Targeted detection of mutations associated with imatinib-resistance

Strategies to increase sensitivity and specificity

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2005/2006

Master thesis in pharmacology

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Acknowledgments

This work was performed at the Department of Pharmacology, Institute of Pharmacy, University of Tromsø in 2005/2006.

First I would like to thank my supervisor Ingvild Mikkola and co-supervisor Franz Gruber for all the training and help in the lab, for guidance during the study, as well as in the writing-process of the thesis.

I would also like to thank all the people in the lab for their guidance and help with lab-related problems, and the sequencing-lab at the University of Tromsø for sequencing of my plasmids.

Finally, I would like to thank my friends and family for their help and support during the last years. My sister Karina deserves an extra appreciation for reading through the thesis and giving me helpful advice about the writing, and Ronny for all the patience and support lately.

Tromsø, 2006

Linda Eilen Johansen

Contents

Abbreviations

ABL	Abelson tyrosine kinase
ALL	Acute Lymphocytic Leukemia
A-loop	Activation Loop
ARMS	Amplification Refraction Mutation System
ASO	Allele-Specific Oligonucleotide
ATP	Adenosine Triphosphate
BCR	Breakpoint Cluster Region
bp	Base Pair
BSA	Bovine Serum Albumine
cDNA	Complementary DNA
CML	Chronic Myelogenous Leukemia
Ct	Threshold Cycle
D	Discriminating
ddNTP	Dideoxynucleotide Triphosphate
D-HPLC	Denaturing High Performance Liquid Chromatography
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dsDNA	Double-Stranded Deoxyribonucleic Acid
EDTA	Ethylene Diamine Tetra-Acetic acid
kb	Kilo Base
kd	Kilo Dalton
LB	Luria-Bertani
LNA	Locked Nucleic Acid
ND	Non-Discriminating
MAT	Matching
MIS	Mismatching
mRNA	Messenger Ribonucleic Acid
PCR	Polymerase Chain Reaction
P-loop	Phosphate-Binding Loop
PNA	Peptide Nucleic Acid
RQ-PCR	Real-Time PCR
SDS	Sodium Dodecyl Sulfate
SNP	Single Nucleotide Polymorphism
Taq	*Thermus aquaticus*
TBE	Tris-Borate

Summary

Background

The main cause to chronic myeloid leukemia (CML) is a translocation between chromosome 9 and 22, resulting in the Philadelphia chromosome, containing a BCR-ABL fusion-gene that encodes a constitutively active tyrosine kinase. Imatinib, a selective inhibitor of the tyrosine kinase, is the first-line treatment for CML-patients. However, drug-resistance is an increasing problem, and the most important reasons for this are point mutations within the BCR-ABL kinase domain. More than 40 such mutations are described, and because the mutations can have a great impact on clinical decision-making, early detection is important. With new tyrosine kinases developing, the resistance problem seems to be diminished, as some of the new drugs are less prone to resistance. Only the important T315I-mutation seems to remain a problem, which is why this particular mutation should be in focus for developing a more sensitive detection method.

Materials and methods

Plasmids and patient-samples containing wild-type and mutated BCR-ABL sequences were used in an effort to design a sensitive and specific, biased assay for detection of the T315I-mutation in BCR-ABL alleles. By comparing a single-step versus a nested approach, the amplification of long versus short templates, TaqMan versus SYBR Green Master Mixes, and ASO versus LNA and ARMS-primers, a patient-test for detection of the T315I-mutation was developed.

Results

A nested PCR approach using SYBR Green Master Mix and a BCR-ABL-specific primer pair with a LNA-nucleotide incorporated in the mutation-specific reverse primer provided the greatest improvements in sensitivity and specificity for detection of the T315I-mutation in samples from CML-patients.

1 Introduction

1.1 Leukemia

Leukemia is a group of neoplastic disorders that arises in the hematopoietic cells of the bone marrow and leads to an uncontrolled proliferation and accumulation of immature blood cells. This excessive production of blood cells can lead to overcrowding of the bone marrow, and spreading into the peripheral blood and to other organs. The lack of functional blood cells can lead to symptoms like anemia, infections and bleedings. If left untreated, leukemia is fatal, often due to complications resulting from the leukemic infiltration of the bone marrow and replacement of normal hematopoietic precursor-cells (Herfindal et al. 2000).

Traditionally, leukemia was classified as chronic or acute by how fast the disease progressed to a fatal clinical outcome. This has later found to correlate well with the degree of maturation of the predominant malignant cell. The disease is further classified into lymphoid or myeloid leukemia according to the predominant cell-type involved (Henderson et al. 2002).

1.1.1 Acute lymphocytic leukemia

Acute lymphocytic leukaemia (ALL) is the malignant transformation and uncontrolled growth of a B- or T-lineage lymphocytic precursor. The disease usually progresses fast, and immediate treatment is necessary. Symptoms can be bruising, fever, fatigue, loss of appetite, anemia, bone-pain and lymphadenopathy, and there are more than 25 to 30 percent lymphoblasts in the bone marrow (Henderson et al. 2002). In 2003 in Norway, 227 persons were diagnosed with acute leukemia, and approximately 30 of these had ALL (Kreftregisteret 2003; Kreftforeningen 2006).

1.1.2 Chronic myelogenous leukemia

Chronic myelogenous leukemia (CML) predominately affects the granulocytic cell-line. There is often an increased proliferation of granulocytes, but their differentiation is relatively normal. About 50 people in Norway are diagnosed with CML each year, primarily older adults (Kreftforeningen 2006). The disease can be discovered on routine-controls and develops gradually over time. Patients can survive years after the diagnosis, even without treatment, because the maturation-arrest occurs in later differentiation steps. This way, the blood cells are relatively mature and retain much of their normal functions, compared to those of acute leukemia. Over time, the disease progresses to more aggressive forms that are less responsive to treatment (Herfindal et al. 2000).

In the first, chronic phase of CML there is a marked granulocytic expansion, but the differentiation is relatively unaffected. The blood cells still have normal functions, and the symptoms are only mild. After an average of four to five years, the disease progresses to an accelerated phase where the symptoms are more pronounced, and the response to treatment is lower. The blast cells proliferate faster and are less mature. When more than 30 percent of the cells in the blood or bone marrow are leukemic, CML has developed into the blast-phase or blast-crisis. In this phase, the cells are predominantly immature, and the disease can be indistinguishable from an acute leukemia (Henderson et al. 2002).

1.2 The Philadelphia chromosome

A reciprocal translocation between the ABL-gene on chromosome 9 and the BCR-gene on chromosome 22 is the hallmark of CML, since it can be found in more than 95 percent of patients with the disease. This abnormality was first described by a Philadelphia-based group, more than 40 years ago, and is therefore called the Philadelphia chromosome (Nowell et al. 1960; Nowell et al. 1960). The translocation results in the formation of a BCR-ABL fusion-gene on chromosome 22, encoding a constitutive active non-receptor tyrosine kinase (figure 1). CML was the first human cancer to be associated with a chromosomal abnormality, and its discovery was a breakthrough in cancer biology (Sawyers 1999).

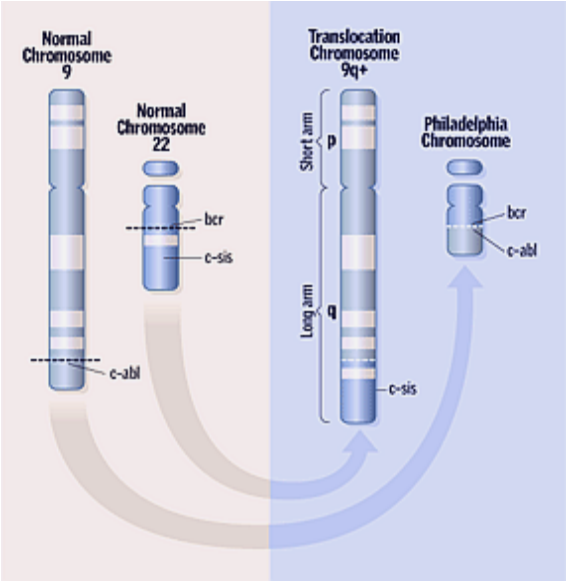

Figure 1. The Philadelphia chromosome. A translocation between chromosome 9 and 22 generates the abnormal Philadelphia chromosome containing the BCR-ABL fusion-gene (Waalen 2001).

The fusion-protein can vary in size from 185 kd to 230 kd, dependent on the breakpoint location in the BCR-gene. In typical chronic phase CML, the 210 kd protein is the most common (Sawyers 1999). Figure 2 describes important features of the most frequent BCR-ABL transcripts. Important mutation sites found in imatinib-resistant patient will be discussed in the following chapters.

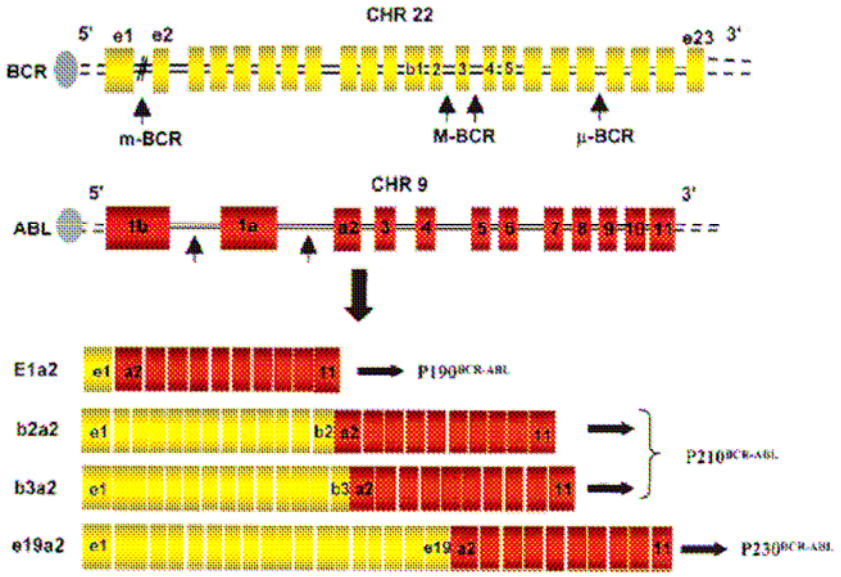

Figure 2. Different breakpoint locations in the BCR- and ABL- genes, and the fusion-proteins derived from them. The most common fusion-protein, the $P210^{BCR-ABL}$, is derived from breakpoints between exons 13 and 14 (b2) or 14 and 15 (b3) of in the major breakpoint cluster region, producing BCR-ABL fusion-genes that transcribe b2a2 or b3a2 mRNA (Salesse et al. 2002).

The increased activity of the tyrosine kinase results in phosphorylation and activation of several substrates involved in signal pathways leading to malignant transformation. Among the cellular processes interfered by this are proliferation, differentiation, adhesion and apoptosis. (Salesse et al. 2002).

1.3 Imatinib

The phenylaminopyrimidine compound imatinib mesylate (Gleevec), seen in figure 3, acts as a potent inhibitor of the tyrosine kinases ABL, PDGFR, c-Kit and ARG. It represents the first selective protein kinase-inhibitor to be developed for targeted cancer therapy (Druker et al. 1996; Deininger et al. 1997), and its superior clinical efficacy was first shown in the treatment of CML (O'Brien et al. 2003). On the molecular level, imatinib acts by binding the catalytic domain of the ABL-protein in its inactive conformation, thereby preventing ATP-binding. Thus, it keeps substrates involved in BCR-ABL downstream signal transduction pathways from phosphorylation and activation (Druker et al. 2002).

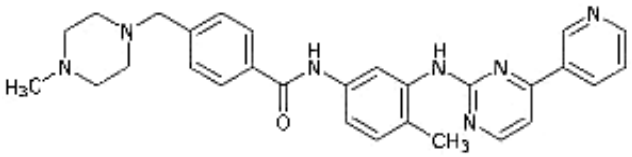

Figure 3. Imatinib mesylate.

1.4 Imatinib-resistance

Patients with CML, especially those in advanced phases, treated with imatinib often experiences relapse due to drug-resistance. The main reason to this is point mutations within the BCR-ABL kinase domain (Gorre et al. 2001; Shah et al. 2002). The mutations induce substitutions of amino acids that either directly interfere with imatinib in the binding pocket (gatekeeper residues), or that are involved in establishing a distinct conformation, which imatinib is unable to bind. Imatinib exclusively binds the inactive conformation of the ABL kinase domain. Thus, if the conformational change of the kinase is hampered by an amino acid change, imatinib-binding may be drastically reduced.

The mutations are, as seen in figure 4, mainly found in four regions of the ABL-kinase domain. The phosphate binding loop (P-loop) that normally surrounds a phosphate group in the depth of the binding pocket, the activation loop (A-loop) that is crucial for the conformation of the kinase, the gatekeeper residues that are directly in contact with imatinib, and other more downstream located residues in the catalytic domain, which may participate to stabilize the A-loop in a certain conformation, like the methionin residue in position 351.

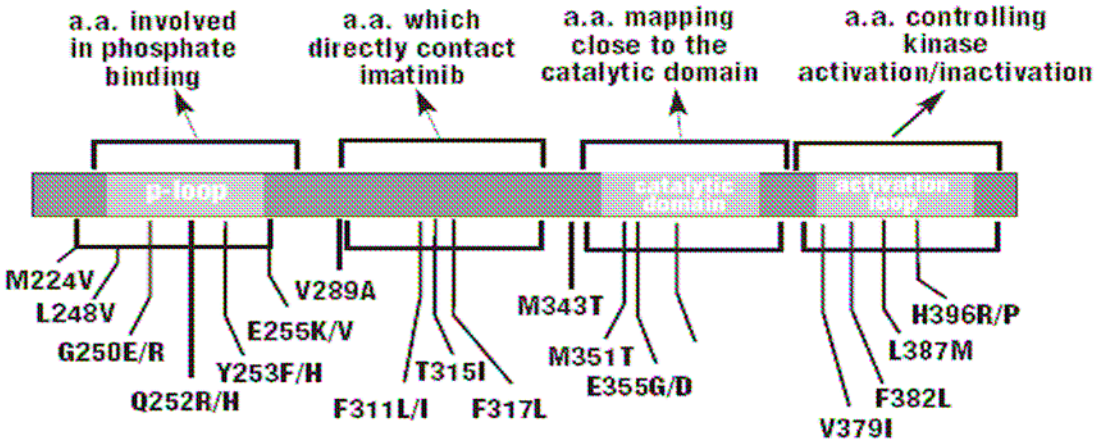

Figure 4. Mutations in the BCR-ABL kinase domain that are associated with imatinib-resistance. The mutations are mainly found in the P-loop, at amino acid residues in direct contact with imatinib, close to the catalytic domain or at the activation loop (Martinelli et al. 2005).

More than 40 different mutations are described in association with imatinib-resistance (Hochhaus et al. 2004; Martinelli et al. 2005; Hughes et al. 2006). It seems that the majority of mutations target amino acid residues involved in conformational change, and only a few residues seem to directly contact the drug in the ABL binding pocket, like the 315 and 317 residues. The T315I-mutation seems to be particularly important since it is frequently detected and confers resistance also to new drugs developed particularly for imatinib-resistance subjects (Shah et al. 2004; Weisberg et al. 2005). Figure 5 shows the ABL kinase domain complexed with imatinib, and illustrates some of the most important mutations associated with resistance.

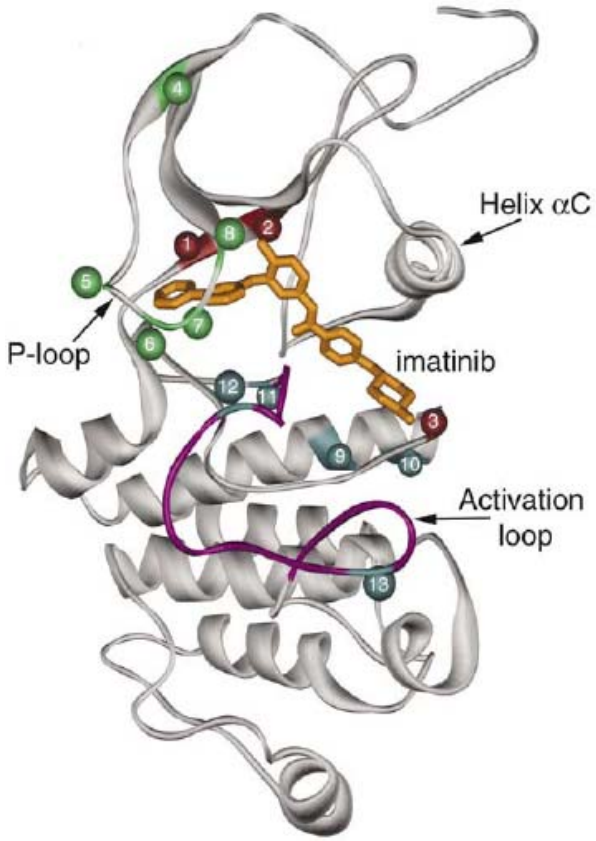

Figure 5: Ribbon illustration of the ABL kinase domain in complex with imatinib. Imatinib is shown in yellow, and the numbers represent amino acid residues involved in resistance. Red spheres (1-3) symbolize mutations that directly affect the binding of imatinib, green spheres (4-8) are P-loop mutations and blue spheres (9-13) are mutations close to the activation loop. The amino acid residues are: 1 F317L; 2 T315I; 3 F359; 4 M244; 5 G250; 6 Q252; 7 Y253; 8 E255; 9 M351; 10 E355, 11 V379; 12 L387; 13 H396 (Shah et al. 2003).

