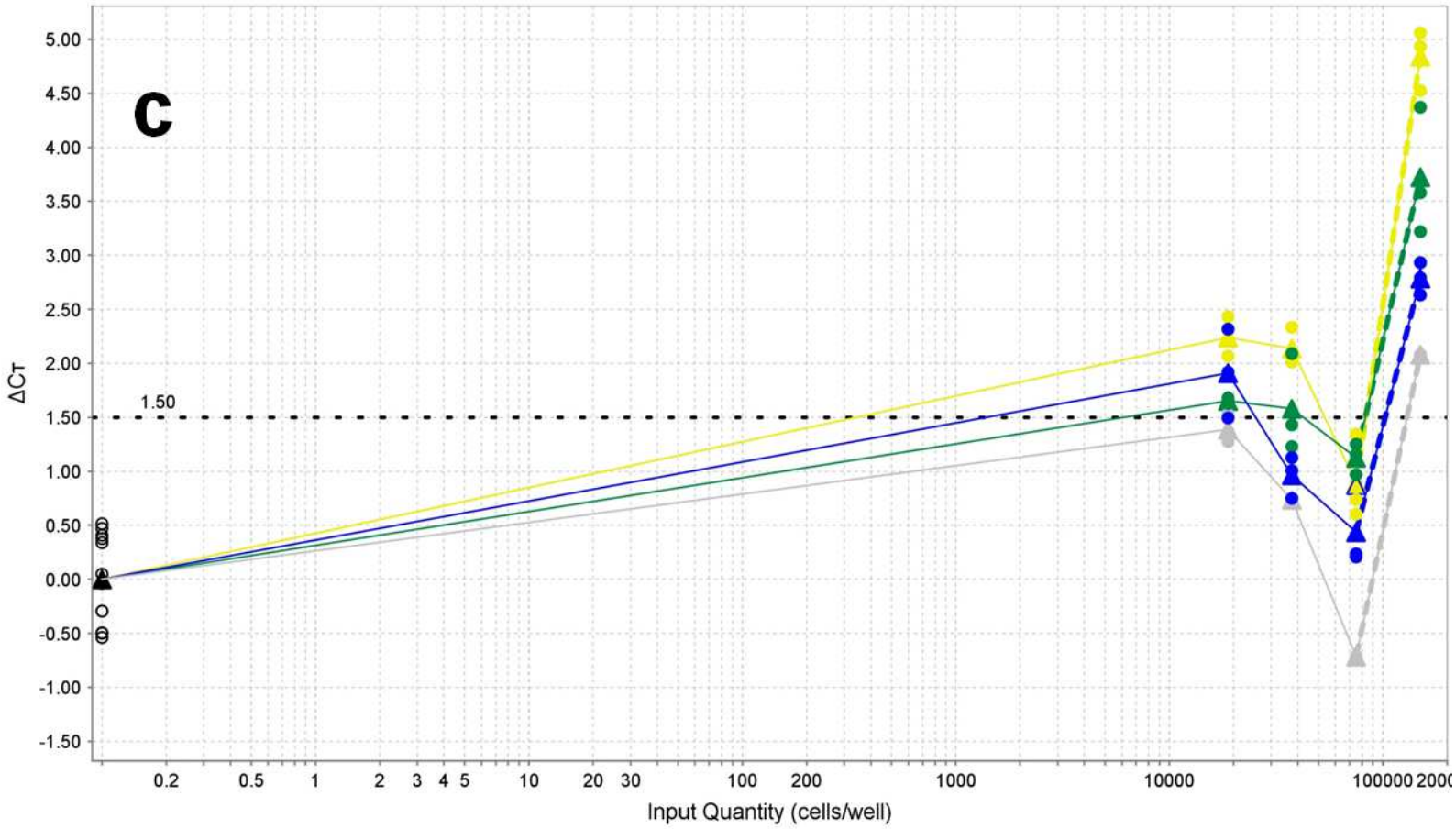


Figure 4.5c. Optimisation of the Taqman protein assay. ΔCT plot obtained from ProteinAssist software showing Taqman protein assay comparing the effect of increased starting concentration of cell lysates and decreased proximity probe concentration - Control cell lysates (grey), 0.5 µM imatinib treated cells (blue), 5 µM imatinib treated cells (green) and 10 µM imatinib treated cells (yellow).

4.5 Conclusion

Overall, the selected antibodies did not appear to be suited for the Taqman protein assay and it was not possible to quantify SLC22A1 protein in K562 cells. Although all three antibodies fulfilled the minimum criteria required for use in the Taqman protein assay and passed the forced proximity probe test, this does not guarantee that antibody epitope recognition will be optimal for the Taqman protein assay. The Taqman protein assay requires antibodies with high binding affinity. Unfortunately, polyclonal antibodies raised against peptide antigens tend to have lower binding affinitiy (personal communication with Dr. Swartzmann, one of the co-developers of the Taqman protein assay).

A key characteristic while choosing the optimal antibody pair for use in the Taqman protein assay is the distance between epitope regions that the respective antibodies recognise on the target protein. The epitopes on target protein should ideally be at a spatial distance of 21 nm (Applied Biosystems). However, this is an inherent antibody characteristic which cannot be optimised in the laboratory while performing the assay. Since none of the antibody epitopes have been mapped, it was not possible to determine their epitope recognition sites. Despite these challenges, the Taqman protein assay is a novel technology which, with suitable antibodies, allows the quantification of protein using real-time PCR. However, one of the major challenges currently, appears to be the availability of suitable antibodies for the target protein.

