MASTER THESIS IN PHARMACY

CLONING AND EXPRESSION OF WILD-TYPE AND MUTATED FORMS OF BCR-ABL IN A MOUSE PRO-B CELL LINE

By

Stine Gangnæs Hammer

2006

Department of Pharmacology, Institute of Pharmacy

Faculty of Medicine

University of Tromsø

Teaching supervisors

Associate professor Ingvild Mikkola, Institute of Pharmacy, UiTø

Medical doctor Franz Gruber, Department of Immunology and transfusion medicine, UNN

ACKNOWLEDGEMENTS

This master thesis in pharmacy was carried out at the Institute of Pharmacy at the University of Tromsø, and is the finalization of an interesting and exciting study.

First of all I would like to thank my two supervisors, Ingvild Mikkola and Franz Gruber.

Ingvild, thank you for your endless suggestions of what to do when things did not go according to plan in the laboratory. Your constructive feedback and good advice in writing this thesis has been invaluable.

Franz, thank you for all encouragement, valuable advice and useful input on the writing of this thesis. I appreciated our many conversations, which were both insightful and entertaining.

Rune Hogseth deserves my thanks for always being helpful for technical and theoretical advice in the lab. He has also assisted me with the electroporation.

Thanks to everyone in the lab for their day-to-day support and conversation.

Thanks to the people at the sequencing lab for sequencing my samples.

I would also like to thank my family and friends for their encouragement and support through all these years, and their assistance in motivating me to achieve my academic goals.

Erik also deserves a great amount of gratitude, not only did you listen to my frustration a long the way, but you have also encouraged and helped me through all these years. Thank you for all your love and understanding.

Tromsø, May 2006 Stine Gangnæs Hammer

ABBREVIATIONS

Abl	Abelson tyrosine kinase
Amp	Ampicillin
ATP	Adenosine triphosphate
Bcr	Breakpoint cluster region
BSA	Bovine serum albumine
CML	Chronic Myelogenous Leukemia
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
GFP	Green Fluorescense Protein
IL-3	Interleukin-3
IPTG	Isopropylthiogalactosidase
IRES	Internal ribosome entry site
Kan	Kanamycin
LB medium	Luria-Bertani Medium
MCS	Molecular Cloning Site
PCR	Polymerase Chain Reaction
Ph chromosome	Philadelphia chromosome
SAP	Shrimp Alkaline Phosphatase
TAE	Tris Acetate EDTA
TBE	Tris Borate EDTA

TABLE OF CONTENTS

ACKNOWLEDGEMENTS **2**

ABBREVIATIONS **3**

TABLE OF CONTENTS **4**

ABSTRACT **6**

1 INTRODUCTION **7**

1.1 THE PHILADELPHIA CHROMOSOME 7

1.2 CHRONIC MYELOGENOUS LEUKEMIA 9

1.3 THE BCR-ABL PROTEIN 10

1.4 IMATINIB, A TARGETED DRUG FOR INHIBITION OF ABL 10

1.5 IMATINIB RESISTANCE 12

1.6 DNA CLONING 14

2 AIM OF THE STUDY **20**

3 MATERIALS & METHODS **21**

3.1 MATERIALS 21

3.1.1 The reagents used in the different methods *21*

3.1.2 The two DNA ladders *27*

3.1.3 Plasmid constructs used in this thesis *28*

3.2 METHODS 30

3.2.1 Digestion of DNA with restriction enzymes *30*

3.2.2 Agarose gel electrophoresis *31*

3.2.3 QIAquick Gel Extraction Kit *33*

3.2.4 DNA ligation *34*

3.2.5 Transformation of DNA into competent E. coli DH5α cells *35*

3.2.6 "Miniprep light" *37*

3.2.7 QIAprep Spin Miniprep Kit *38*

3.2.8 QIAGEN Plasmid Midiprep *39*

3.2.9 Polymerase Chain Reaction (PCR) *41*

3.2.10 Amplifying the Bcr-Abl gene using PCR *42*

3.2.11 Gel –purifying PCR products using crystal violet agarose gel *44*

3.2.12 Cloning of the BC-fragment into pCR®-XL-TOPO® vector *46*

3.2.13 DNA Sequencing *48*

3.2.14 DNA concentration measurements *50*

3.2.15 Application of X-gal and IPTG onto agar plates. *52*

3.2.16 Making freezing stocks *52*

3.2.17 BA/F3 mouse cells *53*

3.2.18 Electroporation of mammalian cells *53*

4 RESULTS 55

4.1 CLONING OF GFP INTO THE PMACS 4-IRES.II VECTOR 55

4.1.1 Optimization of electroporation of BA/F3 mouse cells 60

4.2 CLONING OF BCR-ABL INTO PBLUESCRIPT(KPNI⁻) 61

4.2.1 Construction of pBluescript(KpnI⁻) 64

4.2.2 PCR on Bcr-Abl to generate the A, B and C fragments for cloning. 65

4.2.3 Revised strategy for cloning the A-fragment 68

4.2.4 Cloning the A fragment and the BC fragment into the pBluescript(KpnI⁻) vector 71

5 DISCUSSION 79

5.1.1 Electroporation of pMACS-GFP into BA/F3 cells 80

5.1.2 Amplifying the Bcr-Abl gene using PCR 81

5.1.3 Cloning of long PCR products 82

5.1.4 The position of the restriction sites 83

5.1.5 Future direction 83

6 REFERENCES 84

ABSTRACT

Chronic myelogenous leukaemia is a monoclonal hematopoetic stem cell disorder characterised by the t(9;22) translocation and results in the constitutively activated Bcr-Abl tyrosine kinase. Since the tyrosine kinase activity of the Bcr-Abl fusion protein is the causative molecular event in CML, targeting the tyrosine kinase activity appears to be an attractive therapeutic strategy.

Imatinib, Glivec, is a drug that inhibits the tyrosine kinase activity of Bcr-Abl. By binding to the ATP binding pocket, it prevents ATP from binding and the phosphorylation of downstream substrates is disrupted. Clinical studies have proven imatinib to be highly effective in the treatment of CML and imatinib is now the first-line therapy for all stages of CML However; point mutations have been detected in the ATP binding region of the Abl kinase domain. These mutations alter the conformation of the ATP binding pocket, disturb the binding of imatinib, and lead to imatinib resistance.

We wanted to develop an experimental system where the effects of mutations in Bcr-Abl, leading to imatinib resistance, could be studied and new targets for therapy identified. For this we were going to clone Bcr-Abl into a pMACS 4-IRES.II vector. The Bcr-Abl gene is large, so to get the full-length construct, the cloning strategy involved ligation of PCR fragments in a stepwise order. Once inside the vector, the construct had to be transfected into BA/F3 cells. To study single point mutations some of the relevant point mutations were supposed to be subcloned into the Bcr-Abl construct and expressed in BA/F3 cells.

To monitor the transfection and selection strategy with the pMACS 4-IRES.II vector and the BA/F3 cells, a pilot study was performed. A GFP gene was cloned into the pMACS 4-IRES.II vector and transfected into the BA/F3 cells. Expressed GFP will make fluorescent light that can be observed in a microscope.

In conclusion, the cloning of this long Bcr-Abl gene proved to be more difficult than expected. First, misannealing resulted in an incomplete PCR product, which forced us to develop another strategy for this fragment. The 5’ part and the 3’part of Bcr-Abl was then successfully cloned in two vectors. However, all attempts to try to join the different Bcr-Abl fragments into one vector failed.

1 INTRODUCTION

1.1 The Philadelphia chromosome

Important cellular functions like cell cycling are tightly controlled by multiple regulatory mechanisms. In cancer disease multiple genetic events are needed to destroy the network of cellular control mechanisms [Hanahan and Weinberg 2000]. After intensive studies of those events, many proteins playing major roles in cancer disease have been identified. In an increasing number of diseases this knowledge has lead to development of drugs targeting proteins that are believed to represent the real cause of cancer disease. Chronic myelogenous leukemia was the first malignancy shown to be associated with a specific cytogenetic lesion, the Philadelphia chromosomal translocation [Kabarowski and Witte 2000]. The Philadelphia chromosome was discovered in Philadelphia in 1960 [Nowell and Hungerford 1960]. It took 13 years before J Rowley found that the Philadelphia chromosome results from a reciprocal translocation between the long arms of chromosome 9 and 22 [Rowley 1973]. Another 10 years went before it was shown that the proto-oncogene Abl, normally found on chromosome 9, was translocated to the Philadelphia chromosome in CML cells [Bartram et al. 1983]. We now know that the most important consequence of the Philadelphia chromosome consists in the fusion of the Bcr gene to the tyrosine kinase Abl, encoding the constitutive active fusion protein Bcr-Abl, which is supposed to induce all disease features of CML.

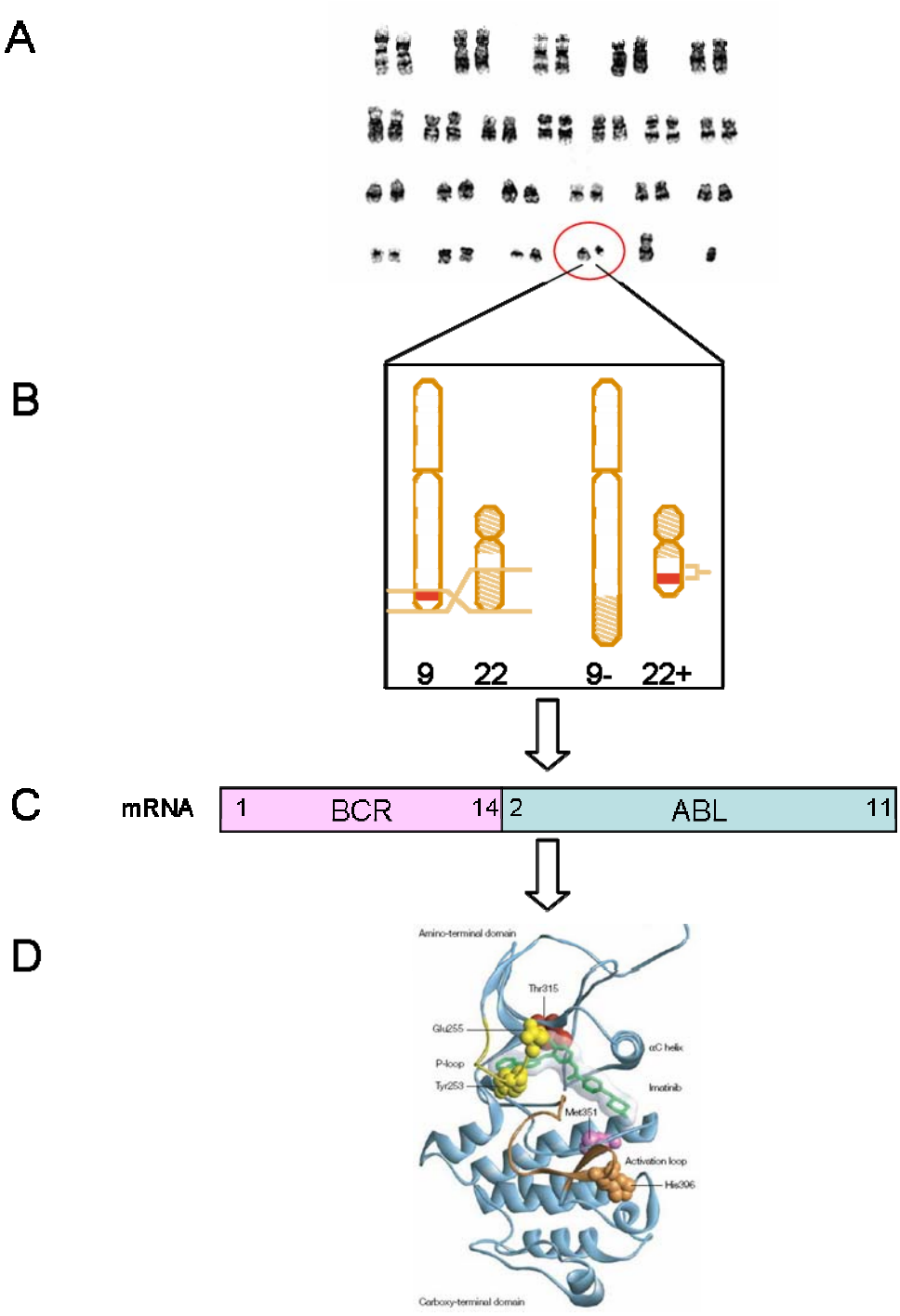


Figure 1. **A.** On the cytogenetic level a minute chromosome can be found in more than 90 percent of patients with CML, which results from a translocation between chromosome 9 and 22, **B.** giving rise to a new cancer specific fusion chromosome stretching Bcr and Abl sequences on chromosome 22+. **C.** The resulting mRNA consists of Bcr fragments (usually exon 1 to 14) and Abl sequences (usually exon 2 until 11). **D.** The fusion protein Bcr-Abl exerts constitutive active tyrosine kinase activity. [For preparation of the figure we used material published in Daub et al 2004]

1.2 Chronic myelogenous leukemia

Chronic myelogenous leukaemia is a monoclonal hematopoetic stem cell disorder characterised by an increase of leukocytes and their ancestors in the peripheral blood [Sawyers 1999]. Clinically, the primary symptoms can be moderate, but most patients complain about fatigue. Usually the spleen is enlarged at diagnosis. Typically, one will find immature bone marrow cells like myelocytes and metamyelocytes in the blood smear.
The diagnosis is finally confirmed by detection of the characteristic translocation t(9;22) by assessment of bone marrow metaphases, detection of a fusion signal by FISH or the amplification of the Bcr-Abl transcript by PCR [Hjort-Hansen et al. 2004].
Over ninety percent of cases of chronic myelogenous leukemia are associated with the presence of the Philadelphia chromosome.

Initially, CML is a slowly progressive disease with symptoms that usually develop gradually [Druker et al. 2001]. The disease progresses through three distinct clinical phases - chronic and accelerated phase and blast crisis, during which the leukemic clone progressively loses its ability to differentiate [Druker et al. 2001]. The chronic phase is characterized by accumulation of myeloid precursors and mature cells in bone marrow, peripheral blood and extramedullary sites [Calabretta and Perrotti 2004]. Most patients are diagnosed in the chronic phase. During the chronic phase of the disease, there is massive clonal expansion of myeloid cells, which retain the ability to differentiate [Druker et al. 2001]. This phase can last for several years before symptoms and signs of more aggressive disease appear, the so-called accelerated phase. In the accelerated phase the control of the proliferation becomes much more difficult. Counts rise to high levels, and the risk for tissue infiltration by white blood cells are present [Hillmann 1994].

As the disease progress, the number of blasts in the bone marrow and peripheral blood continues to increase, and the accelerated phase will evolve to an aggressive acute leukemia, referred to as a blast crisis [Hillmann 1994].
In two thirds of patients the blasts are myeloid, and in one third they are lymphoid [Druker et al. 2001].

Untreated the disease is fatal after a variable period of time. Until the late nineties interferon in combination with cytosar, a cytostatic, has been the treatment of choice. Patients survived 5-6 years in average during this treatment [Guilhot et al. 1997]. They usually died after a transformation from a chronic phase to accelerated phase or blast crisis, a terminal disease stage very similar to acute leukaemia [Sawyers 1999; Azam et al. 2003].

Transition to blast crisis is the unavoidable outcome of CML except of patients receiving allogenic bone marrow transplantation early in the chronic phase [Calabretta and Perrotti 2004]. Allogenic bone marrow or stem cell transplantation is still the only treatment known to cure CML. This approach is available only to patients who have a suitable donor and who are young enough to tolerate the procedure and the subsequent toxic effect of allogenic transplant [Herfindal and Gourley 2000]. The goal of therapy for CML in the chronic phase is to prolong survival and minimize symptoms by achieving complete hematologic response and a complete cytogenetic response [Herfindal and Gourley 2000].

1.3 The Bcr-Abl protein

On the protein level the reciprocal translocation of chromosomes 9 and 22 creates a cancer cell specific fusion protein (Bcr-Abl). It has recently been shown that the amino terminal abl sequences are important for auto inhibition of Abl. In case of the Bcr-Abl protein these sequences are replaced by fragments of the Bcr gene resulting in a constitutive active tyrosine kinase [Azam et al. 2003]. In contrast to other receptor tyrosine kinases Abl is located in the cytosol. It contains several domains that are important for cellular interaction. As a consequence, Abl activates multiple signal transduction pathways leading to uncontrolled cell growth, proliferation and apoptosis (reviewed by Sawyers 1999).

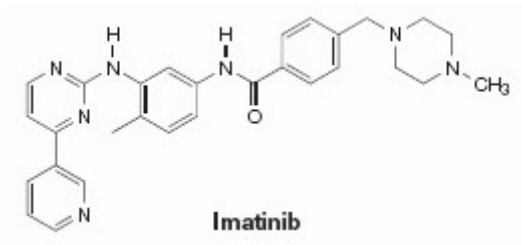
1.4 Imatinib, a targeted drug for inhibition of Abl

From the early nineties it was known that tyrosine kinases play a major role in development of cancer disease [Ullrich and Schlessinger 1990]. Tyrosine kinases are enzymes that transfer phosphate from ATP to tyrosine residues on substrate proteins that in turn regulate different cellular processes. Substances inhibiting tyrosine kinases were therefore systematically screened for their activity in cancer. STI 571, later called imatinib or Glivec (Novartis), was one of the first substances making the way through clinical development [Capdeville et al.

2002], (see Figure 2A). Initially, Imatinib was developed as a specific platelet derived growth factor receptor (PDGFR) inhibitor, but was also found to be a potent and selective inhibitor for Abl tyrosine kinases, including Bcr-Abl, as well as for c-kit and ARG [Mauro and Druker 2001].

Due to its small size imatinib binds to the ATP binding pocket of the inactive form of the Abl kinase domain of Bcr-Abl (see Figure 2B). By preventing ATP from binding the phosphorylation of downstream substrates is disrupted. Signal pathways constitutively activated in CML cells are again shut down. Initial clinical studies demonstrated that imatinib treatment clearly induced a cytogenetic response in substantially more patients than standard treatment. Imatinib was, therefore rapidly drawn through the registration process [Capdeville et al. 2002] and now represents the first-line therapy for all stages of CML.

A.



B.

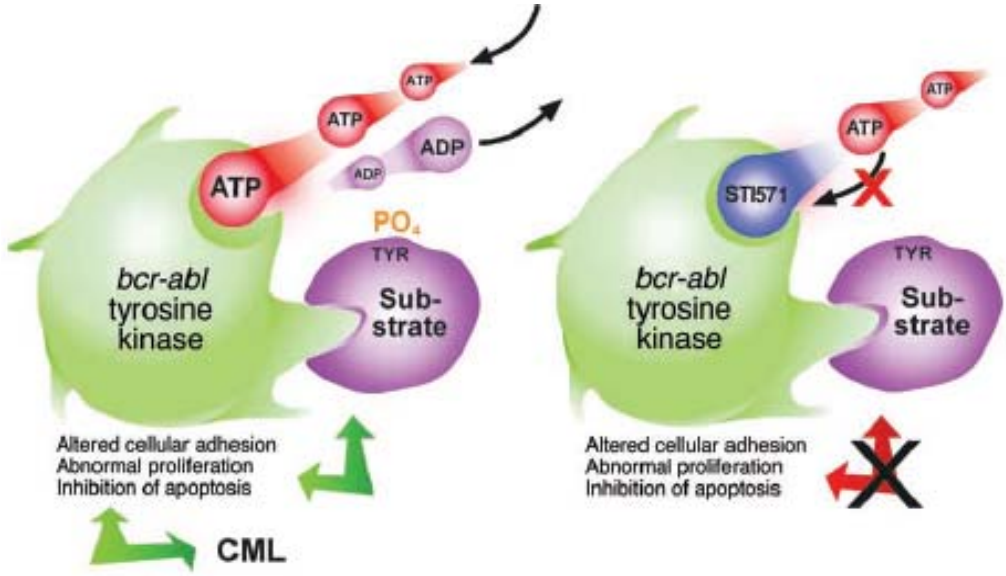


Figure 2 Imatinib (STI571) is a small tyrosine kinase inhibiting molecule binding the ATP binding pocket of Bcr-Abl. A. A sketch showing the molecular formula of the tyrosine kinase inhibitor STI571, later called imatinib (Glivec) for the treatment of chronic myelogenous leukemia. Imatinib belongs to a class of compounds known as the 2-phenylaminopyrimidines, and is a small molecule not unlike the structure of ATP. **B.** Schematic representation of the mechanism of action of imatinib. The Bcr-Abl tyrosine kinase is a constitutively active kinase that functions by binding ATP and transferring phosphate from ATP to tyrosine residues on various substrates. This causes the excess proliferation of myeloid cells characteristic of CML. Imatinib functions by blocking the binding of ATP to the Bcr-Abl tyrosine kinase, inhibiting its activity. In the absence of tyrosine kinase activity, substrates required for Bcr-Abl function cannot be phosphorylated and subsequent cellular events are abrogated. From reference [Mauro and Druker 2001].

1.5 Imatinib resistance

In 2001 Mercedes Gorre and co-workers [Gorre et al. 2001] introduced a novel mechanism of resistance to imatinib after molecular analysis of 11 imatinib resistant, advanced phase patients. In 8 of these 11 subjects an identical A>T point mutation was identified in the Abl kinase domain, resulting in a substitution of threonin in position 315 by isoleucin (T315I) [Gorre et al. 2001]. In addition they showed that Bcr-Abl was reactivated in these subjects,

pointing out that Bcr-Abl represents the driving oncogene also in these advanced phase patients

Over 40 different mutations in the Abl kinase domain have since been identified in imatinib resistant patients inducing substitutions of 17 different amino acid residues [Martinelli et al. 2005]. These residues are clustered within the Abl kinase domain (see Figure 3A). The most important locations concern the phosphate binding loop (amino acid position 248 to 255), residues around position 315 and the activation loop (position 350 to 400). Some residues like the T315 exert a very intimate contact to imatinib in the ATP binding pocket; whereas other residues are located far from the binding pocket and will not have any contact with imatinib (see Figure 3B). The latter group of mutations represents the majority of mutations identified in imatinib resistant patients.

