UPTEC X 03 013
APR 2003

ISSN 1401-2138

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Preparative plasmid purification by means of triple helix hybridisation

Master’s degree project

UPPSALA UNIVERSITET

Molecular Biotechnology Programme

Uppsala University School of Engineering

UPTEC X 03 013	**Date of issue 2003-04**

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Title (English)

Preparative plasmid purification by means of triple helix hybridisation

Title (Swedish)

Abstract

A method for plasmid purification built on triple helix formation was developed. The project was divided into two parts. The first part involved evaluation of an already existing method and testing its yield capacity and stability. The second part involved modifying the method by using other materials in order to scale up the purification process while keeping the costs low.

Keywords

Triple helix, magnetic beads, polystyrene beads, pluronic, PDS, biotin, thiol

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Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **23**

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Preparative plasmid purification by means of triple helix hybridisation

Sophia Bengtsson

Sammanfattning

Genterapi är ett nytt område inom medicinen som man hoppas kunna behandla många idag svårbehandlade sjukdomar med. Det kan vara så att kroppen inte producerar ett ämne som är viktigt för att individen ska fungera som en följd av att en gen är defekt. Genom genterapi kan man föra in en ”frisk” kopia av genen i cellen och därigenom kompensera för den defekta genen. För att föra in genen i cellen har man använt sig av plasmider, ringformiga DNA-strukturer som finns naturligt hos vissa bakteriearter. En nackdel med plasmiderna är att en ytterst liten del accepteras av cellen (ungefär 1 på 1000). Det finns därför ett stort behov av storskalig framrening av plasmider till ett relativt lågt pris.

I detta examensarbete undersöktes en metod för att rena fram plasmider på ett relativt enkelt sätt. Metoden bygger på s.k. trippelhelixformation. En trippelhelix kan bildas mellan en enkelsträngad DNA-sträng och en dubbelsträngad DNA-sträng som har vissa passande bassekvenser. Genom att ha en ”fiskarkrok” av enkelsträngad DNA, kan man fånga in den dubbelsträngade plasmiden ur en soppa av olika bakteriecellkomponenter. Metoden testades först i sitt befintliga skick enligt beskrivningar i diverse vetenskapliga artiklar, därefter testades andra material som passade ihop med våra egna önskemål om storskalighet och pris.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet April 2003

CONTENTS

1 Introduction

The need to produce large quantities of supercoiled plasmid DNA has recently increased as a result of the rapid evolution of gene therapy [1]. In gene therapy, nucleic acids are introduced into human cells in order to modify their genome for medical purposes. Generally, the nucleic acid is double stranded DNA encoding a therapeutic, destructive or marker protein. The most critical step in gene therapy is delivering the nucleic acids efficiently into the targeted cells. Both viral and non-viral vectors have been developed for this purpose. One big drawback with using viral vectors is their toxicity and immunogenicity, as well as the possible activation of oncogenes or deactivation of tumour-suppressor genes. For these reasons, non-viral delivery systems have become more interesting as vectors of gene-transfer systems for commercial products. Plasmid DNA has been used to express specific antigens on cell membranes, which can stimulate and enhance the immune system's response. Getting the plasmids into the cells is however another matter, plasmid-DNA-based delivery vectors are less effective at transfecting cells than the viral vectors. It is estimated that only 1 in every 1000 plasmid molecules presented to the cells reaches the nucleus and is expressed. This means that full treatments require milligram quantities of plasmid DNA. Therefore large-scale plasmid-DNA-manufacturing processes must be developed before gene therapy can be used as general tools for treating diseases. Also the purity of the plasmids has to be evaluated, as very low or no traces of bacterial DNA, RNA or endotoxins can be accepted for insertion into human cells.

1.1 Aim of study

The purpose of this project was to find a method to purify plasmids in large scale in a manner that would be relatively easy, automatic, cheap, reusable and produce a high yield of pure plasmids.

1.2 Previous publications

In the search for specific DNA interaction, purification by the use of DNA-binding proteins or by single stranded DNA binding to a specific double strand sequence in a so-called triple-helix structure was investigated. The search was conducted primarily in Medline. No satisfying results were found on DNA purification by the help of DNA binding proteins. However, several different research groups especially in the mid-nineties have investigated triple-helix structures and its use in capturing DNA. One group in particular had a detailed description of the exact procedure of plasmid purification by the use of triple-helix formation [2]. A drawback of the method described in the article was the cost of materials, to scale up, the materials would have to be much cheaper than the ones used there. A search for registered patents showed that a patent from another group had been accepted in the US in 2001, the patent was on triple-helix-mediated plasmid purification [3]. However, to avoid the patented work would be a later problem. The first thing was to show that the whole procedure worked as described in the article and then modify it to serve our purposes and needs.

1.3 Basic concept

The three basic components of the experiment is a solid phase- the bead, a fishing probe- the single stranded oligo and a target- the double stranded plasmid. The solid phase keeps the plasmid in a stable place while the surrounding liquid is changed (washing with a different buffer etc). Choosing beads instead of a compact block, as the solid phase, enhances the surface area on a given volume to which the probe can bind. The probe is attached to the bead through a strong bond (e.g. disulphide bridge or streptavidin-biotin bond) that can withstand changes in pH and temperature. A bead binds several probes, which each can bind a target plasmid. The ideal is to have an automated loop, where the bead-oligo complex is following the loop multiple times and only new plasmid solution and washing buffers are added (see Figure 1)

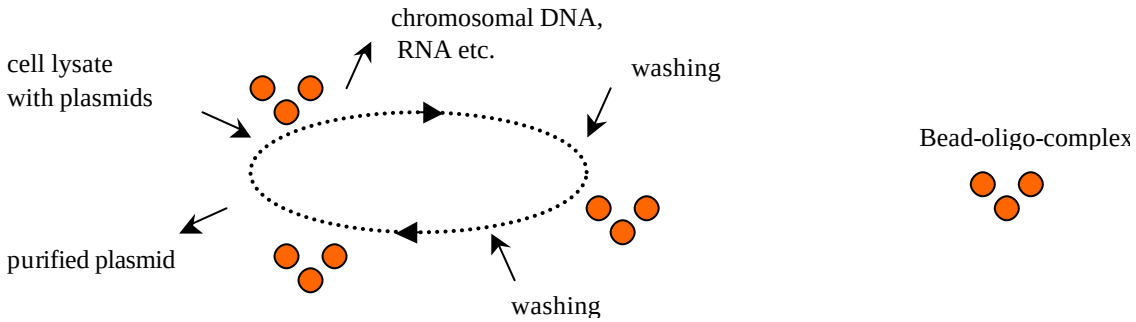

Figure 1. Schematic representation of the ideal automatic loop of the basic purification procedure. The bead-oligo-complex is going around the loop where components are either added or removed at specific stations.