CHAPTER 5

CONCLUSION

5.1 Conclusion

The use of imatinib as first-line therapy for the treatment of chronic myeloid leukaemia (CML) has resulted in excellent response rates and prolonged survival in the majority of CML patients worldwide. Nevertheless, a few patients respond suboptimally to treatment and are at risk of disease progression. Decreased cellular uptake of imatinib into leukemic cells has been identified as the cause of suboptimal response (White *et al*., 2006). Since the uptake of imatinib into leukemic cells is facilitated by the influx transporter, SLC22A1, its activity is considered an important prognostic marker for treatment response.

White *et al.* (2007) suggested that *SLC22A1* mRNA is not a reliable prognostic marker for imatinib since the expression of *SLC22A1* may be affected by imatinib. Although several studies have speculated that imatinib may potentially influence *SLC22A1* expression, there is very little research to conclusively substantiate this hypothesis (Crossman *et al*., 2005; Engler *et al*., 2011; Gromicho *et al.*, 2013). The current study investigated the impact of varying doses of imatinib on *SLC22A1* gene expression at 24 hours, 48 hours and 72 hours. A striking observation was that imatinib influenced expression of *SLC22A1* in a non-linear dose-dependent manner, with the greatest increase observed at 0.5 µM, 5 µM and 10 µM imatinib.

The use of an *in vitro* platform in this study helped to elucidate the impact of imatinib on *SLC22A1* expression uniformly across all concentrations by eliminating factors that may contribute to differences in expression as a result of inherent

inter-patient variability. Studies have reported a heterogeneous expression of *SLC22A1* in CML patients on imatinib. In several studies it has been observed that some patients showed an increase in *SLC22A1* expression after being treated with imatinib (Crossman *et al*., 2005; Engler *et al*., 2011). Results from our study demonstrate that expression of *SLC22A1* is dependent on the concentration of imatinib. This may explain why some patients show an increase in *SLC22A1* expression which is dependent on the intracellular levels of imatinib achieved by each patient regardless of the administered dose.

Several papers have suggested that long term exposure to imatinib contributes to a change in *SLC22A1* expression. In contrast, the results from this study suggest that while expression of *SLC22A1* is dependent on imatinib concentration, no significant increase in expression was observed after 24 hours up to 72 hours. Thus, the differential expression of *SLC22A1* observed in CML patients is most likely as a result of plasma concentrations of imatinib achieved within the first 24 hours of therapy and not a cumulative effect of exposure time to imatinib.

This study also sought to determine the impact of imatinib on SLC22A1 protein to better understand the effect of levels of mRNA at the protein level. The Taqman protein assay was used to quantify levels of SLC22A1 protein in imatinib treated cells compared to control. One of the key determinants of the success of this assay is the antibodies against the target protein which must fulfil stringent criteria including binding to target protein within a spatial distance of 21 nm, antibodies

should display high affinity and specificity to target protein and the antibody solution must be free of amine buffers and carrier proteins. Only three commercially available SLC22A1 antibodies met the minimum criteria for use in this study. Despite various efforts focused at optimising the assay with regard to antibody concentration in assay, amount of target protein in assay and amount of proximity probe in reaction, it was not possible to successfully quantify levels of SLC22A1 protein.

In conclusion, the findings from this study suggest that imatinib does affect the expression of *SLC22A1*. This is the first study to demonstrate that there is a dose-dependent non-linear correlation between *SLC22A1* expression and imatinib concentration. Furthermore, it appears that the relationship between *SLC22A1* expression and imatinib is not time dependent after 24 hours. Although it would have been of added value to compare levels of *SLC22A1* mRNA to protein to better understand the molecular mechanisms underlying the regulation of SLC22A1, nevertheless, the implications of the results obtained from *SLC22A1* mRNA expression analysis in this study are of importance in the context of CML patients treated with imatinib. While this study does not invalidate the use of levels of *SLC22A1* mRNA as a prognostic marker for treatment response to imatinib, as suggested by White *et al.* (2010), the data obtained in this study demonstrated that only levels of *SLC22A1* determined in previously untreated and/or imatinib-naive CML patients can be used as a measure of SLC22A1 activity and treatment response.

SUMMARY

Chronic myeloid leukaemia (CML) is a haematopoietic stem cell disorder characterised by the *BCR-ABL* fusion gene. The *BCR-ABL* fusion gene encodes a constitutively active BCR-ABL tyrosine kinase, which is the driving force of the malignancy. Otherwise fatal, the use of imatinib mesylate has proved highly effective in the treatment of this disease in up to 85% of CML patients. However, approximately 25% of CML patients appear to respond suboptimally or experience treatment failure with imatinib. Suboptimal response in CML patients has been attributed to inadequate BCR-ABL kinase inhibition as a result of reduced intracellular accumulation of imatinib in target leukemic cells. The cellular influx of imatinib is mediated by the influx transport protein, SLC22A1. Therefore, its activity is considered a clinical determinant of imatinib uptake, and hence patients response to therapy.

A number of studies use levels of *SLC22A1* mRNA as a measure of SLC22A1 activity. It has been reported that cells over expressing levels of *SLC22A1* mRNA showed significantly increased uptake of imatinib, thus, suggesting that levels of *SLC22A1* mRNA can be used as a measure of SLC22A1 activity. However, there is a concern that imatinib may affect *SLC22A1* expression. This consideration, however, is based on two studies involving a limited patient cohort and although widely accepted, has not been proven conclusively. Should it be proven that imatinib does influence *SLC22A1* expression, levels of *SLC22A1* mRNA may not

be a reliable indicator of SLC22A1 activity. It is therefore important to understand the effect of treatment with imatinib on *SLC22A1* gene expression.