In conclusion, in the majority of patients developing resistance to imatinib we will find a Bcr-Abl cell clone containing mutations in the kinase domain. These mutations will distort imatinib binding by sterical hindrance, or by trapping Abl in a conformation imatinib will not bind to. The majority of the more than 40 different mutations described in resistant patients belong to the second group. Therefore, given that imatinib exclusively binds to the inactive conformation of Abl, this might represent a major pitfall for the drug since it opens the possibility for resistance. This observation has also been important for the targeted development of second-generation tyrosine kinase inhibitors in CML, because drugs binding the active conformation of Abl could represent a choice in order to overcome resistance to imatinib. This idea directed the focus on combined src/abl inhibitors that were supposed to bind active conformation abl since this conformation is very close to Src. Multiple substances have been screened by several groups and at least three drugs are on their way into clinical practice. Dasatinib (Bristol-Myers Squibb) has almost reached registration after its superior effect on mutated Bcr-Abl has been shown both in vitro and in vivo [Shah et al. 2002]. In this respect we will in the future be faced with several drugs targeting Bcr-Abl. These drugs will be used sequentially on the basis of their resistance profile or a priori as a combination treatment used as a strategy to avoid resistance.

A. **B.**

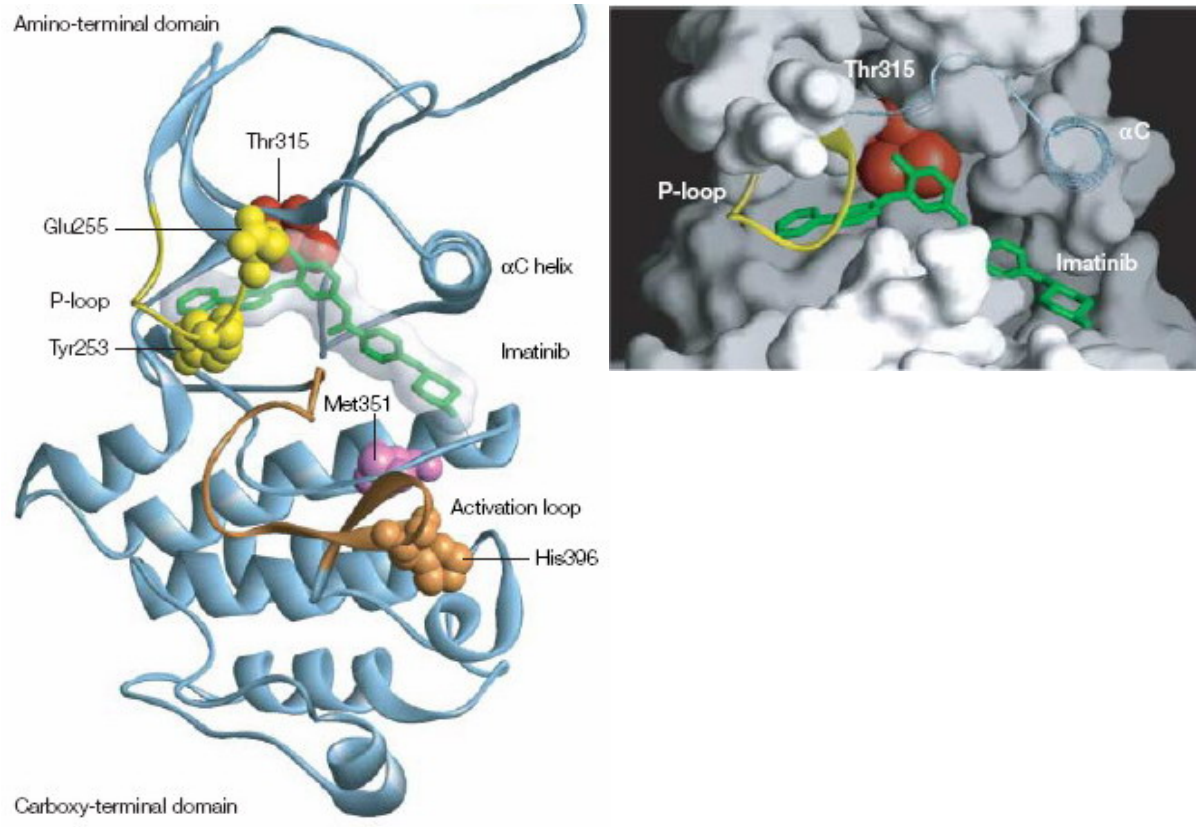


Figure 3. Different point mutations in the Abl kinase domain prevent imatinib from binding in the ATP binding pocket. A. A sketch of the Abl kinase domain showing where in Bcr-Abl the different point mutations have been identified. The most important mutations are found in the p-loop, in residues around position 315 and in the activation loop. These point mutations altering the conformation of the ATP-binding pocket such that imatinib no longer can bind. **B** Imatinib bound to the ATP-binding site on Bcr-Abl. [Daub et al. 2004].

1.6 DNA cloning

Techniques for DNA cloning have opened incredible opportunities to identify or study the genes involved in almost every known biological process [Herfindal and Gourley 2000]. DNA cloning is the basis for other related technologies, such as gene therapy and genetic engineering of organisms. DNA cloning also made it possible to do genome sequencing.

DNA cloning is a technique for isolating and reproducing a large numbers of identical DNA fragments. By introducing recombinant DNA into host cells the foreign DNA is reproduced along with the host cells.

Cloning of DNA molecules from genome can be achieved by two different approaches: Cell based cloning or using polymerase chain reaction (see 3.2.9) [Passarge 2001]. For both these methods the use of restriction enzymes plays an important part.

Restriction enzymes recognize a specific sequence on a DNA strand and cleave the DNA by catalyzing breaks in specific phosphodiester bonds [Trun and Trempy 2003]. The cleavage is on both strands of the DNA so that a double stranded break is made [Trun and Trempy 2003]. This cleavage can give two types of ends, depending upon the specific restriction enzyme. Some restriction enzymes make sticky ends, with protruding single strands, which form hydrogen-bonded base pair with complementary sticky ends of other DNA fragments cut with the same enzyme. Other restriction enzymes make blunt ends; cleave both strands of DNA at the opposing phosphodiester bonds, leaving no unpaired bases on either end [Nelson and Cox 2002]. Any blunt end can be joined to any other blunt end regardless of how the blunt end was generated [Trun and Trempy 2003].

The restriction enzymes were discovered in bacteria in the late 1960s [Campbell et al. 1999]. Bacteria use restriction enzymes as protection against intruding DNA from other organisms, such as viruses and other bacterial cells [Campbell et al. 1999]. There are three classes of restriction enzymes, indicated I, II and III. Type I and III cleave the recognition sequence unspecific and at random sites. Type II restriction enzymes, the type most used within the gene technology, is specific and cleave the DNA within the recognition sequence itself [Nelson and Cox 2002]. The DNA sequence recognized by Type II restriction enzymes is symmetric and usually palindromic. The DNA sequence is between 4 and 8 bp in length, with most restriction enzymes recognizing 4 or 6 bp [Trun and Trempy 2003].
When the plasmid vector and the DNA fragments to be cloned are cut with the same restriction enzymes, the DNA fragments can be ligated into the plasmid vector and form a circular recombinant DNA molecule (see 3.2.4). During the ligation reaction DNA ligase form phosphodiester bonds between the 3'hydroxyl and the 5' phosphate ends of the nucleic acid molecule [Sambrook and Russell 2001].
Cell based DNA cloning involves separating a specific gene or DNA fragment from a larger chromosome using restriction enzymes (see 3.2.1). In order to clone a gene, its DNA sequence needs a carrier that can take it into the cell. There are many different kinds of vectors and most of them are isolated from larger plasmids that occur naturally in bacterial cells [Alberts et al. 2002]. Plasmid vectors are small circular molecules of double-stranded

DNA that can replicate separately from the host chromosome. Generally, a cloning vector contains three elements: a cloning site where the foreign DNA fragment can be inserted, a gene for antibiotics resistance and a replication origin to allow the plasmid to be replicated in the host cell.

When cloning DNA segments amplified by PCR into plasmid vectors there are different ways to do this. One possibility is to introduce specific PCR primers modified at their 5' ends so that they contain a suitable recognition site for restriction enzymes. By doing this the amplified DNA fragment of interest will carry restriction sites at its termini that can be used for further cloning into plasmid vectors. The vector and the amplified DNA fragment can thus be cleaved with appropriate restriction enzymes and ligated together [Sambrook and Russell 2001] (see Figure 4).

Another method is TA cloning (see 3.2.12) a much more efficient cloning strategy than blunt-ended ligation and useful when compatible restriction sites are not available for subcloning DNA fragments from one vector into another. By using *Taq* polymerase, single deoxyadenosine is added to the 3' ends of the PCR fragments. This can be exploited by cloning the PCR product into linearized T vector, fitted with single overhanging 3' deoxythymidine residues at each of its 3'termini. PCR fragments that carry unpaired deoxyadenosin residues at their 3'termini can easily be cloned into vectors with single unpaired deoxythymidine residues at its 3'termini [Sambrook and Russell 2001], a fact that several commercial companies have taken advantage of.

The recombinant DNA generated by PCR or cell based cloning is then transformed into suitable host cells and reproduced along with the host cell DNA. Bacteria are most often used as host cells, and strains of *Escherichia coli* (*E. coli)* cells are the most common cell to use because they are easy to transform with DNA plasmid [Sambrook and Russell 2001] and its metabolism are well understood [Nelson and Cox 2002]. To be able to take up foreign DNA, the bacteria cells need to be made competent. This is often achieved by treating them with divalent cations under cold conditions.

For *E. coli* transformation with plasmid the DNA needs assistance to pass through the cell membranes and to reach the site where it can be expressed and replicated. The plasmids can be introduced chemical or by electroporation (see 3.2.18) [Sambrook and Russell 2001].

Chemical transformation involves a short heat shock of the solution to induce the cells to take up the DNA. In electroporation a brief electrical pulse is applied to the solution containing the cells and the DNA fragments to be inserted. In both cases the intention is to simplify the penetration through the cell membranes.

When recombinant DNA is transferred into bacterial cells (see 3.2.5) the recombinant DNA replicate independently of the cells genome. The host cells transformed by recombinant DNA are grown in culture and as the bacterium grows, the new recombinant DNA molecule is copied by DNA replication and, as the cell divides, the number of cells carrying the recombinant molecule increases [McPherson and Møller 2000].

To distinguish bacteria transformed by recombinant plasmids from bacteria that have no recombinant plasmid different methods have been developed. One way to do this selection is to use vectors carrying genes for antibiotic resistance. Most plasmid vectors contain antibiotic resistance gene that will change the antibiotic resistant of the bacteria when a fragment is transformed. This allows selection for recombinant plasmids because only those bacteria that have been successfully transformed with the desired recombinant plasmid molecules will grow on agar plates in the presence of that bacterium.

To select for clones that have insert in their vectors a so-called blue-white screening can be done (see 3.2.15). This is a non-destructive histochemical procedure to detect β-galactosidase activity in transformed bacteria [Sambrook and Russell 2001]. However, this requires the use of plasmid vectors that carry unique restriction sites that serve as a marker with a marker gene. When DNA fragments are incorporated these marker genes are disrupted [Passarge 2001].
Using α–complementation (blue-white selection), the β-galactosidase enzyme will not be produced when the lacZ gene in the plasmid vector is disrupted, but a plasmid without an insert will still produce β-galactosidase because the lacZ gene is still intact [Passarge 2001]. β-Galactosidase cleaves a synthetic sugar, X-gal, that is similar to lactose, into two sugar components, galactose and glucose [Nelson and Cox 2002], one of which is blue [Passarge 2001]. Therefore, colonies containing plasmid vectors without insert will turn blue; on the other hand colonies containing plasmid vectors with insert will remain white [Passarge 2001]).

To identify clones carrying the recombinant plasmid, with the desired DNA insert, colonies are picked and grown in culture containing the appropriate antibiotic. The plasmid DNA is extracted by the standard minipreparation (see 3.2.7) technique and analysed by restriction digest. The size of the insert or the orientation of the insert can be verified by restriction analysis of minipreps of vector DNA. After digesting the DNA, the samples are run on a 0,7 % agarose gel (see 3.2.2) and the band sizes formed are compared with DNA fragments of known size. To confirm that the cloned DNA fragments are correct sequencing need to be done. This is especially important when the cloned DNA fragment is generated by PCR, since the polymerase can make mistakes, incorporating wrong nucleotides.

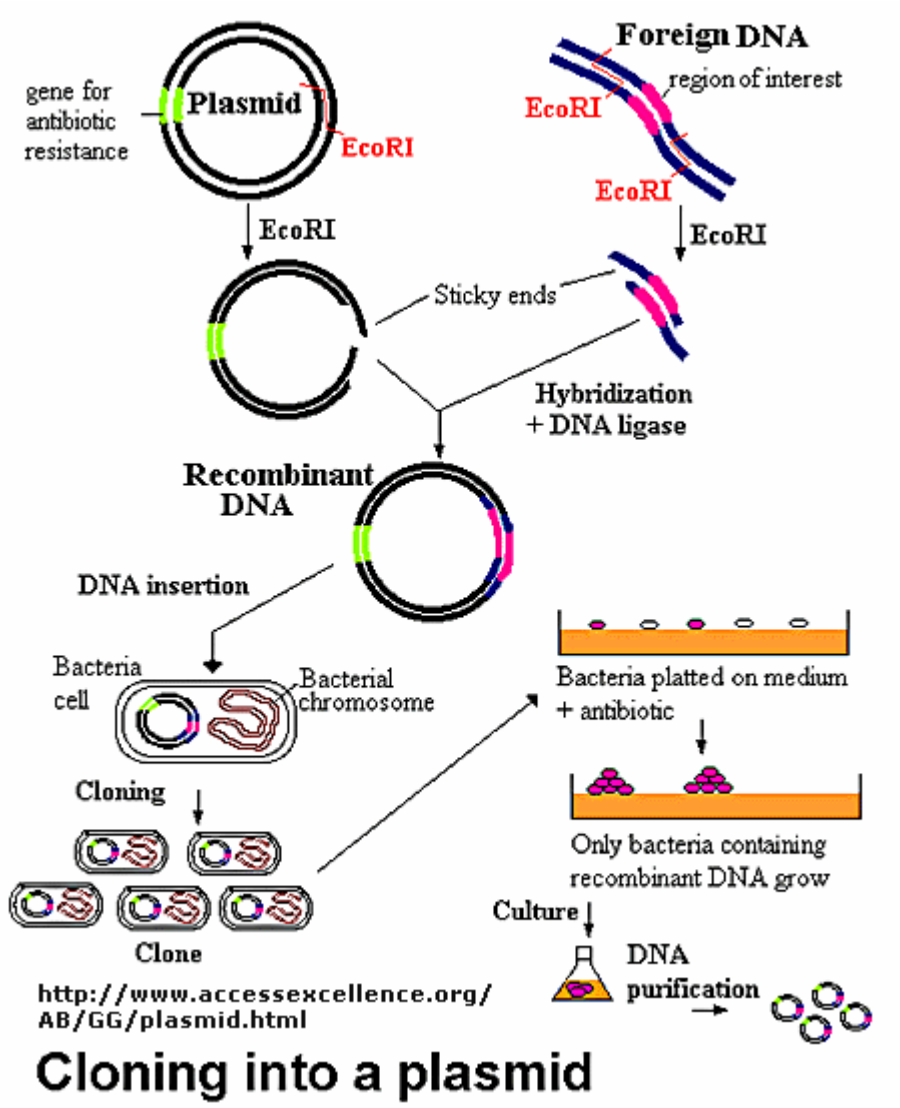


Figure 4. The different methods used when cloning a DNA fragment into a plasmid.

DNA cloning involves separating a specific gene from a larger chromosome and incorporating it to a plasmid vector, before the recombinant DNA is transformed into host cells. The host cells transformed by recombinant DNA are grown in culture and the recombinant DNA molecule is replicated and reproduced along with the host cell DNA. Then the cells is plated onto agar plates containing the appropriate antibiotic. To identify clones carrying the recombinant plasmid, with the desired DNA insert, colonies are picked and grown in culture containing the appropriate antibiotic before the plasmid DNA is extracted and purified (http://employees.csbsju.edu/hjakubowski/classes/ch331/dna/oldnalanguage.html).

2 AIM OF THE STUDY

The main purpose of this thesis is to develop an experimental system where the molecular and biological effects of the Glivec-resistant mutations in Bcr-Abl can be studied.

To do this several sub-goals has to be achieved:

- Cloning of GFP into the pMACS 4-IRES.II expression vector to test the transfection and selection of mouse BA/F3 cells.

- Cloning of Bcr-Abl into a modified pBluescript vector for later use in easy transfer of Glivec-resistant mutations.

- Cloning of Bcr-Abl from the modified pBluescript vector into the pMACS 4-IRES.II expression vector and transfection into mouse BA/F3 cells.

3 MATERIALS & METHODS

3.1 Materials

3.1.1 The reagents used in the different methods

Used in method 3.2.1				
Enzyme	**Recognition sequence**	**Buffer**	**BSA 2 %**	**Manufacturer**
EcoRI	G/AATTC	*10 X TA, Neb 4 and Neb EcoRI	+	New England Biolabs
EcoRV	GAT/ATC	10 X TA, Neb 2, Neb 4 and Neb EcoRI	+	New England Biolabs
FseI	GGCCGG/CC	Neb 4	+	New England Biolabs
KpnI	GGTAC/C	10 X TA	-	Promega
NdeI	CA/TATG	10 X TA	-	New England Biolabs
NheI	G/CTAGC	10 X TA	-	New England Biolabs
SacI	GAGCT/C	Neb 2 and Neb 4	+	New England Biolabs

*330 mM Tris acetate (pH 7.9), 660 mM KoAc, 100 mM $Mg(OAc)_2$

Generate blunt ends		
T4 DNA polymerase		New England Biolabs
2mM dNTP		Takara

Dephosphorylation		
SAP	Shrimp Alkaline Phosphatase	Promega

Used in method 3.2.2		
Reagents	**Specifications**	**Manufacturer**
Agarose	SeaKem® LE Agarose	Cambrex
10X TBE buffer	108 g Tris-base 55 g boronic acid 40 ml 0,5 M EDTA (pH 8.0) Add dH_2O up to 1 liter	
Ethidium bromide	10 mg/ml	GIBCO BRL®
6 x Loading buffer	0,25 % bromphenole blue 60mM Na_2EDTA (pH 8.0) 0,6 % SDS 40 % (w/v) sucrose in water	
1 kb DNA ladder	1 part 1 µg/ml ladder 24 parts TE-buffer 5 parts 6 X T	Invitrogen
1 kb plus DNA ladder	1 part 1 µg/ml ladder 24 parts TE-buffer 5 parts 6 X T	Invitrogen

Used in method 3.2.3		
Reagents	**Specifications**	**Producer information**
QIAquick Gel Extraction Kit	Catalogue no. 28704	QIAgen
Buffer QG	From kit (Solubilization buffer)	QIAgen
Isopropanol	Increase the yield	Arcus AS
Buffer PE	From kit (Wash buffer added ethanol)	QIAgen
Buffer EB	From kit (Elution buffer)	QIAgen

Used in method 3.2.4		
Reagents	**Specifications**	**Manufacturer**
5 X ligation buffer	150 mM 1 M Trios (pH 7.6) 25 mM 1 M $MgCl_2$ 2,5 mM 0,1 ATP 50 mM 1 M DTT 24 % 40 % PEG (8000) 50µg/ml 10 mg/ml BSA add dH_2O up to 1000 ml	
T4 DNA ligase		Invitrogen

Used in method 3.2.5		
Reagents	**Specifications**	**Manufacturer**
E. coli DH5α competent bacterial cells	Genotype: DH5á™-T1R: F-ö80lacZ.M15 .(lacZYA-argF)U169 recA1 endA 1hsdR17(rk-, mk+) phoA SupE44 thi-1 gyrA96 relA1 tonA	Invitrogen Life Technologies
SOC	20 g/l bacto-tryptone 5 g/l bacto yeast extract 8,6 mM NaCl 2,5 mM KCl 20 mM glucose pH adjusted to 7.0	

Used in method 3.2.6		
Reagents	**Specifications**	**Manufacturer**
Buffer P1	From kit (resuspension buffer added RNase A)	QIAgen
Buffer P2	From kit (Lysis buffer)	QIAgen
BufferN3	From kit (Neutralization buffer)	QIAgen
Isopropanol		Arcus AS
70 % ethanol		Arcus AS
TE-buffer	10 mM TrisHCl (pH 8.0) 1 mM EDTA H_2O adjusted to a total volume of 100 ml	

Used in method 3.2.7		
Reagents	**Specifications**	**Producer information**
QIAprep Spin Miniprep Kit	Catalogue no. 27106	QIAgen
Buffer P1	From kit (resuspension buffer added RNase A)	QIAgen
Buffer P2	From kit (Lysis buffer)	QIAgen
BufferN3	From kit (Neutralization buffer)	QIAgen
Buffer PB	From kit (Binding buffer)	QIAgen
Buffer PE	From kit (Wash buffer)	QIAgen
Buffer EB	From kit (Elution buffer)	QIAgen

Used in method 3.2.8		
Reagents	**Specifications**	**Manufacturer**
QIAgen Plasmid Midi Kit	Catalogue no. 12145	QIAgen
Buffer P1	From kit (resuspension buffer added RNase A)	QIAgen
Buffer P2	From kit (Lysis buffer)	QIAgen
Buffer P3	From kit (Neutralization buffer)	QIAgen
Buffer QBT	Equilibration buffer	QIAgen
Buffer QC	From kit (Wash buffer)	QIAgen
Buffer QF	From kit (Elution buffer)	QIAgen
Isopropanol	Precipitation	Arcus AS
70% ethanol	Wash	Arcus AS
TE-buffer	10 mM TrisHCl (pH 8.0) 1 mM EDTA H_2O adjusted to a total volume of 100 ml	

Growth medium	
Reagents	**Specifications**
LB medium with 50 μg/ml ampicillin or kanamysin	10 g/l bacto-tryptone 5 g/l bacto-yeast extract 171 mM NaCl 50 μg/ml with the appropriate antibiotic pH adjusted to 7.0