According to a well-established model (Roche-Lestienne et al. 2003), mutations may pre-exist imatinib treatment in a single cell, conferring a selective advantage as soon as imatinib treatment is initiated. If these cells then are able to proliferate, e.g. as part of the stem cell pool, a new clone of imatinib-resistant cells may be selected during treatment, and after a while induce relapse. Interestingly, some of these mutations have a more unfavorable clinical impact, and it has recently been shown that patients with P-loop mutations die earlier than imatinib-resistant patients with mutations outside the P-loop (Branford et al. 2003; Soverini et al. 2005). Because of this, recent CML treatment programs recommend immediate stop of imatinib-treatment if P-loop mutated clones are

detected (Hjorth-Hansen et al. 2004). Other mutations, like the M351T, have been shown to respond to a dose-escalation in vitro. More recent drugs are also on the way into clinical use, and many of the mutated imatinib-resistant BCR-ABL isoforms are sensitive to these, as recently shown in in vitro experiments (Burgess et al. 2005). Considering the latest developments in CML-monotherapy and the introduction of new drugs, detection of mutations in the ABL kinase domain is getting more important. Some of the methods used for detection of mutations are described in the following sections.

1.5 Methods for detection of mutations in the ABL kinase domain

1.5.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is a process used for amplification of specific DNA sequences, and in CML-patients it can be used to confirm the existence of the BCR-ABL gene, to quantify the level of BCR-ABL transcripts and to detect mutations in the ABL kinase domain. The process includes a thermostable DNA polymerase that adds deoxynucleotides (dNTPs) to the free 3`-OH group of an oligonucleotide primer. By repeating the process for several cycles in a thermal cycler, the number of copies of the amplified gene grows exponentially. Real-time PCR (RQ-PCR), as opposed to regular end-point PCR, allows for continuously monitoring of the amplification, thereby giving an opportunity to perform quantitative analysis. If the PCR was 100 percent effective, it would generate about 10^6 copies of each template molecule, but usually the efficiency is only 60 to 80 percent (McPherson et al. 2000). The sensitivity and specificity of the reaction can however be increased in several ways, e.g. by using nested approaches, or primers of ASO-, ARMS- or LNA-type as described in the following.

Nested PCR is an approach used to increase the sensitivity by adding a PCR-step. The product from the first PCR is used as template for a second PCR, with primers within the region of the original amplified product. An estimated 10^4-fold increase in sensitivity of detection of the true product over non-specific products can be expected with this method

(McPherson et al. 2000). However, because a nested approach consists of several steps, the probability of contamination of the samples and detection of false positive results increases.

Allele-specific oligonucleotide (ASO) primers are used to detect single nucleotide polymorphisms (SNPs), and is based upon matching the 3´-base of a PCR-primer with a SNP-allele and mismatching it with the other allele (Gibbs et al. 1989; Bottema et al. 1993). The mismatched primer-template complex has a lower melting temperature than the perfectly matched complexes, and this promotes selective amplification of the correctly matched primer-templates. When using a DNA polymerase that lacks 5´ → 3´ exonuclease activity, such as *Taq* polymerase, the mismatch cannot be repaired, and the polymerase will be unable to extend the mismatched primer with the same efficiency as the matched primer (Ayyadevara et al. 2000; Logan et al. 2004).

Even though the 3´-terminal base is mismatched, amplification in some cases still proceeds. By using the amplification refractory mutation system (ARMS), additional mismatches are included near the 3´-terminal mismatched primer, and the specificity and discrimination-power of the primer can be increased (Newton et al. 1989; Logan et al. 2004). This is because the additional mismatch makes the already mismatched primer too destabilized for extension by the polymerase. The effect of the destabilization will increase the closer to the 3´-terminus the additional mismatch is (Newton et al. 1989). As with ASO-primers, the DNA polymerase cannot have any 3´-exonucleolytic proofreading activity.

Another way of increasing the sensitivity and specificity of PCR is by replacing one of the DNA-nucleotides in the primer with a locked nucleic acid (LNA) as seen in figure 6. The LNA-base is a nucleic acid analog with a 2´-O, 4´-C methylene bridge that locks the ribose moiety into a C3´-endo conformation. Suggested mechanisms for the increased specificity LNA-primers have for SNP-alleles, are that LNA-primers bind with higher thermal stability to their targets (Kumar et al. 1998) and that they are superior substrates for the *Taq* DNA polymerase (Latorra et al. 2003; Latorra et al. 2003). The slower degradation by the 3´→5´ proofreading exonuclease activity of the DNA polymerase can also help explain why LNA-primers discriminate better between matched and mismatched templates, compared to DNA-primers (Di Giusto et al. 2004).

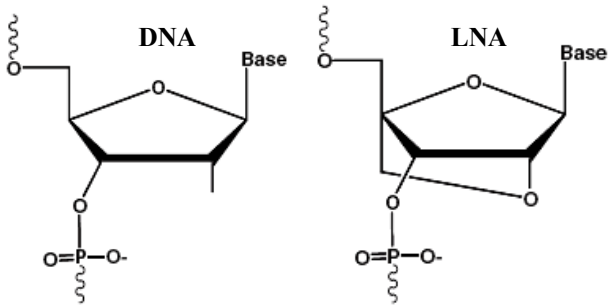

Figure 6. The molecular structure of DNA and LNA. The LNA-nucleotide have a 2´-O, 4´-C methylene bridge that locks the ribose moiety into the C3`-endo conformation.

1.5.2 Biased versus unbiased approaches

Sequencing of PCR products has been the method of choice for detection of mutations in BCR-ABL, since the first description of a mutation causing imatinib-resistance (Gorre et al. 2001). Several different sequencing approaches have been described, but all mutational screening by sequencing have actually been done using a nested PCR approach for selective amplification of the BCR-ABL allele. Differences between the different strategies comprise the length of the sequenced fragment. There is an increasing body of evidence suggesting that, in addition to the main cluster of mutations between amino acids 244 and 396, substitutions of a few residues down to position 580 might also be involved in causing imatinib resistance (Branford et al. 2006; Hughes et al. 2006). An appropriate sequencing strategy for detection of imatinib-resistance associated mutations should therefore cover a DNA fragment including at least amino acid residues 244 until 600.

Though sequencing is conducted BCR-ABL-specific, the procedure is hampered by low sensitivity and a high workload. Alternatively, mutations could be detected by denaturing high performance liquid chromatography (D-HPLC) elution of PCR products (Deininger et al. 2004; Soverini et al. 2004) where mutations appear as additional peaks in the chromatogram due to formation of thermally less stable heteroduplices (Premstaller et al. 2002). Several protocols have been described over the last few years, and this procedure has shown to be around 10 times more sensitive than sequencing. However, in case of a positive finding, the actual type of mutations has to be confirmed by sequencing. Both sequencing and D-HPLC are unbiased approaches, able to identify new mutations as well as known mutation located in the area of interest.

Other, more sensitive methods have been developed primarily to increase the sensitivity of mutation-detection. Many of these techniques target only known mutations and are therefore biased, e.g. they just find what they are looking for. Most of these procedures combine the PCR-principle with strategies to selectively increase the detection of the mutated allele versus the wild-type allele. Recent examples are as seen in table 1

pyrosequencing that detects the light from the reaction cascade after pyrophosphate is released during nucleotide incorporation (Ronaghi et al. 1998; Khorashad et al. 2006), Double-Gradient-Denaturing-Gradient Gel Electrophoresis (DG-DGGE) where mutations are visualized in a double-gradient denaturing gel due to wild-type/mutant heteroduplex formation (Sorel et al. 2005), the PCR clamping technique where PNA-binding prevents amplification of either the wild-type or the mutated allele (Kreuzer et al. 2003), Restriction Fragment Length Polymorphism (RFLP) where an introduced Taq I restriction site ensures amplification of mutated sequences only (Liu et al. 2003), and variations of ARMS-PCR (Gruber et al. 2005; Willis et al. 2005).

Table 1. Some of the technologies available for identification and quantification of mutations in the BCR-ABL kinase domain. A biased technology is designed to only detect specific mutations (Hughes et al. 2006).

Technology	Sensitivity	Bias
Direct sequencing	15-25%	No
Subcloning and sequencing	9%	No
Denaturing high performance liquid chromatography (D-HPLC)	0.1-10%	No
Pyrosequencing	5%	No
Double gradient denaturing electrophoresis	5%	No
Fluorescence PCR and PNA clamping	0.2%	Yes
Allele specific oligonucleotide PCR (ASO-PCR)	0.01%	Yes

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1.6 Basis for quantitative assessment of patient-samples

Real-time PCR, as described earlier, can be used in quantitative assays. Figure 7 shows a typical real-time PCR amplification profile for amplification of a mutated template, using a non-discriminating (ND) and a discriminating (D) primer. The template that is perfectly hybridized to the D-primer is called the matching template (MAT), and the template that is mismatched with the 3´-end of the D-primer is called the mismatching template (MIS). The ND-primer does not discriminate between MAT- and MIS-templates, and if the same

amount of each template is used, the amplification plots of MAT and MIS will be identical. The D-primer will delay the amplification of mismatching templates, because the 3´-end mismatch will interfere with primer extension by the DNA polymerase. The threshold is defined as the cycle where a threshold level of PCR product is detected. The amplification curve of the matching (often the mutated) template can give a measure of the sensitivity of the mutation-detection. The earlier this curve appears in the plot, the more sensitive the mutation-detection is. This curve, together with the curve of the mismatching template, decides the specificity of the mutation-specific approach for amplification of the mutated template. The bigger the difference between the amplification curves of MAT and MIS is, the greater the specificity is (expressed as ΔCt) (Thomassin et al. 2004).

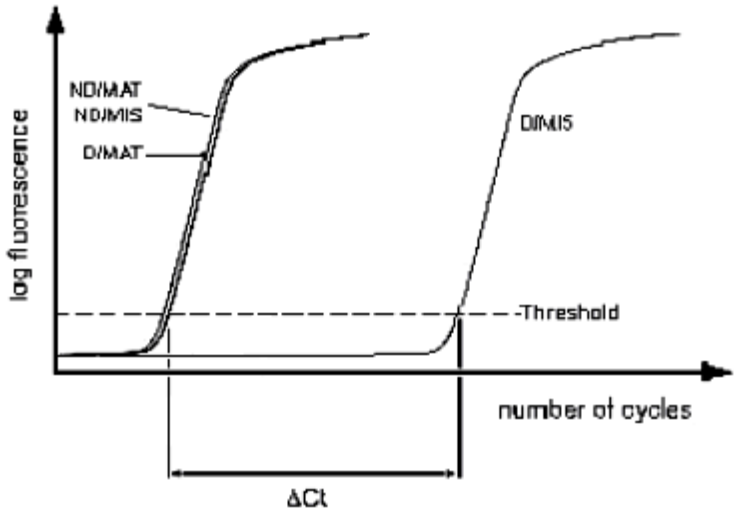

Figure 7. A typical real-time PCR amplification profile. The amplification of a matching (MAT) and a mismatching (MIS) template using a non-discriminating (ND) and a discriminating (D) primer is shown in an amplification profile where the logarithm of the fluorescence (amount of PCR product) is plotted as a function of the number of cycles. As seen, ND/MAT is identical to ND/MIS because the primer does not discriminate between the templates, whereas D/MIS is shifted to the right, giving rise to the ΔCt between MIS and MAT (Thomassin et al. 2004).

1.7 Future direction of CML-treatment

The development of new tyrosine kinase-inhibitors will certainly influence the resistance field, since early in vitro data indicates that each substance might have its distinct mutational resistance profile. There is a clear trend in current papers showing that the group of Src/ABL inhibitors is less prone to resistance. Dasatinib and AMN107 seems to be resistant to only a few mutations, and if a combination of imatinib and dasatinib is used, only the important T315I-substitution will be able to make resistant clones (Burgess et al. 2005). These data are at present only based on in vitro experiments and will have to be confirmed in clinical studies, but combination-treatment has already been pronounced as the future treatment of CML (Burgess et al. 2005; O'Hare et al. 2005).

In this scenario, much of the workload and costs actually spent on mutational screening should rather be spent on strategies to increase the detection of the T315I-mutation, since the other described clones will probably not appear during combination-treatment. Several groups have recently identified a third generation of drugs targeting the T315I BCR-ABL isoform (Gumireddy et al. 2005; Young et al. 2006), therefore early detection of this mutation would be important for clinical decision making.

1.8 Aim of the study

Examples of biased mutation-detection have been published before, as described in table 1 (Hughes et al. 2006). In particular, assays based on the use of primer-sets manipulated with mismatching mutations have shown to selectively detect mutated templates (Gruber et al. 2005; Willis et al. 2005). In those experiments, weak mismatches located 2 to 3 nucleotides from the 3´-end of the primer conferred maximal sensitivity and selectivity (Gruber et al. 2005). However, the described assay is based on TaqMan chemistry, allowing template fragment length of up to 150 base pairs. In the case of T315I, the target mutation is located more than 800 bp from the BCR-ABL breakpoint, meaning that the TaqMan-based assay could not be designed for specific amplification of BCR-ABL templates. In this project, the primary aim was to design a sensitive, biased assay for detection of the T315I-associated mutation of the BCR-ABL allele. Therefore, we tried to expand the described ARMS-assay (Gruber et al. 2005) to a selectively BCR-ABL targeting assay. In addition to the ARMS-principle, LNA-nucleotides were also used in order to increase the selectivity of mutation-specific primers. As illustrated in figure 8, the following approaches were tested:

- The use of long PCR assays, which render the targeted mutation-detection BCR-ABL-specific.
- Different Master Mixes for detection of the amplification during the PCR (SYBR Green, FullVelocity).
- Different approaches of primer-manipulation to increase the amplification of mutated alleles.
- The use of a pre-amplification step in combination with targeted detection.

According to our hypothesis, the application of the described techniques would increase the detection of the targeted mutation. To test the hypothesis, appropriate dilutions of plasmids were used, and in addition, the experiences from the plasmid system were directly transferred into a patient test for detection of the T315I BCR-ABL isoform.

2 Materials

Table 2. Reagents used in method 3.2 (Amplification of cDNA by PCR). The primers are written in the 5´ to 3´ direction.

Reagents	**Specifications**	**Producer**
TOPO® TA Cloning kit	Catalog no. K4520-01	Invitrogen
Template cDNA BCR-ABL	From CML-patient	-
Template cDNA T315I BCR-ABL	From CML-patient	-
Template cDNA wild-type BCR-ABL	From plasmid	-
10× PCR Buffer	From the kit 100 mM Tris-HCl (pH 8.3) 500 mM KCl 25 mM $MgCl_2$ 0,01 % gelatine	Invitrogen
50 mM dNTP mix	From the kit 12,5 mM of each nucleotide (pH 8.0)	Invitrogen
Primer BCR(13)-F	TGTGAAACTCCAGACTGTCCACA	Eurogentec
Primer A7-	AGACGTCGGACTTGATGGAGAACT	Eurogentec
Sterile water	From the kit	Invitrogen
HotStarTaq™ DNA Polymerase	5 U/µl	QIAgen

Table 3 Reagents used in method 3.3 (Gel electrophoresis)

Reagents	**Specifications**	**Producer**
SeaKem® LE Agarose	Product number 50005	MedProbe
1× TBE buffer	108 g Tris base 55 g boric acid 40 ml 0.5 M EDTA (pH 8.0) Sterile water adjusted to a volume of 10 l	-
Ethidium bromide	10 mg/ml	Sigma
6× loading buffer	0.25 % bromophenol blue 0.25 % xylene cyanol FF 40 % sucrose in H_2O 1 ml 1M Tris-HCl (pH 8.0)	-
100 bp DNA Ladder	Cat. no. 15628-019 (figure 10A)	Invitrogen
1 kb DNA Ladder	Cat. no. 15615-016 (figure 10B)	Invitrogen
1 kb Plus DNA Ladder	Cat. no. 10787-018 (figure 10C)	Invitrogen

Table 4. Reagents used in method 3.4 (Cloning)

Reagents	**Specifications**	**Producer**
Salt solution	1.2 M NaCl, 0.06 M $MgCl_2$	Invitrogen
pCR®2.1-TOPO® vector	10 ng/µl plasmid DNA in buffer	Invitrogen

Table 5. Reagents used in method 3.5 (Transformation)

Reagents	Specifications	Producer
One Shot® DH5α™-T1 competent *E. coli* cells	From the cloning kit	Invitrogen
SOC medium	2 % Tryptone 0.5 % Yeast extract 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4$ 20 mM glucose	Invitrogen
LB+ medium with 50 mg/ml ampicillin	Per 1000 ml: 10 g tryptone 5 g Yeast extract 10 g NaCl 1.1 g Glucose 50 mg/ml ampicillin Adjust pH to 7.0 with 5 N NaOH Sterile water adjusted to a volume at 1 l	-
X-gal	Catalog no. 15520-034	Invitrogen

Table 6. Reagents used in method 3.6 (Isolation of plasmids)

Reagents	Specifications	Producer
QIAprep® Spin Miniprep kit	Catalog no. 27106	QIAgen
Buffer P1	Resuspension buffer	QIAgen
Buffer P2	Lysis buffer	QIAgen
Buffer N3	Neutralizing buffer	QIAgen
Buffer PE	Washing buffer	QIAgen
Buffer EB	Elution buffer	QIAgen

Table 7. Reagents used in method 3.8 (Sequencing PCR). The primers are written in the 5′ to 3′ direction.

