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DESCRIPTION

FIELD OF THE INVENTION

[0001] The invention relates to novel processes for the production of oligonucleotides that are suitable for use in the production of chemically modified oligonucleotides, such as those used in therapy.

BACKGROUND TO THE INVENTION

[0002] The chemical synthesis of oligonucleotides and modified oligonucleotides *via* phosphoramidite chemistry is well established and has been the method of choice for synthesizing these defined sequence biopolymers for several decades. The synthetic process is usually run as a solid phase synthesis whereby single nucleotides are added sequentially with the addition of each nucleotide requiring a cycle of several chemical steps to add and deprotect the growing oligonucleotide ("oligo") in preparation for the subsequent step. At the end of the sequential addition of nucleotides the oligo is released from the solid phase support, further deprotection takes place, and then the crude oligonucleotide is further purified by column chromatography.

[0003] While this method may be considered routine and can be automated, there are several shortcomings to this methodology, especially if the goal is to prepare oligonucleotides at large scale as would be needed for oligonucleotide therapeutics. These shortcomings include, but are not limited to:

1. 1) Practical limitations inherent in the use of chromatography making it unsuitable for purifying large quantities of oligonucleotide. The use of chromatography at large scale is expensive and is difficult to achieve due to the limitations on column size and performance.
2. 2) The number of errors accumulates with the length of the oligonucleotide being synthesized. Accordingly, the linear sequential nature of the current process results in a geometric decrease in yield. For example, if the yield for each cycle of nucleotide addition is 99% then the yield of a 20 mer would be 83%.
3. 3) Scale limitations with synthetic oligonucleotide synthesizers and downstream purification and isolation equipment: at present the maximum amount of product that can be produced in a single batch is in the order of 10kg.

[0004] There is a need, therefore, to both reduce (or ideally eliminate) column chromatography and perform the synthesis in a way which is not purely sequential in order to increase yield.

[0005] DNA polymerase is often used to synthesize oligonucleotides for use in molecular biology and similar applications. However, DNA polymerase is unsuitable for synthesizing therapeutic oligonucleotides because of both the relatively short lengths of the oligonucleotides and the need to discriminate between nucleotides with different deoxyribose or ribose modifications. For example, therapeutic oligonucleotides are often in the range of 20 to 25 nucleotides. DNA polymerase needs at least 7 or 8 nucleotides, and optimally 18 to 22 nucleotides, as a primer in each direction so there is little to be gained in trying to synthesize a therapeutic oligo if the primers are similar in size to the desired product. Also, DNA polymerase requires all nucleotides to be present in the reaction and it relies on Watson-Crick base pairing to align incoming nucleotides. Thus It is unable to discriminate between any ordering of deoxyribose or ribose modifications, such as those required by a gapmer, and the result would be a mix of deoxyribose or ribose modifications at a given position.

SUMMARY OF THE INVENTION

[0006] The invention provides a process for producing a single stranded oligonucleotide product having at least one modified nucleotide residue, wherein the modification is selected from the group consisting of modification at the 2' position of the sugar moiety, modification of the nucleobase, and modification of the backbone, and wherein the product is produced at gram or kilogram scale, or greater, and/or the process is carried out in a 1 L or larger reactor, comprising:

1. a) providing a template oligonucleotide (I) complementary to the sequence of the product, said template having properties that allow it to be separated from the product;
2. b) providing a pool of oligonucleotides (II) containing oligonucleotides that are segments of the product sequence, wherein at least one segment contains at least one modified nucleotide residue and wherein the modification is selected from the

group consisting of modification at the 2' position of the sugar moiety, modification of the nucleobase, and modification of the backbone;

3. c) contacting (I) and (II) in conditions to allow annealing;
4. d) joining the segment oligonucleotides by enzymatic ligation with a ligase to form the product;
5. e) changing the conditions to separate any impurities, comprising denaturing the annealed template and impurity oligonucleotide strands and separating the impurities;
6. f) changing the conditions to separate the product, comprising denaturing the annealed template and product oligonucleotide strands and separating the product; and
7. g) recycling the template for use in future reactions.