Used in method 3.2.9		
Reagents	**Specifications**	**Manufacturer**
Elongase 5X Buffer A		Invitrogen
Elongase 5X Buffer B		Invitrogen
XL Control PCR Template	25 ng/μl in TE Buffer	Invitrogen
XL Control PCR Primer	0,2 ng/μl in TE Buffer	Invitrogen
50 mM dNTP Mix	12,5 mM dATP 12,5 mM dCTP 12,5 mM dGTP 12,5 mM dTTP neutralized at pH 8.0 in water	Invitrogen
Sterile water	From kit	Invitrogen
Elongase™ Polymerase Mix		Invitrogen

Used in method 3.2.10		
Primer	**Recognition sequences**	**Manufacturer**
Abl-R (EcoRI)	5'-CCG-GAA-TTC-CTG-CAG-GCA-GCT-CCG-ACG-3'	Eurogentec
Bcr-F (EcoRV)	5'-CAG-GAT-ATC-CCG-GCC-GCG-CCA-TGG-TGG-3'	Eurogentec
SacI-R	5'-GAA-TCT-CGT-AGA-GCT-CAG-GCA-3'	Eurogentec
SacI-F	5'-TGC-CTG-AGC-TCT-ACG-AGA-TTC-3'	Eurogentec
AatII-R	5'-TGC-CCA-GAC-GTC-CGA-CTT-GA-3'	Eurogentec
AatII-F	5'-TCA-AGT-CCG-ACG-TCT-GGG-CA-3'	Eurogentec
Bcr-fp (EcoRV)	5'-CAG-GAT-ATC-GCA-GGT-AAG-GCC-GGC-CGC-G-3'	Eurogentec
Bcr-Abl template pEYKBA	Includes 10061 bp of the Bcr-Abl gene	Eugenia Dikovskaia, MIT

Used in method 3.2.11		
Reagents	**Specifications**	**Manufacturer**
Agarose	Sea®Kem LE Agarose	Cambrex
1X TAE buffer		
Crystal Violet Solution	2 mg/ml	Invitrogen
6X Crystal Violet Loading Buffer	30 % Glycerol 20 mM EDTA 100 μg/ml Crystal Violet	Invitrogen
Excising		
6,6 M Sodium iodide	6,6 M Sodium iodide 16 mM Sodium sulfite	Invitrogen
Binding Buffer	7 M Guanidinium HCl	Invitrogen
Isolating		
1X Final Wash	100 mM NaCl	Invitrogen
TE-buffer	10 mM TrisHCl (pH 8.0) 1 mM EDTA H_2O adjusted to a total volume of 100 ml	

Used in method 3.2.12		
Reagents	**Specifications**	**Manufacturer**
TOPO®XL PCR Cloning Kit	Catalogue no. K4700-10	Invitrogen
pCR®-XL-TOPO vector	10 ng/µl plasmid DNA in: 50 % glycerol 50 mM Tris-HCl (pH 7.4) 1 mM EDTA 2 mM DTT 0,1% Triton X-100 100 µg/ml BSA Phenol red	Invitrogen
6X TOPO® Cloning Stop Solution	0,3 M NaCl 0,06 M $MgCl_2$	Invitrogen
One Shot® TOP10 Electrocompetent *E. coli*	Catalogue no. 4040-50	Invitrogen
SOC	20 g/l bacto-tryptone 5 g/l bacto yeast extract 8,6 mM NaCl 2,5 mM KCl 20 mM glucose pH adjusted to 7.0	

Used in method 3.2.13		
Reagents	**Specifications**	**Manufacturer**
sodiumacetat (NaOAc)	3 M, pH 5,2	
100 % ethanol		Arcus AS
70 % ethanol		Arcus AS
Big Dye version 3.1		Applied Biosystem
Primer T7, T6, T3, CMV FW	10mM	Operon
5x Sequencing buffer	400 mM TrisHCl 10 mM $MgCl_2$ pH adjusted to 9.0	
EDTA	125 mM 23,3g diNaethylene-diaminetetraacetic * 2 H_2O pH adjusted to 8.0 with NaOH	

Used in method 3.2.15		
Reagents	**Specifications**	**Manufacturer**
X-Gal	50mg/ml	Promega
IPTG	50 µl 100 mM	Promega

Used in method 3.2.16	
Reagents	**Specifications**
50 % glycerol	

Used in method 3.2.17 and 3.2.18		
Reagents	**Specifications**	**Manufacturer**
BA/F3 cells	Mouse pro B cell DSMZ no.: ACC 300	Depositor: Obtained from RIKEN Cell Bank (RCB0805), Tsubuka Science City, Ibaraki, Japan
Growth medium	90 % RPMI 1640 10 % FBS 100 µl (10 µg/ml) IL-3	
Opti-MEM®		GIBCO™, Invitrogen life technologies

3.1.2 The two DNA ladders

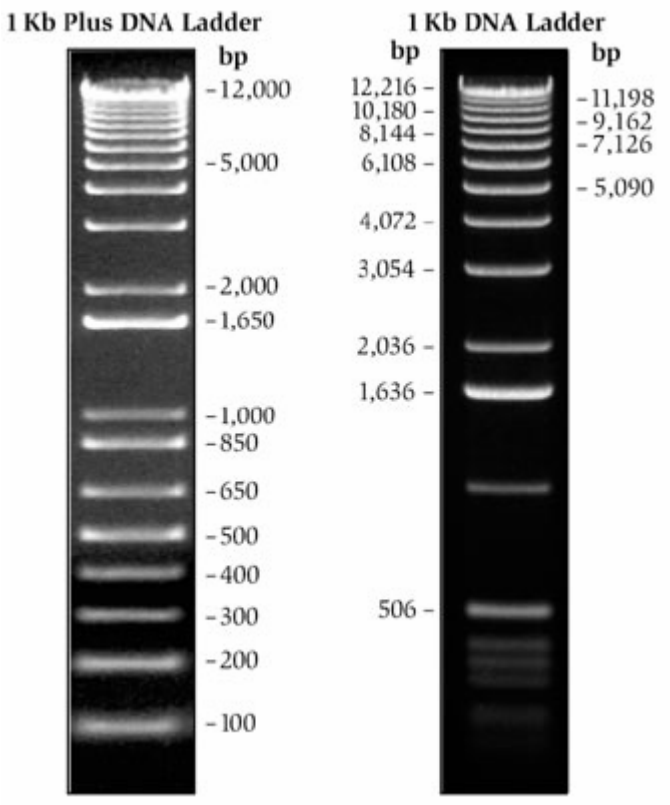


Figure 5. Sketch of the 1 Kb Plus DNA Ladder and the 1 Kb DNA Ladder (Invitrogen)

3.1.3 Plasmid constructs used in this thesis

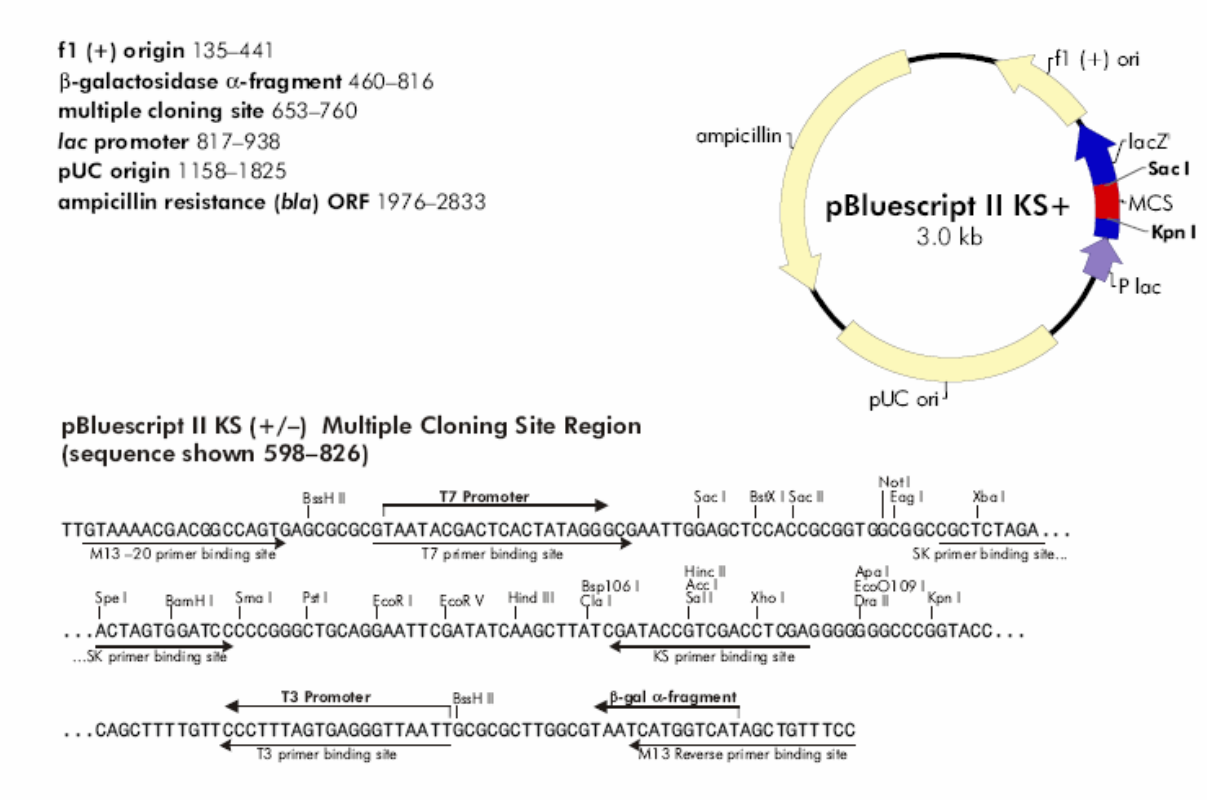


Figure 6. A sketch showing the pBluescript II KS+ vector and the multiple cloning site region (Stratagene).

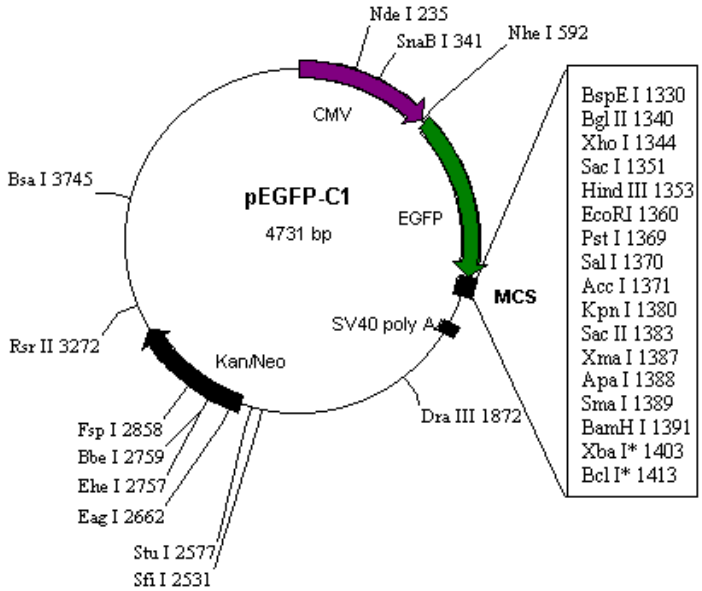


Figure 7. A sketch showing the pEGFP-C1 vector and the multiple cloning site region (Clonetech).

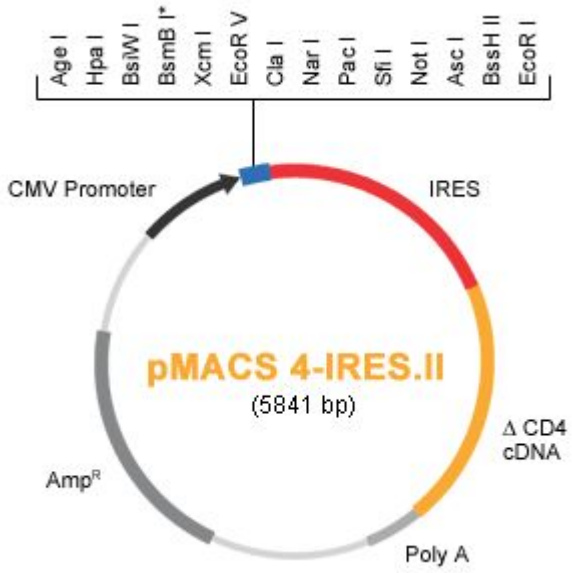


Figure 8. A sketch showing the pMACS 4-IRES.II vector and the multiple cloning site region (Miltenyi Biotec).

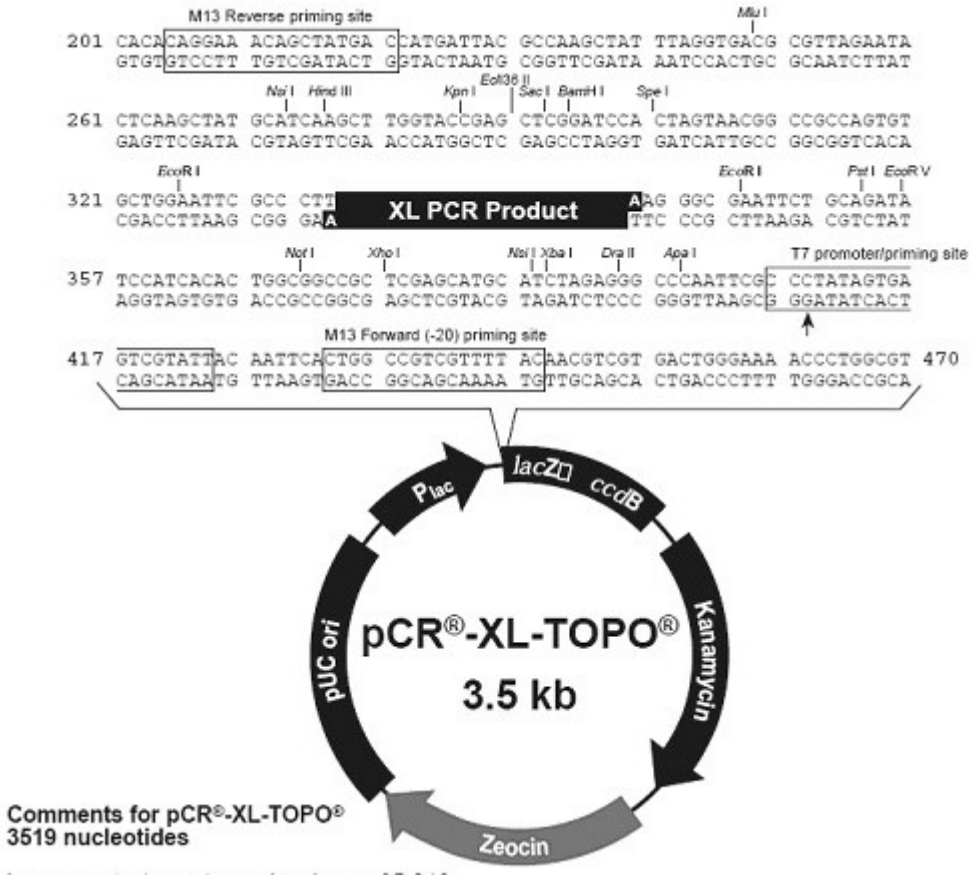


Figure 9. A sketch showing the pCR®-XL-TOPO® vector and the multiple cloning site region (Invitrogen).

3.2 Methods

3.2.1 Digestion of DNA with restriction enzymes

In separate tubes, the plasmid vector and the DNA were digested with the appropriate restriction enzyme(s).

In a microfuge tube, the following mixture was set up for each digesting reaction:

DNA (1-2 μg)	x μl
10x restriction enzyme buffer	2 μl
Restriction enzyme(s)	0,5 μl
dH_2O to a final volume of	20 μl

A control reaction was set up containing all the reagents listed above except the restriction enzyme(s).
The reaction was performed in microfuge tubes at 37°C for 1-2 hours.

When digestion was completed 4 μl 6 x Gel loading buffer was added to the reaction.

<u>To generate blunt ends</u>
If it is not possible to make complementary sticky ends, making blunt ends is an alternative. These ends can be joined to any other blunt end regardless of how the blunt end was generated. Blunt-end cloning is inefficient and blunt ligation gives no occasion to affect the orientation of the DNA fragment within the vector.

T4 DNA polymerase convert protruding 3' ends to blunt ends in the presence of high concentrations of dNTPs. Protruding 3'ends are removed from double-stranded DNA by 3'→5' exonuclease activity of bacteriophage T4 DNA polymerase [Sambrook and Russell 2001].

After 1,5 hours at 37°C the reaction was added:

T4 DNA polymerase	1 μl
2 mM dNTP	1 μl

Thereafter the reaction was incubated at 37 °C for 20 minutes, then 20 minutes at 70 °C to inactivate the DNA polymerase.
Next the appropriate restriction enzyme that generates the second end was added.
Lastly, the reaction was incubated at 37 °C for 1,5 hours.
When digestion was completed 6 x Gel loading buffer was added (4 µl for the 20 µl reaction volume).

Dephosphorylation of plasmid vectors with alkaline phosphatase

To prevent self-ligation of plasmid vectors in ligation reaction the terminal 5'-phosphate groups may be removed. Using alkaline phosphatase, the terminal 5'-phosphate residues from single- or double stranded DNA or RNA will be removed. This dephosphorylation reaction suppresses self-ligation of vector molecules and will decrease the number of "empty" vectors. A foreign DNA with intact 5'-terminal phosphate residues can be ligated efficiently into the dephosphorylated plasmid DNA. Shrimp alkaline phosphatase (SAP) is isolated from arctic shrimp and is one of the most widely used phosphatases in molecular cloning [Sambrook and Russell 2001].

Closed circular plasmid DNA was digested with the desired restriction enzyme as described above for 1,5 hours at 37°C.
Thereafter 1 µl of 10 x SAP buffer was added to the linearized plasmid and incubated for another 30 minutes at 37°C before the digestion was completed and 4 µl 6 x Gel loading buffer was added to the reaction.

3.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate and identify DNA on the basis of their size and rate of movement through a gel under the influence of an electric field. Since the nucleic acids are negatively charged due to the negatively charged phosphate group, they will migrate to the positively charged electrode (anode). Distinct bands, based on the molecule size, will form on the gel. Shorter DNA molecules will move faster than longer, since they are able to slip through the matrix more easily. The band can be visualized by staining the DNA with ethidium bromide, which causes the DNA to fluoresce in ultra violet

light. Ethidium bromide is a ring-formed structure that intercalates between the base pairs in the DNA double helix. It is a mutagen and was handled with care.

Preparing the gel

1. A 0,7% agarose gel was made by mixing 0,7 g agarose with 90 ml distilled water and 10 ml 10X TBE buffer.

2. The mixture was heated in a microwave oven until all agarose had melted and the solution had started to boil.

3. The gel solution was then left to cool (to approximately 65 °C).

4. 10 µl of ethidium bromide were added after the mixture had cooled, and gently mixed into the agar.

5. The gel was poured slowly into a gel rack, the comb was set at one side of the gel, and any bubbles in the solution removed. The gel was allowed to set (20 to 30 minutes).

6. After 20 minutes, when the gel had solidified, the comb was removed, and the gel, together with the rack, was soaked into a chamber with 1X TBE gel running buffer. The gel was placed with the wells facing the electrode that provide the negative current (cathode).

Loading and running the gel

Loading buffers were added to the DNA samples in order to visualize it and sediment it the gel wells (6 X T, added at the end of the cutting).

1. A DNA ladder, a mixture of DNA fragments of known size, was loaded into the first well. This was used to determine the absolute size of the separated DNA strand by comparing their migration with that of the ladder (see Figure 5).

2. The samples were loaded into the wells and the lid of the electrophoresis chamber was closed and the current was applied. The gel was run at 90 volts (usually 30 minutes to 1 hour).

3. The ethidium bromide stained gel was visualised under UV light and photographed. Eventually the required DNA band was cut out and a QIAquick Gel Extraction was done (see 3.2.3).

3.2.3 QIAquick Gel Extraction Kit

Bands cut from an agarose gel were purified from the gel matrix using QIAquick Gel Extraction Kit.

1. The required DNA fragment was excised from the agarose gel using a scalpel and transferred to a microcentrifuge tube.

2. The size of the gel slice was determined by weight and 3 volumes of Buffer QG were added to 1 volume of gel (100 mg ≈100 µl).

3. The tube was incubated at 50°C until the gel slice had dissolved completely. To help dissolve the gel, the tube was mixed by vortexing several times during the incubation.

4. After the gel slice had dissolved, 1 gel volume of isopropanol was added to the sample and mixed.

5. To bind DNA, the sample was applied to a QIAquick column, and centrifuged at 13000 rpm for 1 minute in a Microcentrifuge, Mini Spin (eppendorf).

6. The flow-through was discarded.

7. To remove all traces of agarose, 0,5 ml Buffer QG was added to the column and centrifuged for 1 minute in a Microcentrifuge, Mini Spin (eppendorf). The flow-through was discarded.

8. The column was washed by adding 0,75 ml Buffer PE and centrifuged for 1 minute in a Microcentrifuge, Mini Spin (eppendorf).

9. The flow-through was discarded and the column was centrifuged for and additional minute in a Microcentrifuge, Mini Spin (eppendorf).

10. The QIAprep spin column was placed in a clean 1,5 ml microcentrifuge tube and the DNA was eluted by adding 50 µl Buffer EB to the centre of the membrane before it was centrifuged for 1 minute in a Heraeus, Biofuge pico.

3.2.4 DNA ligation

Ligation is the process in which an insert are annealed into a vector by a covalent bond called phosphodiester bond. DNA ligases catalyze the end-to-end joining of the DNA by forming a phosphodiester bond between the 3'hydroxyl and the 5'phosphate ends of nucleic acid molecules [Sambrook and Russell 2001].

When cutting with restriction enzymes a break is formed in the phosphodiester back bond.
This nick, a broken phosphodiester bond leaving a free 3' OH and a free 5' phosphate, is sealed by DNA ligase [Nelson and Cox 2002].
Ligation is an energy requiring reaction that occurs in three distinct steps [Trun and Trempy 2003]. In the first step the adenyl group from ATP is covalently attached to ligase and inorganic phosphate is released. Next, the adenyl group is transferred from ligase to the 5'-phosphate of the DNA in the nick. Lastly, the phosphodiester bond is formed when the 3'hydroxyl end in the nick attacks the activated 5'phosphate. AMP is released in the process [Trun and Trempy 2003].