Reagents	**Specifications**	**Producer**
BigDye Terminator v3.1 Cycle Sequencing Kit	Part number 4337455	Applied Biosystems
BigDye® Terminator v3.1 5× Sequencing Buffer	From the kit	Applied Biosystems
Terminator Ready Reaction Mix (BigDye v3.1)	From the kit	Applied Biosystems
Primer M13F	GTAAAACGACGGCCAG	Eurogentec
Primer M13R	CAGGAAACAGCTATGAC	Eurogentec
Primer AN4+	TGGTTCATCATCATTCAACGGTGG	Eurogentec

Table 8. Reagents used in method 3.9 (Linearization)

Reagents	**Specifications**	**Producer**
BamHI	20 000 U/ml Catalog number R01365	New England BioLabs
10× NEBuffer for *BamHI*	1× NEBuffer (pH 7.9) contains: 10 mM Tris-HCl 150 mM NaCl 10 mM $MgCl_2$ 1 mM dithiothreitol	New England BioLabs
100× BSA	100 µg/ml	New England BioLabs
HindIII	20 000 U/ml Catalog number R0104S	New England BioLabs
NEBuffer 2	1× NEBuffer 2 (pH 7.9) contains: 10 mM Tris-HCl 50 mM NaCl 10 mM $MgCl_2$ 1 mM dithiothreitol	New England BioLabs

Table 9. Reagents used in method 3.10 (Purification of plasmids)

Reagents	**Specifications**	**Producer**
GFX PCR DNA and Gel Band Purification Kit	Product code 27-9602-01	Amersham Biosciences
Capture buffer	From the kit	Amersham Biosciences
Wash buffer	From the kit	Amersham Biosciences
Elution buffer	From the kit	Amersham Biosciences

Table 10. Reagents used in method 3.11 (Standard curves). The primers are written in the 5´ to 3´ direction.

Reagents	**Specifications** A/T/C/G: Mismatch	**Producer**
TaqMan® Universal PCR Master Mix	With ApliTaq Gold® DNA Polymerase Part number 4304437	Applied Biosystems
Probe 315 pro	ACCCTAACCTAGTGCAGCTCCTT	Eurogentec
Primer 315 F	TGCAGTCATGAAAGAGATCAAA	Eurogentec
Primer A315 mut	TCCCGTAGGTCATGAATTCAA	Eurogentec
Primer T315I ND	CCGTAGGTCATGAACTCA	Eurogentec

Table 11. Reagents used in method 3.12 (Detection of mutated BCR-ABL). The primers are written in the 5´ to 3´ direction.

Reagents	**Specifications** **A/T/C/G**: LNA nucleotide A/T/C/G: Mismatch	**Producer**
TaqMan Universal PCR Master Mix	With ApliTaq Gold® DNA Polymerase Part number 4304437	Applied Biosystems
2× Brilliant® SYBR® Green QPCR Master Mix	Includes SureStart® *Taq* DNA polymerase and SYBR Green I dye	Stratagene
2× FullVelocity™ QPCR Master Mix	Contains an optimized PCR buffer, $MgCl_2$, nucleotides and stabilizers.	Stratagene

HotStarTaq™ DNA Polymerase	5 U/µl	QIAgen
10× PCR Buffer	200 mM Tris-HCl (pH 8.4) 500 mM KCl 15 mM $MgCl_2$	Invitrogen
50 mM dNTP mix	12.5 mM of each nucleotide (pH 8.0)	Invitrogen
FullVelocity™ enzyme	2.5 U/µl	Stratagene
Template BCR-ABL	Plasmid derived from method 3.1-3.9	-
Template T315I BCR-ABL	Plasmid derived from method 3.1-3.9	-
Template RG04	Shorter BCR-ABL plasmid with exon 1-6 of BCR and exon 2-7 of ABL	-
Template cDNA BCR-ABL	From CML-patients	-
Probe 315 pro	ACCCTAACCTAGTGCAGCTCCTT	Eurogentec
Probe ABL 2 pro	CCCTTCAGCGGCCAGTAGCATCTGA	Eurogentec
Primer BCR(13)-F	TGTGGAAACTCCAGACTGTCCACA	Eurogentec
Primer BCR13F	TCCGCTGACCATCAAYAAGGA	Eurogentec
Primer A7-	AGACGTCGGACTTGATGGAGAACT	Eurogentec
Primer T315I mut	TCCCGTAGGTCATGAACTCAA	Eurogentec
Primer T315I ND	CCGTAGGTCATGAACTCA	Eurogentec
Primer A315 mut	TCCCGTAGGTCATGAATTCAA	Eurogentec
Primer ABL-R	CACTCAGACCCTGAGGCTCAA	Eurogentec
Primer T315I-1R	CGTAGGTCATGAACTC**A**A	Eurogentec
Primer T315I-2R	CGTAGGTCATGAACT**C**AA	Eurogentec
Primer T315I-3R	CGTAGGTCATGAAC**T**CAA	Eurogentec
Primer T315I-xR	CGTAGGTCATGAACTCA**A**	Eurogentec
Primer T315I-1xR	CGTAGGTCATGAACTCG**A**	Eurogentec
Primer T315I-2xR	CGTAGGTCATGAACTTA**A**	Eurogentec
Primer T315I-3xR	CGTAGGTCATGAACCCA**A**	Eurogentec

Primer T315I-4xR	CTGAGGTCATGAATTCA**A**	Eurogentec
Primer G250E-xR	ACCTCCCCGTACTGGCCC**T**	Eurogentec
Primer Y253H-xR	CCTCGTACACCTCCCCGT**G**	Eurogentec
Primer Q252Hc-xR	CTCGTACACCTCCCCGTA**G**	Eurogentec
Primer M351T-xR	TTTCTTCTCCAGGTACTCC**G**	Eurogentec
Primer E255K-xR	ACACGCCCTCGTACACCT**T**	Eurogentec

3 Methods

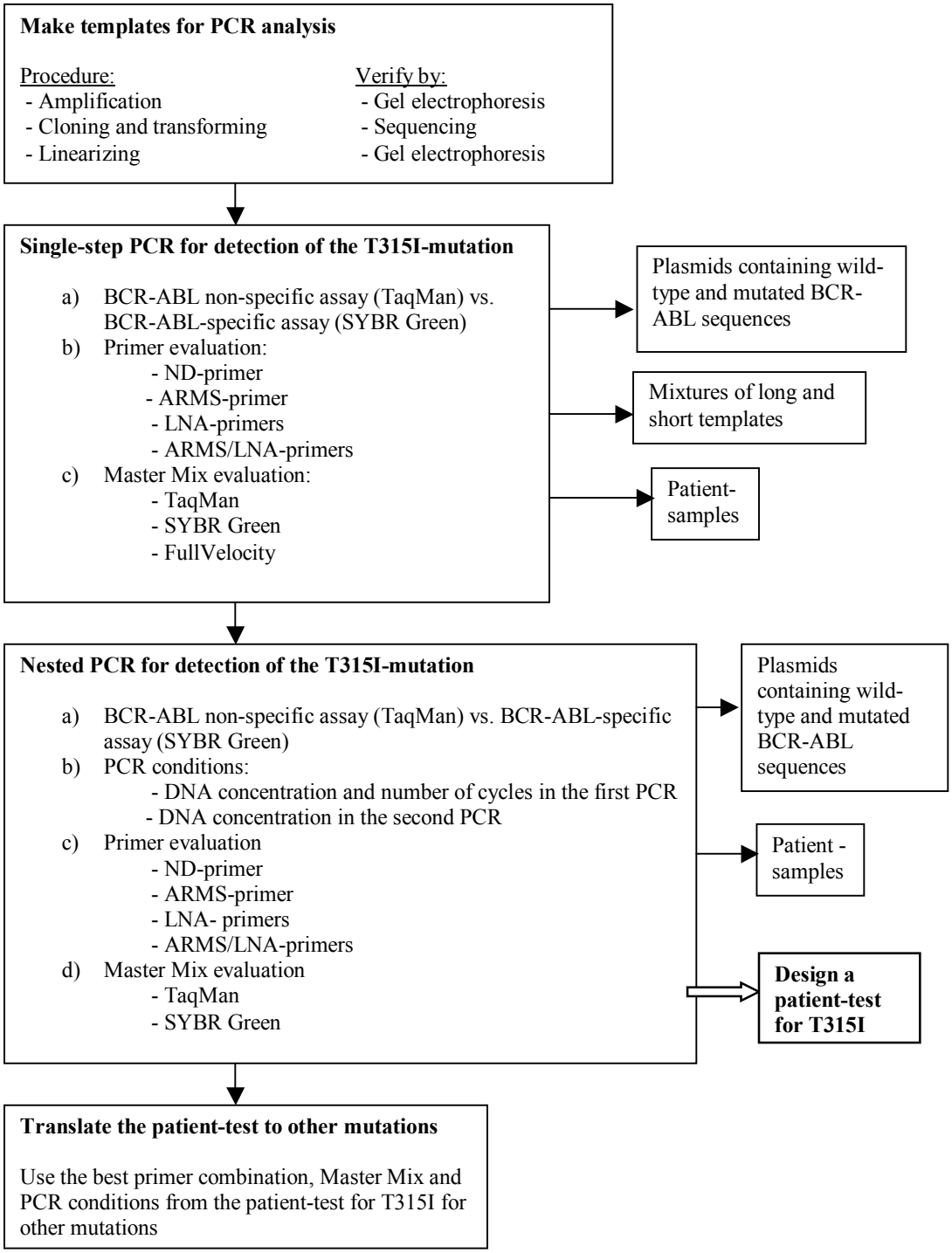

Figure 8: The study procedures.

3.1 Primers used for detection of the T315I-associated mutation

Figure 9 gives an overview of primers and probes used for detection of the T315I-mutation. The primer BCR(13)-F was used in combination with A7- for cloning of the plasmids containing wild-type sequences and the mutation coding for T315I. A recently published article described an ARMS-based TaqMan assay for detection of the T315I-mutation (Gruber et al. 2005), where the primer 315F was used in combination with the probe 315pro and A315 mut. In this study, the ARMS-assay was compared with several other primer combinations, using mutation-specific primers destabilized by incorporation of ARMS- and LNA-nucleotides (T315I-xR, -1R, -2R, -3R, -1xR, -2xR, -3xR and -4xR). These primers were used both in a TaqMan-based assay together with the 315F-primer and 315pro, amplifying templates of 101 bp, and in a SYBR Green assay in combination with the primer BCR(13)-F, amplifying BCR-ABL-specific templates of 882 bp.

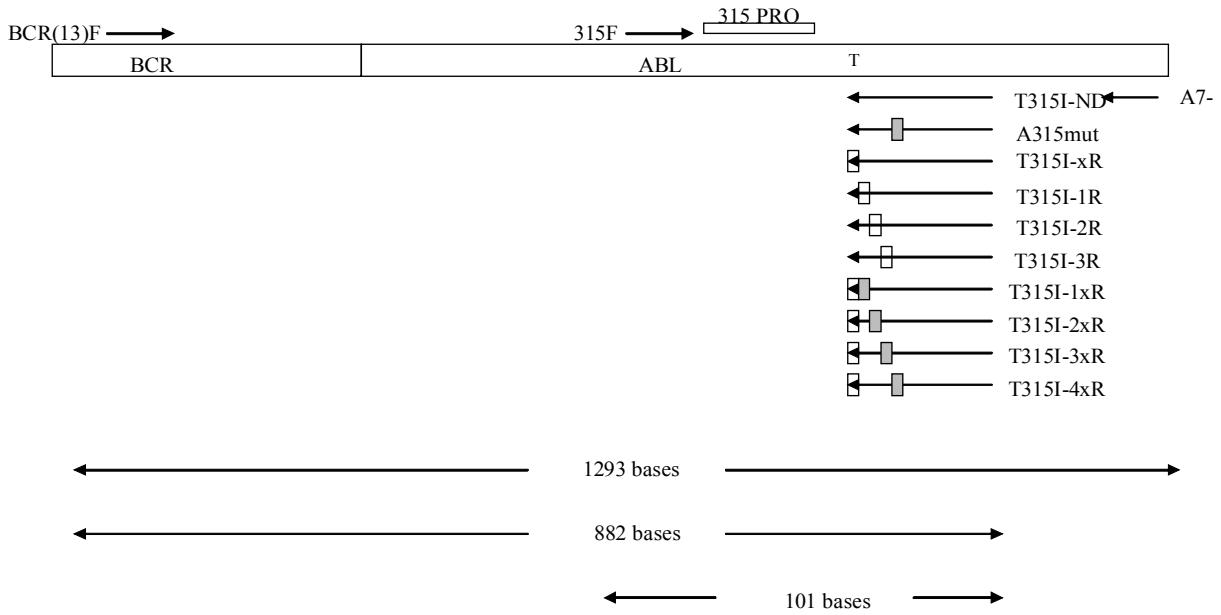

Figure 9: Primers used in the experiments. The figure shows the BCR-ABL allele, the primers used in the study, the relative positions where the primers anneal, and the length of the amplified templates. The LNA-nucleotides in the reverse primers are marked with white boxes, while the additional mismatches in the ARMS-primers are marked with grey boxes. The primers BCR(13)-F and A7- were used for cloning of the plasmids with wild-type and T315I-mutated sequences, creating templates of 1293 bp. The primer 315F and the probe 315pro were used in combination with the ARMS- and LNA-primers in TaqMan-based assays, creating templates of 101 bases. The primer BCR(13)-F was used together with the ARMS- and LNA-primers in BCR-ABL-specific SYBR Green based assays, creating templates of 882 bp.

3.2 Amplification of complementary DNA by PCR

The cDNA containing the gene to be cloned had to be amplified before recombination. This was done by using the TOPO® TA Cloning kit from Invitrogen, including a HotStarTaq™ DNA Polymerase with terminal transferase activity that adds a deoxyadenosine to the 3´-ends of PCR products to create 3´-A overhangs in the PCR product (Invitrogen 2005). In addition to patient-samples with wild-type and mutated BCR-ABL, a plasmid containing wild-type sequences was also amplified as a control.

PCR reaction:

cDNA template (10-100 ng)		2 µl
10× PCR buffer		5 µl
50 mM dNTPs		0.5 µl
Primers BCR(13)-F and A7-		5 µl (100 ng) of each
Sterile water	to a final volume of	49 µl
HotStarTaq™ DNA Polymerase		1 µl
Total volume		50 µl

PCR conditions:

- Initial denaturing at 94°C for 15 minutes to activate the polymerase
- 2 cycles of: Denaturing at 94°C for 1 minute
  Annealing at 50°C for 30 seconds
  Extension at 72°C for 30 seconds
- 43 cycles of: Denaturing at 94°C for 1 minute
  Annealing at 60°C for 30 seconds
  Extension at 72°C for 30 seconds
- Final extension at 72°C for 7 minutes
- 4°C ∞

3.3 Gel electrophoresis

To verify that the PCR was successful, a gel electrophoresis was done. This method separates, identifies and purifies DNA-fragments on the basis of physical properties that influence how rapidly an electric current move them through the pores of a gel matrix. Small, charged molecules move more easily through the gel, and migrate longer towards the positive pole than larger ones. By applying a loading buffer in the samples, the migration can be followed on the gel. Ethidium bromide incorporated in the gel binds to the DNA-fragments, and fluorize when exposed to ultraviolet light. The size of the DNA-fragments can be determined by comparing them to DNA-ladders with fragments of known size (figure 10).

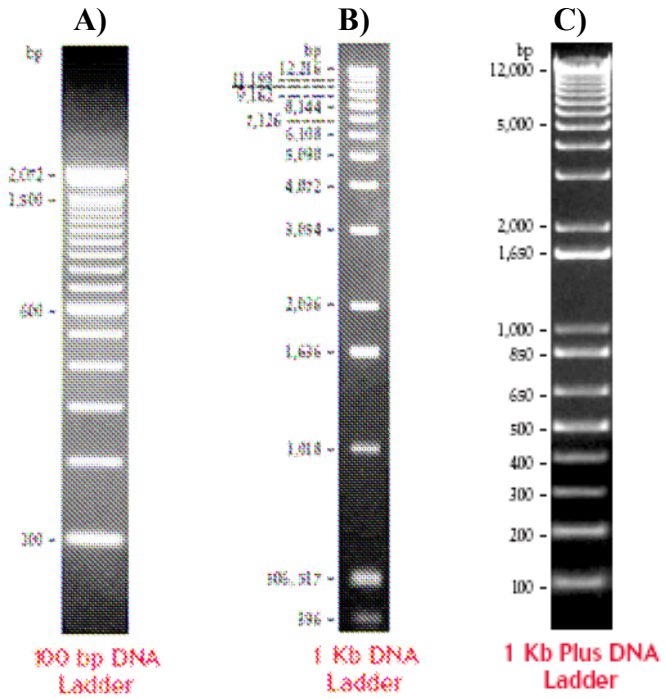

Figure 10: The DNA Ladders used in the experiments. A) The 100 bp DNA Ladder; B) The 1 Kb DNA Ladder; C) The 1 Kb Plus DNA Ladder. All the ladders were from Invitrogen (Invitrogen 2006).