[0007] Such a process may be used to generate a single stranded oligonucleotide product and isolate it from impurities, e.g. as a manufacturing and purification process.

[0008] WO01/64864A2 discloses single stranded nucleic acid template mediated recombination, WO01/71037A1 discloses methods of detecting polynucleotide Kinase, US6004826A discloses repair-mediated process for amplifying and detecting nucleic acid sequences and WO2009/097673A1 discloses the synthesis of single stranded DNA

DESCRIPTION OF FIGURES

[0009]

FIG. 1 Schematic of the process of the invention, including a step of changing the conditions to remove impurities, e.g. a washing step.

FIG. 2 Schematic of the process of the invention, including the steps of joining/ligating the segment oligonucleotides to form the product and changing the conditions to remove impurities.

FIG. 3 Schematic of multiple template configurations.

FIG. 4 Basic schematic of the process of the invention being carried out in a flow system.

FIG. 5 Detailed schematic of the process of the invention being carried out in a flow system: a) ligation chemistry section, b) ligation purification section, and c) alternative ligation chemistry section, and d) alternative purification section. N.B. sections a) and b) (alternatively c) and d)) can be performed in a single step e.g. collection vessel in a) = output from ligation step in b).

FIG. 6 Examples of impurities which may be generated during the process of the invention.

FIG. 7 Chromatogram showing the results of a ligation reaction using commercial NEB T4 ligase (SEQ ID NO:3) and unmodified DNA (Example 1).

FIG. 8 Chromatogram showing the results of a ligation reaction using PERLOZA bound T4 ligase (SEQ ID NO:4) and unmodified DNA (Example 1).

FIG. 9 Chromatogram showing the results of a ligation reaction using PERLOZA bound T4 ligase expressed according to Example 2 and 2'-OMe modified oligonucleotide fragments.

FIG. 10 HPLC traces for Example 4: Upper trace (a) - No ligase control. Lower trace (b) - done A4. Product and template co-elute in this HPLC method. Two intermediate ligation fragments (segments) can be seen in the Clone A4 trace at 10.3 and 11.2 minutes.

FIG. 11 Schematic of the "tri-template hub" used in Example 13, comprising a support material referred to as the "hub" and three template sequences.

FIG. 12 Schematic of the semi-continuous ligation rig used in Example 13.

FIG. 13 Schematic of the dead-end filtration rig used in Example 14.

FIG. 14 Schematic of the cross-flow filtration rig used in Example 14.

FIG. 15 Chromatograms showing the results from the dead end filtration experiments using the 10 kDa MWCO NADIR membrane at 60 °C in Example 14. Figure a) is a chromatogram of the retentate solution, which remained in the filtration cell and contained mainly tri-template hub, after two diafiltration volumes; and chromatogram b) is of the permeate, solution

enriched in the produce after two diafiltration volumes.

FIG. 16 Chromatograms showing the results from the cross-flow filtration experiments using the 5 kDa MWCO Snyder membrane at 50 °C and 3,0 bar pressure, in Example 14, Figure a) is a chromatogram of the retentate solution, which contained mainly tri-template hub and product, after 20 diafiltration volumes; and Figure b) is a chromatogram of the permeate, which contained mainly segment oligonucleotides, after 20 diafiltration volumes.

FIG. 17 Chromatograms showing the results from the cross-flow filtration experiments using the 5 kDaMWCO Snyder membrane at 80 °C and 3.1 bar pressure, in Example 14. Figure 17 shows a) a chromatogram of the retentate solution, which contained tri-template hub only, after 20 diafiltration volumes; and Figure b) is a chromatogram of the permeate solution, which contained the product only, after 2 diafiltration volumes.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

[0010] As used herein, the term "oligonucleotide", or "oligo" for short, means a polymer of nucleotide residues, either deoxyribonucleotides (wherein the resulting oligonucleotide is DNA), ribonucleotides (wherein the resulting oligonucleotide is RNA), or a mixture thereof. An oligonucleotide may be entirely composed of nucleotide residues as found in nature or may contain at least one nucleotide, or at least one linkage between nucleotides, that has been modified. Oligonucleotides can be single stranded or double stranded. An oligonucleotide of the invention may be conjugated to another molecule, e.g. N-Acetylgalactosamine (GalNAc) or multiples thereof (GalNAc clusters).