The data from this study demonstrated that imatinib induces expression of *SLC22A1* mRNA in a non-linear dose dependent manner. It was also observed that expression of *SLC22A1* was not dependent on time of exposure to imatinib. These results explain the differential expression of *SLC22A1* mRNA reported in CML patients on a standard dose of 400 mg/day of imatinib. The trough plasma levels of imatinib achieved between patients after 24 hours of exposure to the same dose of imatinib may vary owing to inter individual differences. Since *SLC22A1* expression is dependent on plasma levels of imatinib, therefore, patients administered the same dose of imatinib may show differential expression of *SLC22A1*. These findings suggest that imatinib does affect *SLC22A1* mRNA expression and that the change in *SLC22A1* expression observed at any particular time is dependent on the intracellular levels of imatinib achieved in CML patients within 24 hours of exposure to the drug.

One of the challenges in this study was the availability of suitably qualified SLC22A1 antibodies for use in the Taqman protein assay to quantify SLC22A1 protein. Antibodies used in the Taqman assay have to fulfil specific criteria and out of 55 commercially available antibodies, only three SLC22A1 antibodies met the minimum requirements for use in the assay. However, despite various efforts focused at optimising the assay, the range of the assay was very limited and hence it was not possible to quantify SLC22A1 protein. We hypothesize that one

of the reasons for assay failure could be as a result of antibodies not binding to the target protein at the required spatial distance to facilitate amplification by real-time PCR. Since the antibodies used in the assay have not been epitope mapped, it is uncertain whether they fulfil this requirement. Future research will be aimed at antibody production for manufacturing SLC22A1 antibodies suitable for use in the Taqman protein assay to enable successful quantification of SLC22A1 protein.

In conclusion, this is the first study which specifically aimed to investigate the influence of imatinib on *SLC22A1* expression. This is also the first study to demonstrate that expression of *SLC22A1* is not time dependent, but follows a non-linear correlation to imatinib concentration. Although it would have been useful to investigate the effect of increasing levels of *SLC22A1* mRNA on intracellular uptake of imatinib in K562 cells, unfortunately, the latter technique requires the use of radio-labelled imatinib and specialized equipment which made it a limiting factor for use in this study. While this study does not invalidate the use of levels of *SLC22A1* mRNA as a prognostic marker for treatment outcome, these findings suggest that levels of *SLC22A1* mRNA as a measure of SLC22A1 activity is only applicable to newly diagnosed imatinib naive or previously untreated CML patients.

KEY WORDS

Chronic myeloid leukaemia, BCR-ABL, imatinib, SLC22A1, gene expression, Taqman protein assay.

OPSOMMING

Chroniese meyloid leukemie (CML) is ’n hematopoïetiese stamsel siekte wat gekenmerk word deur die *BCR-ABL* fusie onkogeen. Hierdie *BCR-ABL* geen kodeer vir ’n aktiewe tiroksienkinase wat die maligniteit veroorsaak. Die gebruik van imatinib is doeltreffend in ongeveer 85% van CML pasiënte, waarsonder CML ‘n dodelike siekte is. Tog blyk dit dat 25% van CML pasiënte nie optimaal teenoor behandeling reageer nie. Sub-optimale response in CML pasiënte is toegeskryf aan ondoeltreffende inhibisie van BCR-ABL-kinase as gevolg van verlaagde intrasellulêre vlakke van imatinib in teiken leukemie selle. Die opname van imatinib binne die sel word bepaal deur die vervoer proteïen, SLC22A1. Die aktiwiteit van hierdie proteïen word dus gebruik as ’n maatstaf van hoe pasiënte gaan reageer teenoor behandeling met imatinib.

Verskeie studies beskryf die gebruik van die vlak van *SLC22A1* boodskapper-ribo-nukleïensuur (bRNS) as ’n aanduiding van SLC22A1 aktiwiteit. Daar is gevind dat selle wat *SLC22A1* in oormaat uitdruk verhoogde opname van imatinib ondergaan wat die veronderstelling steun dat *SLC22A1* boodskapper-ribo-nukleïensuur as ’n aanduiding van SLC22A1 aktiwiteit beskou kan word. Tog is daar aanduidings dat imatinib die geenuitdrukking van *SLC22A1* mag beïnvloed. Hierdie oorweging is net op twee studies met ‘n beperkte aantal pasiënte gebaseer, en alhoewel dit algemeen aanvaar word, is dit nie onweerlegbaar bewys nie. Sou dit wel bewys word dat imatinib die geenuitdrukking van *SLC22A1* beïnvloed, mag dit ook beteken dat vlakke van *SLC22A1* boodskapper-ribo-nukleïensuur nie as ‘n

aanduiding van SLC22A1 aktiwiteit kan dien nie. As gevolg hiervan is dit dus belangrik om die invloed van imatinib op *SLC22A1* geenuitdrukking te verstaan.

Die resultate van hierdie studie het gewys dat *SLC22A1* geenuitdrukking wel deur imatinib beïnvloed word deur ’n ongelinieerde verwantskap. Dit is ook gevind dat die invloed van imatinib op *SLC22A1* geenuitdrukking nie van die tyd van blootstelling afhanklik is nie. Hierdie resultate verduidelik die differensiële vlakke van *SLC22A1* aangedui in pasiënte uit verskillende studies. Dus mag die plasmavlakke van imatinib van pasiënte op dieselfde dosering wissel as gevolg van verskille tussen individue. Gevolglik toon pasiënte op dieselfde dosering van imatinib verskillende vlakke van *SLC22A1* geenuitdrukking . Dus word die voorstelling gemaak dat imatinib die wel die geenuitdrukking van *SLC22A1* beïnvloed en dat dit na 24 uur nie afhanklik is van tydsduur van blootstelling nie.