1.4 The triple-helix structure and Hogsteen bonding

DNA consists of the four bases: adenine (A), thymine (T), cytosine (C) and guanine (G). They are all ring formed structures where T and C have the same basic ring structure and are called pyrimidines (Py) and A and G, which have a slightly larger structure, are called purines (Pu). Base pairs are formed between A and T, C and G. The bases form a ladder structure where the backbone consists of sugar and phosphate. The ladder has a helical structure where the side of the base pair that binds the sugars has a smaller angle and is called the minor groove, the side with the larger angle is called the major groove [4] (Figure 2a). The triple-helix structure (triplex for short) is formed when single stranded DNA forms hydrogen bonds in the major groove of a double stranded DNA strand. The triplet can consist of Py-PuPy (The base in the single strand is a pyrimidine which binds to the purine in the double strand) or Pu-PuPy (The single strand base is a purine which binds to the purine in the double strand). When the third strand consists mainly of pyrimidines (Py-PuPy), Hoogsteen type base triplets (T-AT and C^+-GC) are formed and the third strand is parallel to the purine strand of the target duplex (see Figure 2b, 2c). When the third strand is predominantly purines (Pu-PuPy), reverse Hogsteen type base triplets (G-GC and A-AT) are formed and the third strand runs antiparallel to the purine strand [5]. For this project, only the Py-PuPy type was used. When cytosine is the third base, it has to be protonated to be able to form a Hogsteen bond, which makes the bonding much weaker than the A-TA triplet. This phenomenon can be turned into an advantage since the triplex formation will become pH dependent (pH low-C protonated, triplex formed, pH high-C not protonated, triplex released).

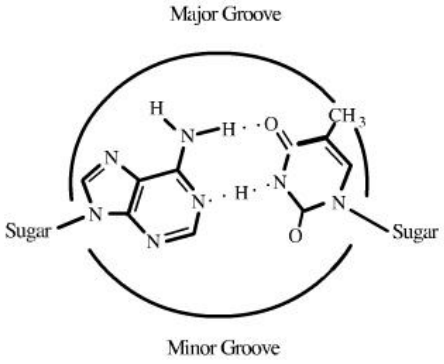

Figure 2a. The base pair is angled, where the side that binds the sugars of the backbone forms a smaller angle -the minor groove, and the other side forms a larger angle - the major groove.

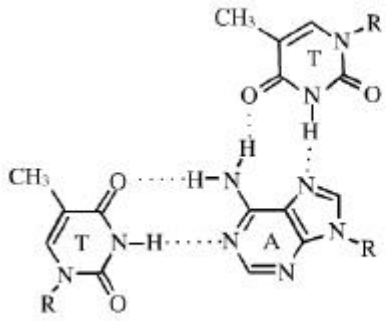

Figure 2b. Hogsteen bond T-AT triplet.

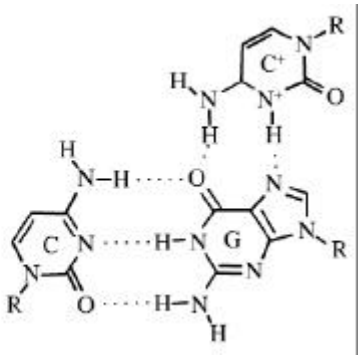

Figure 2c. Hogsteen bond C^+-GC triplet.

1.5 Selecting the plasmids with insert (X-gal, IPTG)

The plasmid used for cloning had the cloning cassette in *lac Z*. The gene product of *lac Z* is β-galactosidase. By adding the inducer IPTG, β-galactosidase can be transcribed and cleave the substrate X-gal which then forms a clear blue color. When the insert is cloned successfully, the color will not appear. This is a good indication for which colonies to be used for further experiments [4].

1.6 Streptavidin coated magnetic beads

For the first part of the project, when proving the concept, the same materials and methods were used as described in the article mentioned in Introduction 1.2 [2]. The triplex-forming oligos were attached to magnetic beads which were 2.8 µm in diameter and had a coating of covalently bound streptavidin. (Dynabeads M-280, Dynal Biotech, Norway). Streptavidin is a protein which has four binding sites for the small ligand biotin. The bond is noncovalent but still extremelly strong, it is stable over a wide range of pH and temperature. The magnetic beads are basically polystyrene beads with an iron core (Fe_2O_3 and Fe_3O_4), the magnetic features of the iron oxide makes it easy to separate the beads from other components in a mixture by the help of a magnet. 1 mg beads (100 µL) will bind 200 pmol of biotinylated single stranded oligo [6] (see Figure 3).

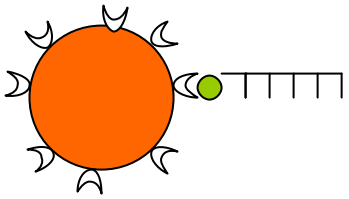

Figure 3. Magnetic bead with streptavidin covalently attached is binding a biotin tagged oligo.

1.7 Triple-Helix-Mediated Affinity Capture (TAC) DNA purification

The procedure is composed of three main steps: cell lysis, affinity capture, and elution. The exact procedure and components are described in Materials and methods 2.8. After being harvested and resuspended, the bacterial cells are lysed in NaOH and SDS, SDS denatures the cellular proteins while the alkaline condition causes the chromosomal and plasmid DNA to become denatured. The lysate is neutralized by addition of high concentration, acidic (pH ~5) KAc. The high salt concentration causes denatured proteins, chromosomal DNA, cellular debris and SDS to precipitate, while the small plasmid DNA stays in solution. The precipitated material is removed by centrifugation, leaving a clear lysate for affinity capture. The cleared lysate contains plasmid DNA as well as contaminants including RNA, unprecipitated chromosomal DNA, polysaccarides, proteins and other cellular components. The triplex beads specifically bind to the plasmid DNA containing the appropriate homopyridine-homopurine insert, allowing its separation and purification. The acidic cell lysate causes the cytosines to become protonated and so a stable triplex can be formed. The lysate is directly mixed with the triplex beads. After a brief incubation, the beads are washed once with high-salt binding buffer and once with low-salt washing buffer which stabilises the triplex structure and minimises non-specific binding. The low buffer strength of the wash buffer also helps to obtain a high pH in the subsequent elution step. The captured plasmids are released from the beads by incubation in a high-pH buffer which disrupts the triplex without disrupting the double stranded plasmid DNA. The eluate contains a highly pure DNA solution which can be used for gene therapy or other purposes. (Modified description from article [2]).

1.8 Pluronic and polystyrene beads

For the second part of the project, the TAC purification method was slightly modified. If the method should be used in large scale, it would have to involve cheaper materials than magnetic beads. The magnetic separation method may also be hard to utilise in a tank of several litres of bacteria lysate. The materials chosen for this modified method also attach to each other with a bond that is not mentioned in the patent on triple helix. Instead of streptavidin coated magnetic beads, polystyrene beads with pluronic molecules adsorbed with a hydrophobic interaction were used. Pluronics are composed of long chains of polyethylene oxide-, polypropylene oxide-, polyethylene oxide- triblock copolymers (PEO-PPO-PEO). The pluronics used were modified with a PDS (pyridyl disulfide) attached to one of its two ends (Figure 4a). The oligo used had a thiol tag which reacts with the PDS. PDS is spliced off and the thiol-oligo forms a disulphide bridge with the pluronic [8] (Figure 4b).

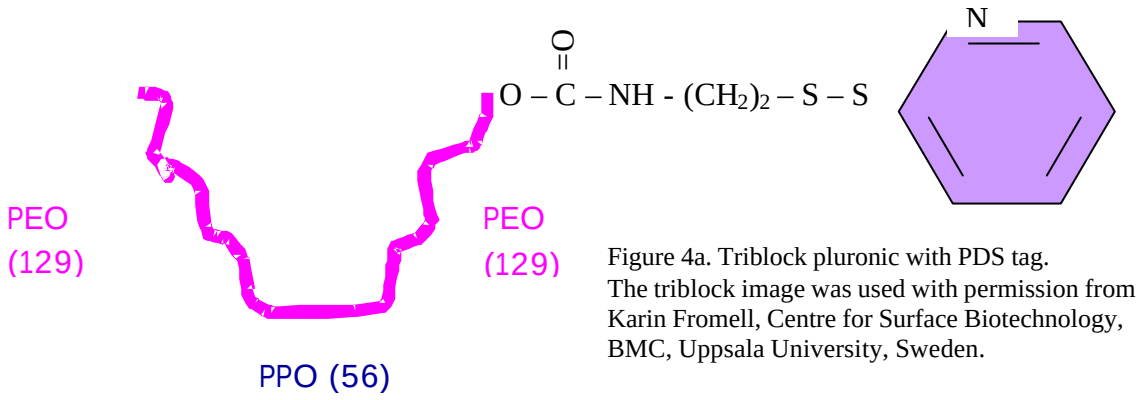

Figure 4a. Triblock pluronic with PDS tag.
The triblock image was used with permission from Karin Fromell, Centre for Surface Biotechnology, BMC, Uppsala University, Sweden.