[0011] As used herein, the term "therapeutic oligonucleotide" means an oligonucleotide that has a therapeutic application. Such an oligonucleotide typically contains one or more modified nucleotide residues or linkages. Therapeutic oligonucleotides act via one of several different mechanisms, including, but not limited to, antisense, splice-switching or exon-skipping, immunostimulation and RNA interference (RNAi), e.g. via microRNA (miRNA) and small interfering RNA (siRNA). A therapeutic oligonucleotide may be an aptamer. Therapeutic oligonucleotides will usually, but not always, have a defined sequence.

[0012] As used herein, the term "template" means an oligonucleotide with a sequence that is 100% complementary to the sequence of the target (or product) oligonucleotide.

[0013] Unless otherwise specified, as used herein, the term "complementary" means 100% complementary.

[0014] As used herein, the term "product" means the desired oligonucleotide, having a specific sequence, also referred to herein as a "target oligonucleotide".

[0015] As used herein, the term "pool" refers to a group of oligonucleotides that may vary in sequence, may be shorter or longer than the target sequence, and may not have the same sequence as the target sequence. The pool of oligonucleotides may be the product of oligonucleotide synthesis, e.g. solid phase chemical synthesis via phosphoramidite chemistry, used with or without purification. The pool of oligonucleotides may be composed of segments of the target sequence. Each segment itself may be present as a pool of that segment and may be the product of oligonucleotide synthesis, e.g. solid phase chemical synthesis via phosphoramidite chemistry.

[0016] As used herein, the term "annealing" means the hybridisation of complementary oligonucleotides in a sequence specific manner. "Conditions to allow for annealing" will depend on the T_m of the hybridised complementary oligonucleotides and will be readily apparent to a person skilled in the art. For example, the temperature for annealing may be below the T_m of the hybridised oligonucleotides. Alternatively, the temperature for annealing may be close to the T_m of the hybridised oligonucleotides, e.g. +/- 1, 2 or 3 °C. The temperature for annealing is, in general, not higher than 10 °C above the T_m of the hybridised oligonucleotides. Specific conditions to allow for annealing are as outlined in the examples section.

[0017] As used herein, the term "denaturing" in relation to a double stranded oligonucleotide is used to mean that the complementary strands are no longer annealed. Denaturing occurs as a result of changing the conditions and is sometimes referred to herein as separating the oligonucleotide strands. . Such strand separation can be done for example, by raising the temperature, changing the pH, or changing the salt concentration of the buffering solution. Denaturing a double stranded

oligonucleotide (a "duplex") results in a single stranded oligonucleotide which could be a product or impurity being "released" from the template.

[0018] As used herein, the term "impurity" or "impurities" means oligonucleotides that do not have the desired product sequence. These oligonucleotides may include oligonucleotides that are shorter than the product (for example 1, 2, 3, 4, 5 or more nucleotide residues shorter), or that are longer than the product (for example 1, 2, 3, 4, 5 or more nucleotide residues longer). Where the production process includes a step whereby linkages are formed between segments, impurities include oligonucleotides that are remaining if one or more of the linkages fail to form. Impurities also include oligonucleotides where incorrect nucleotides have been incorporated, resulting in a mis-match when compared to the template. An impurity may have one or more of the characteristics described above. Figure 6 shows some of the impurities that may occur.

[0019] As used herein, the term "segment" is a smaller portion of a longer oligonucleotide, in particular a smaller portion of a product or target oligonucleotide. For a given product, when all of its segments are annealed to its template and ligated together, the product is formed.

[0020] As used herein, the term "enzymatic ligation" means that the link between two adjacent nucleotides is formed enzymatically. This linkage may be a naturally occurring phosphodiester bond (PO), or a modified linkage including, but not limited to, phosphorothioate (PS) or phosphoramidate (PA).