Een van die uitdagings in hierdie studie is die beskikbaarheid van bruikbare antiliggame vir gebruik in die “Taqman” proteïen-toets. Die antiliggame wat gebruik word moet voldoen aan spesifieke vereistes en uit die 55 wat beskikbaar is, het slegs drie aan die nodige vereistes voldoen. Alhoewel daar sorg geneem is om die toets te optimaliseer, was dit steeds nie moontlik om die hoeveelheid SLC22A1 proteïen te bepaal nie. Ons vermoed dat die rede hiervoor die onvermoë van die antiliggame is om binne die vereiste afstand op die teiken proteïen te bind. Omdat die antiliggame wat in hierdie toets gebruik is nog nie teenoor die epitoop gekarteer is nie, was dit onmoontlik om bindings inligting te

bekom. Toekomstige navorsing moet gemik word op die ontwikkeling van toepaslike antiliggame vir gebruik in die “Taqman”-proteïen-toets.

Ten slote, hierdie is die eerste studie wat probeer het om die invloed van imatinib op *SLC22A1* geenuitdrukking te bestudeer. Dit is ook die eerste studie wat bewys dat die invloed van imatinib op *SLC22A1* geenuitdrukking nie verbonde is aan tyd van blootstelling deur ’n ongelinieer verwantskap nie. Dit sou toepaslik gewees het om die invloed van *SLC22A1* bRNS vlakke op die opname van imatinib te bestudeer, maar die metode daarvoor was egter nie beskikbaar vir hierdie studie nie. Alhoewel hierdie studie nie die gebruik van *SLC22A1* bRNS as prognostiese merker in CML pasiënte uitsluit nie, is hierdie bevindings wel aanduidend dat dit toepaslik is as ‘n maatstaf van SLC22A1 aktiwiteit slegs op pasiënte wat nie voorheen met imatinib behandel is nie.

KERNWOORDE

CML, BCR-ABL, imatinib, SLC22A1, geenuitdrukking, “Taqman”-proteïen-toets.

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APPENDIX A (CHAPTER 2)