Preparation of a 0.7 percent agarose gel:

1. 0.7 g of agarose was dissolved in 100 ml 1× TBE buffer, by boiling in a microwave oven.
2. The solution was cooled a few minutes, and 10 µl ethidium bromide (10 mg/ml) was added.
3. The solution was poured into a gel rack, and allowed to polymerize further for 30 minutes.
4. The gel was placed in a chamber filled with 1× TBE, and a DNA ladder and the samples were added.
5. The electrophoresis ran for 30 minutes at 90 V.

DNA bands were visualized in the gel by the use of a UV lamp, and a picture was taken. When necessary, the bands of the correct size was cut out from the gel and purified with a gel band purification kit.

3.4 Cloning of the PCR product into a pCR®2.1-TOPO® vector

The *Taq* polymerase-amplified PCR products of the two patient-samples were cloned into the plasmid vector pCR®2.1-TOPO®, seen in figure 11. The vector contains genes for ampicillin and kanamycin resistance that make it possible to select for transformed bacteria. The M13 primer site in the LacZα fragment makes it easy to sequence inserts. The linear vector contains a 3´-T overhang complementary to the 3´-A overhang of the *Taq*-amplified PCR product which allows PCR inserts to ligate efficiently with the vector (Invitrogen 2005). The energy from the broken phosphodiester backbone is conserved by a reversible covalent binding of the enzyme Topoisomerase I.

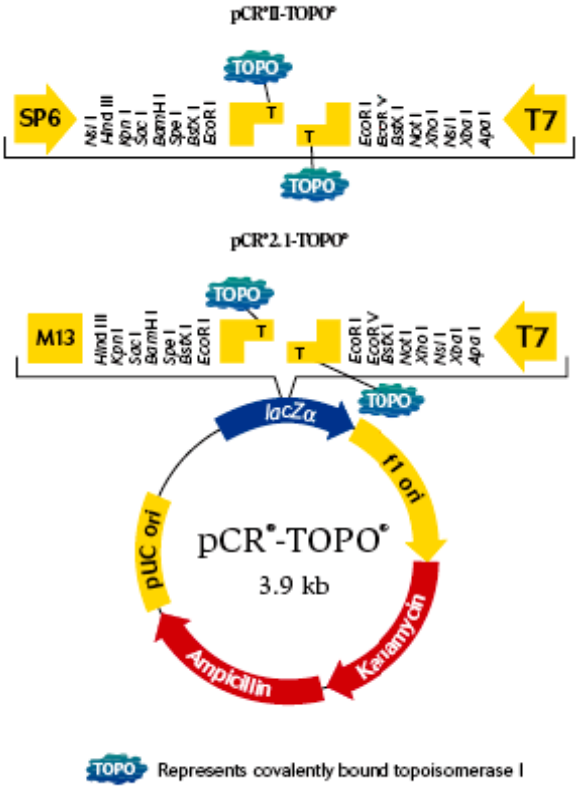

Figure 11. The pCR®2.1-TOPO® vector from Invitrogen (Invitrogen 2005).

The cloning reaction:

PCR product	4 µl
Salt solution	1 µl
TOPO vector	1 µl
Total volume	6 µl

The reaction was set up, mixed and incubated at room temperature for 5 minutes, then placed on ice until transformation.

3.5 Transformation of the pCR®2.1-TOPO® construct into bacterial cells

To be able to produce large quantities of the plasmid, it was transformed into One Shot® DH5α™-T1 competent *Escherichia coli* cells with high transformation efficiency. By using the chromogenic substrate X-gal, colonies with the insert could be separated from colonies without an insert by blue-white screening. Insertion of foreign DNA inactivates α-complementation, the formation of a functional β-galactosidase that cleaves X-gal, and produces a stable blue compound (Sambrook et al. 2001). The blue colonies of vectors without the insert are easily recognizable from the white colonies with vectors containing the insert.

Transformation reaction:

1. 2 µl of the TOPO® cloning reaction was added to a tube with One Shot® DH5α™-T1 competent *E. coli* cells from Invitrogen, and mixed gently.
2. The reaction was incubated on ice for 5 to 30 minutes.
3. The cells were heat-shocked for 30 seconds at 42°C without shaking.
4. The tubes were immediately transferred to ice.
5. 250 µl of room tempered S.O.C. medium was added.
6. The tubes were capped and shaken horizontally at 200 rpm and 37°C for 1 hour.
7. 10 to 50 µl from each transformation was spread on preheated selective plates with 50 mg/ml ampicillin and 40 µl X-gal, and incubated overnight at 37°C.
8. Plates with colonies were stored at 4°C.

3.6 Isolating the plasmids using QIAprep® Spin Miniprep kit from QIAGEN:

The procedure is based on lysis of bacterial cells under alkaline conditions in NaOH/SDS (Buffer P2), leading to opening of the cell wall, denaturing and release of the cell contents. Optimal lysis time is important to allow maximum release of plasmid DNA, without release of chromosomal DNA. Addition of Buffer N3 neutralizes the lysate, and

adjusts it to high-salt binding conditions. Chromosomal DNA, denatured proteins, SDS and cellular debris will precipitate, and leave only plasmid DNA in solution to adsorb to the membrane of the QIAgen columns. The final step in the procedure is washing with Buffer PE, and elution of plasmid DNA with Buffer EB.

1. Four white colonies from the two agar plates were transferred to a Falcon tube each (A, B, C and D), containing 2 ml LB-medium with 50 mg/ml ampicillin, and incubated overnight at 37°C.
2. 1.5 ml of the overnight culture was centrifuged for 30 seconds at 12000 rpm in a MiniSpin microcentrifuge from Eppendorf.
3. The supernatant was removed and the bacterial cells were resuspended in 250 µl Buffer P1.
4. 250 µl Buffer P2 was added and the tube was gently inverted 4 to 6 times to mix, then left for 2 to 5 minutes in room temperature.
5. 350 µl Buffer N3 was added, and the tube was inverted immediately 4 to 6 times.
6. The tube was centrifuged for 10 minutes at 13000 rpm and the supernatant transferred to a QIAprep spin column.
7. The tube was centrifuged for 30 to 60 seconds at 13000 rpm, and the flow-through discarded.
8. The column was washed by adding 0.75 ml Buffer PE, and centrifuged for 30 to 60 seconds at 13000 rpm.
9. The flow-through was discarded, and the tube centrifuged for 1 minute at 13000 rpm to remove residual wash buffer.
10. The column was placed in a clean 1.5 ml microcentrifuge tube. 50 µl Buffer EB was added to the centre of the column, incubated for 1 minute, and then centrifuged 1 minute at 13000 rpm.
11. The eluted DNA was kept on ice until further use.

3.7 Determining the DNA copy number

The DNA content or copy number of the plasmids was determined by measuring their absorbance at 260 nm with the NanoDrop® ND-1000 Spectrophotometer from NanoDrop Technologies. An absorbance of 1 at 260 nm equals a concentration of dsDNA of approximately 50 µg/ml (Sambrook et al. 2001).

1) The sample pedestals were cleaned with water.
2) A water sample was loaded onto the lower pedestal and by pressing "start", the measuring initiated.
3) 1 µl of water was measured as a blank.
4) 1 µl of the sample was pipetted onto the lower measurement pedestal, the sampling arm was closed, and the sample measured.
5) The upper and lower pedestals were wiped with a soft laboratory wipe between each measuring to remove the last sample.
6) The number of copies of DNA per µl was then calculated by the formula:

$$\text{Copies/µl} = \frac{\text{OD260}}{\text{bp}} \times 5.302 \times 10^{13}$$

OD260 is the absorbance measured at 260 nm, bp is the number of base pairs in the template, and the number 5.302×10^{13} is a factor derived from the molecular mass of an average base pair, the concentration of the template from the OD260-measurement, and the Avogadro constant (Fronhoffs et al. 2002).

A stock solution of 1×10^8 copies/µl was made using the formula:

$$C_0 \times V_0 = C_1 \times V_1$$

C_0 is the measured concentration, V_0 is the start volume, C_1 is the wanted concentration and V_1 is the volume after the dilution.

3.8 Sequencing

To verify that the plasmids were correct, their nucleotide sequence was determined by PCR-sequencing. This method uses both regular deoxynucleotides (dNTPs) and a small proportion of fluorescent dideoxynucleotides (ddNTPs) labelled with fluorescent dyes. When a ddNTP is incorporated, the DNA replication is terminated because the absence of a 3´-OH group prevents the formation of a phosphodiester bond. The incorporation of a ddNTP can occur at any position in the template strand, and with several cycles there will be fragments of all possible lengths of the plasmid (Sambrook et al. 2001). The sequencing lab at the University separates the fragments by length, and determines the DNA sequence by measuring the fluorescent colour from the ddNTPs using a 3130*xl* Genetic Analyzer from Applied Biosystems.

Sequencing PCR:

5x sequencing buffer	4.0 µl
Premix (Big Dye v3.1)	2.0 µl
Primer (M13F, M13R and AN4+)	3.4 µl
Purified template (45 ng)	1.0 – 1.5 µl
Sterile water	9.1 – 9.6 µl
Total volume	20.0 µl

PCR conditions:

- Initial denaturing at 95°C for 15 minutes to activate the polymerase
- 25 cycles of: Denaturing at 95°C for 10 seconds
  Annealing at 50°C for 5 seconds
  Extension at 60°C for 4 minutes
- Final extension at 60°C for 4 minutes
- 4°C ∞

3.9 Linearization with BamHI and HindIII

Because PCR analysis of linear templates is more efficient than using circular templates, the plasmids were digested with the two restriction enzymes *HindIII* and *BamHI*. After restriction, the results were confirmed on an agarose gel.

1. The two reaction mixtures:

A. Sterile water	41.0 µl
NEBuffer for *BamHI*	5.0 µl
Bovine serum albumine (BSA)	0.5 µl
BamH1	0.5 µl
Circular template DNA (1×10^8 copies/µl)	3.0 µl
Total volume	50.0 µl
B. Sterile water	41.5 µl
1× NEBuffer 2	5.0 µl
HindIII	0.5 µl
Circular template DNA (1×10^8 copies/µl)	3.0 µl
Total volume	50.0 µl

2. The mixtures were incubated one hour at 37°C.
3. An agarose gel was made as describes in method 3.2, but with 1.6 g agarose in the first step, giving a final concentration of 1.6%. The samples run for 60 minutes at 90 V.

3.10 Purification of plasmids

The plasmids on the gel that were cut with *HindIII* were purified with GFX PCR DNA and Gel Band Purification Kit from Amersham Biosciences following the protocol (Amersham Biosciences 2004):

1. An empty 1.5 ml microcentrifuge tube was weighed to the nearest 10 mg.
2. The agarose containing the DNA band to be purified was cut out, cut into several smaller pieces, and then transferred to the pre-weighted microcentrifuge tube.
3. The tube with the agarose was weighed to the nearest 10 mg, and the weight of the empty tube subtracted.
4. 10 µl of capture buffer was added for each 10 mg gel.
5. The tube was closed and then mixed by vortexing. It was incubated at 60°C until the agarose was completely dissolved (5 to 15 minutes).
6. The tube was centrifuged in a MiniSpin microcentrifuge from Eppendorf to collect the sample at the bottom of the tube.
7. The sample was transferred to a GFX column in a collection tube, and incubated at room temperature for 1 minute before 30 seconds of centrifuging at full speed.
8. The flow-through was discarded, and the column placed back in the tube.
9. 500 µl of wash buffer was added, and the tubes centrifuged for 30 seconds.
10. The collection tube was discarded, and the column transferred to a clean 1.5 ml microcentrifuge tube.
11. 50 µl of elution buffer was applied, and then incubated at room temperature for 1 minute before centrifuging at full speed for 1 minute.

After elution, the DNA copy number of the plasmids was determined by NanoDrop as described in method 3.7, and a stock solution of 1×10^8 copies/µl of each plasmid was made and aliquoted in smaller bathes stored at -20°C. Before each analysis, a new batch was thawed and diluted 10-fold in sterile water to the proper concentration.

3.11 Standard curves

To see if the PCR was efficient, and in case quantification of unknown samples was to be done later, standard curves were made. The T315I BCR-ABL plasmid was analyzed by single step real-time PCR with TaqMan Master Mix in a series of 10-fold dilutions from 1×10^6 to 1×10^1 copies/µl with 315F, 315 pro and A315 mut as discriminating primer and T315I ND as non-discriminating primer.

The reaction setup:

TaqMan Universal PCR Master Mix	12.5 µl
Primers 315F and A315 mut or T315I ND (2 µM)	2.5 µl of each
Probe 315 pro (2 µM)	2.5 µl
Sterile water	to 23.0 µl
Template DNA (dilution series)	2.0 µl
Total volume	25.0 µl

After filling the vessels and closing the plate with the appropriate caps or foil, the plate was centrifuged for about 1 minute at 4000 rpm in the 5810R centrifuge from Eppendorf. The analysis was performed on an ABI Prism® 7900HT Fast Real-Time System from Applied Biosystems.

PCR conditions:

- 50°C for 2 minutes
- 95°C for 10 minutes
- 40-45 cycles of: 95°C for 15 seconds
  60°C for 1 minute

3.12 Detection of mutations in BCR-ABL

3.12.1 Single step real-time PCR

The real-time PCR system is based upon detection and quantification of a fluorescent reporter that increases proportionally to the total nucleic acid concentration. The fluorescent reporter can be in the form of a TaqMan hydrolysis probe that anneal to an internal sequence within the DNA fragment. As the *Taq* DNA polymerase replicates the template, its 5´ → 3´ exonuclease activity cleaves the probe, separates the fluorophore on the 5´ end from the quencher on the 3´ end, and fluorescence is released (Stratagene 2002; Logan et al. 2004). One can also use SYBR Green, a fluorescent dye that specifically binds the minor groove of double stranded DNA (dsDNA). The binding triggers a conformational change that results in greatly increased fluorescence, and accumulated PCR product can be detected at the end of each elongation step in the PCR. Since the dye binds all dsDNA and there are no target-specific probes, the specificity of the reaction is determined only by the specificity of the primers (Logan et al. 2004; Stratagene 2004).

- The wild-type and the mutated plasmids were diluted tenfold in sterile water to a concentration of 10^5 copies/µl and analyzed by single-step real-time PCR.
- The reactions were set up the same way as in method 3.11.
- The following reverse primers were used:
    - T315I-ND
    - The ARMS-primer A315 mut
    - The LNA-primers T315I-xR, -1R, -2R and -3R
    - The ARMS/LNA-primers T315I-1xR, -2xR, -3xR and -4xR.
- When using the TaqMan Universal PCR Master Mix, the primer 315F, which anneals to the ABL-part of the template and creates short amplicons, and the probe 315 pro was used, and the analysis was performed on the ABI Prism®

7900HT Fast Real-Time System from Applied Biosystems with the same PCR-conditions that were used in method 3.11.

- When using the 2×Brilliant® SYBR® Green QPCR Master Mix, the BCR(13)-F primer that anneals to the BCR-part of the template and creates long BCR-ABL-specific amplicons was used. No probe was added, and the amount of water was adjusted accordingly. The analysis was performed on the Mx3000P® QPCR System from Stratagene.

Temperature profile SYBR Green:

- Initial denaturing at 95°C for 10 minutes
- 40-50 cycles of: Denaturing at 95°C for 30 seconds
  Annealing at 55°C for 1 minute
  Extension at 72°C for 30 seconds
- 1 cycle of: Denaturing at 95°C for 1 minute
  Annealing at 55°C for 30 seconds
  Extension at 95°C for 30 seconds

3.12.2 FullVelocity PCR

The FullVelocity technology from Stratagene uses a DNA polymerase that allows for shorter PCR-steps and that tolerates temperature changes better than the *Taq* DNA polymerase. This makes it possible to reduce the amplification-time considerably (Stratagene 2004). The reaction was set up similar to method 3.10, with the 2× FullVelocity™ QPCR Master Mix and 0.5 µl FullVelocity™ enzyme per vessel, and run on an ABI Prism® 7900HT Fast Real-Time System from Applied Biosystems.

Primer/probe combinations:

1. BCR13F , ABL-R and ABL 2 pro
2. BCR(13)-F, T315I-ND and 315 pro
3. BCR(13)-F, A315 mut and 315 pro

PCR temperature profiles:

A. The conventional profile as used in method 3.11 (TaqMan).
B. Fast PCR program: 95°C for 5 minutes, then 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds.
C. Very fast PCR program: 95°C for 2 minutes, then 40 cycles of 95°C for 1 second and 60°C for 20 seconds.

3.12.3 Template mixtures

To make a more complex mixture, the T315I BCR-ABL template was mixed with a shorter BCR-ABL template (RG04) that only contained exon 1 to 6 of BCR and exon 2 to 7 of ABL. Since RG04 lacked the exon where the primer BCR(13)-F bound in the long assay, the amplification would be further specific towards the T315I BCR-ABL template.