The enzyme used to ligate DNA fragments is an enzyme from bacteriophage T4 called T4 DNA ligase [Sambrook and Russell 2001]. This is a monomeric protein of 487 amino acids.

In a microfuge tube, the following ligation mixture was set up:

Plasmid vector	x µl
DNA insert	x µl
5 x ligation buffer	4 µl
T4 DNA ligase	1 µl
dH_2O to a final volume of	20 µl

To do a self-ligation test, a control reaction containing all the reagents listed above except the DNA insert was set up.

The ligation mixture was set to incubation overnight at 14°C.

3.2.5 Transformation of DNA into competent *E. coli* DH5α cells

Transformation is the introduction of a plasmid into a competent cell. A competent cell is a cell that is chemically treated to allow its membrane to be permeated by plasmids. The plasmid will be replicated in the bacteria, which will copy the DNA fragment of interest. Often the plasmid carries a gene that can make the bacteria resistant to an antibiotic. Only the bacteria that carry the plasmid will grow.

All steps in this protocol were carried out as sterile as possible.

1. Frozen competent *E. coli* DH5α cells were removed from the –80 °C freezer and thawed on ice at room temperature.

2. The ligation mixture was diluted to100 µl with distilled water.

3. 300 µl competent *E. coli* DH5α cells and 100 µl off the diluted ligation mix were dispensed into sterile falcon tubes chilled on ice.

4. The tube was incubated on ice for 30 minutes

5. The cells were heat shocked at 37°C for 2 minutes

6. Immediately 5 ml of SOC. was added and the mixture was incubated at 37°C with vigorous shaking at 225 rpm for 45 minutes.

7. After incubation the tube was centrifuged for 5 minutes at 2500 rpm in a Centrifuge 5810 R (eppendorf).

8. The supernatant was poured off and the pellet was resuspended in the remaining supernatant (approximately 0,5 ml).

9. 100 µl was transferred to the centre of an agar plate containing appropriate antibiotic, and a sterile spreader sealed in a flame was used to spread the solution over the entire surface of the plate.

10. The plate was stored at room temperature until the liquid had been absorbed.

11. The plate was inverted and incubated overnight at 37 °C.

After an overnight incubation at 37 °C the numbers of antibiotic-resistant colonies were counted. The self-ligation test should yield few if any colonies, whereas the ligation mixture should yield increasing numbers of colonies containing recombinant plasmid molecules.

The plates were stored at 4 °C.

Extraction of plasmids from bacterial cells

Three different methods were used for preparation of plasmid DNA from *E. coli,* dependent on the requirement for quality and quantity. When purifying where of no importance the "Miniprep light method" was used. This method is a simple and relatively low cost variant where no column and fewer amounts of buffers are used. A variety of kits for plasmid purification are available from commercial vendors. The "QIAprep Spin Miniprep Kit" was chosen when the DNA was to be used in cloning or in the DNA sequencing process. For larger quantity where the quality of DNA was of importance the DNA was purified using the "QIAGEN Plasmid Midiprep". This method yields DNA that is clean enough for transfection of mammalian cells and all enzymatic reactions, including DNA sequencing.

3.2.6 ”Miniprep light”

1. 1,5 ml overnight culture of *E. coli* in LB medium was transferred to a microcentrifuge tube and centrifuged 30 seconds. The supernatant was discarded.

2. The pelleted bacterial cells were resuspended in 100 µl Buffer P1 by vortexing.

3. 100 µl Buffer P2 was added and mixed carefully. For 5 minutes the solution was incubated at room temperature.

4. 140 µl Buffer N3 was added and mixed immediately, but thoroughly by inverting the tube.

5. The tube was centrifuged for 10 minutes at 13000 rpm in a Microcentrifuge, Mini Spin (eppendorf). A compact white pellet formed.

6. The supernatant from step 5 was applied to a new microcentrifuge tube.

7. 350 µl of isopropanol was added and the tube was inverted.

8. For 5 minutes the tube was incubated at room temperature.

9. The tube was centrifuged for 10 minutes at 13000 rpm. The supernatant was discarded.

10. The solution was washed by adding 400 µl of 70% ethanol

11. The tube was centrifuged for 5 minutes at 13000 rpm in a Heraeus, Biofuge pico. The supernatant was discarded.

12. The tube was air-dried for 20 minutes in room temperature.

13. The DNA was resuspended in 50 µl TE-buffer

3.2.7 QIAprep Spin Miniprep Kit

Isolation of plasmid DNA was performed using the QIAprep Spin Miniprep Kit.

To screen the colonies for correct insert a selection of colonies from the transformation procedure was picked, by stabbing a sterile toothpick into the soft agar. The inoculums were transferred into tubes of LB medium containing the appropriate antibiotic before the tubes were incubated at 37°C with vigorous shaking overnight.
Another starting point was overnight cultures started from freezing stocks.

1. 1,5 ml overnight culture of *E. coli* in LB medium was transferred to a microcentrifuge tube and centrifuged 30 seconds at 13000rpm in a Microcentrifuge, Mini Spin (eppendorf). The supernatant was discarded.

2. The pelleted bacterial cells were resuspended in 250 µl Buffer P1 by vortexing.

3. 250 µl Buffer P2 (lysis buffer) was added and mixed carefully. The solution became viscous and slightly clear. For 5 minutes the solution was incubated at room temperature.

4. 350 µl Buffer N3 (neutralization buffer) was added and mixed immediately, but thoroughly by inverting the tube.

5. The solution was centrifuged for 10 minutes at 13000 rpm in a Heraeus, Biofuge pico. A compact white pellet formed.

6. The supernatants from step 5 were applied to a QIAprep spin column by decanting and pipetting before it was centrifuged for 60 seconds in a Microcentrifuge, Mini Spin (eppendorf). The flow-through was discarded.

7. The QIAprep spin column was washed by adding 0,5 ml Buffer PB and centrifuged for 60 seconds. The flow-through was discarded.

8. Further the QIAprep spin column was washed by adding 0,75 ml Buffer PE and centrifuged for 60 seconds in a Microcentrifuge, Mini Spin (eppendorf).

9. The flow-through was discarded, and centrifuged for an additional minute in a Microcentrifuge, Mini Spin (eppendorf) to remove residual wash buffer.

10. The QIAprep spin column was placed in a clean 1,5 ml microcentrifuge tube and the DNA was eluted from the QIAprep column with 50 µl Buffer EB by adding it to the centre of the membrane. The column was left for 1 minute, before it was centrifuged for 1 minute in a Heraeus, Biofuge pico.

11. The DNA yield of the eluate was then determined using a spectrophotometer to measure absorbance at a wavelength of 260 nm

3.2.8 QIAGEN Plasmid Midiprep

Preparation of DNA plasmid for electroporation was performed using QIAGEN Plasmid Midi Kit.

1. In a 500 ml volumetric flask 0,5 ml of overnight culture previously used for miniprep was diluted into 100 ml selective LB medium. The culture was grown at 37°C over night with vigorous shaking.

2. The dilution was transferred to a centrifuge tube and the bacterial cells were harvested by centrifugation in a Sorvall RC 26 PLUS rotor in a SLA1500 centrifuge at 6250 rpm for 5 minutes at 4°C.

3. The supernatant was discarded. To remove all traces of supernatant the open centrifuge tube was set up side down on a paper until all medium had drained.

4. The bacterial pellet was resuspended in 4 ml Buffer P1 by vortexing until no cell clumps remained.

5. 4 ml of Buffer P2 was added and mixed carefully before it was incubated at room temperature for 5 minutes.

6. 4 ml of chilled Buffer P3 was added and mixed immediately but gently by inverting before it was incubated on ice for 15 minutes.

7. The solution was centrifuged in a Sorvall RC 26 PLUS rotor in a SLA1500 centrifuge at 11500 rpm for 30 minutes at 4°C. The supernatant containing plasmid DNA was removed by filtration.

8. A QIAGEN-tip was equilibrated by applying 8 ml Buffer QBT, and the column was allowed to empty by gravity flow.

9. The supernatant from step 7 was applied to the QIAGEN-tip and allowed to enter the resin by gravity.

10. The QIAGEN-tip was washed with 2 x 10 ml Buffer QC.

11. The DNA was eluted with 5 ml Buffer QF. The eluate was collected in a 10 ml tube.

12. The DNA was precipitated by adding 3,5 ml room temperature isopropanol to the eluted DNA. The solution was mixed and centrifuged in a in a Sorvall RC 26 PLUS rotor in a SS-34 centrifuge at 11200 rpm for 30 minutes at 4°C. The supernatant was carefully decanted.

13. The DNA pellet was washed with 2 ml of room temperature 70% ethanol, and centrifuged in a Sorvall RC 26 PLUS rotor in a SS-34 centrifuge at 11200 rpm for 10 minutes at 4°C. The supernatant was carefully decanted, and the remaining liquid was sucked off without disturbing the pellet.

14. The pellet was air-dried for 5-10 minutes, and the DNA was redissolved in 0,5 ml TE-buffer.

15. The DNA yield was then determined using a spectrophotometer to measure absorbance at a wavelength of 260 nm.

3.2.9 Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a cell-free technique, which is used to amplify a specific DNA sequence. In an exponential manner a small amount of DNA is amplified into a large amount of DNA in a very short time [Passarge 2001].

In order to use PCR, one must know a part of the sequence on either side of the region of interest in DNA to be amplified, because the only DNA segment that can be amplified is the segment between the two primers [Nelson and Cox 2002].

The DNA containing the sequence to be amplified is incubated in a test tube with the primers, each complementary to the ends of the targeted DNA, the four deoxynucleotides and a heat stable DNA polymerase.
The heat stable *Taq* DNA polymerase, from the thermophilic bacterium *Thermus aquaticus,* is not inactivated at the high denaturation.

The PCR process consists of a series of about 25-35 subsequent cycles. Each cycle consists of three precisely time-controlled and temperature-controlled steps – denaturation, annealing and extension.

1) The first step separates the double stranded DNA into two single strands by use of a high temperature, usually 94-95°C, is used. This denaturing step breaks the hydrogen bonds between the two strands.

2) Then the temperature is lowered to 55°C to allow the primers to base pair to their complementary sequences on the template strands.

3) Further the reaction is heated to 72°C, the optimal temperature for the heat stable DNA polymerase to replicate the single stranded DNA segments. The DNA polymerase uses deoxynucleotides as building blocks of the new strands.

The denaturation, annealing and extension steps are then repeated for a fixed set of cycles (25-35).

4) Finally the reaction is cooled down to 4 °C where it can be stored until the user terminates the program [McPherson and Møller 2000].

PCR is called a chain reaction because newly synthesized double-stranded DNA molecules are once again denatured and each single strand acts as a new template for further DNA synthesis [Passarge 2001].

Karl B. Mullis invented PCR in 1983 [Nelson and Cox 2002] and in 1993 he won a Nobel Prize in Chemistry for developing this revolutionary technology. Today PCR has a major impact on biological and medical research and biotechnology. PCR is used for a variety of tasks, such as the detection of hereditary diseases, the identification of genetic fingerprints, and the diagnosis of infectious diseases, the cloning of genes, paternity testing, and DNA computing [Nelson and Cox 2002].

3.2.10 Amplifying the Bcr-Abl gene using PCR

The intention is to isolate and amplify the different fragments in the Bcr-Abl gene using PCR and different primers (see Table 1).

Table 1. Primers and templates used for amplification of the Bcr-Abl gene.

Tube	Fragment	Primers	Templats
1.	Control PCR product	XL Control PCR Primer	XL Control PCR templat
2.	Fragment A	Bcr-*F(EcoRV) + SacI-*R	Template (pEYKBA)
3.	Fragment B	SacI-F + AatII-R	Template (pEYKBA)
4.	Fragment C	AatII-F + Abl-R	Template (pEYKBA)
5.	Fragment BC	SacI-F + Abl-R	Template (pEYKBA)
6.	Fragment ABC	Bcr-F + Abl-R	Template (pEYKBA)

*F = Forward

R = Reverse

The following PCR reaction was set up (tube 1.):

Elongase 5 X Buffer A	5 µl
Elongase 5 X Buffer B	5 µl
XL Control PCR Template	1 µl
XL Control PCR Primers	1 µl
50 mM dNTPs	0,5 µl
Sterile Water to a final volume of	50 µl

To each of the tubes (2-6) there was applied

Elongase 5 X Buffer A	5 µl
Elongase 5 X Buffer B	5 µl
Plasmid Template (pEYKBA)	1 µl
50 mM dNTPs	0,5 µl
Primer F	1µl
Primer R	1 µl
Sterile Water to a final volume of	50 µl

Table 2. The PCR program used to amplify the different fragments in the Bcr-Abl gene.

Step	**Time**	**Temperature**	**Cycles**
1. Initial Denaturation	2 minutes	94°C	1X
2. Denaturation	15 seconds	94°C	25X
3. Annealing	1 minute	56°C	25X
4. Extension	5 minutes	68°C	*25X
5. Final Extension	7 minutes	72°C	1X
6. Storage	For ever	4°C	∞

*Go to step 2. 24 times more

1. The program was started and paused when the heat block reached 94°C.

2. The tubes were placed on the block for 30 seconds before 1 µl of Elongase™ polymerase mix was added.

3. After that the program was continued.

3.2.11 Gel –purifying PCR products using crystal violet agarose gel

In traditional ethidium bromide agarose gel electrophoresis the DNA is exposed to UV light and this may damage the DNA and decrease the cloning efficiency. To avoid damaging the PCR products agarose gel electrophoresis using crystal violet was chosen. Using this method the PCR products can be visualized under normal light as a thin blue band. PCR products can also be visualized while the gel is running and excised as soon as they are sufficiently resolved (TOPO® XL PCR Cloning Kit, Instruction manual).

Preparing the gel

1. A 0,8% agarose gel was made by using 0,4 g of agarose and 50 ml 1X TAE buffer in a volumetric flask.

2. The mixture was placed in the microwave oven and heated until just boiling. The flask was swirled to dissolve the agarose and continued to heat until the agarose was dissolved and the nucleases destroyed.

3. The agarose was cooled for a few minutes before 20µl 2mg/ml Violet solution Crystal was added.

4. The gel was poured into a gel rack and the comb was set in the gel.

5. After 20 minutes, when the gel had solidified, the gel, together with the rack was transferred to a chamber with 1X TAE buffer.

6. The 1X TAE running buffer was made using 90 ml distilled water and 10 ml 10X TAE buffer. There should be enough running buffer to submerge the gel.

Loading and running the gel

1. 8 µl of 6X Crystal Violet Loading buffer was added to 40 µl of the PCR amplification and loaded onto the gel (see 3.2.2).

2. The gel was run at 80 volts until a thin blue PCR product was visible on the gel.

3. The PCR product was excised

Excising the PCR Product

1. The PCR product was excised from the gel using a scalpel and put into a microcentrifuge tube.

2. The size of the gel slice was determined by weight and 2,5 volumes of 6.6 M sodium iodide was added to 1 volume of gel before it was mixed by vortexing.

3. The tube was incubated at 42 to 50 °C until the gel slice had dissolved completely. To help dissolving the gel, the tube was vortexed several times during the incubation.

4. The tube was placed at room temperature and 1,5 volume of Binding Buffer was added before the tube was mixed well.

Isolating the PCR product

1. The mixture from step 4 over was transferred into a S.N.A.P™ Purification column and centrifuged for 30 seconds at 13000 rpm in a Microcentrifuge, Mini Spin (eppendorf).

2. The liquid in the collection vial was poured back onto the column and centrifuged for additional 30 seconds. This step was done twice, to bind the DNA to the column, before the supernatant was discarded.

3. After the last centrifugation 400 µl of 1X Final Wash was added to the column and centrifuged 30 seconds at 13000 rpm in a Microcentrifuge, Mini Spin (eppendorf).

4. Step 3 was repeated and the supernatant was discarded after the final centrifugation.

5 To dry the column resin, the column was centrifuged at 13000 rpm speed for 1 minute before the supernatant was discarded.

6. The column was placed in a clean microcentrifuge tube and 40 µl of TE buffer was added directly to the column and incubated for 1 minute at room temperature.

7. The column was centrifuged for 1 minute at 13000 rpm in the Microcentrifuge, Mini Spin (eppendorf) to elute the DNA into the microcentrifuge tube.

8. The tube was placed on ice.

3.2.12 Cloning of the BC-fragment into pCR®-XL-TOPO® vector

TOPO®XL PCR Cloning is an efficient one-step cloning strategy for the cloning of long PCR products.

1. A 5 µl TOPO®cloning reaction was set up in a microcentrifuge tube:

Gel-purified PCR product	4 µl
pCR®-XL-TOPO® vector	1 µl

2. The reaction was mixed gently and incubated for 5 minutes.

3. After 5 minutes incubation, 1 µl of the 6X TOPO® Cloning Stop Solution was added and mixed.

4. The tube was briefly centrifuged and placed on ice.

Chemical Transformation of TOPO® vector into One Shot® cells

1. 2 µl of the TOPO®cloning reaction was added into a vial of One Shot® cells and mixed gently.

2. The tube was incubated on ice for 30 minutes.

3. The cells were heat-shocked for 30 seconds at 42°C without shaking.

4. The cell tube was transferred back on ice and incubated for 2 minutes.

5. 250 µl of room temperature S. O. C. was added.

6. The tube was set at 37°C with vigorous shaking for 1 hour. After the incubation the tube was placed on ice.

7. 100 µl was spread on a prewarmed kanamycin plate.

8. The plate was incubated overnight at 37°C.

The DNA sequencing process

DNA sequencing, known as dideoxy sequencing, the Sanger method or chain termination method was developed by the British scientist Frederick Sanger in the1970s.
The method is used to determine the exact nucleotide sequence in a given DNA fragment [Campbell et al. 1999].

This technique utilizes 2',3'-dideoxynucleotide triphosphates (ddNTPs), molecules that differ from deoxynucleotides (dNTPs) by lacking a hydrogen atom attached to the 3' carbon rather than a hydroxyl group. These molecules will terminate the DNA chain elongation because they cannot form a phosphodiester bond with the next deoxynucleotide [Passarge 2001].

During the sequencing reaction, when the temperature reach 94 °C, the DNA double helix is separated and the template strand is supplied with

- DNA primers (complementary to the template which is to be sequenced)
- DNA polymerase I (an enzyme that replicates DNA)
- A mixture of all four normal deoxynucleotides (dATP, dGTP, dCTP and dTTP) in ample quantities.
- A mixture of all four dideoxynucleotides (ddATP, ddGTP, ddCTP and ddTTP) in limiting quantities labelled with a "tag" that fluoresces a different colour and different wavelengths.

Because all four normal nucleotides are present, chain elongation proceeds normally until, by chance, DNA polymerase inserts a dideoxynucleotide instead of a normal deoxynucleotide. The competition between dideoxynucleotide and deoxynucleotide for incorporation into the growing chain will lead to a representation of lengths of DNA that correspond to the first 200-500 residues complementary to the template.

The tagged fragments are subsequently separated on a thin column. At the base of the column a laser is located. As the DNA fragments run off the column, they pass through the laser beam, fluoresce, and the wavelength of the fluorescence is recorded and sent to a computer. The order of the fluorescently tagged molecules coming off the column reflects the sequence of the template DNA [Nelson and Cox 2002].

Each of the four dideoxynucleotides fluoresces a different colour when illuminated by a laser beam and an automatic scanner provides a printout of the sequence [Nelson and Cox 2002].

3.2.13 DNA Sequencing

Before sending samples for sequencing they were precipitated. The QIAgen minipreps were used:

In a microcentrifuge tube
5 µl 3M NaOAc (pH 5,2)
150 µl 100% ethanol
was added to 50 µl QIAgen purified DNA.

1. The tube was vortexed and then centrifuged at 13000 rpm for 20 minutes at 4°C in a Heraeus Biofuge fresco centrifuge.

2. The supernatant was sucked off.

3. 0,5 ml 70% ethanol was added and the solution was centrifuged at 13000 rpm for 5 minutes at 4°C in a Heraeus Biofuge fresco centrifuge. .

4. The supernatant was sucked off.

5. The sample was air-dried for 20-30 minutes.

6. The DNA precipitate was resuspended in 50 µl of sterile water

The concentration of DNA was measured by using a nano drop count or a spectrophotometer (see 3.2.14).

A sequencing PCR reaction was set up:

DNA (about 500 ng) in sterile water	6 µl
Big Dye version 3,1	2 µl
Primer (10µM)	1 µl
5 X sequencing buffer	3 µl
Sterile water to a final volume of	20 µl

Table 3. PCR program for plasmid DNA sequencing

Step	**Time**	**Temperature**	**Cycles**
1. Initial Denaturation	2 minutes	94°C	1X
2. Denaturation	1 minute	94°C	*35X
3. Annealing	2 minutes	63°C	35X
4. Replication	2 minutes	72°C	35X
5. Final Extension	10 minutes	72°C	1X
	∞	4°C	-

*Go to step 2. 34 times more

1. After the sequencing PCR the product was precipitated in a microcentrifuge tube as described below.

125 mM EDTA	2 µl
3 M NaOAc (pH 5,2)	2 µl
50 µl 100% ethanol	50 µl
Sequencing PCR product	20 µl

2. The solution was mixed and incubated in room temperature for 15 minutes.

3. The tube was centrifuged at 13000 rpm for 15 minutes in room temperature in a Heraeus, Biofuge pico.

4. The supernatant was removed immediately

5. 150 µl of 70% ethanol was used to wash the DNA precipitate and the tube was centrifuged at 13000 rpm for 5 minutes in room temperature in a Heraeus, Biofuge pico.

6. The supernatant was removed and the DNA precipitate was air-dried for 30 minutes before it was delivered for sequencing. The Sequencing lab uses a 16 capillars machine, 3130*xl* Genetic Analyzer from Applied Biosystem HITACHI.