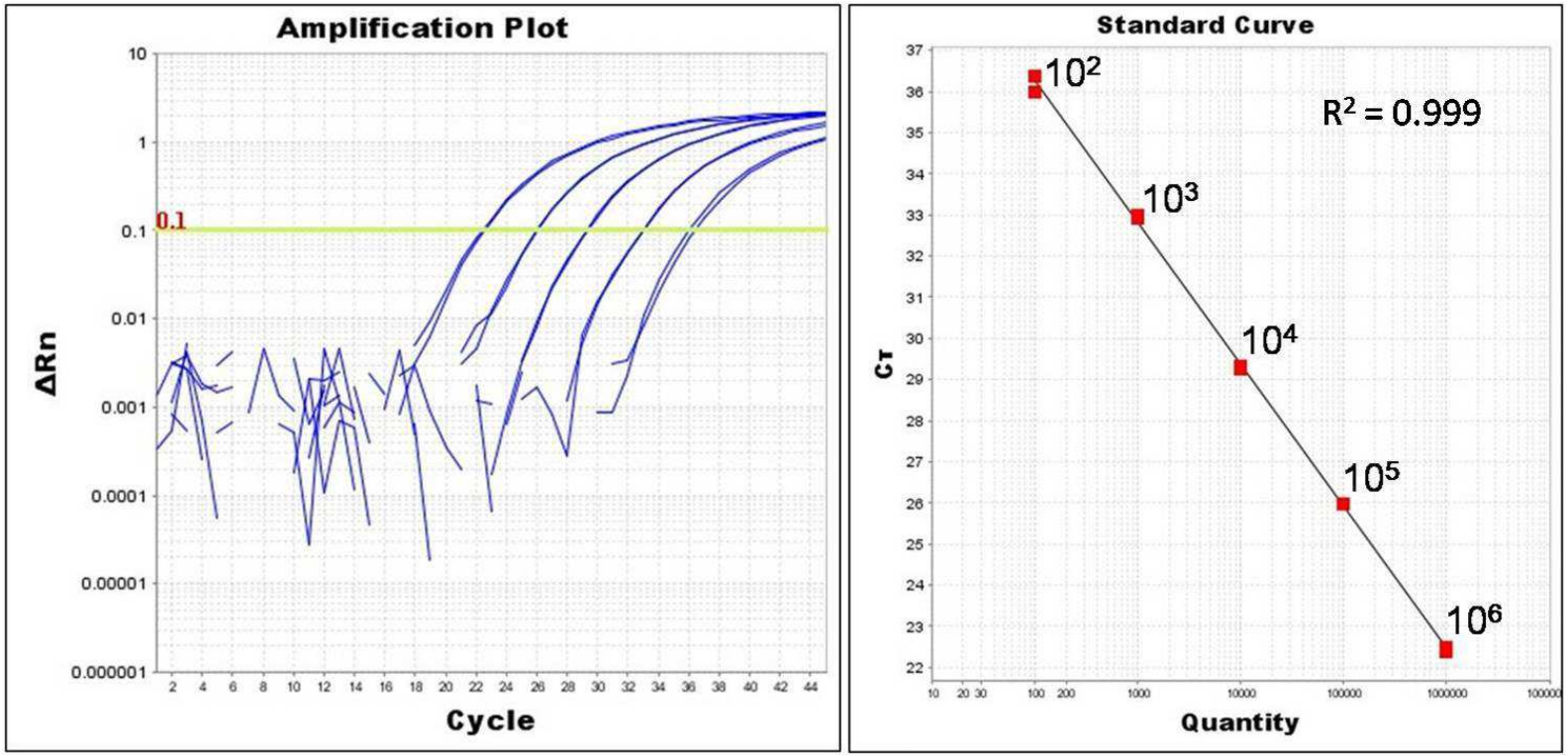


Figure: Example of real-time PCR amplification plot and respective standard curve obtained for *SLC22A1* using serial dilutions of ultramer (10^2, 10^3, 10^4, 10^5, 10^6 copies). The C_T values obtained from the amplification plots for *SLC22A1* were used to plot the standard curve.

SLC22A1	Day 0		
	V	P	I
STD 1 (10^2)	39.38	37.49	37.94
STD 2 (10^3)	35.02	34.75	33.48
STD 3 (10^4)	31.02	30.64	30.36
STD 4 (10^5)	27.52	27.21	26.82
STD 5 (10^6)	23.82	23.81	23.41

SLC22A1	DAY 1						DAY 7					
	4ºC			-20ºC			4ºC			-20ºC		
	V	P	I	V	P	I	V	P	I	V	P	I
STD 1 (10^2)	38.17	38.25	37.00	37.24	38.24	36.9	38.22	39.56	38.04	37.65	38.36	37.48
STD 2 (10^3)	34.66	34.22	33.83	34.8	34.14	33.87	34.97	34.41	33.88	35.34	34.48	33.6
STD 3 (10^4)	31.12	30.68	30.22	31.05	30.91	30.44	31.01	30.74	30.32	31.55	30.84	30.3
STD 4 (10^5)	27.64	27.15	26.74	27.65	27.46	27.09	27.74	27.39	26.89	28.3	27.64	27.47
STD 5 (10^6)	23.87	23.88	23.47	24.17	24.18	23.73	24.04	24.12	23.63	24.67	24.13	24.88

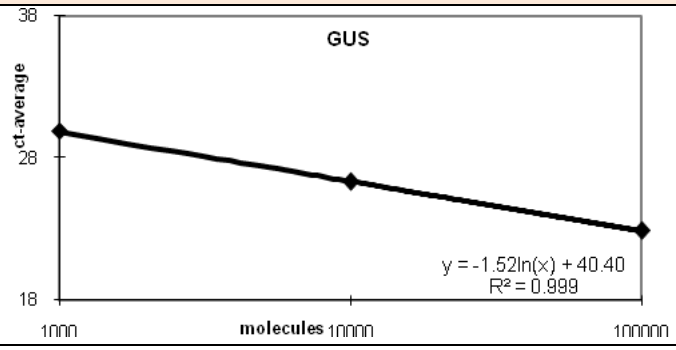
SLC22A1	DAY 15						DAY 30					
	4ºC			-20ºC			4ºC			-20ºC		
	V	P	I	V	P	I	V	P	I	V	P	I
STD 1 (10^2)	38.15	38.67	36.76	36.56	35.46	36.21	38.84	38.07	37.52	36.11	36.06	36.5
STD 2 (10^3)	34.55	34.43	33.3	29.94	32.57	32.38	34.49	34.25	33.63	30.5	34.2	33.69
STD 3 (10^4)	30.9	30.58	30.35	29.29	29.22	29.35	30.95	30.54	30.18	28.89	29.44	31.16
STD 4 (10^5)	27.43	27.22	26.71	26.39	25.94	26.37	27.53	27.29	26.77	26.31	26.46	26.65
STD 5 (10^6)	23.83	23.85	23.23	23.01	22.68	22.88	24.06	24.1	23.57	22.82	24.4	22.84

Table: C_T values of serial dilutions of ultramer copy number standards stored using different mixing methods, at 4ºC and -20ºC, respectively, for up to 30 days.

APPENDIX B (CHAPTER 3)

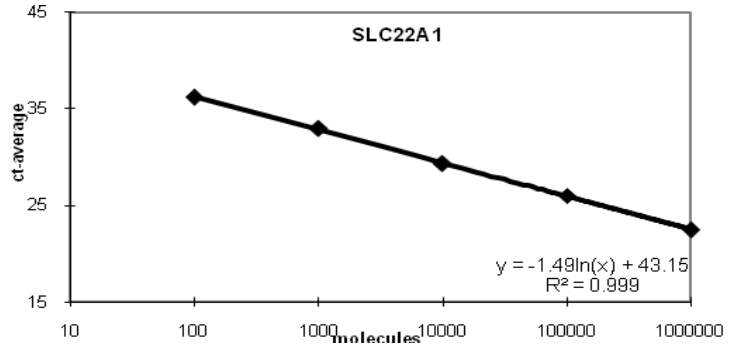
Sample	Replica	C_T	Copy number	C_T-average
GUS - standard 1	a)	30.0	1000	29.92
	b)	29.9		
GUS - standard 2	a)	26.4	10000	26.32
	b)	26.3		
GUS - standard 3	a)	22.8	100000	22.90
	b)	23.0		
SLC22A1 - standard 1	a)	36.4	100	36.19
	b)	36.0		
SLC22A1 - standard 2	a)	32.9	1000	32.94
	b)	33.0		
SLC22A1 - standard 3	a)	29.2	10000	29.29
	b)	29.3		
SLC22A1 - standard 4	a)	26.0	100000	25.98
	b)	26.0		
SLC22A1 - standard 5	a)	22.4	1000000	22.43
	b)	22.5		