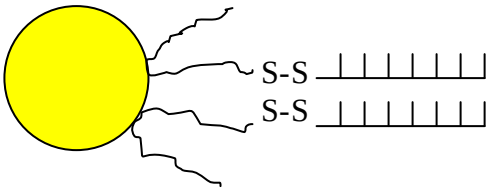

Figure 4b. Oligo attached to a pluronic through a disulphide bridge.

1.9 Calculating the amount of oligo that can bind

Polystyrene beads of two different diameters were used, 240 nm and 20 µm (Bangs labs, USA). Each pluronic takes up 10.2 nm^2 of bead surface when the supply of pluronic is saturated [9]. Each 240-nm bead had a surface area of ~$1.8x10^5$ ($4pr^2$). 100 µL of beads contained $1.322x10^{12}$ beads (Bangs Labs order specifications) this gave a total area of $2.4x10^{17}$ nm^2 in 100 µL water. For some reason only one of the two polyethylene ends binds the modification group PDS [9]. Therefore 100 µL beads could bind $2.4x10^{16}$ oligos. Dividing this number with Avogadro´s constant ($6.022x10^{23}$) gives 40 nmol oligo / 100 µL beads. For the 20 µm beads, the surface area per gram beads was according to the company $2.886x10^{11}$ μm^2/g. 100 µL solution contained 10 mg beads (concentration 100 mg/mL). 100 µL beads could then bind (with each pluronic taking up a space of 10.2 nm^2) $2.9x10^{13}$ oligos, i.e. 0.48 nmol oligo / 100 µL beads.

1.10 Measuring PDS as indication of bonded oligo

For each bonded oligo, a PDS molecule will be cleaved off. In the preparation of bead-pluronic-oligo-complex, the excess of both pluronic and oligo are removed by centrifugation. The first excess centrifugation of oligo will contain the free PDS molecules. Since the PDS molecule is a ringformed aromatic structure they will absorb UV light, more precise at the wavelength 343 nm (in their unbound state). By measuring the amount of free PDS molecules in the excess sample with a spectrophotometer, one can determine the amount of oligo bound to the beads [10]. The connection between absorption and DNA concentration can be calculated by using Lambert- Beer's law: A = ebc, where A is absorbance, b is the path length of the light in cm (cuvette width), c is concentration of the sample in mol/L and e is the extinction coefficient (unique for each type of molecule). For unbound PDS: e = 8080 / M cm.

1.11 DyNA Quant and Hoechst dye

The amount of double stranded DNA in a solution can be measured with a spectrophotometer, at an absorbtion wavelength of 260 nm. There can be difficulties in measuring small amounts of DNA though, since the sample has to be diluted multiple times to fill up the measurement cuvette. In this case a fluorometer and a dying assay was used instead. The main component of the assay, Bisbenzimide or Hoechst dye, sticks to the major groove in the double stranded DNA. In the absence of DNA, the excitation of the dye peaks at 356 nm and the emission spectrum peaks weakly at 492 nm. When the dye binds to DNA, these peaks shift to 365 nm for excitation and 458 nm for emission. The sample is exposed to filtered light (365 ± 7 nm) from a mercury lamp. This light excites the DNA-dye complex, causing light that peaks at 458 nm to be emitted. An emission filter in front of the photodetector allows only fluorescence at 460 ± 15 nm to register. Thus the measured fluorescence is a direct indicator of the DNA concentration in the sample [11].

2 Materials and Methods

2.1 Plasmid with target sequence and probe used

The same base pairing sequences were used for probe oligo and plasmid target sequence as in the article mentioned in Introduction 1.2 [2].

The probe was a 25 base single stranded oligo where seven bases were 5-methylcytosines. The methylation helps to increase the stability of the Hogsteen paired triple-helix strand [2]. The 5'-end had a tag attached. Two different tags were used, biotin for magnetic bead experiment and thiol for polystyrene bead-pluronic experiment. See Figure 5a.

The triplex target sequence (to be cloned into the plasmid) was 48 bp, where a cleavage site for Sca I had been introduced in the non-triplex forming region. See Figure 5b.

The plasmid vector was included in the cloning kit, it contained one cleavage site for Sca I at base 1266. The plasmid had a blunt cloning site at EcoRV, base 101. See Figure 5c.

3´-TTTCCTTCTTTCTCTCTTCTGATCA-biotin/thiol-5´
mm m m m m m

Figure 5a. Single stranded oligo used for triplex formation. The m means that the cytosine is methylated, the last five bases at the 5'-end works as a spacer between triplex and tag.

3´-TGAATTCA TGAAGAAGAGGAAAGAGAAGAAAGGAAGAAAGAGAGAAGA -5´
5´-ACTTAAGT ACTT CTT CT CCTTT CT CTT CTTT CCTT CTTT CTCT CTT CT -3´

Figure 5b. Double stranded target sequence where the Sca I cleaving site has been marked.

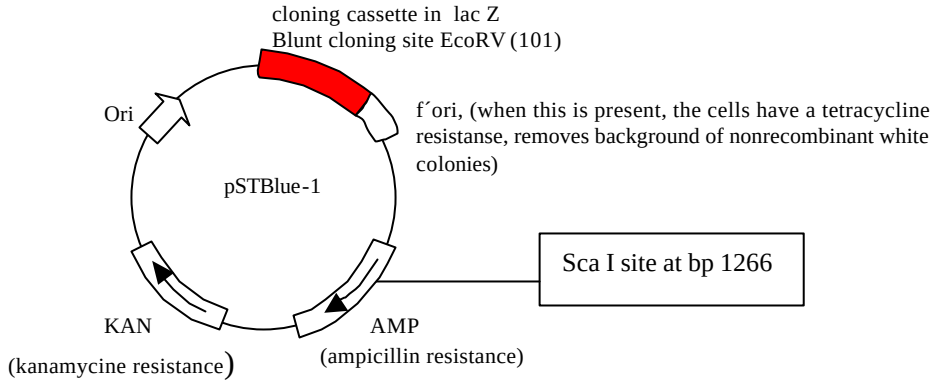

Figure 5c. Plasmid used for cloning (The image was adapted from [10]).