- The shorter BCR-ABL template RG04 was OD-measured and diluted to 5×10^8 as in method 3.7, then further diluted to 5×10^7 copies/µl.
- 10 µl of the T315I-template (1×10^8 copies/µl) was diluted in 90 µl of the RG04-template (5×10^7 copies/µl), and the mixture was further diluted 10-fold in the RG04-template, to a concentration of 1×10^1 copies/µl of the T315I-template.
- The dilution series of the mixture were analyzed by real-time PCR, as in method 3.12.1.
- Primer/probe combinations:
    - SYBR Green: BCR(13)-F, A315 mut, T315I-1R and T315I-2R (long, BCR-ABL-specific amplicons)
    - TaqMan: 315F, 315 pro, A315 mut, T315I-1R and T315I-2R (short, BCR-ABL non-specific amplicons)
- The SYBR Green assay was used with the same PCR-conditions as in method 3.12.1.
- Temperature profile TaqMan:
    - Initial denaturing at 95°C for 10 minutes

- 40-50 cycles of: Denaturing at 95°C for 30 seconds
  Annealing at 60°C for 1 minute
  Extension at 72°C for 30 seconds

3.12.4 Nested PCR

To improve the sensitivity of the detection of the T315I mutation, a nested approach was used. The first PCR amplified the BCR-ABL templates, before the mutation specific PCR was done. Several different setups were done to find the optimal template concentration and number of cycles in the first PCR.

First PCR:

10× PCR buffer	5 µl
2 mM dNTP-mix	5 µl
2 µM Primers (BCR(13)-F and A7-)	5 µl of each
Template ($1\times10^{4\text{-}5}$ copies/µl)	5 µl
Sterile water	24.5 µl
HotStarTaq™ DNA polymerase	0.5 µl
Total volume	50 µl

PCR conditions:

- Initial denaturing at 95°C for 10 minutes
- 2 cycles of: Denaturing at 94°C for 1 minute
  Annealing at 50°C for 30 seconds
  Extension at 72°C for 30 seconds
- 18-38 cycles of: Denaturing at 94°C for 1 minute
  Annealing at 60°C for 30 seconds
  Extension at 72°C for 30 seconds
- Final extension at 72°C for 7 minutes
- 4°C ∞

Some of the PCR product was analysed by gel electrophoresis as in method 3.3, while the rest was purified as in method 3.6. The DNA copy number was determined by NanoDrop, and the plasmids diluted as in method 3.7. The plasmids were then analyzed by real-time PCR with TaqMan and SYBR Green I chemistry as in method 3.12.1.

3.12.5 Creating a patient-test for detection of the T315I-mutation

Since mutation-detection in patient-samples can be different than in plasmids, several samples from CML-patients containing wild-type and T315I BCR-ABL were analysed by some of the earlier used methods. In the single-step procedures, both the TaqMan and the SYBR Green Master Mix were used, with primers creating short and long amplicons, respectively. In the nested approach, only the SYBR Green Master Mix was used, due to the results from the plasmids, where this Master Mix combined with primers creating long BCR-ABL-specific amplicons seemed to give the greatest sensitivity and specificity.

The single-step procedure (as in method 3.12.1):

- Two different samples, one with wild-type BCR-ABL and one with T315I BCR-ABL were analysed undiluted, with the same primer pairs as in method 3.12.1.

The nested SYBR Green procedure (as in method 3.12.4):

- Three patient-samples containing T315I BCR-ABL and one containing wild-type BCR-ABL were used in this nested approach.
- Dilution of the samples to 100%, 10%, 1% and 0.1% BCR-ABL before the first PCR indicated that the undiluted samples gave best results.
- The number of cycles in the first PCR was varied from 20 to 40, and 40 cycles seemed to give the best results.
- After gel electrophoresis and purification of the PCR product, it was diluted to a concentrations of 1×10^7 templates/µl. The second PCR was performed with SYBR Green master mix and the primers BCR(13)-F, A315 mut, T315I-xR, -1R,

-2R and -3R. The primers with combined LNA- and ARMS-technology were excluded because of the poor sensitivity shown in earlier tests.

3.12.6 Translating the patient-test to other mutations than T315I

To see if the results derived from detection of the T315I-mutation in patient-samples could be used for detection of other mutations, samples from CML-patients with different mutations were analyzed in a nested approach like in method 3.12.4. As in method 3.12.5, using undiluted samples and 40 cycles in the first PCR gave the best results.

- The first PCR was carried out with undiluted samples, and with 40 cycles.
- The first PCR product was analyzed by gel electrophoresis as in method 3.3 and purified as in method 3.6, before dilution to 1×10^7 copies/µl.
- The second PCR was performed with SYBR Green Master Mix with the primers G250E-xR, Q252Hc-xR, Y253H-xR, E255K-xR and M351T-xR.
- All the primers had a LNA-nucleotide in 3`-position and no additional mismatches, because this primer (T315I-xR) gave the greatest sensitivity and specificity in method 3.12.5.

4 Results

4.1 Amplification of cDNA by PCR and cloning of the PCR product

In order to obtain templates for PCR cloning of BCR-ABL fragments, cDNA-samples from two patients were amplified. Samples from patient A contained wild-type BCR-ABL sequences, whereas samples B contained BCR-ABL with the C → T mutation encoding T315I. As positive control a plasmid containing BCR-ABL was used. The PCR products were then analyzed on an agarose gel to confirm that the lengths of the fragments were correct. Figure 12 shows that all the samples were amplified, giving bands of the expected size, 1293 bp.

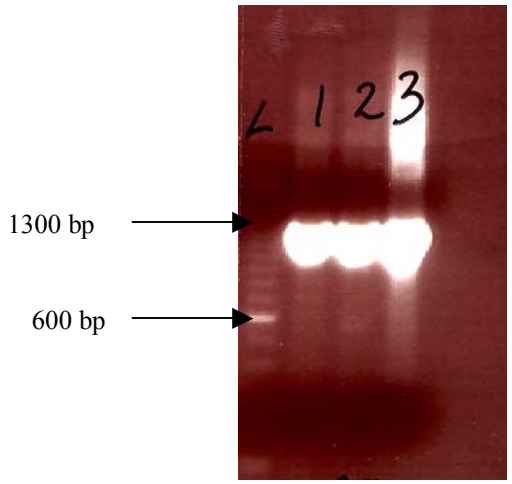

Figure 12: Gel electrophoresis of the first PCR product confirmed that the correct products of 1293 bp had been amplified. L) The 100 bp DNA Ladder; 1) sample A with wild-type BCR-ABL; 2) sample B with T315I BCR-ABL; 3) positive control, a BCR-ABL plasmid. The PCR primers BCR(13)-F and A7- (table 2) were used, creating products of 1293 bp. The PCR products were run for 30 minutes at 90 V on a 0.7% agarose gel.

After the PCR products were verified by gel electrophoresis, sample 1 (from patient A) and 2 (from patient B) were cloned into the pCR®2.1-TOPO® vector by T/A cloning and transformed into the bacteria *E. coli* DH5α. Because the vector contained genes for

ampicillin-resistance, only bacteria with the vector inserted grew on agar plates with ampicillin. The use of X-gal made it easy to separate colonies containing vector with insert (white) from colonies containing a vector without insert (blue). Four white colonies from each of the two plates were picked and cultivated in LB+ medium, then purified by the QIAprep Spin Miniprep Kit from QIAGEN. The plasmids were OD-measured at 260 nm to determine their DNA content (table 12). To confirm that they had the BCR-ABL inserts, they were in addition sequenced with M13F, M13R and AN4+ primers. Figure 13 shows critical parts of the correct sequence of the wild-type and the T315I BCR-ABL template.

Table 12: The results from the OD-measuring of the plasmids. Samples 1A - 1D contain the wild-type sequence from patient A, and samples 2A - 2D contain the T315I-mutated sequence from patient B.

Sample	Absorption at 260 nm	Content of dsDNA (μg/ml)	Copy count/μl
1A	0.232	232	8.21×10^{9}
1B	0.208	208	7.36×10^{9}
1C	0.370	370	1.31×10^{10}
1D	0.388	388	1.37×10^{10}
2A	0.334	334	1.18×10^{10}
2B	0.372	372	1.32×10^{10}
2C	0.376	376	1.33×10^{10}
2D	0.357	357	1.26×10^{10}

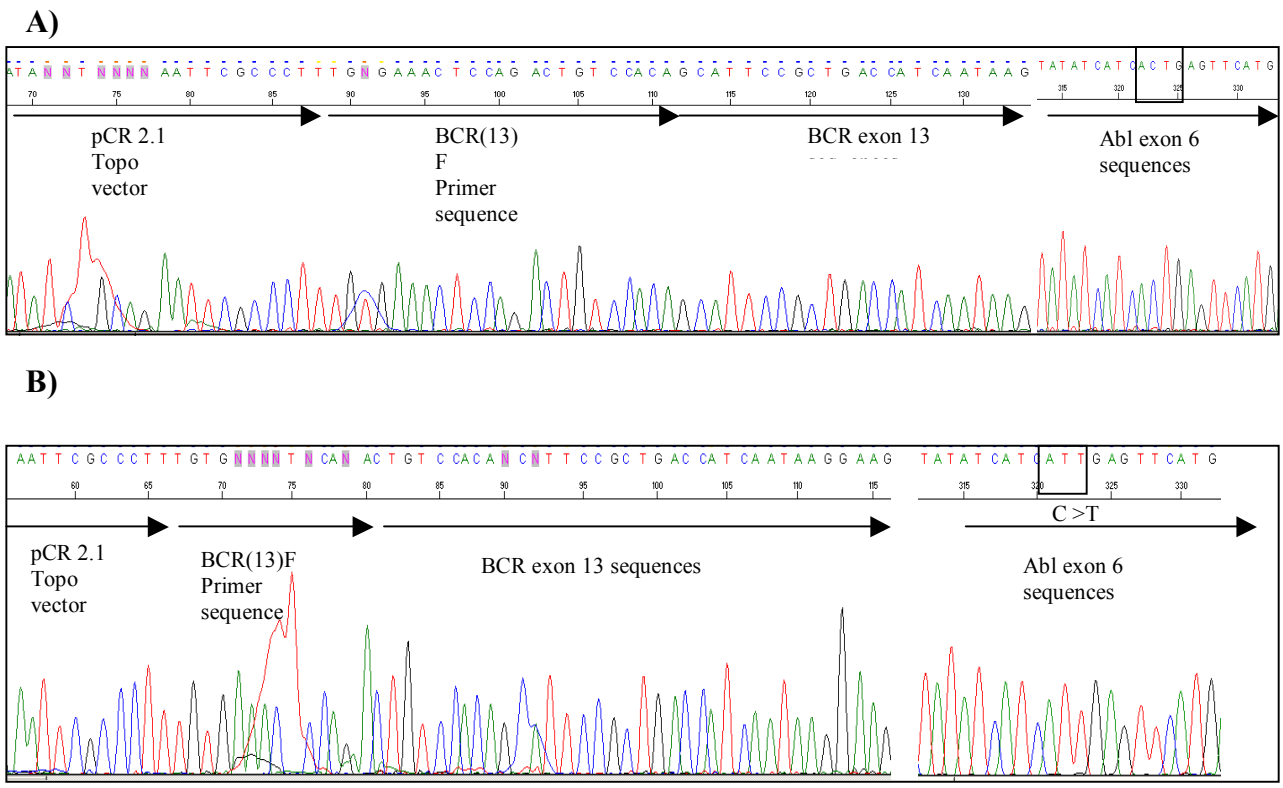

Figure 13: Critical parts of the sequence of the BCR-ABL templates. The figure shows the sequence where the pCR 2.1 Topo vector goes over into the BCR-ABL insert, the sequence where the BCR(13)-F primer binds, a part of BCR exon 13, and the part of exon 6 in ABL where the important C→T substitution in base number 1308 can occur, inducing the T315I-mutation. A) Sequences of the wild-type plasmid 1A. As seen in the box in the ABL exon 6 sequence, the base 1308C is un-substituted. B) Sequences of the 2A-plasmid containing the T315I-mutation. Base number 1308 is substituted with a T, as seen in the box in the ABL exon 6 sequence, giving rise to the T315I-mutation.

4.2 Linearization of the plasmids with BamHI and HindIII

The polymerase chain reaction is more efficient when using linear templates rather than circular ones, therefore the plasmids made from patient samples A and B had to be linearized. The restriction enzymes *HindIII* and *BamHI* were used to linearize the plasmids. Gel electrophoresis (figure 14) showed that the cutting with *HindIII* was successful, creating one band of approximately the expected size of 4600 bp, while *BamHI* had not cut all of the plasmids. Faster migrating bands below the bands of the linear plasmid indicated that some of the plasmids were uncut and perhaps supercoiled.

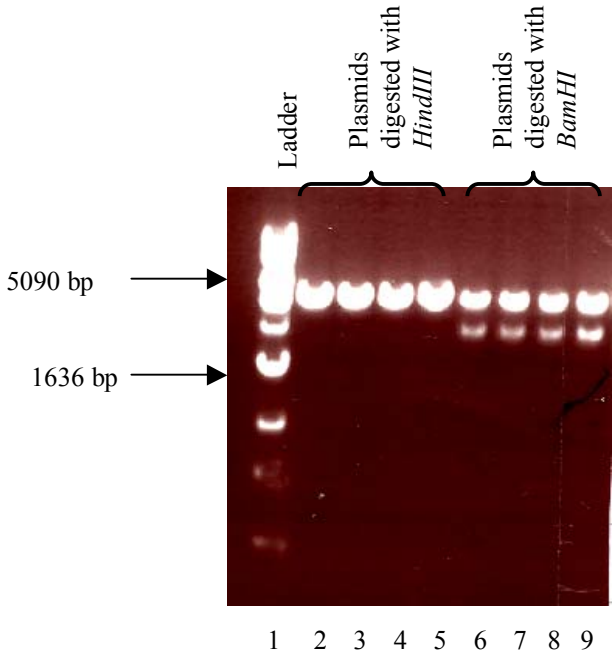

Figure 14: The linearization of the plasmids was confirmed with gel electrophoresis. Lane 1 contains the 1 kb DNA Ladder. The single bands in lane 2 to 5 represent plasmids cut with *HindIII* (plasmid 1A, 1B, 2A and 2B) giving a band of approximately 4600 bp, and the double bands in lane 6 to 9 represent plasmids cut with *BamHI* (plasmid 1A, 1B, 2A and 2B). The faster migrating bands in lane 6 to 9 could be un-cut plasmids in supercoils. The linearization products were run on a 1.6 % agarose gel for 60 minutes at 90 V.

The gel-signals derived from PCR products digested with *HindIII* were cut out and purified using the GFX PCR DNA and Gel Band Purification Kit from Amersham Biosciences. The resulting batch of linearized plasmid was again measured for OD260 and the copy number was calculated as described in method 3.7. The linear plasmids were in the following used as templates for T315I-mutation targeted assays.

4.3 Standard curves

To find out if the PCR was efficient, and in case later quantifying should be necessary, standard curves were made (figure 15). A series of 10-fold dilutions from 1×10^6 to 1×10^1 copies/µl for the mutated template were used in a real-time PCR assay using a discriminating (D) and a non-discriminating (ND) primer.

If the PCR was 100 percent effective, the number of templates would double each cycle, and it would take approximately 3.3 cycles to increase the number of templates by a factor of ten. The slope of the standard curve should therefore ideally be -3.3. Figure 15 shows that these standard curves had a slope of -3.6, indicating that the PCR was close to, but not 100 percent efficient.