[0021] As used herein, the term "ligase" means an enzyme that catalyses the joining, *i.e.* covalent joining, of two oligonucleotide molecules, *e.g.* by formation of a phosphodiester bond between the 3' end of one oligonucleotide (or segment) and the 5' end of the same or another oligonucleotide (or segment). These enzymes are often referred to as DNA ligases or RNA ligases and utilise cofactors: ATP (eukaryotic, viral and archael DNA ligases) or NAD (prokaryotic DNA ligases). Despite their occurrence in all organisms, DNA ligases show a wide diversity of amino acid sequences, molecular sizes and properties (Nucleic Acids Research, 2000, Vol. 28, No. 21, 4051-4058). They are usually members of the Enzyme Class EC 6.5 as defined by the International Union of Biochemistry and Molecular Biology, *i.e.* ligases used to form phosphoric ester bonds. Within the scope of the invention is a ligase capable of joining an unmodified oligonucleotide to another unmodified oligonucleotide, a ligase capable of joining an unmodified oligonucleotide to a modified oligonucleotide (*i.e.* a modified 5' oligonucleotide to an unmodified 3' oligonucleotide, and an unmodified 5' oligonucleotide to a modified 3' oligonucleotide), as well as a ligase capable of joining a modified oligonucleotide to another modified oligonucleotide.

[0022] As used herein, a "thermostable ligase" is a ligase that is active at elevated temperatures, *i.e.* above human body temperature, *i.e.* above 37 °C. A thermostable ligase may be active at, for example, 40 °C - 65 °C; or 40 °C - 90 °C; and so forth.

[0023] As used herein, the term "modified nucleotide residue" or "modified oligonucleotide" means a nucleotide residue or oligonucleotide which contains at least one aspect of its chemistry that differs from a naturally occurring nucleotide residue or oligonucleotide. Such modifications can occur in any part of the nucleotide residue, *e.g.* sugar, base or phosphate. Examples of modifications of nucleotides are disclosed below.

[0024] As used herein, the term "modified ligase" means a ligase which differs from a naturally occurring, "wild-type", ligase by one or more amino acid residues. Such ligases are not found in nature. Such ligases are useful in the novel processes of the invention. Examples of modified ligases are disclosed below. The terms "modified ligase" and "mutant ligase" are used interchangeably.

[0025] As used herein, the term "gapmer" means an oligonucleotide having an internal "gap segment" flanked by two external "wing segments", wherein the gap segment consists of a plurality of nucleotides that support RNase H cleavage and each wing segment consists of one or more nucleotides that are chemically distinct to the nucleotides within the gap segment.

[0026] As used herein, the term "support material" means a high molecular weight compound or material that increases the molecular weight of the template, thereby allowing it to be retained when the impurities and products are separated from the reaction mixture.

[0027] As used herein "Percent identity" between a query nucleic acid sequence and a subject nucleic acid sequence is the "Identities" value, expressed as a percentage, that is calculated by the BLASTN algorithm when a subject nucleic acid sequence has 100% query coverage with a query nucleic acid sequence after a pair-wise BLASTN alignment is performed. Such pair-wise BLASTN alignments between a query nucleic acid sequence and a subject nucleic acid sequence are performed by using the default settings of the BLASTN algorithm available on the National Center for Biotechnology Institute's website with the filter for low complexity regions turned off. Importantly, a query nucleic acid sequence may be described by a nucleic acid sequence identified in one or more claims herein. The query sequence may be 100% identical to the subject sequence, or it may include

up to a certain integer number of nucleotide alterations as compared to the subject sequence such that the % identity is less than 100%. For example, the query sequence is at least 80, 85, 90, 95, 96, 97, 98, or 99% identical to the subject sequence.

STATEMENT OF THE INVENTION

[0028] in one aspect of the invention, there is provided a process for producing a single stranded oligonucleotide product having at least one modified nucleotide residue, wherein the modification is selected from the group consisting of modification at the 2' position of the sugar moiety, modification of the nucleobase, and modification of the backbone, and wherein the product is produced at gram or kilogram scale, or greater, and/or the process is carried out in a 1L or larger reactor, comprising:

1. a) providing a template oligonucleotide (I) complimentary to the sequence of the product, said template having properties that allow it to be separated from the product;
2. b) providing a pool of oligonucleotides (II) containing oligonucleotides that are segments of the product sequence, wherein at least one segment contains at least one modified nucleotide residue and wherein the modification is selected from the group consisting of modification at the 2' position of the sugar moiety, modification of the nucleobase, and modification of the backbone;
3. c) contacting (I) and (II) in conditions to allow annealing;
4. d) joining the segment oligonucleotides by enzymatic ligation with a ligase to form the product;
5. e) changing the conditions to separate any impurities, comprising denaturing the annealed template and impurity oligonucleotide strands and separating the impurities;
6. f) changing the conditions to separate the product, comprising denaturing the annealed template and product oligonucleotide strands and separating the product; and
7. g) recycling the template for use in future reactions.