2.2 Cloning of 48-bp insert into competent cells

Materials: 48bp cloning insert (from Thermohybaid, Germany), Ampicillin plates (15 µg/mL)
Solutions: TE-buffer pH 7.6 (10 mM Tris-HCL, pH 7.4, 1 mM EDTA, pH 8.0), Tetracycline (10 mg/mL), IPTG (100 mM), X-gal (50 mg/mL)

The cloning was performed using the Perfectly Blunt cloning kit pSTBlue-1 from Novagen. The kit was stored at -80°C until use. Components were kept on ice during the cloning

experiment. The 48 mer (delivered dry frozen) was resuspended in TE-buffer to a concentration of 100 pmol/µL. Cloning was performed in three steps; End conversion (to activate the insert with triphosphate), ligation and transformation. End conversion: 1 µL diluted insert (0.05 pmol), 4 µL sterile water and 5 µL End conversion mix was added to a PCR tube and mixed gently by pipetting up and down. To check the capacity of the kit a control reaction was also performed, mixing 2 µL control insert (4.5 ng / µL), 3 µL sterile water and 5 µL End conversion mix. The reactions were performed at 22°C for 15 min and stopped by raising the temperature to 75°C for 5min. The tubes were then put on ice for 2 min followed by a brief centrifugation in order to collect condensed material. Ligation: 1 µL Blunt vector and 1 µL T4 DNA Ligase was added to both tubes, mixed with pipette and incubated at 22 °C for 15min. Transformation: Competent cells were put on ice for approx 5 min until thawed, 1µL ligation product was added. The tubes were put back on the ice for 5 min, heated to 42°C for 30 sec and then put on ice again for 2 min. Finally, 250 µL SOC Medium was added. Transformed cells were grown on ampicillin plates with an addition of 75 µL tetracycline and a 200 µL mix containing 20 µL IPTG, 40 µL X-gal and 140 µL LB (Luria-Bertani) to each plate. A total of four such plates were prepared, 50 µL and 250 µL cells were spread for both control insert and real insert. Plates were incubated up side down in 37° C for 18 h and then put in cold room for storage.

2.3 Growth of overnight cultures

Solutions: 20 % glucose in sterile water, tetracycline (10 mg/mL), carbinicillin (50 mg/mL), liquid LB medium.

Working under sterile conditions, 1 mL glucose, 100 µL of tetracycline and 100 µL of carbinicillin (can be used instead of Amp) were added to an E-flask containing 100 mL of LB medium. The bacterial colony was picked with a sterile metal wire loop. The E-flask was kept on shaking at 37 °C over night.

2.4 Conventional plasmid purification (QIAprep Miniprep kit from Qiagen)

Solutions: All included in the QIAprep kit.

1 mL of cells from overnight cultures (Materials and methods 2.3) were centrifuged at 13000 rpm for 5 min.The bacterial pellet was resuspended by pipetting up and down in 250 µL buffer P1 (containing RNase A) and transferred to a microcentrifuge tube. 250 µL of buffer P2 was added. The tube was gently inverted a couple of times until the solution became clear and viscous. 350 µL of buffer N3 was added and the tube inverted again until a cloudy appearance. The tube was centrifuged for 10 min at 13000 rpm. The supernatant was applied to a QIAprep column. It was centrifuged for 30-60 s and the flow-through discarded. The column was washed twice by first adding 0.8 mL buffer PB, centrifuging for 30-60 s and discarding the flow-through and then again the same procedure with 0.75 mL buffer PE. To remove the last traces of washing buffer, the column was then centrifuged again for an additional 1 min. To elute the plasmid DNA, the column was placed in an eppendorf tube and 50 µL buffer EB (10 mM Tris-HCl, pH 8.5, preheated to 60°C) was added. The solution was left on the bench for 1 min and centrifuged at 13000 rpm for 1 min.

2.5 Sca I cleavage of plasmid

Solutions: Sterile water containing the plasmids, restriction enzyme Sca I and 10x restriction buffer (from Sigma-Aldrich, Germany).

The recipe used was for an approximate DNA amount of 0.2-1 µg. 18 µL of the plasmid solution was mixed with 2 µL restriction buffer and 1 µL restriction enzyme. The reaction was left at 37°C for 3 h before analysis with electrophoresis [12].

2.6 Electrophoresis (on a 1 % Agarose gel)

Materials: 1 g Agarose powder. Solutions: 5 x stock solution of TBE running buffer (54 g Tris, 27.5 g boric acid, 20 mL EDTA (0.5 M, pH 8.0), 880 mL distilled water), concentrated ethidium bromide.

1 % Agarose gel is used for DNA fragments in the range of 0.5-7 kb. The agarose powder was dissolved in 100 mL 1 x TBE running buffer by using a magnetic stirrer and heating the solution. The solution became completely clear when ready. It was then slightly cooled and poured into the gel tray (covered with tape on the two open sides) until a height of approx 5-10 mm. The comb was put in the right position and the gel was allowed to cool completely. The tape was removed and the tray was placed in the electrophoresis container, TBE running buffer was poured into the container until it covered the gel completely.

As a standard 2 µL loading buffer was used together with 5 µL of sample in each well. The electroforesis was run on 150 V, 200 mA and 30 W for approx 1.5 h. The gel was transferred to a small container together with some of the running buffer and stained with 5 µL concentrated ethidium bromide. It was left for approx 15 min and could then be examined under UV light [12].

2.7 Making of triplex beads (bead-oligo-complex)

Materials: Single stranded oligo (Thermohybaid, Germany), magnetic beads (Dynabeads M-280, Dynal Biotech). Solutions: TE-buffer (10 mM Tris-HCL, pH 7.4, 1 mM EDTA, pH 8.0), PBS (10 mM Na_2HPO_4, pH 7.5, 0.15 M NaCl).

5 mg beads was prepared for a total of 5 DNA purifications. The oligo (delivered dry frozen) was resuspended in TE-buffer to a concentration of 100 pmol / µL upon arrival and stored at -20° C. 500 µL of beads was transferred to an eppendorf tube, washed twice with 500 µL PBS and resuspended in 500 µL of PBS. 4 µL (400 pmol) oligo was added and the tube put in room temperature for 30 min. The beads were then washed twice with PBS, twice with EB and twice with BB. It was stored at -20° C until use.

2.8 Standard protocol for magnetic beads purification

Materials: Magnetic stand, triplex beads (1mg for each purification). Solutions: GET (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCL, pH 8.0), BB (1.0 M KAc, pH 5.0), WB (10 mM NaAc, pH 5.8, 100 mM $MgCl_2$), EB (50 mM Tris-HCL, pH 9.0), NaOH/1% SDS (0.2 M), KAc (3 M, pH 5).

An overnight culture was prepared (Materials and methods 2.3). The cells were kept on shaking at 37°C until OD_{600} had reached 2 (approx 16h for Novagen cells). A volume of 1.5 mL of the overnight culture (used as a standard starting volume for each magnetic bead purification procedure) was transferred to an eppendorf tube and centrifuged for 5 min at 13000 rpm. The supernatant was discarded and the cell pellet resuspended in 100 µL of GET solution by vortexing until completely dissolved. 200 µL NaOH/1 % SDS solution was added and after mixing by inverting the tube was put on ice for 5 min. 150 µL of cold 3 M KAc was added and the tube inverted a few times before returning to ice for another 5 min. The tube was then centrifuged for 5 min at 13000 rpm. The supernatant was transferred to a new tube. To bind, 100 µL of Triplex beads was added and the tube left in room temperature for 15 min. To wash, beads were immobilized with a magnetic stand and washed once with 100 µL of BB and once with 100 µL of WB. The liquid was carefully removed by pipette. To elute, 50 µL of EB was added and the tube left in room temperature for 10 min. The tube was then again placed on the magnetic stand and the liquid now containing the purified plasmid could be transferred to a new tube. For both washing and elution, the liquid and the triplex beads were mixed by pipetting up and down until an even suspension could be observed. See the whole procedure in Figure 6.