3.2.14 DNA concentration measurements

Ultrospec 2000 spectrophotometer from Pharmacia Biotech was used to measure the DNA concentration. The apparatus was calibrated and adjusted on the right wavelength according to the user manual. DNA is measured at a wavelength of 260 nm. A solution with an A_{260} of 1 contains about 50 µg of DNA/ml [Sambrook and Russell 2001].

1. The DNA sample was diluted: 3 µl DNA + 57 µl sterile water

2. A blank sample containing only 60 µl with sterile water was loaded into the cuvette. Then the "Set ref" button was pressed.

3. After the blank measurement, the DNA sample was pipetted into the cuvette.

4. After the measurement the sample was sucked up again.

5. Step 3 and 4 was repeated for each sample.

6. After use the cuvette was cleaned with sterile water.

Using the NanoDrop® ND-1000 Spectrophotometer was another apparatus used to measure the DNA concentration. One advantage with this method is that it is not necessary to dilute the sample before measuring.

The “nucleic acid” was selected on the application module and the further measurements were performed according to the user manual.

1. First, the instrument was made ready for use by loading a water sample onto the lower measurement pedestal.

2. Before a sample measurement, a blank sample was loaded. A 1 µl blank sample with the same solvent used in the sample was loaded onto the lower measurement pedestal and the sampling arm was closed. Then the “Blank” button was clicked.

3. The pedestals were wiped using a laboratory wipe.

4. After making the initial blank measurement a 1 µl sample was pipetted onto the lower pedestal and the sampling arm was closed. Then the “Measure” button was clicked.

5. When the measurement was completed the sampling arm was opened and the sample was wiped from the upper and lower pedestals using a laboratory wipe.

6. Step 4 and 5 was repeated for each sample.

3.2.15 Application of X-gal and IPTG onto agar plates.

This is a method for identifying recombinant plasmids. Using X-gal that is converted by β-galactosidase into an insoluble dense blue compound [Sambrook and Russell 2001] it will be possible to distinguish bacteria transformed by recombinant plasmids from empty plasmids (see introduction).

1. 20 µl of 50mg/ml of X-gal and 50 µl of 100 mM IPTG was transferred to the centre of an agar plate containing appropriate antibiotic.

2. A sterile spreader sealed in a flame was used to spread the solution over the entire surface of the plate.

3. The plate was incubated for 30 minutes at 37 °C before the bacteria from the transformation procedure was applied (see 3.2.5).

4. After an overnight incubation at 37 °C the plate was removed from the incubator and stored at 4 °C for several hours for the blue colour to develop.

Blue colonies will contain plasmid vectors without insert, while white colonies contain plasmid vectors with insert.

3.2.16 Making freezing stocks

1 µl from the miniprep confirmed by sequencing containing the plasmid with the right insert and
200 µl *E. coli* DH5α bacterial cells (see 3.2.5) were dispensed into a sterile falcon tube.

100 µl was spread onto an agar plate containing the right antibiotic and incubated at 37°C overnight.
One colony was picked and transferred into a tube of 3 ml LB medium containing the appropriate antibiotic before the tube was incubated at 37°C with vigorous shaking (225 rpm) over night.

1.2 ml overnight culture was transferred into a cryon tube before 0.3 ml 50 % glycerol was added.

The tube was stored at the –80 °C freezer

3.2.17 BA/F3 mouse cells

The BA/F3 cell line is a mouse pro B cell type. They have their origin from IL-3 dependent murine pro B cell line established from peripheral blood; apparently derived from BALB/c mouse. Their appearance is mostly single, round cells in suspension. The saturated culture is split 1:10 in fresh medium (see materials) every 3 days, and incubated at 37 ˚C in a humidified atmosphere with 5 % CO_2.

3.2.18 Electroporation of mammalian cells

Electroporation is a mechanical method used to introduce DNA into a cell through the cell membrane. Techniques in molecular biology require a foreign gene or protein material to be inserted into a host cell. The phospholipid bilayer of the plasma membrane is amphipathic, meaning it has both a hydrophilic region and a hydrophobic region. The hydrophobic core of the membrane will make polar molecules, including DNA, unable to freely pass through the membrane [Campbell et al. 1999].

To deal with this problem scientists have developed this method for introducing DNA into cells. In electroporation, a brief electrical pulse is applied to a solution containing the host cells and the molecules to be inserted into these cells. This electrical pulse disturbs the phospholipid bilayer of the membrane and creates temporarily aqueous pores in the cell's plasma membranes, through which DNA or other molecules can enter [Campbell et al. 1999].

As molecules flow through the pores, the cell membrane discharges and the pores quickly close, and the phospholipid bilayer reassembles. Once inside the cell, DNA has a chance to be incorporated into the cell's DNA by natural genetic recombination [Campbell et al. 1999].

Procedure:

1. Two days prior to electroporation the cells were diluted into fresh growth medium (see 3.2.17).

2. At the day of electroporation the cell cultures were transferred to a centrifuge tube and pelleted by centrifugation at 1000 rpm for 4 minutes in a Heraeus, Labofuge 400 (FUNCTION Line).

3. The supernatant was removed and the cell pellet was washed in electroporation buffer (Opti-MEM) and centrifuged one more time at 1000 rpm for 4 minutes.

4. The buffer was removed and the cell suspension was diluted in Opti-MEM to the calculated density of cells/ml.

5. The calculated amount of plasmid DNA was added to the electroporation cuvettes (0,2 cm gap), thereafter the cell suspension was added

6. The desired pulse condition on the Gene Pulser Xcell™ was set.

7. The cuvette was tapped on the side to mix before it was placed in the electroporation device.

8. A pulse of electricity was delivered to the cells once.

9. Immediately after the pulse the cuvettes were rinsed with growth media and the cells were transferred onto the cell culture dishes.

10. The dishes were incubated at 37°C in a humidified atmosphere, and checked for transfection efficiency and viability after 24 hours.

4 RESULTS

As described in the "aim of the study" the main purpose of this thesis was to develop an experimental system where the molecular and biological effects of the Glivec-resistant mutations in the Bcr-Abl could be studied. To do this we intended to use the MACSelect transfected cell selection and the mouse pro–B cell-line BA/F3 was used.

To be sure that the system would work as intended, a pilot study with GFP cloned into the pMACS 4-IRES.II vector (see Figure 8) was performed.

In the light of this the thesis is divided into two sections:

- Cloning of GFP into the pMACS 4-IRES.II vector and electroporation of BA/F3 cells.
- Stepwise cloning of the Bcr-Abl gene into BA/F3 cells.

4.1 Cloning of GFP into the pMACS 4-IRES.II vector

The pMACS 4-IRES.II is an expression vector that contains an internal ribosome entry site (IRES) and a ΔCD4 encoding gene in addition to a multiple cloning site where the gene of interest can be cloned. The ΔCD4 gene encodes a transmembrane receptor with a truncated cytoplasmic domain. It has no possibilities to participate in signal transduction, but cells transfected with the cloned vector will co-express the CD4 markers on the cell-surface. By the use of magnetic beads with antibodies recognising the CD4 receptors one can select the transfected cells that co-express ΔCD4 along with the cloned gene of interest. When Green fluorescence protein (GFP) is cloned into the pMACS 4-IRES.II vector (see Figure 10), CD4 positive cells will make fluorescent light that can be observed in a microscope with UV filter. The purpose of cloning GFP into this vector is to be able to easily monitor the transfection efficiency and the magnetic bead selection process.

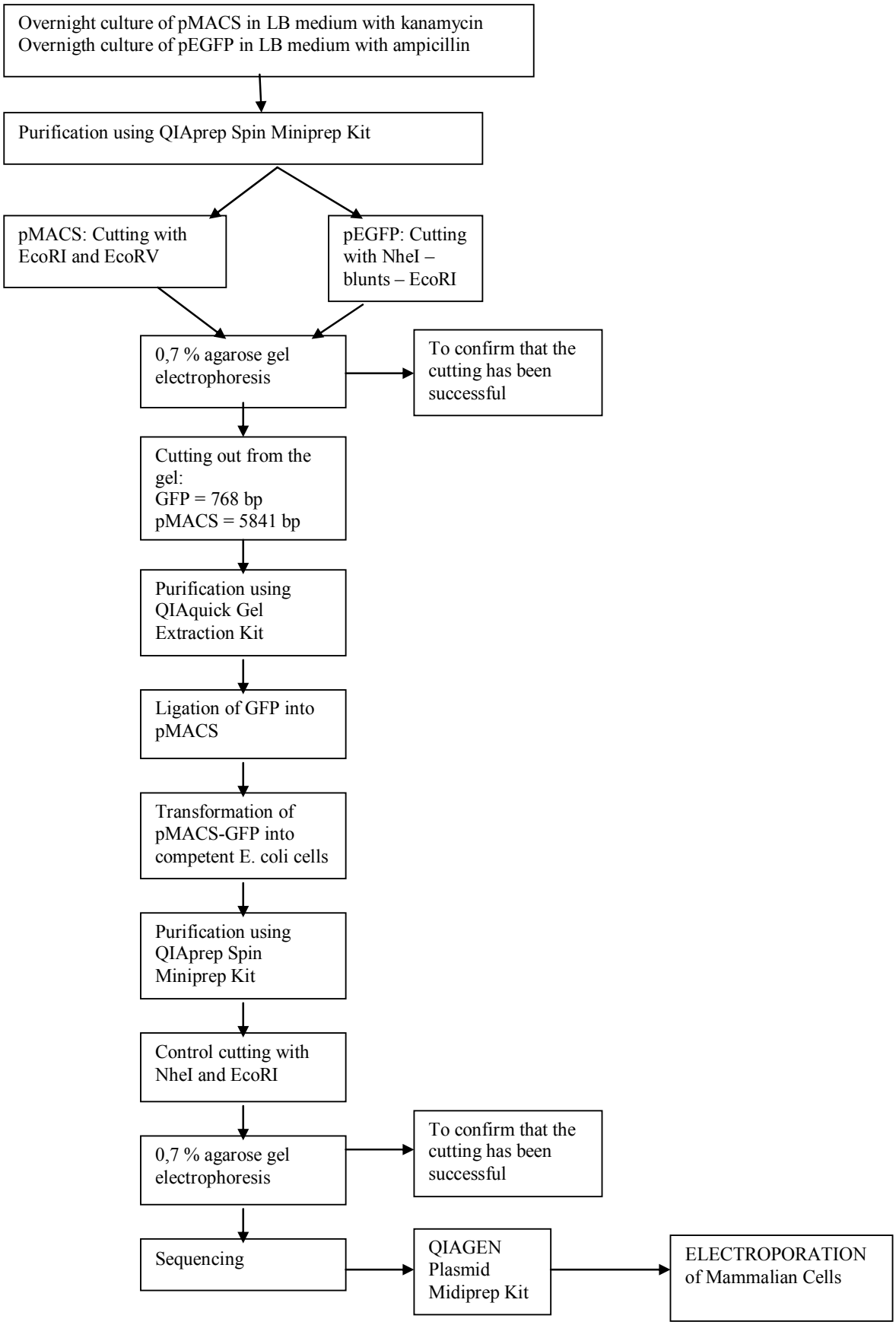


Figure 10. The cloning strategy of the GFP gene into the pMACS 4-IRES.II vector

Bacteria stocks containing either the pEGFP-C1 plasmid or the pMACS 4-IRES.II plasmid was grown in LB medium with appropriate antibiotics, and DNA minipreps were prepared as described in method 3.2.7.

To cut out the GFP from the pEGFP-C1 vector (see Figure 7) the vector was first cut with NheI (see 3.2.1). Thereafter the fragment was made blunt-ended by filling the 5' overhang with nucleotides, using T4 DNA polymerase (see 3.2.1). This would make it possible for the 5' end to ligate with the blunt end of the pMACS 4-IRES.II cut with EcoRV. Further the DNA fragment was cut with EcoRI. This separated the 760 bp NheI /EcoRI fragment from the pEGFP-C1 vector backbone. The pMACS 4-IRES.II vector was cut with EcoRI and EcoRV for 1,5 hours at 37°C before the vector was SAP treated to prevent religation of the vector in the ligation reaction (see 3.2.1). The DNA was separated on a 0,7 % agarose gel (see 3.2.2) for about 30 minutes (see Figure 11). Two bands were observed for pEGFP-C1. The upper band indicated rest of the pEGFP-C1 vector, while the lower band indicated the fragment containing the fragment of interest, the GFP gene. This 760 bp GFP-fragment with a blunt end and a staggered EcoRI 3' end, and the linearized pMACS 4-IRES.II vector on 5841 bp was cut out and purified using QIAquick Gel Extraction Kit (see 3.2.3).

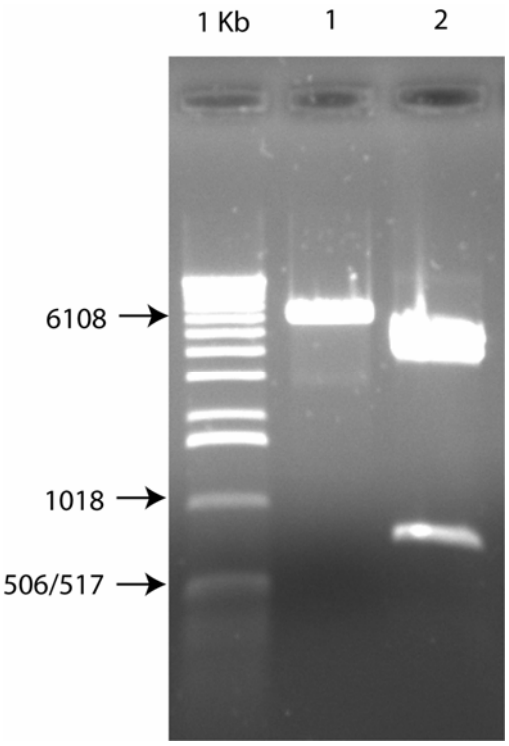
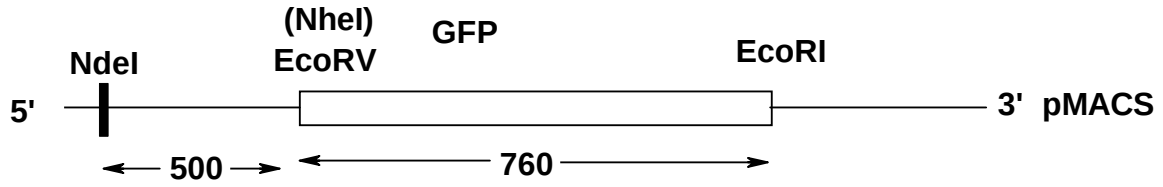


Figure 11. The GFP cut out from the pEGFP-C1 vector. The GFP in lane 2 is cut out from the pEGFP-C1 vector with NheI and EcoRI. Two bands are formed, the upper band indicate rest of the pEGFP-C1 vector on about 4000 bp, while the lower band on 760 bp indicate the GFP insert. The band formed in lane 1, on almost 6000 bp, indicates the pMACS 4-IRES.II vector cut with EcoRI and EcoRV. The pMACS vector and the GFP fragment were cut out from the gel and purified.

The purified GFP-NheI/EcoRI fragment was then ligated into the pMACS 4-IRES.II- EcoRI / EcoRV vector (see 3.2.4). The ligation reaction was transformed into competent *E. coli* DH5α cells as described in method 3.2.5, and plated onto media with ampicillin.

Single colonies were picked from the plates and were grown over night. Plasmid DNA was purified using Miniprep Kit (see 3.2.7). To test for plasmid DNA with correct insert, purified DNA was digested with NdeI and EcoRI. The NdeI recognition site is located 500 bp from the insert (see Figure 12). The resulting DNA fragments were separated on a 0,7 % agarose gel (see 3.2.2) to confirm whether the cloning of GFP into pMACS 4-IRES.II vector had been successful. It was expected to see two bands, one band on about 1300 bp and one band on about 6000 bp. The results show that four of the seven minipreps (lanes 3, 4, 5 and 7) have bands on the right size, 1268 bp and 5841 bp (see Figure 12). The minipreps with other band sizes are probably supercoiled uncut plasmid. Lane 6 contains a religated vector with no insert.

A.



B.

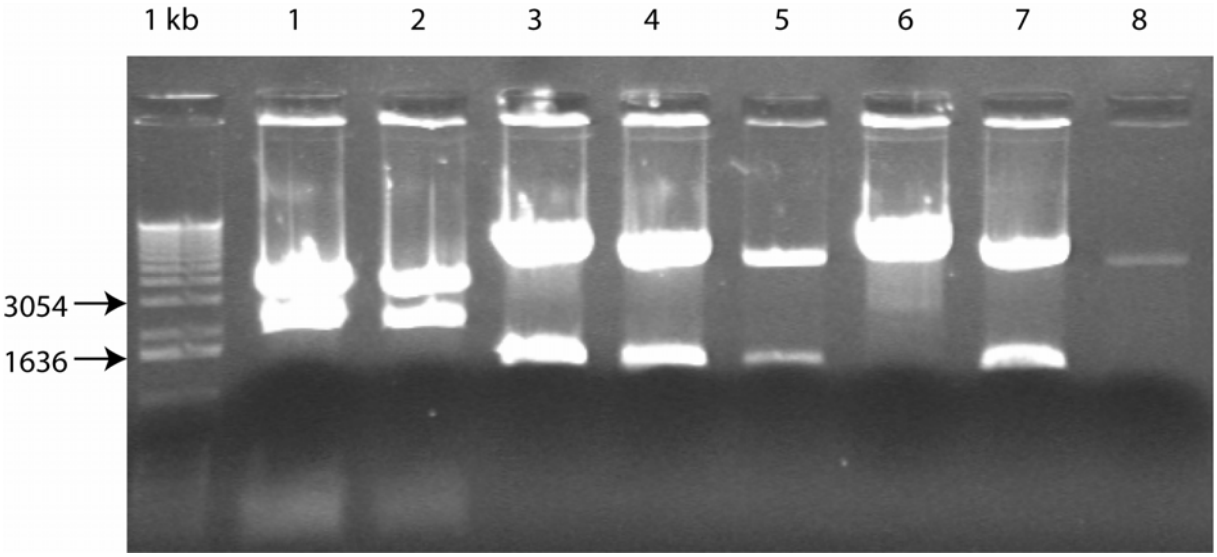


Figure 12. Cloning of GFP into pMACS 4-IRES.II. A. A sketch showing the GFP gene cloned into the pMACS 4-IRES.II vector with the NdeI restriction site located 500 bp from the insert. The plasmid is drawn as a line, while the incorporated GFP gene is drawn as a rectangle. Restriction enzyme put in parenthesis indicates that the end is made blunt. **B.** The GFP insert is cut out from the pMACS 4-IRES.II vector with NdeI and EcoRI. The NdeI sequence site is located 500 bp from the insert and will form the GFP band on about 1300 bp, while the pMACS vector is expected to form a band on about 6000 bp. The results show that the samples in lane 3, 4, 5 and 7 have an insert on the expected size.

Minipreps 3 and 4 were sent for sequencing (see 3.2.13) and confirmed positive before freezing stocks were made and stored at the –80 °C freezer (see 3.2.16).

Based on the overnight cultures used to make miniprep 3 with correct insert, preparation of DNA plasmids for electroporation was performed using QIAGEN Plasmid Midi Kit (see 3.2.8).

4.1.1 Optimization of electroporation of BA/F3 mouse cells

We wanted to test the electroporation protocol for electroporation of BA/F3 cells with pMACS-GFP and pEGFP-C1.

For a long time it was a problem that the transfection method did not work as intended. pEGFP-C1 was successfully transfected into the cells producing fluorescent cells, while the pMACS-GFP formed no green cells when used for electroporation. To verify that GFP was produced from the pMACS-GFP plasmid HeLa cells were transfected with pMACS-GFP. The transfection efficiency was poor, but green fluorescent cells were observed (result not shown) indicating that the pMACS-GFP plasmid is functional.

After additional optimization of the electroporation method it was still difficult to see green cells when pMACS-GFP was transfected, but the electroporation of pEGFP-C1 must be considered as successful (see Figure 13).

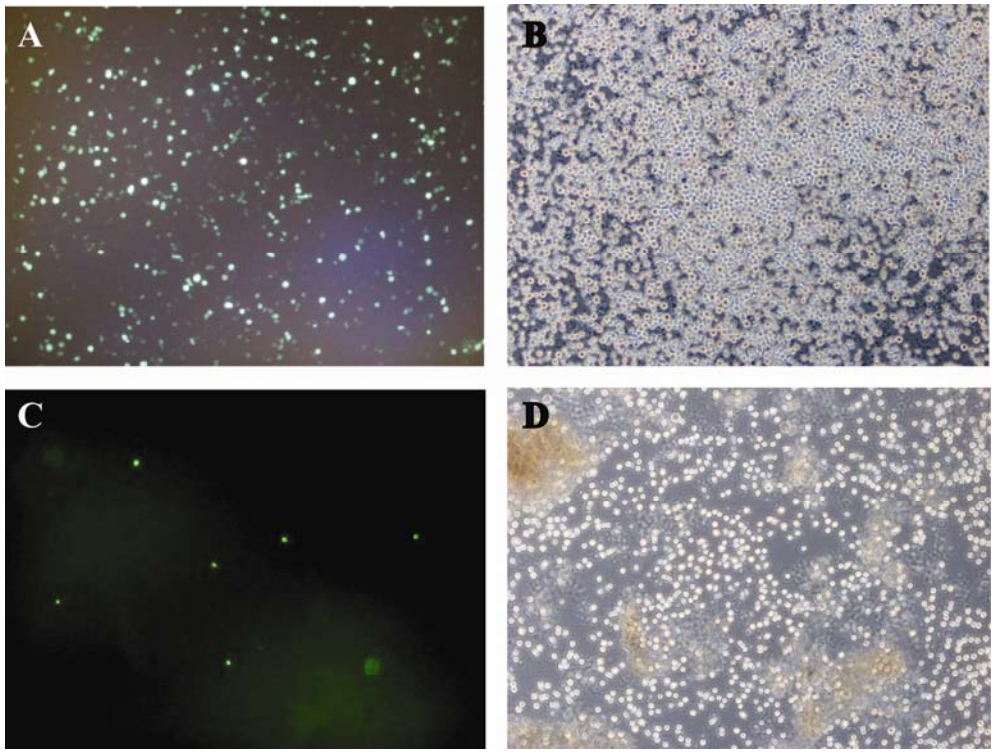


Figure 13. Pictures showing the transfection of pEGFP and pMACS-GFP into BA/F3 mouse cells. A. Fluorescent picture showing the BA/F3 cells successfully transfected with the pEGFP vector. **B.** Brightfield picture showing all the cells independent of whether they are transfected or not. 5 µg plasmid, 1 x 10^{-7} cells in 200 µl cell suspension. **C.** Fluorescent picture showing the BA/F3 cells successfully transfected with the pMACS-GFP vector. **D.** Brightfield pictures showing all the cells independent of whether they are transfected or not. 10 µg plasmid, 1 x 10^{-7} cells in 200 µl cell suspension. The electroporation parameters used: 155 voltages and 1000 µF.