[0029] In an embodiment of the invention, there are substantially no nucleotides in the reaction vessel. In an embodiment of the invention, there are no nucleotides in the reaction vessel. In particular, the reaction vessel does not comprise a pool of nucleotides, *i.e.* the reaction is substantially free, preferably free, of nucleotides.

[0030] Another embodiment of the invention provides a process as previously described herein, whereby the denaturation results from a temperature increase.

[0031] Another embodiment of the invention provides a process as previously disclosed herein, whereby the segment oligonucleotides are joined by chemical ligation. In a further embodiment, the chemical ligation is a click chemistry reaction. In an embodiment of the invention, chemical ligation of segment oligonucleotides takes place in templated reactions that produce phosphoramidate linkages as detailed by Kalinowski et al. in ChemBioChem 2016, 17, 1150 - 1155.

[0032] Yet another embodiment of the invention provides a process as previously disclosed herein, whereby the segment oligonucleotides are 3 to 15 nucleotides long. In a further embodiment of the invention the segments are 5 to 10 nucleotides long. In a further embodiment of the invention the segments are 5 to 8 nucleotides long. In a further embodiment of the invention the segments are 5, 6, 7 or 8 nucleotides long. In a particular embodiment there are three segment oligonucleotides: a 5' segment that is 7 nucleotides long, a central segment that is 6 nucleotides long and a 3' segment that is 7 nucleotides long, which when ligated together form an oligonucleotide that is 20 nucleotides long (a "20-mer"). In a particular embodiment there are three segment oligonucleotides: a 5' segment that is 6 nucleotides long, a central segment that is 8 nucleotides long and a 3' segment that is 6 nucleotides long, which when ligated together form an oligonucleotide that is 20 nucleotides long (a "20-mer"). In a particular embodiment there are three segment oligonucleotides: a 5' segment that is 5 nucleotides long, a central segment that is 10 nucleotides long and a 3' segment that is 5 nucleotides long, which when ligated together form an oligonucleotide that is 20 nucleotides long (a "20-mer"). In a particular embodiment there are four segment oligonucleotides: a 5' segment that is 5 nucleotides long, a 5'-central segment that is 5 nucleotides long, a central-3' segment that is 5 nucleotides long, and a 3' segment that is 5 nucleotides long, which when ligated together form an oligonucleotide that is 20 nucleotides long (a "20-mer").

[0033] One embodiment of the invention provides a process as previously described herein, whereby the product is 10 to 200 nucleotides long. In a further embodiment of the invention the product is 15 to 30 nucleotides long. In an embodiment of the invention the product is 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides long. In an embodiment of the invention the product is 20 nucleotides long, a "20-mer". In an embodiment of the invention the product is 21 nucleotides long, a "21-mer". In an embodiment of the invention the product is 22 nucleotides long, a "22-mer". In an embodiment of the invention the product is 23 nucleotides long, a "23-mer". In an embodiment of the invention the product is 24 nucleotides long, a "24-mer". In an

embodiment of the invention the product is 25 nucleotides long, a "25-mer". In an embodiment of the invention the product is 26 nucleotides long, a "26-mer". In an embodiment of the invention the product is 27 nucleotides long, a "27-mer". In an embodiment of the invention the product is 28 nucleotides long, a "28-mer". In an embodiment of the invention the product is 29 nucleotides long, a "29-mer". In an embodiment of the invention the product is 30 nucleotides long, a "30-mer".