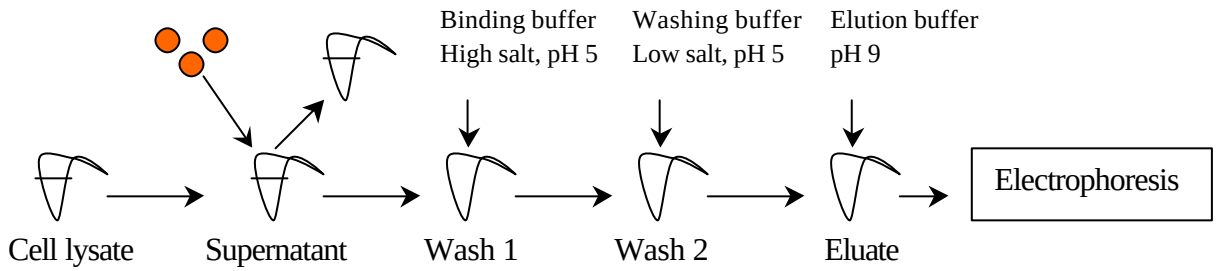

Figure 6. From cell lysate to eluate. Cell lysate- final clear solution of cell components just before adding triplex beads, supernatant- the liquid phase after fishing out plasmids with the triplex beads, wash 1- BB buffer after washing the beads, wash 2- WB buffer after washing the beads, eluate- Tris-HCl with the released plasmids

2.9 Compensating for concentration differences in lysate, supernatant and eluate

Solutions: Tris-HCl (pH 9)

Usually, 100 µL of lysate was kept for comparison on the gel, while 300 µL of the lysate was used for purification. The elution was in a much smaller volume than the supernatant (60 µL compared to 300 µL). These differences had to be adjusted for in order to compare the yield directly on the gel. For lysate and supernatant, only a third of the Tris-HCl volume added to the supernatant was added to the lysate after isopropanol precipitation. The eluate was diluted by adding seven extra volumes of Tris-HCl (5 times to compensate for the concentrated eluate volume and then 3 more times to compensate for the larger starting volume of the supernatant).

2.10 Modifications of TAC purification

2.10.1 Proof of concept

For testing a reverse addition of beads and oligo to the cell lysate, the step of adding triplex beads was substituted with an addition of free oligo to the plasmid solution. The reaction was left for 15 min, followed by the addition of beads and left for another 10 min. Two rounds of

elution were performed. Elution 1 was a regular elution. For elution 2, new elution buffer was added right after the first elution on the same beads, this time the elution buffer was preheated to 50 °C before adding to beads.

2.10.2 Testing different hybridization and elution conditions

Four different versions of the original hybridisation (triple helix formation) method were tested. 1- raised the salt concentration by adding 100 µL 4 M NaCl to the 300 µL 1 M KAc lysate solution and letting the hybridization continue over night. 2- Added 100 µL 4 M NaCl to the 300 µL 1 M KAc and lysate solution and hybridize regular time 15 min. 3- heated the solution to 45 °C during hybridization for 10 min, left in room temperature the last 5 min. 4- regular conditions as a control. 5- the original lysate (to estimate the yield in the elutions). 100 µL MilliQ was added to 3 and 4 to adjust for the extra volume introduced with the NaCl in 1 and 2. To get the plasmids on a straight line on the gel, Tris-HCl was used as the final soluble for all five samples (Materials and methods 2.11). Extra Tris-HCl was added to the eluates to compensate for concentration differences (Materials and methods 2.9).

2.10.3 Interference of other plasmids or bacterias without plasmids in solution

Two different versions of plasmids in a greater mass of bacteria were investigated:

1 part of bacteria containing pSTBlue plasmid + 9 parts of bacteria without plasmid
3 parts of bacteria containing pSTBlue plasmid + 7 parts of bacteria without plasmid

Two versions of pSTBlue plasmid in a solution of other plasmids were investigated:

1 part of bacteria containing pSTBlue plasmid + 9 parts of bacteria with other type of plasmid
3 parts of bacteria containing pSTBlue plasmid + 7 parts of bacteria with other type of plasmid

The TAC purification was performed according to the standard protocol (starting with 1.5 ml of cell culture). For the first two versions, empty Novagen bacteria from the cloning kit were used. For the second two versions, pBR322-containing bacteria was used. The pBR322-plasmid is slightly larger (~ 4360 bp) than the pSTBlue plasmid with insert (3899 bp). Both types of bacteria were collected from the deep freezer (-83 °C), plated on LB plates and grown over night at 37 °C. They were transferred to liquid LB, and grown over night at 37 °C on shaking until they had reached OD_{600}=2. Novagen bacteria containing pSTBlue plasmids were grown according to standard protocol (Materials and methods 2.3) to an OD_{600} = 2. The eluates from the four experiments together with a regular purification (control) were checked by electrophoresis. A size/amount marker (High DNA Mass Ladder from Invitrogen [13]) was used to estimate the plasmid yield in ng. 4 µL of the marker was mixed with 1 µL loading buffer and 4 µL loaded on the gel [13]. To compare with the marker, the same volumes were employed when mixing and loading the eluates. Concentration differences were compensated for (Materials and methods 2.9).

2.10.4 Testing repeated times of elution

A regular TAC purification was performed. After the elution, the old supernatant was added to the beads and another purification round was performed, this was then repeated to a final of four elutions (see Figure 7). The resulting supernatants and eluates were checked by electrophoresis together with original lysate and size/amount marker (High DNA Mass Ladder from Invitrogen [13]). 4 µL of the marker/sample was mixed with 1 µL loading buffer and 4 µL loaded on the gel. Lysate and supernatant were transferred to Tris-HCl buffer with

isopropanol precipitation (Materials and methods 2.11). Concentration differences were compensated for (Materials and methods 2.9).

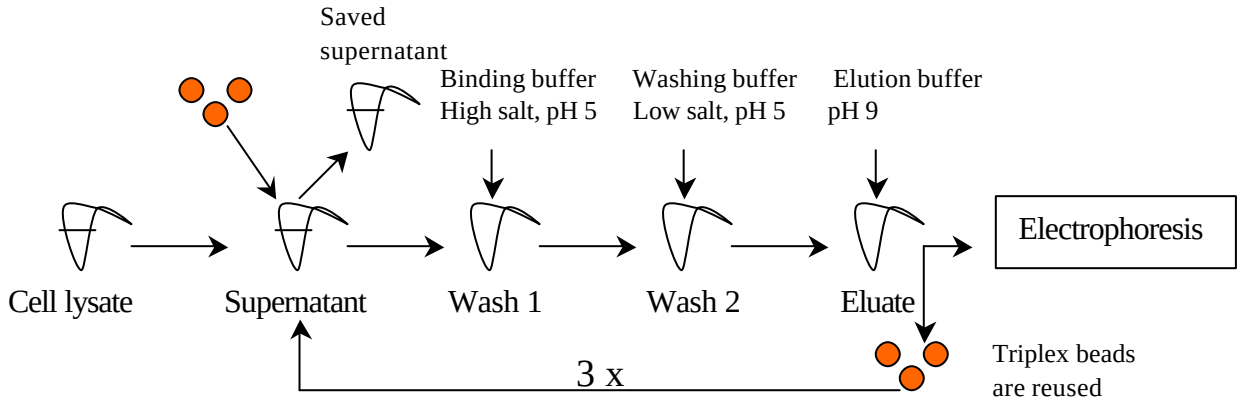

Figure 7. Multiple elutions, where the bead-oligos are added to the saved supernatant again after each elution.

2.11 Changing solute environment by isopropanol precipitation

Solutions: Concentrated isopropanol, NaAc (3 M), sterile water or Tris-HCl (pH 9).

50 µL isopropanol and 50 µL NaAc (1/10 of total volume each) were added to 400 µL DNA containing liquid. It was then kept at –20 °C for at least 30 min. The sample was thawed and centrifuged at 13000 rpm for 30 min. The supernatant was removed carefully and the pellet was resuspended in an equal amount of preferred soluble (sterile water or Tris-HCl).