4.2 Cloning of Bcr-Abl into pBluescript(KpnI$^-$).

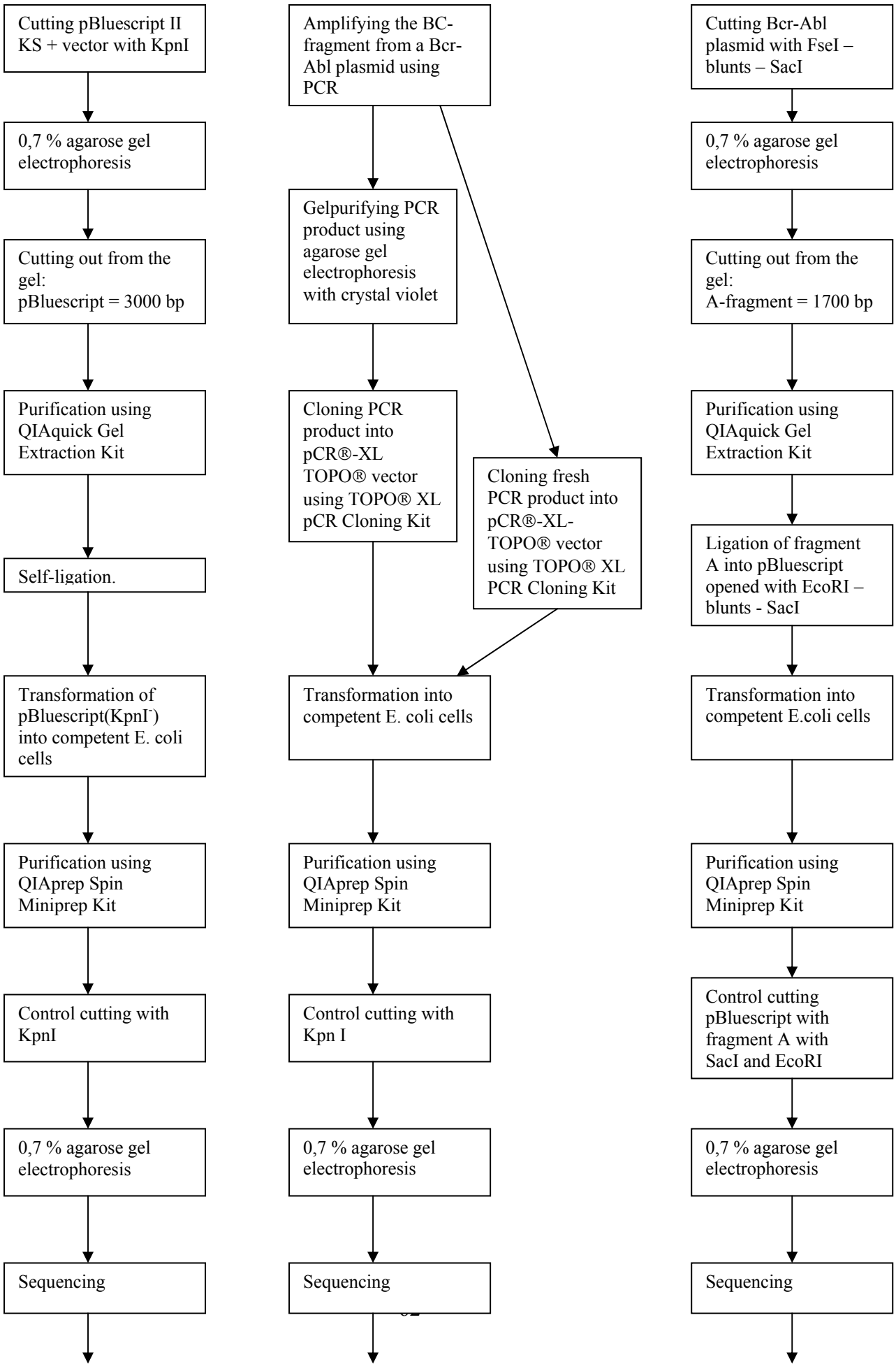
The primary goal of the project was to clone the 6000 bp Bcr-Abl into the pMACS 4-IRES.II vector. Once inside the vector, the construct was going to be transfected into BA/F3 cells. Due to the vector system used, transfected cells that express the Bcr-Abl gene will be selected by the help of the magnetic beads since they co-express the CD4 molecule at the cell surface. This selection will make sure that all the further downstream experiments are done with cells expressing Bcr-Abl, since non-transfected cells are not selected.

Cloning such a large fragment is intricate; in addition, the vector is large and difficult to clone into. So the cloning strategy for the Bcr-Abl gene is first to clone it into pBluescript(KpnI$^-$), and thereafter transfer it into the pMACS 4-IRES.II vector (see Figure 14).

The pBluescript II KS vector (see Figure 6) contains one KpnI sequence in the multiple cloning site. By cutting pBluescript with KpnI and making this site blunt before self-ligation, the KpnI sequence in the MCS region was destroyed. This was done so that the only KpnI site in the Bcr-Abl fragment later can be used, together with AatII, for subcloning of the P-loop mutants. All the mutants we are interested in studying are situated between the KpnI and AatII restriction sites in the Bcr-Abl gene. When the KpnI sequence in the MCS region of the pBluescript is destroyed, it is possible to subclone mutants from patient material into pBluescript(KpnI$^-$)/Bcr-Abl by cutting the mutants with KpnI and AatII. The pBluescript(KpnI$^-$) /Bcr-Abl is opened by cutting with the same restriction enzymes.

Provided that the subcloning into pBluescript(KpnI$^-$) is successful the mutants can be further cloned into the pMACS 4-IRES.II vector by cutting both pMACS 4-IRES.II and pBluescript(KpnI$^-$)/Bcr-Abl with EcoRI and EcoRV.

Because the Bcr-Abl gene is so large the cloning has to be done in a stepwise order.
According to where the restriction sequences were situated, the Bcr-Abl gene was divided into three different fragments, referred to as the A, B and C fragments (see Figure 15).


Cutting pBluescript II KS + vector with KpnI
0,7 % agarose gel electrophoresis
Cutting out from the gel: pBluescript = 3000 bp
Purification using QIAquick Gel Extraction Kit
Self-ligation.
Transformation of pBluescript(KpnI-) into competent E. coli cells
Purification using QIAprep Spin Miniprep Kit
Control cutting with KpnI
0,7 % agarose gel electrophoresis
Sequencing
Amplifying the BC-fragment from a Bcr-Abl plasmid using PCR
Gelpurifying PCR product using agarose gel electrophoresis with crystal violet
Cloning PCR product into pCR®-XL TOPO® vector using TOPO® XL pCR Cloning Kit
Cloning fresh PCR product into pCR®-XL-TOPO® vector using TOPO® XL PCR Cloning Kit
Transformation into competent E. coli cells
Purification using QIAprep Spin Miniprep Kit
Control cutting with Kpn I
0,7 % agarose gel electrophoresis
Sequencing
Cutting Bcr-Abl plasmid with FseI – blunts – SacI
0,7 % agarose gel electrophoresis
Cutting out from the gel: A-fragment = 1700 bp
Purification using QIAquick Gel Extraction Kit
Ligation of fragment A into pBluescript opened with EcoRI – blunts - SacI
Transformation into competent E.coli cells
Purification using QIAprep Spin Miniprep Kit
Control cutting pBluescript with fragment A with SacI and EcoRI
0,7 % agarose gel electrophoresis
Sequencing

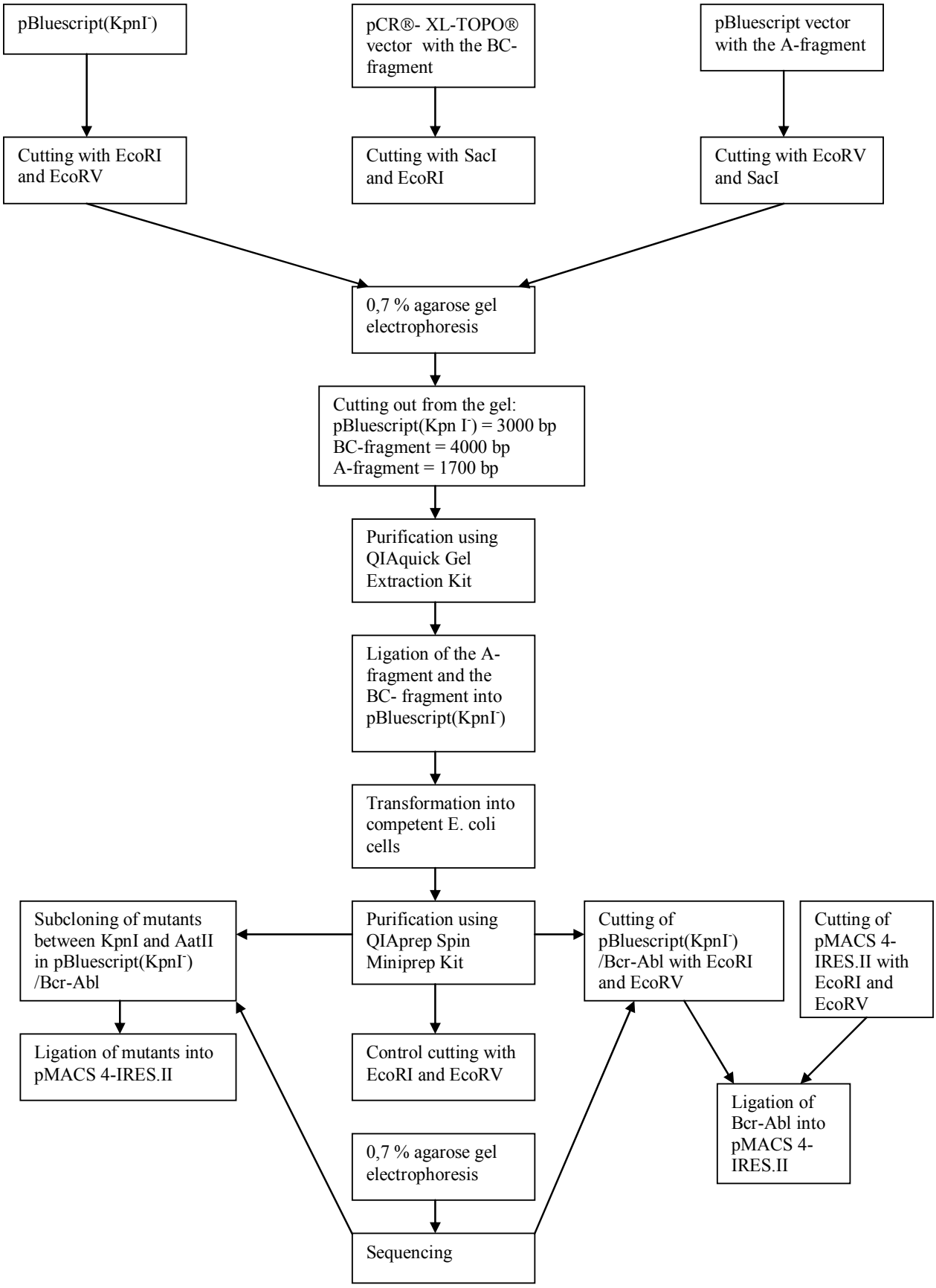


Figure 14. The cloning strategy of the Bcr-Abl into the pMACS 4-IRES.II vector

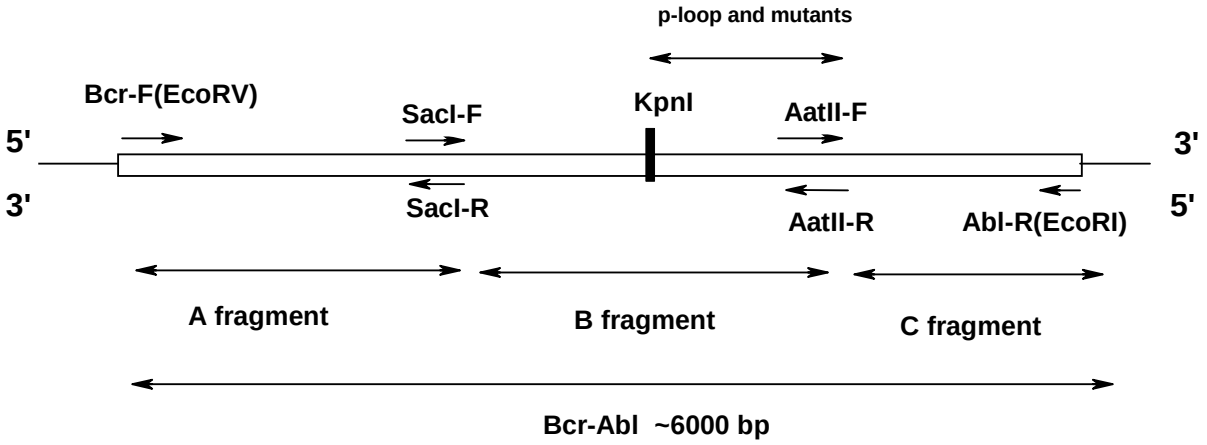


Figure 15. A general drawing of the Bcr-Abl gene showing the different primer combinations, the KpnI restriction site, the division of the three fragments, and the p-loop where the mutants we are interested in studying are situated**.**

4.2.1 Construction of pBluescript(KpnI$^-$)

The pBluescript vector was cut with KpnI, and then made blunt using T4 DNA polymerase (see 3.2.1). This would make it possible for the vector to religate and destroy the original KpnI site thereby creating pBluescript(KpnI$^-$). The vector was run on a 0,7 % agarose gel (results not shown). The band of 3000 bp was cut out from the gel and purified using QIAquick Gel Extraction Kit (see 3.2.3). Thereafter the pBluescript vector was self-ligated to generate pBluescript(KpnI$^-$) (see 3.2.4). The ligation reaction was transformed into competent *E. coli* DH5α cells (see 3.2.5). DNA from ampicillin resistant colonies was purified using standard DNA miniprep before the plasmid was test cut with KpnI. If the cloning has been successful, KpnI will no longer be able to cut the pBluescript vector. The digested DNA preps were separated on a 0,7 % agarose gel. The results shown in Figure 16 show that two of the four minipreps (lane 4 and 5) have the same band size as the control (lane 6), which is uncut pBluescript. The samples in lane 2 and lane 3 have the same band size as the pBluescript cut with KpnI in lane 1.

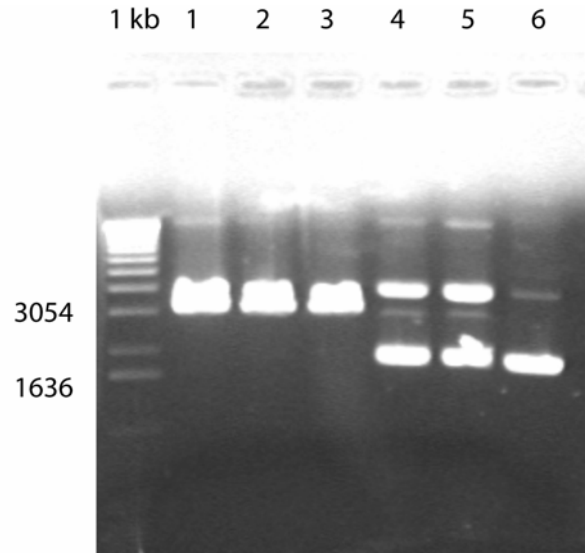


Figure 16. Restriction analysis to confirm the construction of the pBluescript(KpnI-). The pBluescript vector was first cut with KpnI to destroy the KpnI sequence in the MCS region in the vector before it was relegated to construct pBluescript(KpnI$^-$). The pBluescript vector is 3000 bp in size. The results were run on a 0,7 % agarose gel and show that two of the samples have the same band size as the pBluescript cut with KpnI, and the other two samples have the same band size as the uncut pBluescript vector. The samples in lanes 2 and 3 have bands of about 3000 bp, the same size as the pBluescript vector in lane 1 that is cut with KpnI. The samples in lane 4 and lane 5 show two bands with a band size similar to the uncut pBluescript in lane 6. The samples in lane 4 and 5 show the correct bands, KpnI shall no longer be able to cut the pBluescript vector in the KpnI site. This means that the construction of pBluescript have been successful in two of the four minipreps.

To be sure that the samples in lanes 3 and 4 were pBluescript vector with destroyed KpnI sites minipreps of 3 and 4 were sent for sequencing (see 3.2.13). When also sequencing confirmed the pBluescript vector had a non-functional KpnI site, freezing stocks were made and stored in the –80 °C freezer (see 3.2.16).

4.2.2 PCR on Bcr-Abl to generate the A, B and C fragments for cloning.

Primers containing appropriate restriction enzyme sites were generated and used for PCR to amplify the A, B and C fragments of Bcr-Abl. Different primer combinations were used to; if possible, generate larger parts of Bcr-Abl in one single PCR reaction. pEYKBA was used as template. To see which of the fragments that had been successfully amplified the PCR products were separated on a 0,7 % agarose gel for 1 hour. The results showed in Figure 17 show that the B-fragment, the C-fragment and the BC-fragment had been successfully amplified using this method. However, there were no signs neither of the A-fragment nor the whole Bcr-Abl gene. The Bcr-F(EcoRV) primer has misannealed, probably because of repeating sequences in the gene, and a shorter A fragment have been amplified.

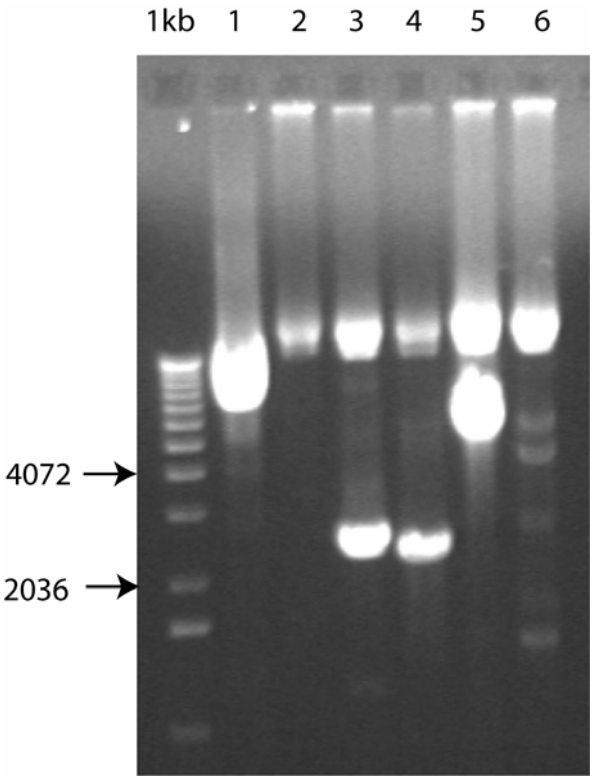


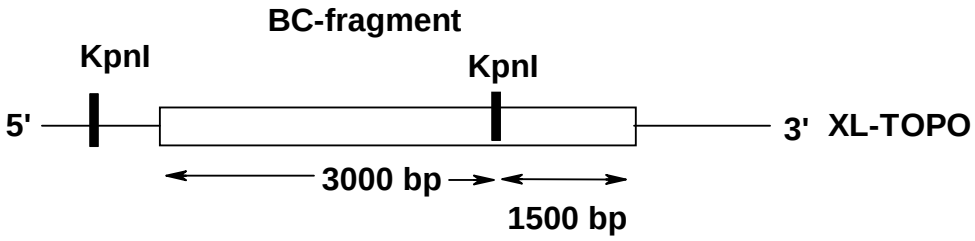
Figure 17. Amplification of the different fragments in the Bcr-Abl gene using different primers. The PCR products from the amplification of the different fragments of Bcr-Abl show that only the B fragment in lane 3, the C fragment in lane 4 and the larger fragment in lane 5 containing both the B and C fragment have been successfully amplified. The A fragment consists of 1700 bp, but there is no sign of the fragment in lane 2. The whole Bcr-Abl gene in lane 6 is not visualized either.

The BC-fragment was cloned directly into the pCR® XL-TOPO® vector, which is recommended for cloning of large PCR products.

Before the BC-fragment could be cloned into the pCR® XL-TOPO® vector (see Figure 9) it had to be gel purified. Here two alternative strategies were considered. To avoid damaging the DNA by UV light the PCR products were run on a 0,8 % agarose gel using crystal violet from the TOPO® XL PCR Cloning Kit (see 3.2.11). This gel was run parallel to a traditional 0,7 % ethidium bromide agarose gel. Using crystal violet the PCR product are visualized on the agarose gel under normal light and can be excised as soon as they are sufficiently resolved. Agarose gel electrophoresis using crystal violet turned out to be easier said than done. No thin blue bands were visualized on the gel so the BC fragment was not possible to excise out from the gel. The result of this was that fresh PCR product, instead of purified PCR product, first was cloned into pCR® XL-TOPO® vector and further chemically transformed into One Shot® bacterial cells (see 3.2.12).

For analysing the clones, colonies were picked and cultured overnight in LB medium. Thereafter the plasmids were purified using Miniprep Kit (see 3.2.7). Finally, the plasmids were cut with KpnI and separated on a 0,7 % agarose gel (see Figure 18) (see 3.2.2). The restriction enzyme KpnI cut once in the vector and once in the inserted BC-fragment. Depending on which way the BC fragment is inserted into the TOPO®-XL vector the KpnI digest will give bands of different sizes. The result gave either one band of about 1500 bp in combination with one band of about 6500 bp, or bigger bands of 3000 and 5000 bp were produced, depending on which way the insert was oriented (see Figure 18). All the minipreps contained inserts, but as seen from the restriction pattern the inserts were oriented different ways in the vector. The orientation of the insert does not matter for the further procedure, so we chose to sequence miniprep 2. As soon as the sequence was verified a freezing stock was made and stored in the –80 °C freezer.