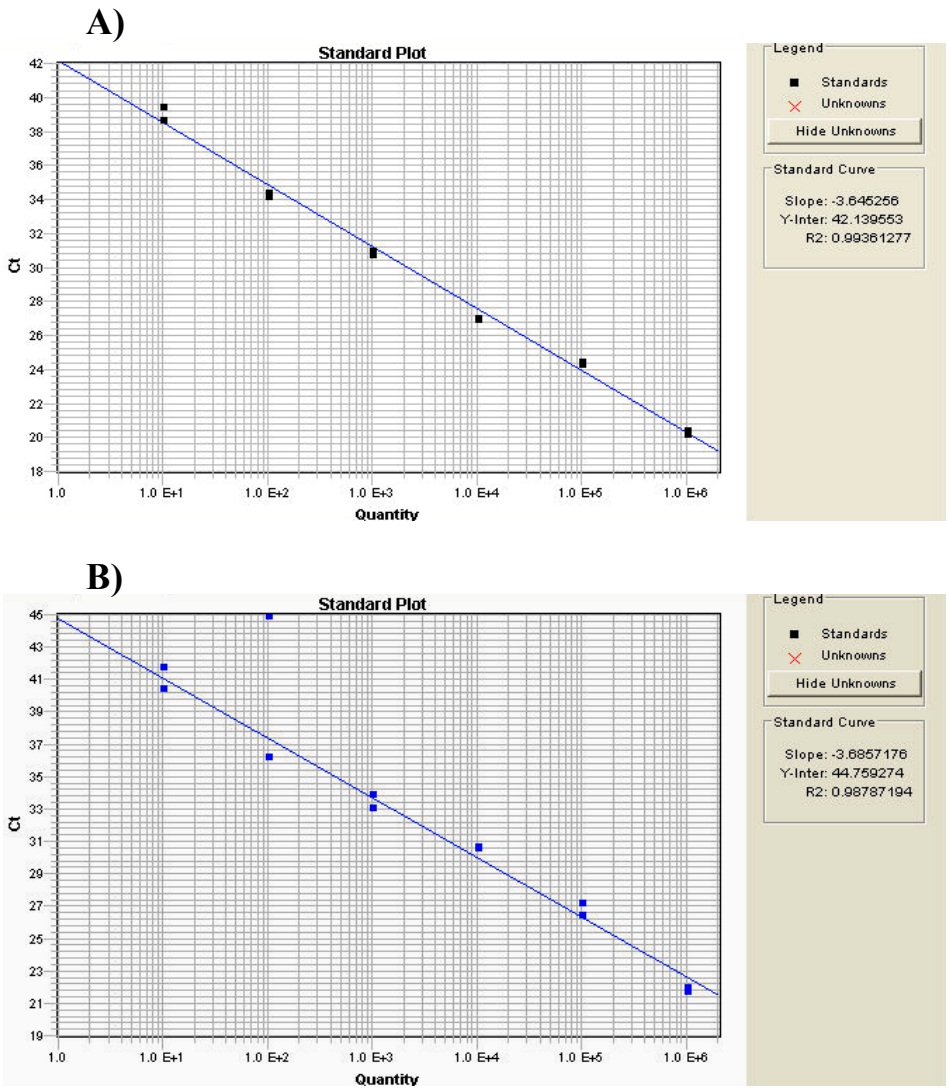

Figure 15: Standard plots. The T315I BCR-ABL template was used in concentrations from 1×10^{6} to 1×10^{1} copies/µl to make standard curves. A) the standard curve using the discriminating primer A315 mut; B) the standard curve using the non-discriminating primer T315I-ND. If the PCR was 100 percent efficient, the slope of the curves would have been -3.3. Both the curves in this experiment had a slope of -3.6, indicating that the PCR was not completely efficient.

4.4 Detection of the T315I-mutation in plasmids

To increase the sensitivity and the specificity of a real-time PCR approach for detection of BCR-ABL mutations, different types of primers were tested in an evaluation system based on the linear plasmids containing wild-type and mutated sequences. As illustrated figure 16, CT_{MAT}, CT_{MIS} and ΔCt were used as primary endpoints in the study. The goal was to maximize the difference in amplification of the matching versus the mismatching template (ΔCt). Primer combinations resulting in maximal ΔCt were evaluated as to be best suited for an assay for detection of the T315I-mutated allele. As a secondary endpoint, different amplicon lengths and PCR Master Mixes, and their influence on the discriminating effect of primer combinations was tested. For further improvement of the sensitivity, a nested approach including an additional PCR step was also tested.

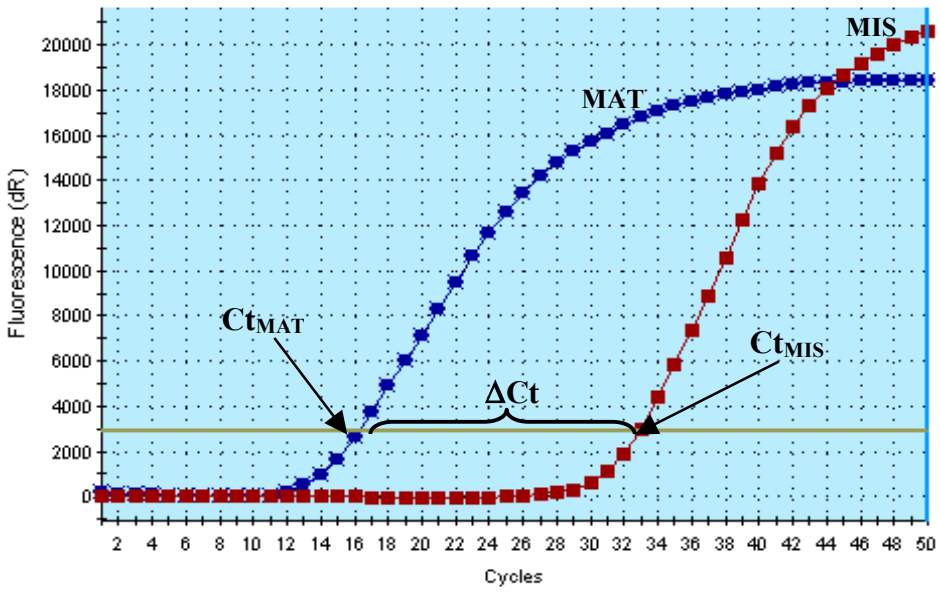

Figure 16. Amplification plot illustrating the primary endpoints in the study. MAT, the amplification curve of the matching template; MIS, the amplification curve of the mismatched template; threshold cycle (Ct), the cycle where fluorescence is statistically above the background; Ct_{MAT}, the cycle where amplification of the matching template reaches Ct; Ct_{MIS}, the cycle where amplification of the mismatching template reaches Ct; ΔC, the difference between amplification of the matching and the mismatching templates (Ct_{MIS} - Ct_{MAT}).

4.4.1 The single-step approach

The plasmids were analysed by real-time PCR with different primer-combinations and Master Mixes. The TaqMan Master Mix was used together with primers pairs that created short amplicons, whereas the SYBR Green Master Mix was used with primer pairs that created long and BCR-ABL-specific amplicons (see figure 9). Table 13 shows the results from the single-step procedures using TaqMan and SYBR Green Master Mixes, with the Ct-values of the matching (MAT, T315I-mutated sequence) and the mismatching (MIS, wild-type sequence) template, as well as the difference between them (ΔCt, also shown in figure 17). A high ΔCt indicates that the primer has great discrimination capacity between the templates, whereas a negative value indicates that the mismatching template was amplified before the matching template.

When using the TaqMan Master Mix, the A315 mut primer gave the best sensitivity. Moving the mismatch closer to the 3´-end of the primer or introducing a LNA-nucleotide in most cases deteriorated the sensitivity, but the specificity (ΔCt) increased, meaning that the modified primers could better discriminate between the mutated and wild-type sequences. When using the SYBR Green Master mix, the sensitivity was reduced compared to the TaqMan Master Mix, but the selectivity was increased for most of the primers.

Table 13: Results from the primer evaluation using the single-step PCR in plasmids. The table gives the Ct value for the matching (MAT, T315I-BCR-ABL) and the mismatching (MIS, wild-type BCR-ABL) templates, and the difference between them (ΔCt). In the TaqMan (BCR-ABL non-specific) assay, the forward primer 315F and the probe 315pro were used. In the SYBR Green (BCR-ABL-specific) assay, the forward primer BCR(13)-F was used. As well as a non-discriminating (ND) reverse primer, several types of discriminating primers were used; LNA-primers, ARMS-primers, and combinations of the two. The PCR was run with a template concentration of 1×10^5 copies/µl for 40 cycles. Each value is the average of two parallels. – indicates that no amplification was detected, and a blank cell indicates that the primer was not used in the particular experiment.

		Single-step TaqMan			**Single-step SYBR Green**		
Primer type	Primer	Ct_{MAT}	Ct_{MIS}	ΔCt	Ct_{MAT}	Ct_{MIS}	ΔCt
ND	T315I-ND				24.8	21.5	-3.3
ARMS	A315 mut	18.9	29.6	10.7	25.1	35.1	10.0
LNA	T315I-xR	27.2	37.3	10.1	35.5	-	-
	T315I-1R	25.5	38.8	13.3	24.6	37.2	12.6
	T315I-2R	19.1	29.2	10.1	25.5	38.8	13.3
	T315I-3R	22.7	32.7	10.0	28.3	-	-
ARMS + LNA	T315I-1xR	27.9	38.9	11.0	-	-	-
	T315I-2xR	-	-	-	-	-	-
	T315I-3xR	28.1	38.8	10.7	36.8	-	-
	T315I-4xR	-	-	-	-	-	-

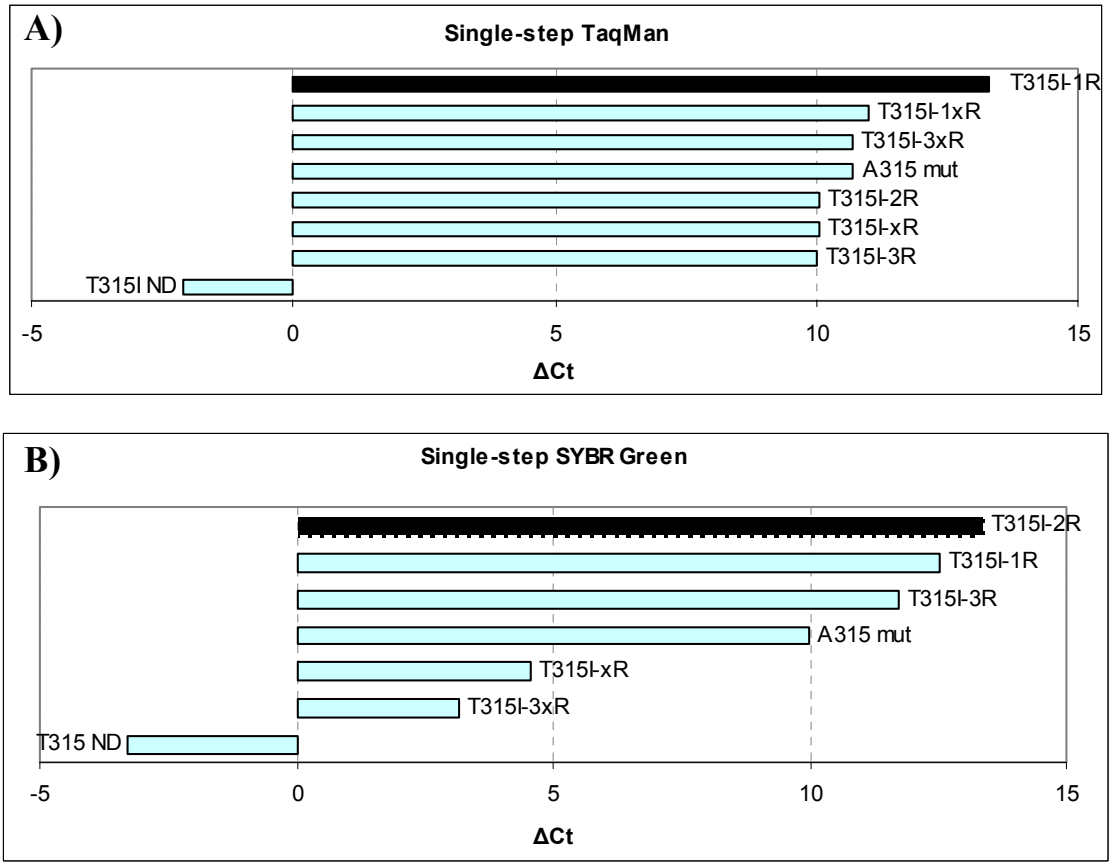

Figure 17: ΔCt values from the primer evaluation with plasmids in a single-step approach. The figure shows the difference in amplification (ΔCt) of the matching (T315I BCR-ABL) and the mismatching (wild-type BCR-ABL) template when using a single-step approach (see table 13). A) The ΔCt-values for the primers when using the TaqMan Master Mix and BCR-ABL non-specific primer pairs, where the LNA-primer T315I-1R gave the highest ΔCt; B) The ΔCt-values for the primers when using SYBR Green Master Mix and BCR-ABL-specific primer pairs, where the LNA-primers T315I-2R, -1R and -3R gave the highest ΔCt. A high ΔCt indicates that the primer has great discrimination capacity between the templates. The primer with highest ΔCt is shown in black. A negative value means that the mismatching template was amplified before the matching one, as with primer T315I-ND, which is a non-discriminating primer.

4.4.2 The nested approach

In an effort to increase the sensitivity, a nested approach with two PCR-steps was tested, with different primer-combinations, amplicon lengths and Master Mixes. The results are shown in table 14, with the Ct-values for the matching (MAT, T315I BCR-ABL) and the mismatching (MIS, wild-type BCR-ABL) templates, and the difference between them (ΔCt, also shown in figure 18). The primer-evaluation showed that the ARMS- and LNA-primers decreased the sensitivity and increased the specificity compared to the A315 mut primer. The SYBR Green Master Mix with the BCR-ABL-specific primer pairs gave a slightly better sensitivity than the TaqMan Master Mix, and the specificity (ΔCt) increased greatly, especially for the LNA-primers.

Table 14: Results from the primer evaluation using the nested approach in plasmids. The table gives the Ct value for the matching (T315I BCR-ABL) and the mismatching (wild-type BCR-ABL) templates, and the difference between them (ΔCt). A template concentration of 1×10^5 copies/µl and 40 cycles was used in the first PCR, with the primers BCR(13)-F and A7-. In the second PCR of 50 cycles, a template concentration of 1×10^7 copies/µl was used with the forward primers 315F creating short amplicons (TaqMan) and BCR(13)-F creating BCR-ABL-specific amplicons (SYBR Green). – indicates that no amplification was detected.

		Nested PCR TaqMan			**Nested PCR SYBR Green**		
Primer type	Primer	Ct_{MAT}	Ct_{MIS}	ΔCt	Ct_{MAT}	Ct_{MIS}	ΔCt
ND	T315I-ND	18.6	17.7	-0.9	17.0	18.6	1.6
ARMS	A315mut	19.6	33.3	13.7	19.8	29.9	10.1
LNA	T315I-xR	27.6	38.3	10.7	25.9	42.0	16.1
	T315I-1R	25.0	38.1	13.1	23.0	36.3	13.3
	T315I-2R	18.7	28.5	9.8	21.3	35.5	14.2
	T315I-3R	23.0	36.6	13.6	22.5	39.4	16.9
ARMS + LNA	T315I-1xR	27.0	37.3	10.3	35.7	-	-
	T315I-2xR	47.0	-	-	43.7	-	-
	T315I-3xR	27.1	38.4	11.3	28.1	41.5	13.4
	T315I-4xR	-	-	-	43.4	-	-

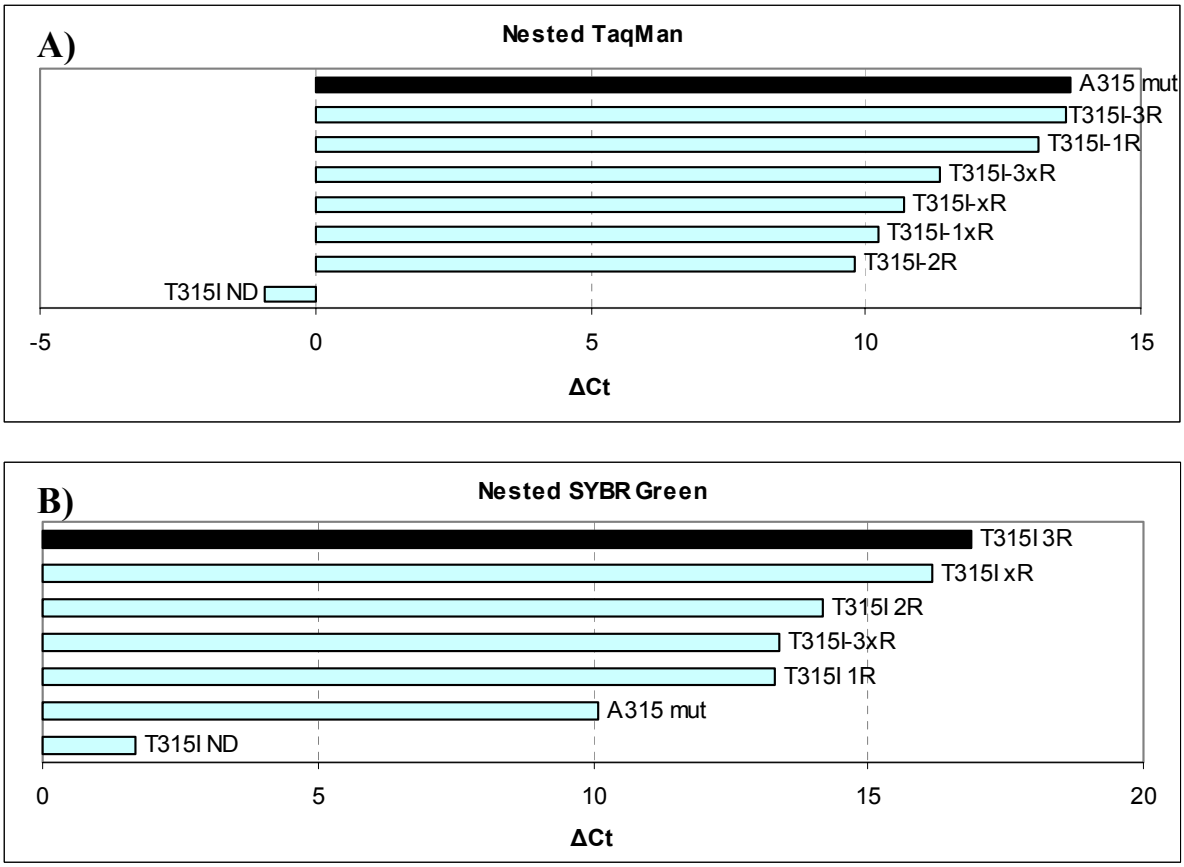

Figure 18: ΔCt values from the primer-evaluation with plasmids in a nested approach. The figure shows the difference in amplification (ΔCt) of the matching (T315I BCR-ABL) and the mismatching (wild-type BCR-ABL) template, when using a nested approach (see table 14). A) The ΔCt-values when using the TaqMan Master Mix and primer pairs creating short amplicons, where the ARMS-primer A315 mut and the LNA-primers T315I-3R and -1R gave the highest ΔCt; B) The ΔCt-values when using the SYBR Green Master Mix and primer pairs creating BCR-ABL-specific amplicons, where the LNA-primers T315I-3R and –xR gave the highest ΔCt. The primer with highest ΔCt is shown in black. A negative value means that the mismatching template was amplified before the matching one, as with primer T315I-ND, which is a non-discriminating primer.