2.12 Connecting oligos to polystyrene beads using a pluronic spacer

Solutions: TE-buffer (Materials and methods 2.7), pluronic in phosphate buffer (F108-PDS from Alvivo, 2 mg/mL), polystyrene beads in MilliQ (1 mg/µL).

The oligo (delivered dry frozen) was resuspended in TE buffer to a concentration of 100 pmol / µL upon arrival and stored at -20° C. The polystyrene beads used were of two sizes, 240 nm and 20 µm in diameter (both from Bangs labs). 0.5 mL of pluronic was added to 100 µL polystyrene beads. The mix was left on shaking for 24 h. The excess of non-binding pluronic was removed by centrifuging three times at 13000 rpm for 20 min. In between each spin, the supernatant was removed and an equal amount of MilliQ was added. The oligo was then added from the stock of 100 pmol / µL. The mix was left on shaking over night. The excess of non-binding oligos was removed following the same procedure as pluronic excess. All 6 excess samples were saved and stored in coldroom for later estimations of binding capacity.

2.13 Estimations of DNA amount with DyNA Quant 200 Fluorometer

Solutions: 10x stock solution of TNE buffer (1.21 g Tris, 0.37 g EDTA (x $2H_2O$), 11.69 g NaCl, pH 7.4 (adjusted with concentrated HCl)), Hoechst dye stock solution (10 mg H 33258 powder in 10 mL of distilled water), deionised water.

The assay used was for a DNA concentration range of 10-500 ng/µL. Hoechst dye is a possible mutagen and gloves were used during the whole preparation and measurement. A mask was used when weighing the powder. The blank assay contained 4 µL Hoechst dye solution, 4 mL 10x TNE buffer and 36 mL deionised water. It was prepared fresh every day

before measuring, TNE buffer and water were filtered before adding Hoechst dye stock solution. The Fluorometer was calibrated with a DNA sample of known concentration before starting measurements on the unknown samples, the assay was zeroed each time before adding DNA. For measuring, 2 µL sample was added to 2 mL of blank assay, which made a 1000 times dilution [11].

2.14 Preparing double stranded oligo for DyNA Quant measurement

Materials: Complementary oligo strand to the thiol tagged probe (ThermoHybaid, Germany), 240 nm polystyrene beads with pluronic in MilliQ (1 mg/ µL). Solutions: TE buffer (Materials and methods 2.7).

The complementary oligo was dissolved in TE buffer to a final concentration of 100 pmol / µL. 40 µL of both single stranded oligos were added to a new test tube and allowed to hybridise for 10 min in room temperature. The new concentration of oligo was now 50 pmol / µL. 40 µL of this double stranded oligo was added together with 5 µL beads-pluronic and 455 µL MilliQ to a new test tube. 460 µL MilliQ was added to the 40 µL oligo left in the test tube. They were both kept on vortex 1 rpm over night. The oligo-bead-pluronic mix was centrifuged for 10 min on 13000 rpm and the excess oligo transferred to a new tube. The oligo in MilliQ was used as a calibrant for the excess oligo from the bead mix. It had a concentration of 50 pmol / µL which equals, taken that one base weighs 330 g / mol and the double stranded oligo was 25 base pair long, 66 ng / µL. 2 µL, 1 µL and 3 µL excess sample was tested on the fluorometer.

3 Results and Discussion

3.1 Colonies to continue with after cloning

Cloning was performed successfully both for the test insert and real insert. After an overnight storage at 8° C, colonies were observed on all four plates. For some reason there were only a few colonies in the middle of the plates, most colonies were found closer to the plate walls. This may be the result of higher concentrations of antibiotics in the middle of the plates. Blue colonies were not observed until several weeks in coldroom storage (8° C). Five colonies were picked from the 50 µL plate, two from the middle and three from the sides (see Figure 8). After a conventional Qiagen plasmid prep the five colonies were run on electrophoresis. The three colonies from the side areas showed plasmid bands while the two lanes with samples from the middle colonies were blank.

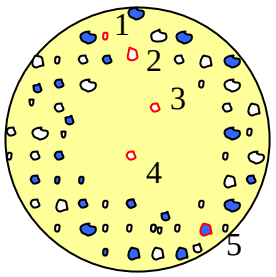

Figure 8. Plate with blue and white colonies. The red borders indicates that these colonies were picked. Note that one of the colonies, colony 5, showed a blue colour after some weeks in coldroom storage.

3.2 Sca I restriction cleavage

A restriction reaction (using 0.5 µL Sca I instead of 1 µL as in standard protocol) was performed on the rest of the samples from the plasmid prep. The results were analyzed by electrophoresis (see Figure 9a). Samples were loaded according to standard protocol (Materials and methods 2.6). The cleavage should result in a fragment of 1200 bases and one of about 2700 bases. All three lanes with uncleaved plasmid show a band in the same range, probably the supercoiled plasmid. The cleaved colonies 1 and 2 show two smaller bands plus a heavier one, this heavier band probably belongs to a linear plasmid that has only been cleaved once. Cleaved colony 5 looks different. When these colonies were picked, the blue colour had not started to appear on the plates. Colony 5 later developed the blue colour and the bands appearing in this lane probably comes from the uncleaved plasmid and the plasmid cleaved only once.

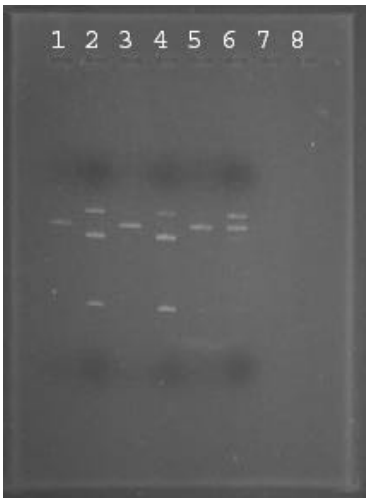

Figure 9a.

lane 1-colony #1 uncleaved
lane 2-colony #1 cleaved
lane 3-colony #2 uncleaved
lane 4-colony #2 cleaved
lane 5-colony #5 uncleaved
lane 6-colony #5 cleaved

The whole procedure from plasmid prep, cleaving and electrophoresis was performed again, this time using 1 µL of Sca I restriction enzyme. An amount marker was also loaded on the gel (READY-LOAD 1 Kb PLUS DNA Ladder from GIBCO BRL Life technologies). 2 µL marker plus 8 µL loading buffer was loaded, the samples were loaded according to standard protocol (Materials and methods 2.6). The marker verified that the suggested origins of the different bands were correct (see Figure 9b). This time colony 5 seemed to be cleaved in two places just like the others. There were faint bands for colony 5 on these places even in the first restriction cleaving and electrophoresis. During prolonged storage at 4° C, white colonies can start to develop a light blue colour and thus a functioning *lac Z*. 90 % of these light blue colonies contains the correct insert (Novagen instruction manual for the perfectly blunt cloning kit) This phenomena together with a very small amount of restriction enzyme added on the first gel might have given rise to the different results for the two Sca I reactions. Further experiments were only performed on colony 1 and 2.