A.



B.

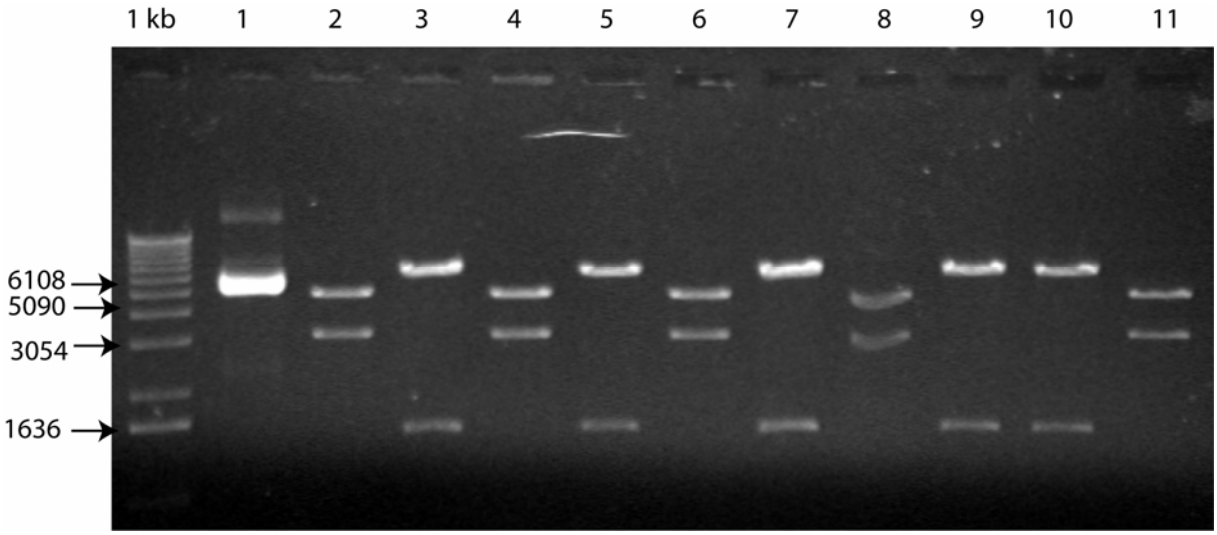


Figure 18. Restriction analysis to confirm the insert of the BC-fragment in pCR® XL-TOPO® vector. **A.** There is a KpnI site localized 50 bp away from the cloning site in the vector. And in the BC fragment there is a KpnI site in nucleotide position 1500. Depending on which way the BC fragment is inserted, the KpnI enzyme will therefore produce fragments of 1500 + 6500 bp, or at 3000 + 5000 bp. The TOPO vector (3500 bp) is drawn as a line, while the BC fragment is drawn like a rectangle. **B.** The DNA minipreps screened for BC-fragment insert in the pCR® XL-TOPO® vector was digested with KpnI and run on a 0,7 % agarose gel. All the minipreps contain inserts, but the restriction pattern is different. This is because the inserts are oriented in two different ways in the vector. Minipreps 2, 4, 6, 8 and 11 all contain inserts with the same direction, while minipreps 3, 5, 7, 9 and 10 have the fragment inserted in the opposite direction.

4.2.3 Revised strategy for cloning the A-fragment

Since two-thirds of the Bcr-Abl fragment now was successfully cloned, we focused on the remaining part of the gene, the A fragment.

When the first attempt to amplify the A fragment failed, a new PCR was set up, using another forward primer (see 3.2.10). When this amplification also failed a new strategy was made. The Bcr-Abl containing plasmid pEYKBA was cut with FseI since the Bcr-Abl genome sequence shows that there is a FseI site just in front of the start site of transcription (see 3.2.1)

(see Figure 19). Thereafter the fragment was made blunt-ended using T4 DNA polymerase, before it was cut with SacI (see 3.2.1). This should give us a 1700 bp FseI(blunt)-SacI fragment (the A-fragment), which can be ligated to the pBluescript plasmid cut with EcoRI, blunted and thereafter cut with SacI. The fragments were separated on a 0,7 % agarose gel (see Figure 19) . For Bcr-Abl two bands were observed, and the lowest band was cut out from the gel and purified (see 3.2.3).

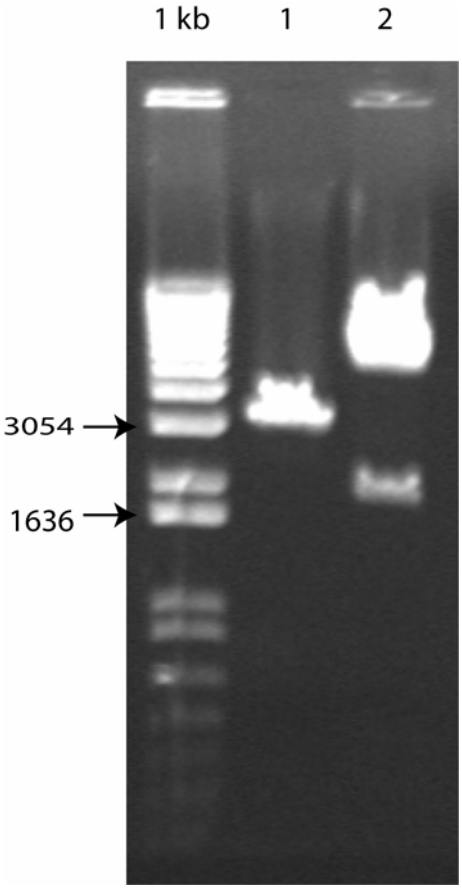
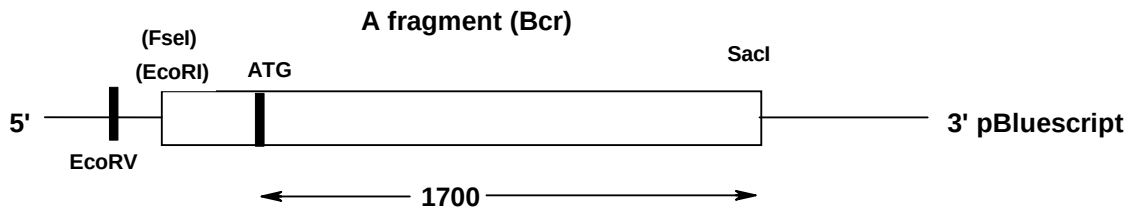


Figure 19. The A fragment cut out from a Bcr-Abl containing plasmid. Lane 2 shows the A fragment of about 1700 bp cut out from the Bcr-Abl plasmid. The Bcr-Abl plasmid was first cut with FseI, thereafter the fragment was made blunt before it was cut with SacI. The pBluescript vector in lane 1 was first cut with EcoRI, made blunt and then cut with SacI. The pBluescript indicating the band on 3000 bp in lane 1 and the A fragment on about 1700 bp in lane 2 was cut out from the gel and purified.

For pBluescript the 3000 bp linearized plasmid was cut out and purified, before the A-fragment was ligated into the pBluescript vector. The ligation reaction was transformed into competent *E. coli* DH5α cells as described in method 3.2.5, and amp-resistant colonies were picked from the plates for screening. Plasmid DNA was purified using Miniprep Kit, before

SacI and EcoRV was used for test cutting. The results were separated on a 0,7 % agarose gel. SacI and EcoRV will cut out the A fragment from the pBluescript vector. As can be seen from Figure 20B, the samples in lane 7 and lane 11 have an insert of the expected size. Miniprep 7 was sent for sequencing, and was confirmed to contain the 5' part of Bcr corresponding to the A fragment. However it turned out that in front of the A fragment, 256 bp of the original Bcr-Abl vector was included. This is because the FseI site did not exist just in front of Bcr-Abl, but about 250 bp further upstream. This may explain why the molecule weights of the A fragment in figure 19 (lane 2) and figure 20 (lane 7 and 11) is a bit higher than expected. Since this does not affect the rest of the cloning strategy we continued as planned.

A.



B.

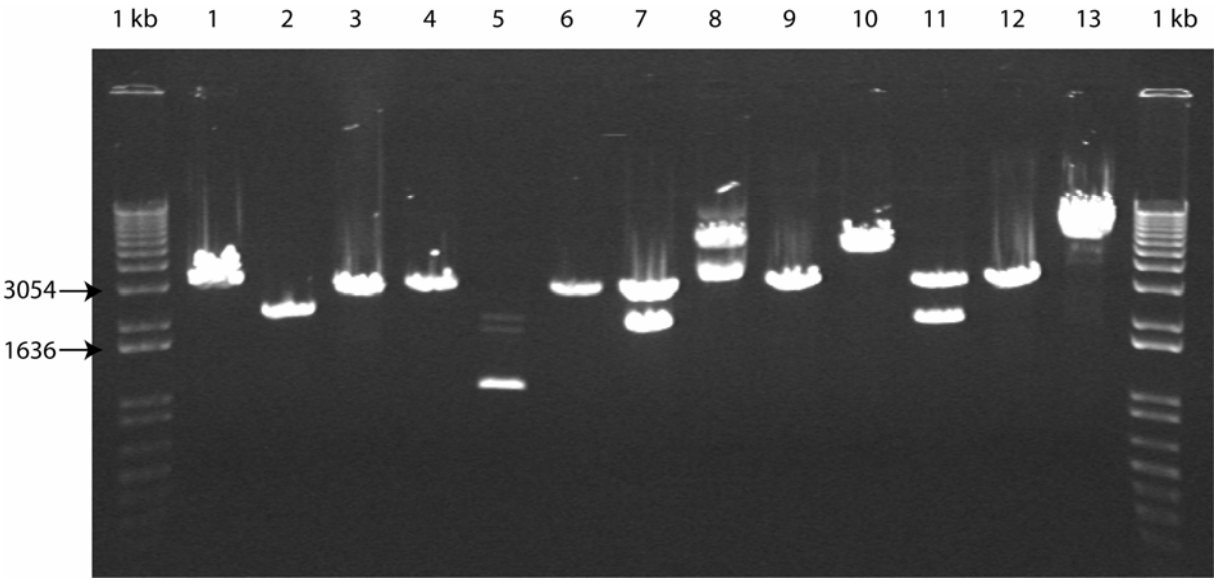


Figure 20. The A fragment cut out from the pBluescript vector. **A**. A sketch showing the A fragment cloned into the pBluescript vector. The vector is drawn as a line, while the A fragment is drawn as a rectangle. The restriction enzymes put in parentheses indicate ends made blunt. **B.** Cutting out the A fragment from the pBluescript vector with SacI and EcoRV. Lane 7 and lane 11 show vectors with insert at the correct band size. The bands shown are about 2000 bp and 3000 bp, indicating the 1700 bp A fragment with the additional 256 bp in front and the pBluescript vector.

4.2.4 Cloning the A fragment and the BC fragment into the pBluescript(KpnI$^-$) vector

Now the A fragment is cloned into the pBluescript vector and the BC fragment is cloned into the pCR® XL-TOPO® vector. The next step will be to cut these two fragments out from their vectors and clone them into the constructed pBluescript(KpnI$^-$) vector so that a continuous Bcr-Abl gene segment is made (as illustrated in Figure 21). The fragments are cloned into their vectors in a way that will make it possible to ligate the two fragments together into the new vector by cutting each fragment with their specific restriction enzymes.

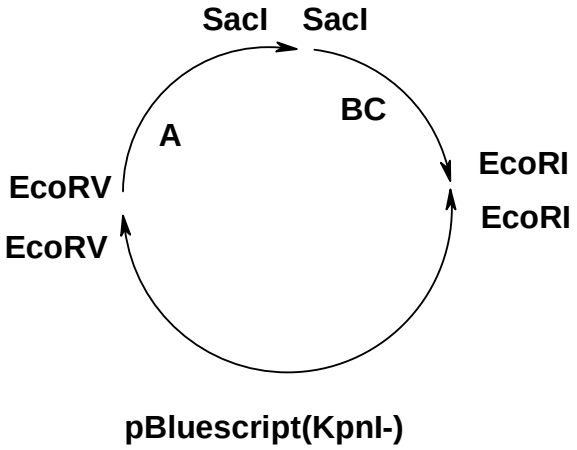


Figure 21. A sketch showing the different restriction sites and how the A fragment and the BC fragment was supposed to be cloned into the pBluescript(KpnI$^-$)vector.

First, the BC fragment was cut out with SacI and EcoRI, the A-fragment was cut with SacI and EcoRV and the pBluescript(KpnI$^-$) was cut with EcoRI and EcoRV. When separated on a 0,7 % agarose gel (see Figure 22) different band sizes were seen. The A fragment in lane 1 formed two bands where the upper band contained the pBluescript vector (3000 bp) and the lower band indicated the A-fragment. Two bands were also formed in lane 2 and lane 3 with the BC fragment. The lower band contained the pCR® XL-TOPO® vector (3500bp) and the upper band indicated the BC-fragment. In lane 4 with the pBluescript three different conformations of the vector were observed, probably due to incomplete digestion of the plasmid DNA. The A fragment, the BC fragment and the pBluescript(KpnI$^-$) vector of about 3000 bp were cut out from the gel and purified (see 3.2.3).

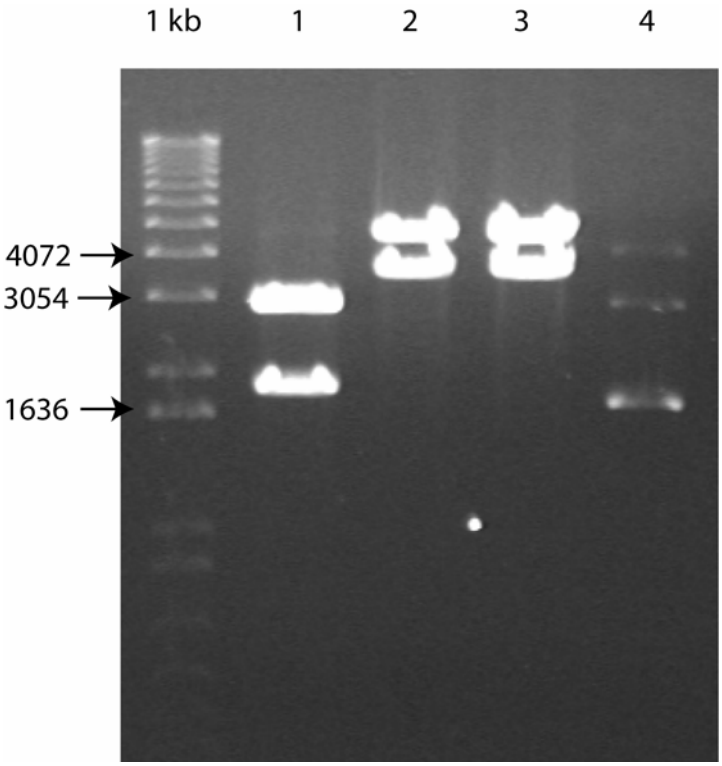


Figure 22. A gel verifying the correct band sizes of the A fragment, the BC fragment and the pBluescript(KpnI-) vector. Lane 1 shows the A fragment cut out from pBluescript with EcoRV and SacI. Two bands are observed, one band at 3000 bp and the other band at about 1700 bp. The upper band indicates the pBluescript vector, while the lower band indicates the A-fragment. The A-fragment was cut out from the gel. Lane 2 and lane 3 indicate the BC-fragment cut out from the XL-TOPO vector with SacI and EcoRI. The upper band on about 4500 bp indicates the BC-fragment, and the lower band on 3500 bp indicates the vector. The BC-fragment was cut out from the gel. When it comes to the three bands in lane 4 they indicate the pBluescript(KpnI-) vector in three different conformations. The pBluescript(KpnI-) band in the middle, on approximately the same level as the pBluescript vector in lane 1, was cut out and purified together with the other band cut out. The upper band > 3000 bp is probably not completely cut plasmid, and the lowest < 3000 bp, supercoiled uncut plasmid.

When it came to the ligation of the A-fragment and the BC-fragment into the pBluescript(KpnI-) it proved to be unexpectedly difficult.

First a ligation mix containing only the two fragments, A and BC, the DNA ligase and the ligase buffer was left for 1 hour in room temperature before the pBluescript(KpnI$^-$) vector was added. This was done in order to increase the chances for ligation between the two fragments. The ligation reaction was left over night at 14 ˚C and transformed into competent *E. coli* DH5α cells and plated onto media with ampicillin. Single colonies were grown and purified using Miniprep Kit (see 3.2.7). To test for plasmid DNA with correct inserts, purified DNA was screened. The agarose gel after the control cutting with EcoRI and EcoRV showed only

one band on 3000 bp (results not shown). It was expected to see two bands, the vector on 3000 bp and the insert on approximately 6000 bp. The vectors are probably relegated.

The pBluescript(KpnI$^-$) was once again cut with EcoRI and EcoRV treated with SAP (see 3.2.1) and purified from agarose gel before ligation in case the observed relegation arose from improper dephosphorylation of the vector.

A new ligation mix with the A fragment and the BC fragment was one more time tried to be ligated with the new pBluescript(KpnI$^-$) vector. The transformation into *E. coli* cells gave good growth and 10 colonies were picked from the plates and grown over night. Plasmid DNA was purified (see 3.2.6) and to test for correct insert the plasmid DNA was digested with EcoRI and EcoRV. The results were analyzed on a 0,7 % agarose gel. Again two bands, of 3000 bp and 6000 bp were expected. The result (not shown) showed only one band on 3000 bp, the same size as the control that indicated the pBluescript(KpnI$^-$). Probably only one of the restriction enzymes had worked and made a linearized plasmid with two complementary ends.

The recognition sites for EcoRI and EcoRV in the pBluescript(KpnI$^-$) vector are very close. Maybe the binding of one enzyme will prevent the binding of the other so that only one of the two restriction enzymes has worked. In that case the vector is only linearized and will have two ends that can base pair with each other. It will be impossible to clone the Bcr-Abl gene cut with EcoRI and EcoRV into the pBluescript(KpnI$^-$) and the likelihood for that the vector will relegate is big. It is not possible to see if both the restriction enzymes have cut on their sequence sites just by looking at the gel.

After a number of unsuccessful attempts to clone the two fragments together in pBluescript(KpnI$^-$) different strategies were tried out.

Since it might be a problem ligating big fragments in one ligation reaction, we wanted to try to clone the A fragment into the pCR® XL-TOPO® vector with the BC fragment instead, and then transfer the ABC fragment to pBluescript(KpnI$^-$) and pMACS 4-IRES.II as first planned.

Since the A fragment had to be cut out from the pBluescript vector with SacI and ligated to the SacI site in the BC fragment, the recognition site for SacI in the MCS of the pCR® XL-

TOPO® vector had to be destroyed. To do this, the BC fragment had to be cut out from the pCR® XL-TOPO® vector with EcoRI, before separating the DNA fragments on a 0,7 % agarose gel. Two bands of 3500 and 4500 bp, indicating the pCR® XL-TOPO® vector and the BC fragment, were observed as expected (results not shown). When cut out from the gel and purified (see 3.2.3) the pCR® XL-TOPO® vector was self-ligated and transformed into competent *E. coli* DH5α cells and then plated onto media with kanamycin. The intention was to purify the XL-TOPO vector and destroy the SacI site before the BC fragment was relegated into the EcoRI site. Then the pCR®-XL-TOPO(SacI$^-$)-BC vector could be opened with SacI and EcoRV, and the A fragment digested with the same enzymes could have been inserted. When the whole Bcr-Abl gene is cloned into the pCR® XL-TOPO® vector and transformed into competent *E. coli* DH5α cells the gene can be transferred to the pMACS 4-IRES.II vector using the restriction enzymes EcoRI and EcoRV.

The problem with this strategy was to grow the colonies containing self-ligated EcoRI digested pCR-XL-TOPO onto medium with kanamycin. After several overnight incubations at 37 ˚C there were no colonies on the plates, indicating that the self-ligation of the vector had failed. The kanamycin containing plates were tested with other bacteria containing kan-resistance, and found to be OK.

Another unsuccessful attempt to clone Bcr-Abl gene into pBluescript was done. This time the A fragment and the BC fragment were ligated before the fragment was amplified using PCR (see 3.2.10). A PCR product would only be seen if the A fragment and the BC fragment had been properly ligated to each other. The PCR products were intended to be cloned into pBluescript following the usual procedure with transformation, purification of the plasmid DNA, digestion of the plasmid DNA and the vector with specific restriction enzymes, gel purification and ligation into the pBluescript(KpnI$^-$). However the PCR product was run on a 0,7 % agarose gel there was no sign of the sample, so the attempt was terminated.

As a final experiment, several ligation reactions were set up in an attempt to increase the number of correct ligation products. The usual ligation procedure was followed and different amounts and concentrations of plasmid vectors and DNA inserts in a total volume of 50 µl were set up (see Table 4).