Standard curve GUS



slope	-1.523
	-3.506
intersect	40.404
efficiency	92.8%
R^2	0.9998
threshold	0.1

Standard curve SLC22A1



slope	-1.497
	-3.448
intersect	43.158
efficiency	95.0%
R^2	0.9997
threshold	0.1

Sample	Replica	C_T	Copy number	% *SLC22A1*	Mean *SLC22A1*	Standard deviation
24H C	GUS a)	18.2	2114638	1.75%	**1.57%**	0.26%
24H C	GUS b)	18.0	2512341	1.39%		
24H C	SLC22A1 a)	27.5	37066			
24H C	SLC22A1 b)	27.6	34909			
24H 0.1	GUS a)	18.9	1392882	2.44%	**2.22%**	0.31%
24H 0.1	GUS b)	18.7	1575521	2.00%		
24H 0.1	SLC22A1 a)	27.6	34027			
24H 0.1	SLC22A1 b)	27.7	31494			
24H 0.2	GUS a)	18.1	2349332	2.86%	**3.02%**	0.22%
24H 0.2	GUS b)	18.1	2265346	3.18%		
24H 0.2	SLC22A1 a)	26.5	67273			
24H 0.2	SLC22A1 b)	26.4	71955			
24H 0.5	GUS a)	19.6	864160	4.37%	**3.95%**	0.60%
24H 0.5	GUS b)	19.3	1033905	3.52%		
24H 0.5	SLC22A1 a)	27.4	37767			
24H 0.5	SLC22A1 b)	27.5	36439			
24H 1.0	GUS a)	18.6	1643441	1.77%	**1.69%**	0.12%
24H 1.0	GUS b)	18.6	1689001	1.61%		
24H 1.0	SLC22A1 a)	27.9	29079			
24H 1.0	SLC22A1 b)	28.0	27125			

Sample	Replica	C_T	Copy number	% *SLC22A1*	Mean *SLC22A1*	Standard deviation
24H 2.0	GUS a)	19.2	1128108	2.12%	**2.08%**	0.06%
24H 2.0	GUS b)	19.1	1200213	2.05%		
24H 2.0	SLC22A1 a)	28.2	23957			
24H 2.0	SLC22A1 b)	28.1	24549			
24H 5.0	GUS a)	19.4	952542	4.58%	**4.38%**	0.28%
24H 5.0	GUS b)	19.2	1107139	4.19%		
24H 5.0	SLC22A1 a)	27.2	43597			
24H 5.0	SLC22A1 b)	27.1	46365			
24H 10.0	GUS a)	19.5	941072	14.16%	**12.54%**	2.30%
24H 10.0	GUS b)	19.3	1031497	10.91%		
24H 10.0	SLC22A1 a)	25.5	133256			
24H 10.0	SLC22A1 b)	25.7	112578			
48H C	GUS a)	18.1	2340028	0.50%	**0.53%**	0.04%
48H C	GUS b)	18.0	2529553	0.55%		
48H C	SLC22A1 a)	29.3	11756			
48H C	SLC22A1 b)	29.0	14019			
48H 0.1	GUS a)	18.7	1534044	1.30%	**1.62%**	0.49%
48H 0.1	GUS b)	18.8	1488243	1.28%		
48H 0.1	SLC22A1 a)	28.4	19952			
48H 0.1	SLC22A1 b)	28.5	19009			
48H 0.2	GUS a)	19.6	838322	1.96%	**1.29%**	0.02%
48H 0.2	GUS b)	19.1	1200482	1.27%		
48H 0.2	SLC22A1 a)	28.7	16441			
48H 0.2	SLC22A1 b)	28.9	15274			
48H 0.5	GUS a)	19.7	793982	2.21%	**2.54%**	0.46%
48H 0.5	GUS b)	19.9	720855	2.87%		
48H 0.5	SLC22A1 a)	28.6	17578			
48H 0.5	SLC22A1 b)	28.4	20673			
48H 1.0	GUS a)	19.6	853022	2.70%	**2.08%**	0.88%
48H 1.0	GUS b)	19.0	1306116	1.46%		
48H 1.0	SLC22A1 a)	28.2	23015			
48H 1.0	SLC22A1 b)	28.5	19012			