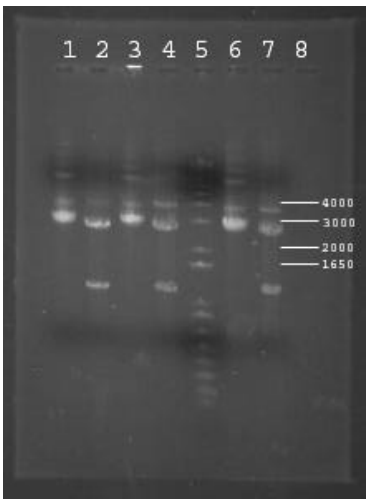

Figure 9b.

lane 1-colony #1 uncleaved
lane 2-colony #1 cleaved
lane 3-colony #2 uncleaved
lane 4-colony #2 cleaved
lane 5-size marker
lane 6-colony #5 uncleaved
lane 7-colony #5 cleaved

3.3 Magnetic beads

3.3.1 Proof of concept

To test if the plasmid purification method worked as in [2], a first purification experiment was set up with the exact same materials and methods as described in the article (Materials and methods 2.8). The resulting lysate, supernatant, eluate 1 and eluate 2 (Materials and methods 2.10.1) were investigated by electrophoresis (see Figure 10, lane 1-4)

Preferably the final purification system would be automated on the most part. In the article by Smith *et al*, the triplex beads (beads and fishing oligo) were formed first and then the plasmid solution was added. This way the triplex beads could be used multiple times, after each time the plasmids had been eluted. However, the possibility of getting a greater yield of plasmids by forming the triple helix between oligo and plasmid before adding beads had to be investigated (Materials and methods 2.10.1) The results from this lysate, supernatant, eluate 1 and eluate 2 were checked by electrophoresis (see Figure 10, lane 5-8).

Both the original method and the new reversed version show clear plasmid bands in the elution lanes. The lysate, supernatant and eluate are all in different buffers, therefore the plasmid line is crooked. The intensity of the elution bands are visually equal and therefore no obvious advantages can be noticed with one over the other. The big white areas in lane 1, 2, 5 and 6 is RNA, in the eluate lanes this RNA contaminant has disappeared. (Note that the eluted sample is concentrated and the amount of purified plasmid cannot be directly determined by inspecting the gel). Further experiments only involved the original binding order.

Conclusion: The order in which the binding of oligo to bead and the binding of plasmid to oligo happens does not have a significant impact on the yield.

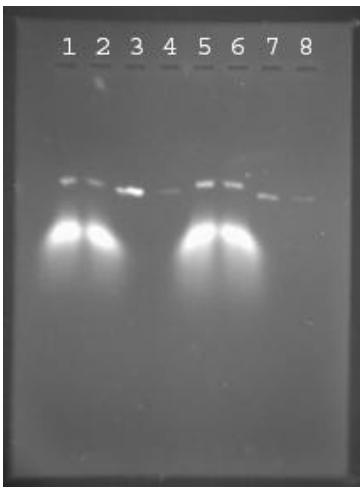

Figure 10.

Regular order of hybridisation
lane 1- lysate
lane 2- supernatant
lane 3- eluate 1
lane 4- eluate 2 (buffer had been heated)
Reverse order of hybridisation
lane 5- lysate
lane 6- supernatant
lane 7- eluate 1
lane 8- eluate 2 (buffer had been heated)

3.3.2 Testing different hybridization and elution conditions

To check if the yield of purified DNA could be increased, some parameters were being changed while keeping the total initial lysate volume constant. The salt concentration for hybridisation was raised and the time of hybridisation was prolonged, also the hybridisation temperature was raised. Three combinations were tested plus a regular hybridisation method and the initial lysate to compare yield (Materials and methods 2.10.2). All five were checked by electrophoresis (see Figure 11). Compensating for concentration differences allowed direct inspection on the gel (Materials and methods 2.9). No special differences in yield can be observed by inspecting the intensity on the gel.

Conclusion: Changing hybridisation conditions including higher salt concentration, heating or longer hybridisation time does not have a significant impact on the yield.

Figure 11.

lane 1- extra salt and over night hybridisation
lane 2- extra salt
lane 3- heating while hybridising
lane 4- usual conditions
lane 5- the original lysate

3.3.3 Interference of other plasmids or bacterias without plasmids in solution

Could the yield of plasmids be influenced by the presence of other plasmids in the lysate or a greater mass of bacteria without plasmids? Four experiments were set up, two of each variant, where one contained a smaller and one a larger part of pSTBlue containing bacteria (Materials and methods 2.10.3). These four versions together with a regular purification were run on an electrophoresis gel together with a size/amount marker (see Figure 12) in a manner which allows direct quantification (Materials and methods 2.10.3). All five versions seems to be in

the 20 ng area, lane eight contains a regular purification with triplex beads prepared at a different time, this plasmid yield seems to lie above the rest, in the 60 ng area. The amount of beads in this purification was slightly greater than in the other cases, probably due to a difficulty of getting an even concentration when suspending the triplex beads in PBS.

Conclusion: The yield of plasmid through TAC purification is not in any great extent influenced by the other components in the cell lysate.

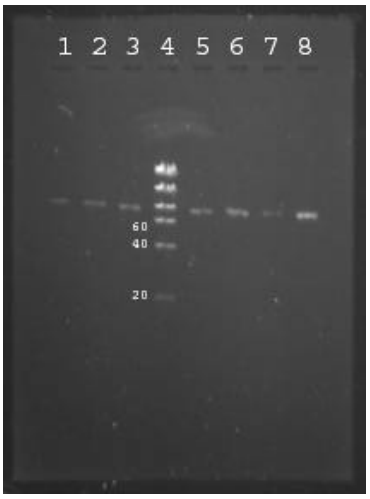

Figure 12.

lane 1- 1 part pSTBlue + 9 parts no plasmid, wrong loading vol.
lane 2- 1 part pSTBlue + 9 parts no plasmid
lane 3- 3 parts pSTBlue + 7 parts no plasmid
lane 4- amount/size marker
lane 5- 1 part pSTBlue + 9 parts pBR322
lane 6- 3 parts pSTBlue + 7 parts pBR322
lane 7- usual, only pSTBlue
lane 8- usual, only pSTBlue, more amount of beads

3.3.4 Testing repeated times of elution

To see how much plasmids could be eluted from the same lysate, a TAC purification experiment was set up with multiple rounds of elutions (Materials and methods 2.10.4). The results were checked by electrophoresis. The samples were loaded together with a size/amount marker (see Figure 13). The plasmid band disappears at elution 2 but reappears in elution 4. If the pH does not rise when elution buffer is added to the beads, the triple helix bond will still keep the plasmids bound to the solid phase. If some traces of the high salt binding buffer is left in the bottom of the test tube with the beads it might hold the pH down in the elution step. Eluate 1 was loaded twice on the gel, the first time no volume adjustments were made (Materials and methods 2.10.4).

Conclusion: The elution step of the TAC purification has to be looked at more closely and maybe revised, in order to make the plasmid yield more stable and reliable.