[0034] In an embodiment of the invention, the process is a process for producing a therapeutic oligonucleotide. In an embodiment of the invention, the process is a process for producing a single stranded therapeutic oligonucleotide. In an embodiment of the invention, the process is a process for producing a double stranded therapeutic oligonucleotide.

[0035] Another embodiment of the invention provides a process as previously disclosed herein, wherein the property that allows the template to be separated from the product is that the template is attached to a support material. In a further embodiment of the invention, the support material is a soluble support material. In a yet further embodiment of the invention the soluble support material is selected from the group consisting of: polyethylene glycol, a soluble organic polymer, DNA, a protein, a dendrimer, a polysaccharide, an oligosaccharide, and a carbohydrate. In an embodiment of the invention the support material is polyethylene glycol (PEG). In a further embodiment of the invention, the support material is an insoluble support material. In a further embodiment of the invention the support material is a solid support material. In a yet further embodiment, the solid support material is selected from the group consisting of: a glass bead, a polymeric bead, a fibrous support, a membrane, a streptavidin coated bead and cellulose. In an embodiment the solid support material is a streptavidin coated bead. In a further embodiment, the solid support material is part of the reaction vessel itself, e.g. a reaction wall.

[0036] One embodiment of the invention provides a process as previously disclosed herein, wherein multiple, repeated copies of the template are attached in a continuous manner via a single attachment point to the support material. The multiple repeated copies of the template may be separated by a linker, e.g. as shown in Figure 3. The multiple repeated copies of the template may be direct repeats, e.g. they are not separated by a linker.

[0037] Yet another embodiment of the invention provides a process as previously disclosed herein, wherein the property that allows the template to be separated from the product is the molecular weight of the template. For example, repeated copies of the template sequence may be present on a single oligonucleotide, with or without a linker sequence.

[0038] Another embodiment of the invention provides a process as previously disclosed herein, wherein the template, or the template and support material, are recycled for use in future reactions, for example as detailed below. Another embodiment of the invention provides a process as previously disclosed herein, wherein the reaction is carried out using a continuous or semi-continuous flow process, for example as shown in Figure 4 or Figure 5.

[0039] As mentioned here-above, the process is for large scale manufacture of oligonucleotides, in particular therapeutic oligonucleotides. In the context of the present invention large scale manufacture of oligonucleotides means manufacture at a scale greater than or equal to 1 litre, e.g. the process is carried out in a 1 L or larger reactor. Alternatively or in addition, in the context of the present invention large scale manufacture of oligonucleotides means manufacture at gram scale of product, in particular the production of greater than or equal to 10 grams of product. In an embodiment of the invention, the amount of oligonucleotide product produced is at gram scale. In an embodiment of the invention the amount of product produced is greater than or equal to: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100 grams. In an embodiment of the invention, the amount of oligonucleotide product produced is greater than or equal to: 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950 grams. In an embodiment of the invention, the amount of oligonucleotide product produced is 500 grams or greater. In an embodiment of the invention, the oligonucleotide product produced is at kilogram scale. In an embodiment of the invention, the amount of oligonucleotide product produced is 1 kg or more. In an embodiment of the invention, the amount of oligonucleotide product produced is greater than or equal to: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 kg. In an embodiment of the invention, the amount of oligonucleotide product produced is greater than or equal to: 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 kg.

[0040] In an embodiment of the invention, the amount of product produced is between 10 grams and 100 kg. In an embodiment of the invention, the amount of product produced is between 10 grams and 50 kg. In an embodiment of the invention, the amount of product produced is between 100 grams and 100 kg. In an embodiment of the invention, the amount of product produced is between 100 grams and 50 kg. In an embodiment of the invention, the amount of product produced is between 500 grams and 100 kg. In an embodiment of the invention, the amount of product produced is between 500 grams and 50 kg. In an embodiment of the invention, the amount of product produced is between 1 kg and 50 kg. In an embodiment of the invention, the amount of product produced is between 10 kg and 50 kg.