Sample	Replica	C_T	Copy number	% *SLC22A1*	Mean *SLC22A1*	Standard deviation
48H 2.0	GUS a)	18.8	1488427	0.92%	**0.90%**	0.02%
48H 2.0	GUS b)	18.7	1532874	0.89%		
48H 2.0	SLC22A1 a)	29.0	13689			
48H 2.0	SLC22A1 b)	29.0	13616			
48H 5.0	GUS a)	20.5	477662	5.49%	**5.64%**	0.21%
48H 5.0	GUS b)	20.4	491277	5.78%		
48H 5.0	SLC22A1 a)	28.0	26239			
48H 5.0	SLC22A1 b)	27.9	28417			
48H 10.0	GUS a)	19.9	707351	12.11%	**11.74%**	0.51%
48H 10.0	GUS b)	19.7	787341	11.38%		
48H 10.0	SLC22A1 a)	26.2	85631			
48H 10.0	SLC22A1 b)	26.1	89596			
72H C	GUS a)	17.1	4393513	0.20%	**0.21%**	0.01%
72H C	GUS b)	17.4	3579621	0.22%		
72H C	SLC22A1 a)	29.7	8906			
72H C	SLC22A1 b)	29.9	7971			
72H 0.1	GUS a)	18.6	1695844	0.70%	**0.61%**	0.12%
72H 0.1	GUS b)	18.5	1783073	0.52%		
72H 0.1	SLC22A1 a)	29.3	11789			
72H 0.1	SLC22A1 b)	29.6	9297			
72H 0.2	GUS a)	19.2	1089779	1.68%	**1.68%**	0.00%
72H 0.2	GUS b)	19.1	1152064	1.68%		
72H 0.2	SLC22A1 a)	28.6	18330			
72H 0.2	SLC22A1 b)	28.5	19339			
72H 0.5	GUS a)	21.3	303464	4.41%	**3.83%**	0.82%
72H 0.5	GUS b)	20.9	404674	3.25%		
72H 0.5	SLC22A1 a)	28.9	13376			
72H 0.5	SLC22A1 b)	29.0	13150			
72H 1.0	GUS a)	20.7	454643	2.75%	**2.82%**	0.09%
72H 1.0	GUS b)	20.6	473456	2.88%		
72H 1.0	SLC22A1 a)	29.0	12511			
72H 1.0	SLC22A1 b)	28.9	13628			

Sample	Replica	C_T	Copy number	% *SLC22A1*	Mean *SLC22A1*	Standard deviation
72H 2.0	GUS a)	19.9	775965	0.73%	**0.80%**	0.10%
72H 2.0	GUS b)	19.9	764625	0.88%		
72H 2.0	SLC22A1 a)	30.2	5664			
72H 2.0	SLC22A1 b)	30.0	6694			
72H 5.0	GUS a)	20.1	651932	2.59%	**2.42%**	0.24%
72H 5.0	GUS b)	20.1	665593	2.25%		
72H 5.0	SLC22A1 a)	28.6	16891			
72H 5.0	SLC22A1 b)	28.8	14956			
72H 10.0	GUS a)	23.5	70716	14.31%	**13.15%**	1.63%
72H 10.0	GUS b)	23.2	85290	12.00%		
72H 10.0	SLC22A1 a)	29.3	10118			
72H 10.0	SLC22A1 b)	29.3	10234			

NTC GUS	Replica 1	Undetermined
	Replica 2	Undetermined
NTC SLC22A1	Replica 1	Undetermined
	Replica 2	Undetermined

Table: *SLC22A1* gene expression analysis sheet: 24H – 24 hours; 48H – 48 hours; 72 H – 72 hours; C - control cells, NTC – No template control.

APPENDIX C (CHAPTER 4)

Experiment 1						
[Imatinib]	**Absorbance at 570 nm**			**Absorbance at 600 nm**		
0 µM	0.552	0.528	0.538	0.423	0.399	0.435
0.1 µM	0.534	0.533	0.531	0.466	0.475	0.471
0.2 µM	0.490	0.477	0.435	0.452	0.446	0.410
0.5 µM	0.482	0.386	0.521	0.459	0.460	0.518
1 µM	0.405	0.439	0.471	0.466	0.475	0.471
2 µM	0.354	0.350	0.410	0.356	0.396	0.408
5 µM	0.234	0.219	0.277	0.254	0.272	0.249
10 µM	0.227	0.226	0.239	0.240	0.239	0.253
Experiment 2						
[Imatinib]	**Absorbance at 570 nm**			**Absorbance at 600 nm**		
0 µM	0.432	0.495	0.488	0.595	0.692	0.541
0.1 µM	0.921	0.990	0.971	0.564	0.557	0.563
0.2 µM	0.981	0.945	0.952	0.593	0.613	0.598
0.5 µM	0.721	0.730	0.692	0.527	0.505	0.511
1 µM	0.640	0.633	0.597	0.489	0.517	0.48
2 µM	0.599	0.536	0.540	0.409	0.422	0.461
5 µM	0.504	0.459	0.487	0.371	0.365	0.321
10 µM	0.487	0.459	0.471	0.363	0.321	0.362

Table: Cytotoxicty assay: Absorbance values of Alamar Blue obtained at 570 nm and 600 nm for two independent experiments performed in triplicate to determine cell viability in imatinib-treated cells compared to control.

Imatinib	Experiment 1		
	Numerator value	Denominator value	% reduction compared to control
0.1 µM	24507.91	30540.75	80.25
0.2 µM	19643.45	30540.75	64.32
0.5 µM	15670.31	30540.75	51.31
1 µM	14256.37	30540.75	46.68
2 µM	12366.29	30540.75	40.49
5 µM	7704.51	30540.75	25.23
10 µM	7374.88	30540.75	24.15
Experiment 2			
0.1 µM	67386.01	87948.33	76.62
0.2 µM	63850.48	87948.33	72.60
0.5 µM	42223.99	87948.33	48.01
1 µM	33130.13	87948.33	37.67
2 µM	30984.2	87948.33	35.23
5 µM	28486.46	87948.33	32.39
10 µM	27272.78	87948.33	31.01

Figure: Cytotoxicty assay: Calculated percentage reduction of Alamar Blue in imatinib treated K562 cells compared to the control.