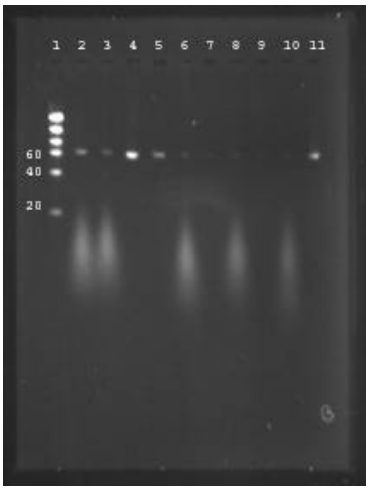

Figure 13.

lane 1- size/amount marker
lane 2- lysate
lane 3- supernatant 1
lane 4- eluate 1 (not adjusted volume)
lane 5- eluate 1
lane 6- supernatant 2
lane 7- eluate 2
lane 8- supernatant 3
lane 9- eluate 3
lane 10- supernatant 4
lane 11- eluate 4

3.4 Polystyrene beads

3.4.1 Testing different washing buffers

A TAC purification was performed with a slight modification. Since the polystyrene beads are not magnetic, centrifugation was used as an alternative separation method. Beads of two different diameters were tested, one ten times smaller (240 nm) and one ten times larger (20 µm) than the magnetic beads. 1 mg beads of each type were added in the two parallel experiments. After addition of washing buffer however, the beads seemed to gather along the surface of the liquid phase. 1 M KAc has a density of ~1.06 g/cm^3 which is very close to the bead density (~1.05 g/cm^3). Different experiments where the KAc concentration was lowered to 0.5 M or substituted with HCl pH 5 were tried. The beads formed a pellet on the bottom of the tube as usual but no plasmid bands could be observed on an electrophoresis gel.

Conclusion: Before performing more TAC purification experiments, it has to be investigated if the prepared bead-pluronic-oligo complex is functioning correctly.

3.4.2 Testing if probe oligo has bonded

3.4.2.1 Spectrophotometer

To investigate how much oligo had bonded to the pluronics, the excess of spliced PDS molecules in spin 1 with excess oligo was measured at wavelength 343 nm (Materials and methods 2.12) However, the results did not seem trustworthy, on several occasions the amount of oligo bonded exceeded the amount that had originally been added to the pluronic-bead. Also direct measurement of oligo was performed, the excess oligo from spin 1 was recovered by isopropanol precipitation (Materials and methods 2.11), redissolved in MilliQ and measured at 260 nm. The absorbance values were very low, almost as low as for the blank, MilliQ. Both smaller (500 µL) and larger (1.5 mL) quarts cuvettes were used.

Conclusion: Another method where the sample would not have to be so diluted is needed.

3.4.2.2 Fluorometric DyNA Quant 200

A complementary strain to the thiol oligo was allowed to hybridise and the excess oligo from spin one was measured. A total of 2 nmol oligo was added. The fluorometer gave the values directly in ng / µL. The calibrant (2 nmol double stranded oligo with the same concentration as the sample oligo before the addition of beads) had a concentration of 66 ng / µL. The amount of beads (240 nm diameter) added to the oligo solution had a binding capacity of 2 nmol i.e. if all the oligos added would bind, there would be no oligos left in solution (Materials and methods 2.15). However, the fluorometer measurements showed a sample concentration of 68 ng / µL. Half of the sample volume and one and a half of the volume were also tested, they gave results which strengthened the initial volume result (see Table 1).

	Volume of sample added	DyNA Quant signal	Adjusted signal (all volumes 2 µL)	Bonded oligo (unreacted-excess)
Unreacted oligo	2 µL	66 ng / µL	66 ng / µL	-
Excess oligo from oligo-bead-pluronic mix	1 µL	~ 35 ng / µL	~ 70 ng / µL	~ 0 ng / µL
	2 µL	~ 68 ng / µL	~ 68 ng / µL	~ 0 ng / µL
	3 µL	~ 100 ng / µL	~ 66 ng / µL	~ 0 ng / µL

Table 1. Comparison of unreacted oligo and excess oligo after over night reaction with beads-pluronics.

Conclusion: The binding between pluronic and oligo does not seem to work. Although the amount of bead-pluronics added to the oligo solution should be sufficient to bind all oligos, there was no differens in unbound oligo in solution between the unreacted oligo sample (calibrant) and the excess of oligos in solution after an over night reaction. Since people in the department have successfully carried out the adsorption of pluronic to bead before, the most possible reason is an inactivity of the thiol tag on the oligos.

4 Conclusions

The magnetic bead-based purification of plasmids seems to work as described in [2]. In a possible automated procedure it is preferable to have the oligo attached to the bead first and then the triplex hybridisation between oligo and plasmid, which allows for the reuse of bead-oligo-complex several times. Experiments showed that the order of bonding between beads to oligo and oligo to plasmid does not have a significant impact on the plasmid yield.

The presence of a large amount of other bacteria in the original cell culture does not have a great impact on the yield, nor does the presence of a different plasmid without the specific triplex forming sequence.

The elution step in the purification procedure is problematic, on several occasions the plasmids were not released from the beads, probably because of difficulties of getting the right elution pH, the step would have to be modified in order to get a stability to the purification procedure.

Using magnetic beads has several drawbacks when it comes to scaling up the method:

- The beads are much too expensive (Dynal biotech, 1854 Norwegian crowns for 20 mg beads, October 2002)

- The magnetic separation might be hard to carry out in a large container of several litres of liquid.

By using regular polystyrene beads, the price would fall and a separation by centrifugation could be used instead. The bonding of the pluronic to the bead through adsorption is an alternative to the covalent bonding used to connect the different components in the patent found [3].

The separation did not work for the polystyrene-pluronic-oligo-complex. A reason for this could be the thiol tag on the oligos. Oxygen present in the water solution, in which the oligos

are stored, can react with the thiol group and thus prevent them from forming the disulphide bridge to the pluronic.

Further experiments would involve ordering fresh thiol oligos, which would be diluted in buffer in an oxygen-lacking environment upon arrival or treating the existing oligos with some reagent to remove the supposed oxygen bonded to the thiol groups. Another solution might be to use the streptavidin-biotin connection instead of a disulphide bridge since this bond seemed to work successfully in the magnetic beads case.

5 Acknowledgements

I would like to thank the following people at the Centre for Surface Biotechnology:

Professor Karin Caldwell who has given me support and input on how to tackle some of the problems I have encountered.

Karin Fromell for her help with materials and knowledge about pluronics and polystyrene beads.

Karine Ehlin and Margareta Andersson for troubleshooting and help with recipe for preparing the bead-pluronic-oligo-complex, as well as the spectrophotometer measurements.

My three supervisors Andras Ballagi (who together with Professor Karin Caldwell initiated the project), Mikael Berg (thank you for being so calm and reasonable) and Anders Elfwing (who put a lot of time and effort into this project).

And also Anita and Jan whom I shared office with, thank you for helping me with getting to know the lab and for making me feel welcome, which is something that everyone at the centre has contributed to, a nice and welcoming atmosphere.

And last but not least, thank you Brian for those groovy images :0) and your support!

6 References

[1] Ferreira, G.N.M. et al. (2000) *Tibtech*.**18**, 380-387.

[2] Ji, H., Smith, L.M. (1993) *Anal. Chem.* **65**, 1323-1328.

[3] Crouzet et al. (2001) United States patent 6319672.

[4] Stryer, L. Biocehmistry 4th Ed. W.H. Freeman and company, New York.

[5] Jayasina, S.D., Johnston, B.H. (1992) *Nucleic Acids Res.* **20**, 5279-5288.

[6] Dynal biotech homepage: www.Dynalbiotech.com (4 Apr. 2003).

[7] Personal communication with Karin Caldwell, Centre for Surface Biotechnology, BMC, Uppsala University, Sweden.

[8] Personal communication with Karin Fromell, Center for Surface Biotechnology, BMC, Uppsala University, Sweden.

[9] Personal communication with Karine Ehlin, Center for Surface Biotechnology, BMC, Uppsala University, Sweden.

[10] Novagen Homepage: www.novagen.com (4 Apr. 2003).

[11] Hoefer DyNA Quant 200 fluorometer, Instruction Manual (1995) Hoefer Pharmacia Biotech.

[12] Maniatis, T. et al. (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York.

[13] High DNA Mass Ladder, Instruction manual (2003) Invitrogen life technologies.