[0041] In an embodiment of the invention, oligonucleotide manufacture takes place at a scale greater than or equal to: 2, 3, 4, 5, 6, 7, 8, 9, 10 litres, e.g. in a 2, 3, 4, 5, 6, 7, 8, 9 or 10 L reactor. In an embodiment of the invention, oligonucleotide

manufacture takes place at a scale greater than or equal to: 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 70, 75, 80, 85, 90, 95, 100 litres, e.g. in a 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 70, 75, 80, 85, 90, 95, 100 L reactor. In an embodiment of the invention, oligonucleotide manufacture takes place at a scale greater than or equal to: 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 litres, e.g. in 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 L reactor.

[0042] In an embodiment of the invention, the reactor volume is about 10,000 L, about 5000 L, about 2000 L, about 1000 L, about 500 L, about 125 L, about 50 L, about 20 L, about 10 L, or about 5 L.

[0043] In an embodiment of the invention, the reactor volume is between 5 and 10,000 L, between 10 and 5000 L, between 20 and 2000 L, or between 50 and 1000 L.

[0044] An oligonucleotide in accordance with the present invention has at least one backbone modification, and/or at least one sugar modification and/or at least one base modification compared to an RNA or DNA-based oligonucleotide.

[0045] The invention provides a process as previously disclosed herein, wherein the product contains at least 1 modified nucleotide residue. In a further embodiment the modification is at the 2' position of the sugar moiety.

[0046] Oligonucleotides used in the process of the invention may include sugar modifications, *i.e.* a modified version of the ribosyl moiety, such as 2'-O-modified RNA such as 2'-O-alkyl or 2'-O-(substituted)alkyl *e.g.* 2'-O-methyl, 2'-O-(2-cyanoethyl), 2'-O-(2-methoxy)ethyl (2'-MOE), 2'-O-(2-thiomethyl)ethyl, 2'-O-butyryl, 2'-O-propargyl, 2'-O-allyl, 2'-O-(3-amino)propyl, 2'-O-(3-(dimethylamino)propyl), 2'-O-(2-amino)ethyl, 2'-O-(2-(dimethylamino)ethyl); 2'-deoxy (DNA); 2'-O-(haloalkoxy)methyl (Arai K. et al. Bioorg. Med. Chem. 2011, 21, 6285) *e.g.* 2'-O-(2-chloroethoxy)methyl (MCEM), 2'-O-(2, 2-dichloroethoxy)methyl (DCEM); 2'-O-alkoxycarbonyl *e.g.* 2'-O-[2-(methoxycarbonyl)ethyl] (MOCE), 2'-O-[2-(N-methylcarbamoyl)ethyl] (MCE), 2'-O-[2-(N,N-dimethylcarbamoyl)ethyl] (DCME); 2'-halo *e.g.* 2'-F, FANA (2'-F arabinosyl nucleic acid); carbasugar and azasugar modifications; 3'-O-alkyl *e.g.* 3'-O-methyl, 3'-O-butyryl, 3'-O-propargyl; and their derivatives.

[0047] In an embodiment of the invention, the sugar modification is selected from the group consisting of 2'-Fluoro (2'-F), 2'-O-methyl (2'-OMe), 2'-O-methoxyethyl (2'-MOE), and 2'-amino. In a yet further embodiment, the modification is 2'-MOE.

[0048] Other sugar modifications include "bridged" or "bicyclic" nucleic acid (BNA), *e.g.* locked nucleic acid (LNA), xylo-LNA, α -L-LNA, β -D-LNA, cEt (2'-O,4'-C constrained ethyl) LNA, cMOEt (2'-O,4'-C constrained methoxyethyl) LNA, ethylene-bridged nucleic acid (ENA), tricyclo DNA; unlocked nucleic acid (UNA); cyclohexenyl nucleic acid (CeNA), altrial nucleic acid (ANA), hexitol nucleic acid (HNA), fluorinated HNA (F-HNA), pyranosyl-RNA (p-RNA), 3'-deoxypyranosyl-DNA (p-DNA); morpholino (as *e.g.* in PMO, PPMO, PMOPlus, PMO-X); and their derivatives.

[0049] Oligonucleotides used in the process of the invention may include other modifications, such as peptide-base nucleic acid (PNA), boron modified PNA, pyrrolidine-based oxy-peptide nucleic acid (POPNA), glycol- or glycerol-based nucleic acid (GNA), threose-based nucleic acid (TNA), acyclic threoninol-based nucleic acid (aTNA), oligonucleotides with integrated bases and backbones (ONIBs), pyrrolidine-amide oligonucleotides (POMs); and their derivatives.