Catalog number	Reason for unsuitability in TaqMan assay
Abcam	
2C5-ab107246	Provided as an ascitic fluid - Not purified
ab118539	Antibody against isoform b of SLC22A1
ab123128	Contains 0.09% Sodium azide preservative
ab55916	Not affinity purified - Protein A column purified
Abgent	
AP13944b	Contains 0.09% Sodium azide preservative
Abnova	
MAB10321	Provided as an ascitic fluid - Not purified
PAB15968	Raised against very short peptide sequence
PAB22359	Antibody concentration is too low
Acris Antibodies GmBH	
AM06232SU-N	Recognizes Syk (72 kDa) NOT SLC22A1
AM06553SU-N	Provided as an ascitic fluid - Not purified
Antibodies-online.com	
ABIN203396	Not affinity purified - Protein A purified
ABIN203397	Not affinity purified - Protein A purified and contains KLH-carrier protein
ABIN203397	Protein A column and antibody raised against isoform b of SLC22A1
ABIN310169	Not affinity purified - Protein A purified
ABIN310188	Protein A column and antibody raised against isoform b of SLC22A1
ABIN630301	Not affinity purified - Protein A purified and contains KLH-carrier protein
ABIN630309	Not affinity purified - Protein A purified
ABIN657865	Contains 0.09% sodium azide and KLH-carrier protein

ABIN865491	Provided as an ascitic fluid - Not purified
ABIN957574	DISCONTINUED
ABIN969400	Provided as an ascitic fluid - Not purified
Aviva Systems Biology	
ARP41516_T100	Not affinity purified - Protein A purified
ARP41640_T100	Antibody against isoform b of SLC22A1
Biorbyt	
orb121416	Raised against very short peptide sequence
orb32029	Antibody concentration is too low
Creative Biomart	
CABT-27704MH	Wrong protein description
CPBT-Y35700RH	Antibody concentration is too low
CPBT-46330RH	Antibody against isoform b of SLC22A1
CPBT-57719RH	Antibody against isoform b of SLC22A1
DPABT-H13181	Antibody concentration is too low,0.02% sodium azide
DPABT-H14761	Antibody concentration is too low, 0.02% sodium azide
Genway biotech	
18-003-43706	Antibody against isoform b of SLC22A1
LifeSpan Biosciences	
LS-C108732	Recognizes Syk protein and NOT SLC22A1
LS-C144187	Not affinity purified - Protein A purified
LS-C31870	Not affinity purified - Protein A purified
LS-C31871	Protein A column and antibody raised against isoform b of SLC22A1
MyBioSource	
MBS850022	Product description is about Syk kinase but NCBI accession number is of SLC22A1.

MBS850496	Provided as an ascitic fluid - Not purified
NovateinBio	
AB-A102681	Provided as an ascitic fluid - Not purified
Novus Biologicals	
NBP1-51684	Provided as an ascitic fluid - Not purified
NBP1-54502	The uniprot ID (P14859) provided in product description is that of POU2F1 (OCT-1) protein
NBP1-59419	Antibody raised against isoform b of SLC22A1
NBP1-59464	Not available as affinity purified
NBP1-89418	Antibody concentration is too low; 0.02& Sodium azide
ProMab	
30140	Provided as an ascitic fluid - Not purified
R&D Systems	
MAB6469	Not affinity purified - Protein A/G column purified
Santa Cruz Biotechnology	
sc-133866	Not affinity purified - Protein A column purified
Sigma-Aldrich	
AV41516-100UG	Antibody raised against isoform b of SLC22A1
AV41640-100UG	Antibody raised against isoform b of SLC22A1
Thermo scientific pierce antibodies	
MA5-15406	Provided as an ascitic fluid - Not purified
MA5-15730	Provided as an ascitic fluid - Not purified
USBIO	
S5329-36D	Not affinity purified - Protein A column purified

Taqman protein assay: Commercially available SLC22A1 not suitable for use in the Taqman protein assay (section 4.3.2).

MPTVDDILEQ VGESGWFQKQ AFLILCLLSA AFAPICVGIV FLGFTPDHHC QSPGVAELSQ RCGWSPAEEL NYTVPGLGPA GEAFLGQCRR YEV**DWNQSAL SCVDPLASL**A TNRSHLPLGP CQDGWVYDTP GSSIVTEFNL VCADSWKLDL FQSCLNAGFL FGSLGVGYFA DRFGRKLCLL GTVLVNAVSG VLMAFSPNYM SMLLFRLLQG LVSKGNWMAG YTLITEFVGS GSRRTVAIMY QMAFTVGLVA LTGLAYALPH WRWLQLAVSL PTFLFLLYYW CVPES**PRWLL SQKRNTEAIK IMDHIAQKNG KLPPADLKML SLEEDVTEKL SPSFADLFRT PRLRKRTFIL** MYLWFTDSVL YQGLILHMGA TSGNLYLDFL YSALVEIPGA FIALITIDRV GRIYPMAMSN LLAGAACLVM IFISPDLHWL NIIIMCVGRM GITIAIQMIC LVNAELYPTF V*RNLGVMVCS SLCDIGGIIT PFIVFRLREV WQALPLILFA VLGLLAAGVT LLLPETKGVA LPETMKDAEN LGRKAKPKEN TIYLKVQTSE PSGT

SLC22A1 antibodies used in Taqman protein assay (section 4.3.2): Peptide sequence (554 aa) of SLC22A1 protein isoform a (NCBI accession number NP_003048.1) highlighting immunogenic sequences used to raise specific antibodies used in this study to perform Taqman protein assay for the quantification of SLC22A1 protein. approximate immunogen sequences are indicated for the respective antibodies (Red dotted line – Santa Cruz antibody; blue straight line – Abcam antibody; green double line – Sigma Aldrich antibody). The amino acid position from which SLC22A1 isoform a and isoform b differ is indicated with red asterisk.

“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 ~