Table 4. The different amounts and concentrations used in the ligation reactions

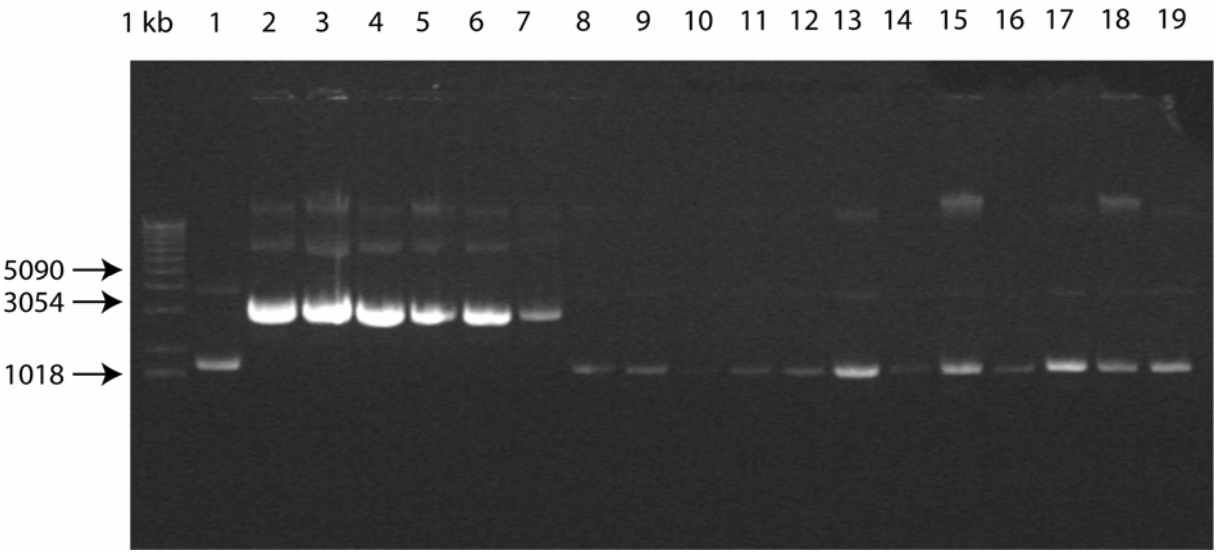
	1	**2**	**3**	**4**	**5**	**6**
A (9,3 ng/µl)	2 µl	3,5 µl	2 µl	3,5 µl	3,5 µl	3,5 µl
BC (8,6 ng/µl))	4 µl	-	4 µl	8 µl	-	-
BC (7,1 ng/µl))	-	8,5 µl	-	-	8,5 µl	8,5 µl
pBluescript(KpnI$^-$) (0,7 ng/µl))	14 µl	14 µl	-	-	-	-
pBluescript(KpnI$^-$) (13,5 ng/µl))	-	-	1 µl	-	2,5 µl	-
pBluescript(KpnI$^-$) (28,3 ng/µl))	-	-	-	1,5 µl	-	2 µl

To increase the chances for ligation of the A and BC fragments with each other, the fragments were incubated with ligase for 1 hour before the vector was added.

An overnight ligation was set up and the ligation mixes were transformed into competent DH5α *E. coli* cells. It was growth on all the plates, but the plates with the vectors with the highest concentration had most colonies. Three colonies were picked from each plate and purified. The purified DNA plasmids were run on a 0,7 % agarose gel to find out which of the plasmids that had an insert. A sample with pBluescript(KpnI$^-$) was used as a control. All the minipreps originating from ligation mix 1 and 2 (see Table 4) migrated more slowly in the gel than the vector control did, indicating that they contained an insert. The other twelve minipreps formed band at the same size as the pBluescript(KpnI$^-$), showing that the vector probably had religated containing no insert (see Figure 23A). The six minipreps from ligation mix 1 and 2 were test cut with (a) EcoRI and EcoRV to cut out the insert and (b) only with the EcoRI to linearize the vector with the insert. After digestion, the minipreps were run on a 0,7 % agarose gel. As can be seen from Figure 23B the bands formed in lane 2 to lane 7 are about 5000 bp indicating that the vectors are linearized and contain an insert on about 2000 bp. The Bcr-Abl gene is 6000 bp so it cannot be the whole gene, but it might be the A fragment. In lane 8 to lane 13 two bands are formed. The upper band indicated nicked plasmid, while the lower band indicated supercoiled plasmid. The EcoRI has not worked so the vector is still circular. One of the minipreps in lane 2-7 was sent for sequencing and it was confirmed that it was only the A fragment that was cloned into the pBluescript(KpnI$^-$) vector, and that the EcoRI site was destroyed. The pBluescript vector was cut with EcoRI and EcoRV, and the A

fragment was cut out from the pBluescript with EcoRV and SacI. Theoretically these two fragments are not supposed to ligate and form a circular plasmid. In a way the SacI end of the fragment must have been made blunt, and so must the other ends as well. To select for clones that had insert in their vectors a so-called blue-white screening was done (see 3.2.15). After an overnight incubation there were a lot of colonies on the plate, but none of them had turned blue. It was unlikely that all the clones had insert in their vectors. Because of the time limit in this project the practical laboratory work was terminated here.

A.



B.

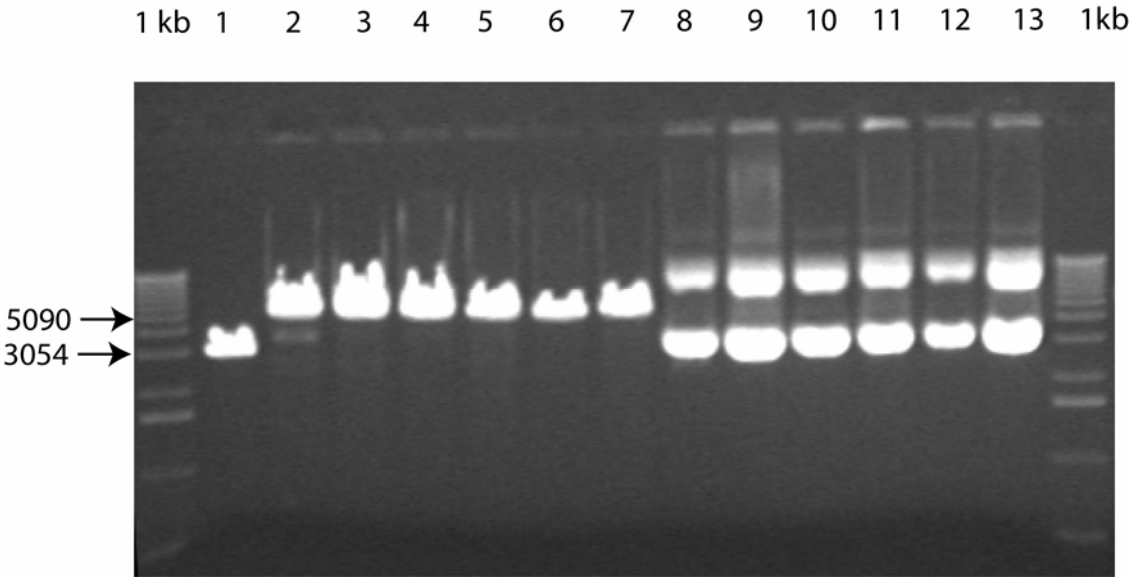


Figure 23. The pBluescript(KpnI⁻) vector controlled for correct insert. A. The minipreps in lane 2-7 (from ligation mix 1 and 2) are different from the control with pBluescript(KpnI⁻) in lane 1 and the other minipreps in lane 8-19. The bands formed in lane 2-7 are about 2000 bp larger than the other minipreps, indicating that they contain an insert. **B.** Lane 1 shows the pBluescript(KpnI⁻) control on 3000 bp. The sample in lanes 2-7 show the minipreps from ligation mix 1 and 2 (Table 4) cut with EcoRI and EcoRV. Bands on about 5000 bp indicate that the vectors are linearized and contain an insert on about 2000 bp. Lane 8 to lane 13 contain minipreps from ligation mix 1 and 2 (Table 4) cut with only EcoRI. Two bands are observed (> 3000 bp and <3000 bp) a typical pattern for undigested plasmids. The upper band indicates nicked plasmid, while the lower band indicates supercoiled plasmid.

As a short summing up, in the pilot study the GFP was successfully cloned into the pMACS 4-IRES.II vector, but the further transfection of pMACS-GFP into the BA/F3 cells was not satisfactory. The only successfully transfection into the BA/F3 cells was the pEGFP-C1 vector used as a control.

When it comes to the Bcr-Abl gene the challenge was to clone this 6000 bp long fragment into the pMACS 4-IRES.II vector. The A fragment and the BC fragment were separately cloned into two different vectors, but the placing of the restriction sites made it difficult to ligate these two fragments into the same vector. The A fragment was cloned into the pBluescript($KpnI^-$) vector, but the EcoRI site was destroyed and made it difficult to clone the BC fragment into the same vector in a next step

5 DISCUSSION

The constitutive tyrosine kinase activity of the Bcr-Abl fusion protein is the causative agent of CML. A small tyrosine kinase inhibitor, imatinib, Glivec, has shown to be remarkably effective in the treatment of the disease; it induces hematologic in over 90 % and cytogenetic remission in over 80% of all CML patients [Kantarjian et al. 2002], but clinical resistance to imatinib treatment has become a significant problem [Shah et al. 2002]. The main reason for resistance is single point mutations in the Abl gene encoding the ATP binding region, which is also the binding site for imatinib. How and why these point mutations arise is not known, but increasing evidence suggests that cells with pre-existing mutations are selected rapidly during imatinib treatment [Shah et al. 2002; Roche-Lestienne et al. 2004]. In addition, a distinct group of patients with P-loop mutations have a poorer prognosis [Branford et al. 2003; Soverini et al. 2005]. Early detection of these mutations and re-evaluation of treatment options are of particular interest for this group of patients. It is still not known if P-loop mutations represent increased fitness mutations rendering Bcr-Abl more aggressive or if the reduced survival is a matter of inadequate treatment. The imatinib resistance due to mutations has led to the development of a second generation of targeted CML drugs [Shah et al. 2002]. In this respect, the emergence of mutated Bcr-Abl clones in the setting of imatinib resistance raised many questions.

Our intention was to look more closely on how the different point mutations affect the gene expression. Thus, the aim of this project was to clone the Bcr-Abl and subsequently subclone some of the interesting point mutations found in Bcr-Abl and expresses them in BA/F3 cells. To identify genes regulated by Bcr-Abl we wanted to employ microarray analysis to compare the wild type and the mutated forms of Bcr-Abl in the mouse pro-B cell line and see if there were any differences in the gene expression profile. Kinase assay or the activation of the different signalling pathways could have been checked by the use of Western blot analysis of phosphotyrosine levels in transfected BA/F3 cells.

The Bcr-Abl tyrosine kinase affects the same pathways in the cells as the abl tyrosine kinase. Among the pathways affected are the Ras, PI3K and JAK/STAT pathways [Hickey and Cotter 2006].

5.1.1 Electroporation of pMACS-GFP into BA/F3 cells

The electroporation of pMACS-GFP into the BA/F3 cells did not work out as expected, but the transfection of pEGFP-C1 into the same cells worked well. The reason for this is difficult to say for sure, but there are a number of factors that can be decisive for the results.

Electroporation is a process in which a high voltage current is used to open pores in the cell membrane in order to introduce nucleic acids, proteins and other membrane-impermeable molecules. The strength of the applied electric pulses can both be too high and too low. The electric pulses must be strong enough to cause permeabilization but not so intense that the membrane fails to reseal and the cell dies [Sambrook and Russell 2001].
Transfection efficiency and cell viability is influenced by a number of factors. In addition to the intensity of the electric pulses, the frequency and pattern of the electric pulses must be optimized to allow enough time for the plasmid to enter, and at the same time be short enough to prevent the cell from losing its contents, which can cause irreversible damage to the membrane. Temperature, the concentration of DNA and other factors can also influence the results during electroporation varying with the type and size of cells used [Sambrook and Russell 2001]. Another factor is that methods from protocols designed to a particular instrument can be difficult to adapt to other instruments.

The pMACS 4-IRES.II vector is a big (5841 bp) vector, and when the GFP is incorporated it is over 6000 bp. When the Bcr-Abl gene is inserted the vector will be even bigger. We do not know if it is the size that is the problem, but if so it can be even more difficult to transfect the BA/F3 cells with the pMACS vector when the Bcr-Abl gene is incorporated. Since the pEGFP-C1 vector (4731 bp), which also is a relatively big vector, has been successfully transfected into the BA/F3 cells, it might be another reason for the unsuccessful attempt in transfecting the pMACS-GFP into the BA/F3 cells, but as discussed above there are several factors that can influence the transfection efficiency.

When we managed to transfect the HeLa cells with the pMACS-GFP we proved that the system with GFP worked. The HeLa cells transfected with the pMACS-GFP made fluorescent light when we observed them in the microscope.

Why it is easier to transfect the HeLa cells compared to the BA/F3 cell is difficult to say, but different cell types takes up foreign DNA to various degrees. While the HeLa cells originate from cancer cells in the uterus, we used BA/F3 pro-B cells similar to the cells where the Bcr-Abl naturally occurs. Another decisive factor for using the BA/F3 cells is that they grow in suspension in difference to the HeLA cells that are adherent. To detach them from the plastic surface trypsin, a protease, which can degrade the CD4 receptors from the cell surface, is used. In suspension the BA/F3 cells are free to co-express the CD4 receptors and are therefore more suitable for our work.

5.1.2 Amplifying the Bcr-Abl gene using PCR

Given the large size of the transcript, the cloning of Bcr-Abl was not expected to be straightforward. Well aware of all the problems cloning of long products could offer, we carefully tried to design a stepwise cloning procedure. The first part consisted of a PCR amplification step, which according to our plans should be carried out in amplification of three fragments, each stretching a little more than 2000 bp. The primers used for amplification of these fragments were located in relation to the start- and stop codons respectively. Each of these primers contained recognition sites for restriction enzymes, which at a later stage would allow cloning the fragments. Furthermore, we identified unique restriction sites within the Bcr and Abl transcript. Additional pairs of forward and reverse primers were designed stretching these sites. According to this strategy PCR products could be digested with the respective enzymes after the amplification step and cloned into a vector, which was opened using the same enzymes. Unfortunately, using this strategy, primers had to be designed for annealing to well-defined sequences, rather than designing primers, which were optimized for maximal efficiency.

This method worked well for amplifying the BC fragment, but in case of the A fragment the PCR primer turned out to be unspecific. Misannealing of the Bcr-F(EcoRV) primer, at the start of the Bcr gene, resulted in a false small PCR product that did not contain the sequences in the beginning of the Bcr gene. Annealing of long strands can lead to unspecific products caused by misannealing often due to repeating CG sequences in the template.

5.1.3 Cloning of long PCR products

The cloning of PCR amplified fragments into a plasmid vector is a routine procedure in recombinant DNA cloning, but all PCR fragments will not clone with the same efficiency, and there are various reasons for this. After searching the Internet for advice and hints about cloning long PCR fragments it is evident that problems with cloning long PCR product is a quite common problem. As already mentioned, the size of the fragment to be cloned is of importance, but also the complexity of the cloning strategy needs to be considered.

"TOPO® Cloning", used when cloning the BC fragment, is an efficient method for cloning PCR products into a plasmid vector and is specifically designed to clone long PCR products. *Taq* DNA polymerase adds a single deoxyadenosine to the 3' ends of the PCR product and produce specific DNA sequences with 3' A overhangs. Using TOPO Cloning the PCR products are ligated into a linearized plasmid vector with 3' deoxythymidine overhangs activated by topoisomerase I. This method worked well for the BC fragment, and if the whole Bcr-Abl had been amplified in one single PCR reaction, we would have cloned the whole gene into a pCR® XL-TOPO® vector

But the misannealing of the A fragment prevented us from isolating and amplifying the full-length Bcr-Abl gene using PCR, and forced us to try another strategy for the cloning of the A fragment. When the use of PCR failed in amplifying the A fragment it had to be cut out from the Bcr-Abl plasmid instead. This resulted in two long fragments to clone into the pBluescript(KpnI$^-$) vector which is much more complicated than cloning just one fragment.

It would probably have been much easier to clone the Bcr-Abl into the pBluescript(KpnI$^-$) if it first was cloned into the XL-TOPO vector in one fragment. This is why we tried to ligate the A fragment into the XL-TOPO vector with the BC fragment. However, this proved to be much more difficult than expected.

Not only was the Bcr-Abl gene to be cloned long, but also the pMACS 4-IRES.II vector is big (see Figure 9). Cloning Bcr-Abl into this vector could also have offered some problems. There were two reasons for using the pBluescript(KpnI$^-$) vector. The pBluescript vector is known to be a vector easy to clone into, and when we destroyed the KpnI site, the

pBluescript(KpnI$^-$)-Bcr-Abl could be used to subclone the mutants by switching the KpnI-AatII fragment between the pBluescript(KpnI$^-$) and the pMACS 4-IRES.II vector.

5.1.4 The position of the restriction sites

When cloning this 6000 bp long fragment into one vector there are, as already mentioned, different challenges to handle. Cloning more than one fragment into one vector is a factor that complicates the strategy. In a ligation reaction where different fragments are supposed to ligate and form a circular DNA molecule the strands cut with the same enzyme will have complentary ends that can base pair and form phosphodiester bonds. The longer the fragments are even more difficult will it be to get the right ends to ligate and orientate.

In our case the recognition sites for EcoRI and EcoRV in the pBluescript(KpnI$^-$) vector were positioned very close to each other. This was probably an affecting factor for the difficulties in cloning the Bcr-Abl into the vector. If the binding of one enzyme prevent the binding of the other enzyme the pBluescript(KpnI$^-$) vector will be opened, but only with one of the restriction enzymes. This will form a linearized vector with two ends that can easily base pair with each other and make a relegated vector containing no insert. Cloning the Bcr-Abl gene cut with EcoRI and EcoRV into this pBluescript vector is theoretically impossible when there is only one matching end. A linearized fragment where the matching end of the insert is ligated with one of the vector ends can form, but the vector cannot ligate and form a circular plasmid

5.1.5 Future direction

As mentioned earlier in this discussion, the preferred strategy would be to amplify the whole Bcr-Abl gene in one single PCR reaction and then clone it directly into the XL-TOPO vector, designed for cloning of long PCR products. Thereafter the Bcr-Abl fragment could have been subcloned into pBluesscript(KpnI$^-$) and pMACS 4-IRES.II. In the future, a possible way to do this could be to design a new specific primer 5’ to the start codon of the Bcr-Abl gene in the pEYKBA vector. Hopefully will the vector-specific primer not misanneal, and a full-length Bcr-Abl PCR product will be generated.

6 REFERENCES

Alberts, B., A. Johnson, et al. (2002). Molecular Biology of the cell. New York, Garland Science.

Azam, M., R. R. Latek, et al. (2003). "Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL." Cell **112**(6): 831-43.

Bartram, C. R., A. de Klein, et al. (1983). "Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia." Nature **306**(5940): 277-80.

Branford, S., Z. Rudzki, et al. (2003). "Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis." Blood **102**(1): 276-83.

Calabretta, B. and D. Perrotti (2004). "The biology of CML blast crisis." Blood **103**(11): 4010-22.

Campbell, N. A., J. B. Reece, et al. (1999). Biology. San Francisco, Benjamin/Cummings.

Capdeville, R., E. Buchdunger, et al. (2002). "Glivec (STI571, imatinib), a rationally developed, targeted anticancer drug." Nat Rev Drug Discov **1**(7): 493-502.

Daub, H., K. Specht, et al. (2004). "Strategies to overcome resistance to targeted protein kinase inhibitors." Nat Rev Drug Discov **3**(12): 1001-10.

Druker, B. J., C. L. Sawyers, et al. (2001). "Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome." N Engl J Med **344**(14): 1038-42.

Druker, B. J., M. Talpaz, et al. (2001). "Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia." N Engl J Med **344**(14): 1031-7.

Gorre, M. E., M. Mohammed, et al. (2001). "Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification." Science **293**(5531): 876-80.

Guilhot, F., C. Chastang, et al. (1997). "Interferon alfa-2b combined with cytarabine versus interferon alone in chronic myelogenous leukemia. French Chronic Myeloid Leukemia Study Group." N Engl J Med **337**(4): 223-9.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." Cell **100**(1): 57-70.

Herfindal, E. T. and D. R. Gourley (2000). Textbook of therapeutics: drug and disease management. USA, Lippincott Williams & Wilkins.

Hickey, F. B. and T. G. Cotter (2006). "Identification of transcriptional targets associated with the expression of p210 Bcr-Abl." Eur J Haematol **76**(5): 369-83.

Hillmann, R. (1994). Hematology in clinical practice. A guide to diagnosis and management. USA, McGraw-Hill Inc.

Hjort-Hansen, H., F. X. Gruber, et al. (2004). Nasjonalt handlingsprogram for kronisk myelogen leukemi. Trondheim.

Kabarowski, J. H. and O. N. Witte (2000). "Consequences of BCR-ABL expression within the hematopoietic stem cell in chronic myeloid leukemia." Stem Cells **18**(6): 399-408.

Kantarjian, H., C. Sawyers, et al. (2002). "Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia." N Engl J Med **346**(9): 645-52.

Martinelli, G., S. Soverini, et al. (2005). "New tyrosine kinase inhibitors in chronic myeloid leukemia." Haematologica **90**(4): 534-41.

Mauro, M. J. and B. J. Druker (2001). "STI571: targeting BCR-ABL as therapy for CML." Oncologist **6**(3): 233-8.

McPherson, M. J. and S. G. Møller (2000). PCR. The Basics from background to bench. New York, Bios Scientific Publisher Ltd.

Nelson, D. L. and M. M. Cox (2002). Lehninger Principles of Biochemistry. New York, Worth.

Nowell, P. C. and D. A. Hungerford (1960). "Chromosome studies on normal and leukemic human leukocytes." J Natl Cancer Inst **25**: 85-109.

Passarge, E. (2001). Color Atlas of Genetics. Stuttgart, New York, Thieme.

Roche-Lestienne, C., F. X. Mahon, et al. (2004). "[Origin of resistance to Imatinib mesylate: lessons learned from this experience]." Med Sci (Paris) **20**(12): 1125-30.

Rowley, J. D. (1973). "Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining." Nature **243**(5405): 290-3.

Sambrook, J. and D. W. Russell (2001). Molecular cloning. A laboratory manual. New York, Cold Spring Harbour Laboratory Press.

Sawyers, C. L. (1999). "Chronic myeloid leukemia." N Engl J Med **340**(17): 1330-40.

Shah, N. P., J. M. Nicoll, et al. (2002). "Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia." Cancer Cell **2**(2): 117-25.

Soverini, S., G. Martinelli, et al. (2005). "ABL mutations in late chronic phase chronic myeloid leukemia patients with up-front cytogenetic resistance to imatinib are

associated with a greater likelihood of progression to blast crisis and shorter survival: a study by the GIMEMA Working Party on Chronic Myeloid Leukemia." J Clin Oncol **23**(18): 4100-9.

Trun, N. and J. Trempy, Eds. (2003). Fundamental bacterial genetics. USA, Blackwell.

Ullrich, A. and J. Schlessinger (1990). "Signal transduction by receptors with tyrosine kinase activity." Cell **61**(2): 203-12.