4.4.3 Comparison between the single-step and the nested approach

To compare the specificity of the single-step and the nested approach, the ΔCt for each primer analysed by single-step PCR was subtracted from the ΔCt for the primers analysed by nested PCR. The resulting number was called ΔΔCt, and describes what can be gained (or loss) by using a nested PCR instead of a single-step PCR (figure 19). A negative

ΔΔCt indicates that the single-step procedure was more specific than the nested approach. The LNA-primer T315I-3R and the ARMS-primer A315 mut gained the most in specificity when changing to a nested approach with the TaqMan assay. The greatest improvement was observed when using the SYBR Green Master Mix. The LNA-primer T315I-xR and the ARMS/LNA-primer T315I-3xR gained the most in specificity when changing to a nested approach with this Master Mix.

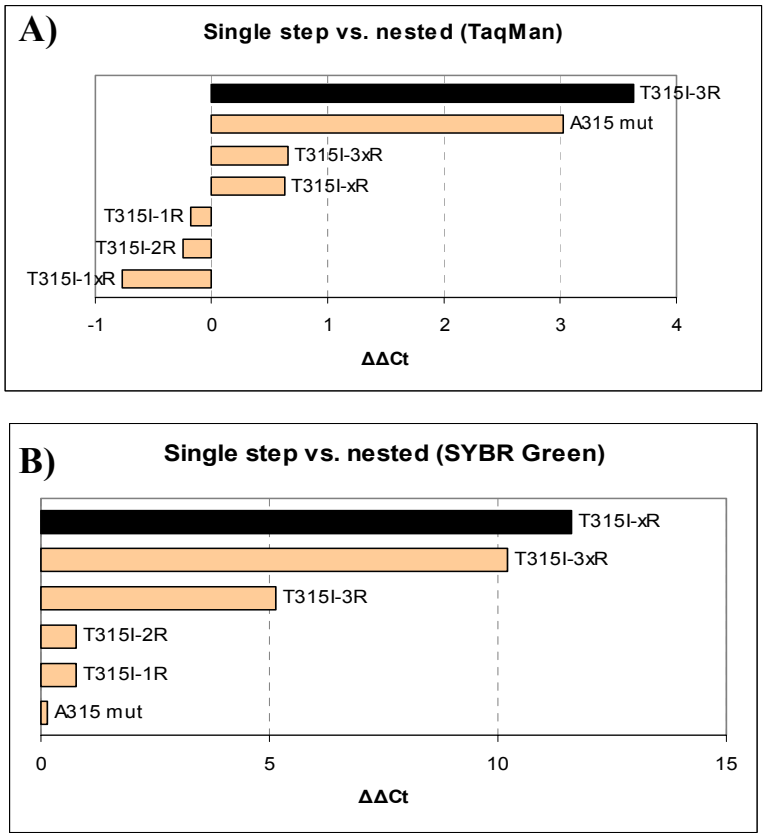

Figure 19. The difference in ΔCt values (ΔΔCt) between the single-step and the nested approach for plasmids. ΔΔCt, the difference in ΔCt from the single-step to the nested approach (see tables 13 and 14), describes what can be gained in specificity when changing to a nested approach. A) The ΔΔCt values with the TaqMan Master Mix and primer pairs creating short amplicons.; B) The ΔΔCt values with the SYBR Green Master Mix and primer pairs creating BCR-ABL-specific amplicons. The primer with the greatest ΔΔCt is shown in black. A negative ΔΔCt indicates that the single-step procedure was more specific than the nested approach.

4.5 Designing a patient-test for detection of the T315I-mutation

Patient-samples were used as templates for detection of the T315I-mutation, to find out if the methods used on plasmids also could be used for patient-samples. Table 15 shows that the single-step BCR-ABL non-specific procedure using TaqMan Master Mix gave an inadequate sensitivity, whereas the single-step BCR-ABL-specific SYBR Green approach gave a better sensitivity, but no improvement in specificity compared to the TaqMan assay. Including an additional step with the nested approach improved both the sensitivity and the specificity greatly, and gave the best results. Since the BCR-ABL-specific assay with SYBR Green Master Mix had given better specificity in patient-samples and plasmids earlier, and because there was not enough patient-material, the short TaqMan assay was not used for analysing patient-samples.

Figure 20 shows the specificity (ΔCt) of the primers in the different experiments. The ARMS-primer A315 mut and the LNA-primer T315I-2R gave the highest ΔCt values in the single-step approaches, while the LNA primers T315I-xR and -3R was gave the best ΔCt with the nested approach.

Table 15: Results from the primer evaluation with patient-samples. The table gives the Ct values for the matching (MAT, T315I BCR-ABL) and the mismatching (MIS, wild-type BCR-ABL), and the difference between them (ΔCt). The forward primers used were 315F for the TaqMan assays, creating short amplicons, and BCR(13)-F for the SYBR Green assays, creating long BCR-ABL-specific amplicons. The single-step PCR was performed with undiluted patient-samples, while the nested PCR was performed with undiluted samples in the first step of 40 cycles, and 2 µl template of 1×10^7 copies/µl in the second step. Each value is the average of two parallels. – indicates that no amplification was detected, and a blank cell indicates that the primer was not used in the particular experiment.

		Single-step TaqMan			**Single-step SYBR Green**			**Nested SYBR Green**		
Primer type	Primer	Ct MAT	Ct MIS	ΔCt	Ct MAT	Ct MIS	ΔCt	Ct MAT	Ct MIS	ΔCt
ND	T315I-ND	24.9	24.4	-0.5	15.5	15.1	-0.3			
ARMS	A315	27.5	43.7	16.2	16.7	27.1	10.4	17.5	32.9	15.4
LNA	T315I-xR	38.5	-	-	30.6	-	-	25.3	44.2	18.9
	T315I-1R	32.6	-	-				17.5	30.8	13.3
	T315I-2R	26.0	40.4	14.4	18.9	30.6	11.7	20.0	35.3	15.3
	T315I-3R	30.4	-	-				21.2	38.7	17.5
ARMS+LNA	T315I-1xR	45.0	-	-						
	T315I-2xR	-	-	-	-	-	-			
	T315I-3xR	41.7	-	-						
	T315I-4xR	-	-	-	-	-	-			

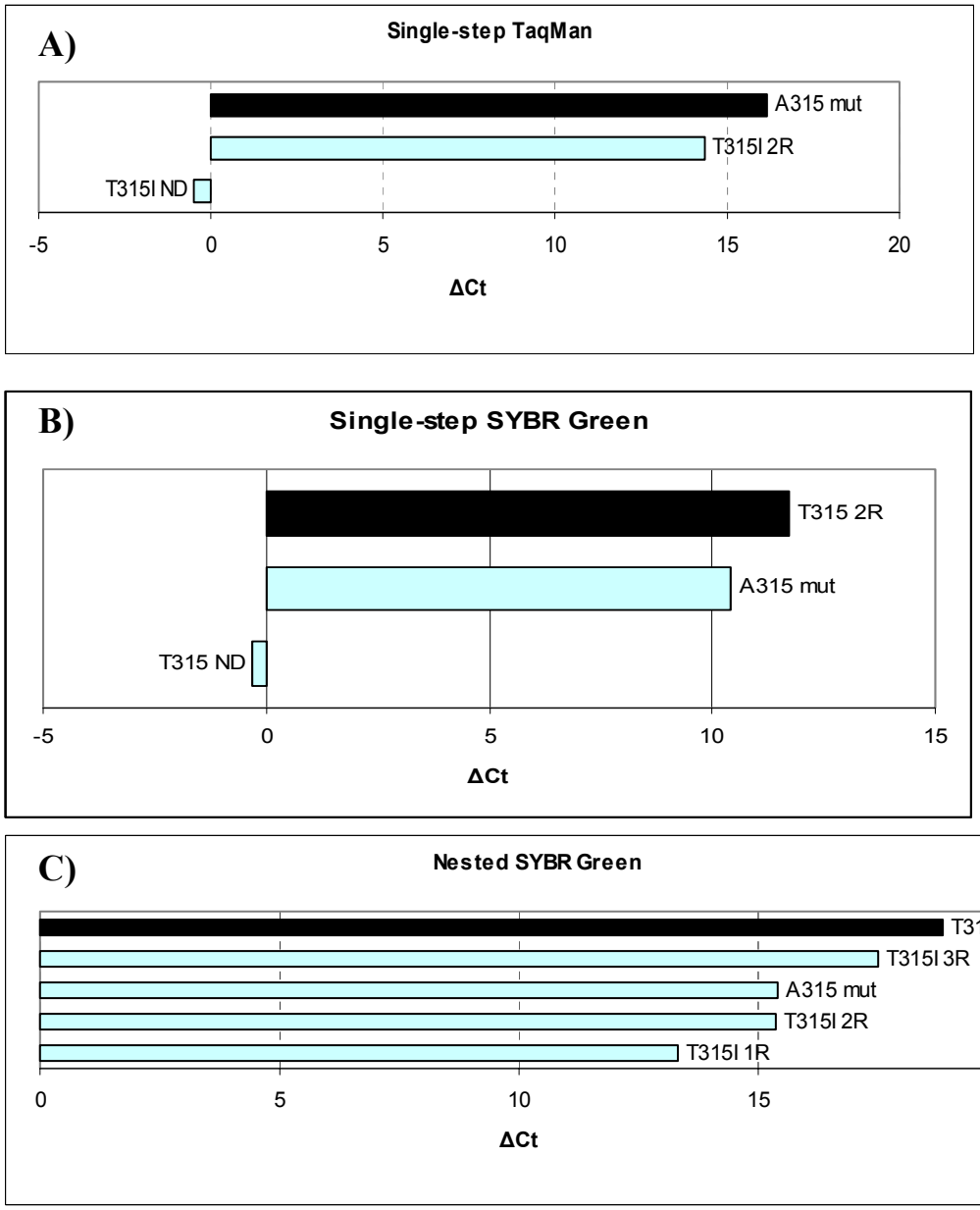

Figure 20: ΔCt values from the primer evaluation using the nested approach in patient-samples. The figure shows the difference in amplification (ΔCt) of the matching (T315I BCR-ABL) and the mismatching (wild-type BCR-ABL) template (template 15). A) The primers A315 mut and T315I-2R gave the highest ΔCt values with the single-step TaqMan (BCR-ABL non-specific) approach; B) The primers T315I-2R and A315 mut gave the greatest ΔCt with the single-step SYBR Green (BCR-ABL-specific) approach; C) The LNA-primers T315I-xR and -3R gave the highest ΔCt values with the nested SYBR Green (BCR-ABL-specific) approach. The primer with highest ΔCt is shown in black. A negative value means that the mismatching template was amplified before the matching one.

4.5 FullVelocity PCR

To reduce the amplification-times of the PCR, so that more samples could be analyzed in shorter time, the FullVelocity™ QRT-PCR Master Mix kit from Stratagene was tested under different PCR-conditions and with different primer combinations. The kit is designed for high-speed, one-step PCR, and promises a more than 40 percent reduction of overall run times (Stratagene 2004). Table 16 shows however, that the FullVelocity kit only gave amplification during conventional PCR-conditions, and not during the faster programs. The reason for this could be that the templates were too long, 1293 bp, when the recommended template length was 90 to 300 bp.

Table 16: Results from PCR using the FullVelocity kit with different PCR-conditions. Amplification was only detected when using the conventional PCR-program, and not with the faster times recommended for the FullVelocity kit. The plasmids were used in concentrations of 1×10^5 copies/µl. – indicates that no amplification could be detected.

Primer/probe combination	Template	PCR conditions	Ct value
BCR(13)-F, 315pro, T315I-ND	T315I-BCR-ABL	Conventional PCR program	18.1
BCR13F, ABL 2 pro, ABL-R	T315I-BCRABL	Conventional PCR program	19,3
BCR(13)-F, 315 pro, T315I-ND	T315I-BCR-ABL	Fast PCR program	-
	Wild-type BCR-ABL	Fast PCR program	-
BCR(13)-F, 315 pro, A315 mut	T315I-BCR-ABL	Fast PCR program	-
	Wild-type BCR-ABL	Fast PCR program	-
BCR(13)-F, 315 pro, T315I-ND	T315I-BCR-ABL	Very fast PCR program	-
	Wild-type BCR-ABL	Very fast PCR program	-
BCR(13)-F, 315 pro, A315I mut	T315I-BCR-ABL	Very fast PCR program	-
	Wild-type BCR-ABL	Very fast PCR program	-

4.6 Detection of the T315I-mutation in mixed templates

To mimic the in vivo situations, a mixture of templates was made in order to create a background in the amplification plots, and to make the samples more complex. This was done by mixing the mutated plasmid with a shorter template, RG04, and analysing dilution series of the mixture with different primer combinations and Master Mixes. The TaqMan Master Mix was used together with the forward primer 315F, which anneals to the ABL-part of the template (see figure 9). In combination with the mutation-specific reverse primers, this will create short and BCR-ABL non-specific amplicons. When the forward primer BCR(13)-F, which anneals to the BCR-part of the template (see figure 9), was used in combination with mutation-specific reverse primers, long and BCR-ABL specific amplicons were produced. In this case, the SYBR Green Master Mix was used, due to the inability of the TaqMan Master Mix to create such long templates. The reason for this is that the exonucleolytic activity of the DNA polymerase on the TaqMan probe would be too slow to complete the amplification before the next step in the PCR starts.

Figure 21 illustrates that the amplification curves of the short amplicons are overlapping, while the amplification curves for the first two concentrations of the long amplicons can be separated from the background. This indicates that by making the amplification BCR-ABL-specific, the sensitivity could be increased by a 2-log factor.

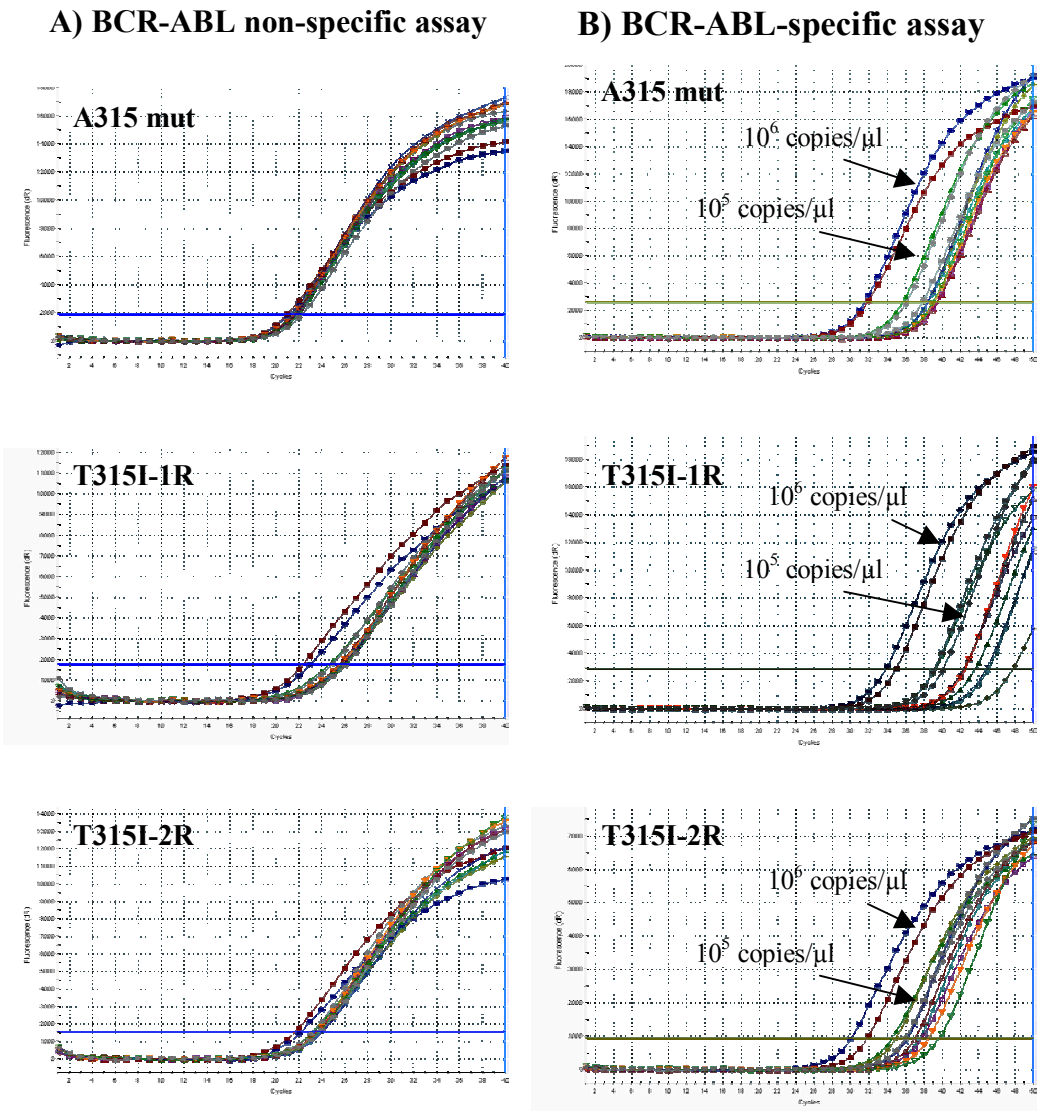

Figure 21. Amplification plots of mixtures of long and short templates. Amplification plots of a 10-fold dilution series of the T315I BCR-ABL template (1×10^6 to 1×10^1 copies/µl) in the RG04 template (1×10^7 copies/µl) using the three different reverse primers A315 mut, T315I-1R and -2R. A) Amplification of short templates with a BCR-ABL non-specific assay using TaqMan Master Mix, the three reverse primers and 315F forward primer that anneals to the ABL-allele. The amplification curves are overlapping, indicating that there is a mis-amplification of non-mutated templates; B) Amplification of long templates with a BCR-ABL-specific assay using SYBR Green Master Mix, the three reverse primers and the BCR(13)-F forward primer that anneals to the BCR-allele. There is a specific amplification of mutated templates at the concentrations 1×10^6 and 1×10^5 copies/µl, indicating that the sensitivity can be increased by a 2-log factor when using a BCR-ABL specific primer pair and SYBR Green technology.

4.7 Detection of other mutations than T315I

The procedure that detected the T315I-mutation with the highest sensitivity and specificity in patient-samples, a nested BCR-ABL-specific approach with a primer containing a LNA-nucleotide in 3` position (see table 15), was also tested in samples containing other mutations. Suitable primer was ordered (E255K-xR, G250E-xR, Q252Hc-xR, Y253H-xR and M351T-xR), and the test was set up with a panel of five unknown patient-samples. The samples were amplified undiluted in the first PCR of 40 cycles, and a part the PCR products was analyzed on an agarose gel seen in figure 22. The rest of the PCR products were purified and diluted to a concentration of 1×10^7 copies/µl. Each sample was then analyzed by real-time PCR with BCR(13)-F, SYBR Green Master Mix and each of the LNA-primers (table 17).

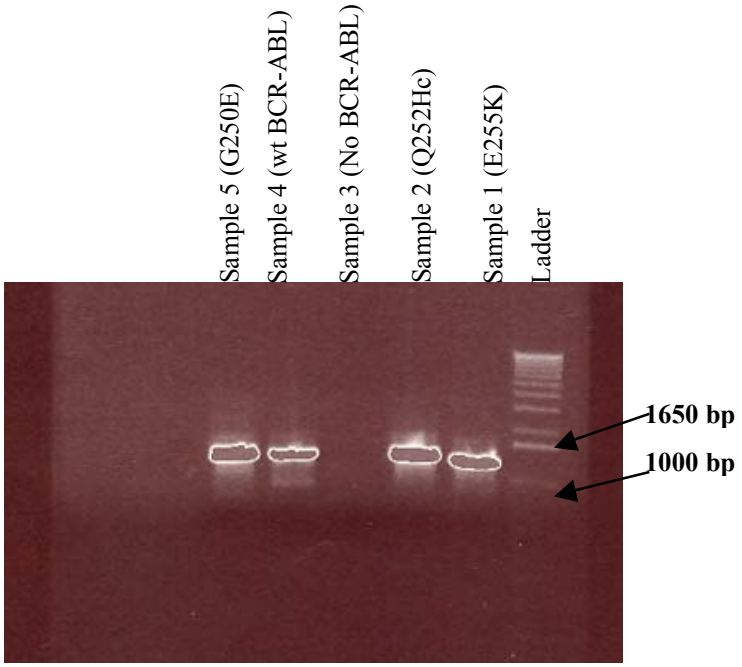

Figure 22: Gel electrophoresis of the patient-samples after the first PCR. Each sample was amplified for 40 cycles with the primers BCR(13)-F and A7- creating amplicons of 1293 bp. Some of the PCR product was run for 30 minutes on a 0.7 % agarose gel at 90 V. Sample 1, 2, 4 and 5 could be seen as single bands of approximately 1293 bp. Sample 3 contained no BCR-ABL and was not amplified.

The results from the second PCR are expressed in table 17 showing the Ct-values for each template-primer combination. The primers differed greatly in their ability to detect the mutation. The G250E-xR primer could detect the G250E-mutation in sample number five, with a ΔCt of 9.5 to the background, given by the other reverse primers used. The E255K-xR primer could barely separate the E255K-mutation from the first of the background curves. The Q252Hc-xR primer was unable to separate the Q252Hc-mutated sequence from the background, suggesting that this type of primer is unsuitable for detecting this particular mutation. No mutations were detected in sample number 4, which contained a wild-type BCR-ABL sequence. Sample 3 had no BCR-ABL gene, seen by the missing band in the gel electrophoresis (figure 22), and the absorbance at 260 nm, measured by NanoDrop, was very low (0.012). The second PCR gave some amplification, but only at high Ct-values, indicating some mis-amplification.

Table 17: Results from the analysis of different patient-samples with LNA-primers. The samples were analyzed undiluted in the first PCR of 40 cycles, and diluted to a concentration of 1×10^7 copies/µl before the second PCR. Each value is the average of two parallels. The values in bold writing represent the correct primer-template combination, where the primer ideally should detect the mutation.

Patient-sample	Ct E255K-xR	Ct G250E-xR	Ct Y253H-xR	Ct Q252Hc-xR	Ct M351T-xR
1 (E255K)	**21.9**	24.2	25.0	42.7	33.5
2 (Q252Hc)	25.8	22.4	23.9	**26.1**	33.2
3 (No BCR-ABL)	41.3	39.7	37.0	-	-
4 (wild-type BCR-ABL)	26.3	24.7	25.6	39.7	31.6
5 (G250E)	26.5	**15.7**	25.2	41.2	33.7

To summarize, the BCR-ABL-specific assay with SYBR Green Master Mix deteriorated the sensitivity of the PCR. However, the specificity of the mutation-detection could be increased, especially with LNA-primers in the second step. By adding a PCR-step in a nested approach, both the specificity and the sensitivity was increased.

5 Discussion

Drug-resistance and relapse can be critical for CML-patients treated with imatinib, therefore it is important to be able to detect mutations in the BCR-ABL gene as early as possible. This is especially crucial when it comes to the T315I-mutation that is unresponsive to both dose-escalation of imatinib and many of the recently developed tyrosine kinases (Walz et al. 2006). The T315I-mutation will probably be an even greater problem as new tyrosine kinases, less prone to all mutations but T315I, are developed. Therefore, it was chosen as the main target for detection in this study, where plasmids containing sequences of wild-type and T315I BCR-ABL were used in different approaches, in an attempt to increase the sensitivity and specificity of a real-time PCR-based assay for detection of the T315I-mutation. Primer manipulation was one of the methods used to achieve this, by using ASO-primers with 3´-ends complementary to the mutated sequence, ARMS-primers with an additional mismatch close to the 3`-end, LNA-primers with a LNA-nucleotide incorporated, and combinations of ARMS- and LNA-primers. A non-discriminating (ND) primer that is unable to distinguish the wild-type from the mutated plasmid was also used. The different reverse primers were tested with both TaqMan and SYBR Green master mixes, which uses different methods to detect the PCR amplification. By using different forward primers, the amplification of long, BCR-ABL-specific templates could be compared to the amplification of shorter BCR-ABL non-specific templates. The tests were, in addition to a single-step procedure, also performed in a nested approach with two PCR steps in an attempt to increase the sensitivity. Using patient-samples with wild-type and mutated BCR-ABL, a patient-test for detection of the T315I-mutation was developed.

5.1 Detection of the T315I-mutation in BCR-ABL

Direct sequencing, the traditional method for detecting mutations in the BCR-ABL gene has several drawbacks. With its multiple steps it is time-consuming and the risk for contamination increases. The sensitivity of the method is about 20 percent (Branford et al. 2003), which means that mutations will not be detected in the early phase of clonal evolution of mutated cells. Subcloning and sequencing, or D-HPLC gives a somewhat

better sensitivity (Shah et al. 2002; Soverini et al. 2004), but not as good as ASO-PCR (Roche-Lestienne et al. 2002; Willis et al. 2005). In addition to good sensitivity, ASO-PCR also has a low contamination-risk and is relatively easy and quick to perform. Therefore, it is an excellent method for detecting mutations during imatinib-treatment. Besides, real-time PCR is also the recommended procedure for monitoring BCR-ABL levels in CML-patients treated with tyrosine kinase inhibitors, and it would be convenient to carry out the mutation-detection with the same technique.

The ASO-PCR method can be further improved by adding mismatches close to the 3`-end of the primer (ARMS) or introducing a LNA-nucleotide close to the 3`-end of the primer (Newton et al. 1989; Latorra et al. 2003). This can improve the sensitivity and specificity of the PCR, so that mutations can be detected earlier. The major disadvantage with these methods is that they are biased, and will only find the mutations included in the test. Because one primer is needed for each mutation, the number of mutations to screen for has to be limited to the most important ones, like the T315I-mutation.

The plasmids used in this study were made by cloning a PCR product, and transforming into bacterial cells. After linearization, the plasmids were quantified, and stock solutions were made. For each new experiment, a batch from this stock solution was diluted to the proper concentration and analysed. Two different approaches (single-step and nested PCR) were tested with primer combinations creating short BCR-ABL non-specific and long BCR-ABL-specific amplicons. Three different Master Mixes were tested (TaqMan, SYBR Green and FullVelocity), together with different types of modified reverse primers (ARMS, LNA and combinations of the two).

The forward primer 315F, which was used in the experiments with the TaqMan Master Mix, anneals to the ABL-part of the template, and creates short amplicons. Plasmids amplified with this primer showed a better sensitivity compared to plasmids amplified with the forward primer BCR(13)-F, used in experiments with the SYBR Green Master Mix. The latter primer anneals to the BCR-part of the template, thereby amplicating longer, BCR-ABL-specific sequences. The longer templates made the PCR less efficient,

which could be a reason for the poorer sensitivity. However, by making the amplification BCR-ABL-specific, the specificity, hence the discrimination-power between mutated and non-mutated alleles, was increased. Especially the LNA-primers offered good results when using the BCR-ABL-specific approach. By combining this with the SYBR Green Master Mix, the PCR-setup was easier and faster, because there was no need for addition of a probe. This would be more important if many samples were to be analysed, to save time and costs.

Real-time PCR analysis of the plasmids showed that, independent of the Master Mix and PCR approach used, modification of primers with LNA and ARMS gave a poorer sensitivity compared to the ARMS-primer used today (A315 mut), but the modification increased the specificity (ΔCt) so that mutations could be detected easier. The LNA-primers generally provided better results than the combination of LNA and ARMS, both for sensitivity and specificity. Some of the ARMS/LNA-primers (T315I-2xR and -4xR gave no amplification in any of the experiments, indicating that they probably were too destabilized.

The nested approach was favourable both for specificity and sensitivity, compared to the single-step PCR, independent of what Master Mix that was used. This is due to the first amplification step where the BCR-ABL sequence was amplified before the second PCR with mutation-specific primers was done. The nested approach however, increases the contamination-risk due to the extra PCR step and the need for purification between the steps.

In these experiments, the FullVelocity kit did not give any amplification with the faster PCR programs, probably because the plasmids were too long, 1293 bp compared to the recommended 90 to 300 bp. When using the conventional amplification-times, some amplification could be seen, but considering that the FullVelocity technology is supposed to reduce the amplification-time, there would be no benefits using this for such long templates compared to the TaqMan Master Mix.

5.2 The transferability of the method from plasmids to patient-samples

The methods that worked well for mutation-detection in plasmids were also tried out in patient-samples, to make a patient-test for detection of the T315I-mutation. The best results were achieved with the nested BCR-ABL-specific PCR with SYBR Green Master Mix and the LNA-primer T315I-xR in the second PCR-step. This procedure improved both the sensitivity and the specificity of the mutation-detection. The patient-test was then translated to other mutations, but this gave variable results. One of the mutations (G250E) was detected with great sensitivity and specificity, while others could not be detected at all. As each mutation seems to require different conditions for optimal detection, a new primer evaluation probably needs to be done for each mutation.

5.3 Clinical importance of mutation-detection

Identifying mutations in the BCR-ABL tyrosine kinase domain can be important for decision-making about further treatment of CML-patients. As earlier mentioned, the different mutations can have quite a different impact on the disease, and the detection of certain mutations implies that the imatinib treatment must be stopped immediately to prevent selection of the mutated clone and progression of the disease (Branford et al. 2003; Hjorth-Hansen et al. 2004). Screening of all CML-patients for all known mutations would neither be practical nor efficient. Besides, the finding of a mutant clone does not necessarily mean that it will expand and cause relapse (Willis et al. 2005). It should rather be concentrated on screening patients at high risk, like the ones with inadequate response to treatment or with rising BCR-ABL transcripts (Hughes et al. 2006), and on detecting the most important mutations, like the P-loop and the gatekeeper mutations. By using a sensitive method like ASO-PCR, mutations can be detected early, and the proper precautions can be initiated. As shown in these experiments, a nested approach using a LNA-primer could further increase the sensitivity and specificity for detection of the T315I-mutation.

The knowledge of the mechanisms of imatinib-resistance has helped create new drugs that are more potent and more effective against imatinib-resistant BCR-ABL clones.

Many of these are in an early state of clinical studies, and more time is needed to determine the effects in vitro, but the results so far have been promising. AMN107, a derivative of imatinib has more than 10-fold increased potency as a BCR-ABL inhibitor, probably because it has higher affinity to the ABL-kinase pocket. It is thought to inhibit several imatinib-resistant BCR-ABL mutations, except for T315I that is resistant even at high doses (Weisberg et al. 2005). Dasatinib (BMS-354825), an inhibitor of the SRC-family kinases, has an increased activity against the ABL-kinase compared to imatinib. The only imatinib-resistant BCR-ABL mutation known to be resistant to this new drug is the T315I-substitution (Shah et al. 2004; Martinelli et al. 2005). The drug is able to bind both the active and inactive conformation of ABL with fewer contact points between it and the kinase (Walz et al. 2006). ON012380 targets the substrate-binding site, in contrast to the ATP-binding site. Due to the different binding site, it can function as an effective agent against imatinib-resistant BCR-ABL proteins, including the T315I-mutant, and together with imatinib the drugs act synergistically (Gumireddy et al. 2005).

5.4 Factors of uncertainty with the method

Several factors of uncertainties could have affected the results in these experiments and contributed to variety. The PCR amplification process itself can actually be a source of variety, since small variations during the early stages in the PCR can greatly influence the final yield of the amplified product (Bustin 2000). It is therefore important that the PCR is consistent and optimized concerning reagent concentration, primer and probe design, and temperature program among other things, to be able to produce accurate and reproducible results.

The OD-measuring, done by NanoDrop, is a factor that could give rise to uncertain results. The absorbance of a sample could be different if it was measured several times, which means that the true absorbance would be different from the measured one. This also means that there is an uncertainty in the calculation of copy number. The dilution-series are another factor contributing to uncertainty, because variations in the early dilutions would have a great impact further down in the dilution-series.

Each PCR was performed with two parallels of each template-primer combination. This could have contributed to uncertainty since more parallels would help discover any outliers, and make the results less variable and more reliable.

A no-template control to screen for contamination of reagents or false amplification was not used. Internal or external standards could have been included to control for false negatives that could have occurred through failure of a reagent or the thermal cycling process, or through the presence of inhibitors (Logan et al. 2004). The reference dye provided in the different kits for providing a stable baseline was not used either, meaning that any non-PCR related variations in fluorescence could not have been compensated for.

5.5 Future perspectives

In these experiments, the T315I-mutation was detected in three patient-samples with the nested procedure and in two patient-samples with the single-step procedure, as well as in the plasmid-system created. The results indicated that the nested BCR-ABL-specific approach using SYBR Green Master Mix and a LNA-primer was the most sensitive and specific procedure, but to be able to confirm the results, the method should be carried out with more samples. The methods could also be used for detection of other important mutations, like the P-loop mutations, but as the last experiment in this study showed, a new primer evaluation is necessary for each mutation. In future experiments, there should also be a greater focus on quantifying, so that the sensitivity could be calculated more precisely, and for monitoring the presence of mutated clones in patients.

Conclusion

The experiments were performed in order to make a real-time ARMS-PCR based method for detection of the T315I mutation, specific for the BCR-ABL allele, and more sensitive and specificity. The method that seemed to work best for detection of this mutation, both in plasmids and patient-samples, was the approach that included a pre-amplification step, combined with SYBR Green Master Mix and a LNA-primer with the LNA nucleotide in 3`-position. These results are in accordance with recently published, detecting mutant alleles using LNA-nucleotide-containing primers and pre-amplification with high sensitivity (Thomassin et al. 2004).

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