[0050] In an embodiment of the invention, the modified oligonucleotide comprises a phosphorodiamidate morpholino oligomer (PMO), a locked nucleic acid (LNA), a peptide nucleic acid (PNA), a bridged nucleic acid (BNA) such as (S)-cEt-BNA, or a SPIEGELMER.

[0051] In a further embodiment, the modification is in the nucleobase. Base modifications include modified versions of the natural purine and pyrimidine bases (*e.g.* adenine, uracil, guanine, cytosine, and thymine), such as inosine, hypoxanthine, orotic acid, agmatidine, lysidine, 2-thiopyrimidine (*e.g.* 2-thiouracil, 2-thiothymine), G-clamp and its derivatives, 5-substituted pyrimidine (*e.g.* 5-methylcytosine, 5-methyluracil, 5-halouracil, 5-propynyluracil, 5-propynylcytosine, 5-aminomethyluracil, 5-hydroxymethyluracil, 5-aminomethylcytosine, 5-hydroxymethylcytosine, Super T), 2,6-diaminopurine, 7-deazaguanine, 7-deazaadenine, 7-aza-2, 6-diaminopurine, 8-aza-7-deazaguanine, 8-aza-7-deazaadenine, 8-aza-7-deaza-2, 6-diaminopurine, Super G, Super A, and N4-ethylcytosine, or derivatives thereof; N²-cyclopentylguanine (cPent-G), N²-cyclopentyl-2-aminopurine (cPent-AP), and N²-propyl-2-aminopurine (Pr-AP), or derivatives thereof; and degenerate or universal bases, like 2, 6-difluorotoluene or absent bases like abasic sites (*e.g.* 1-deoxyribose, 1,2-dideoxyribose, 1-deoxy-2-O-methylribose; or pyrrolidine derivatives in which the ring oxygen has been replaced with nitrogen (azaribose)). Examples of derivatives of Super A, Super G and Super T can be found in US6683173. cPent-G, cPent-AP and Pr-AP were shown to reduce immunostimulatory effects when incorporated in siRNA (Peacock H. et al. J. Am. Chem. Soc. (2011), 133, 9200).

[0052] In an embodiment of the invention, the nucleobase modification is selected from the group consisting of 5-methyl

pyrimidines, 7-deazaguanosines and abasic nucleotides. In an embodiment, the modification is a 5-methyl cytosine.

[0053] Oligonucleotides used in the process of this invention may include a backbone modification, e.g. a modified version of the phosphodiester present in RNA, such as phosphorothioate (PS), phosphorodithioate (PS2), phosphonoacetate (PACE), phosphonoacetamide (PACA), thiophosphonoacetate, thiophosphonoacetamide, phosphorothioate prodrug, H-phosphonate, methyl phosphonate, methyl phosphonothioate, methyl phosphate, methyl phosphorothioate, ethyl phosphate, ethyl phosphorothioate, boranophosphate, boranophosphorothioate, methyl boranophosphate, methyl boranophosphorothioate, methyl boranophosphonate, methylboranophosphonothioate, and their derivatives. Another modification includes phosphoramidite, phosphoramidate, N3'→P5' phosphoramidate, phosphordiamidate, phosphorothiodiamidate, sulfamate, dimethylenesulfoxide, sulfonate, triazole, oxalyl, carbamate, methyleneimino (MMI), and thioacetamido nucleic acid (TANA); and their derivatives.

[0054] In a further embodiment, the modification is in the backbone and is selected from the group consisting of: phosphorothioate (PS), phosphoramidate (PA) and phosphordiamidate. In an embodiment of the invention, the modified oligonucleotide is a phosphordiamidate morpholino oligomer (PMO). A PMO has a backbone of methylenemorpholine rings with phosphordiamidate linkages. In an embodiment of the invention the product has a phosphorothioate (PS) backbone.

[0055] In an embodiment of the invention, the oligonucleotide comprises a combination of two or more modifications as disclosed above. A person skilled in the art will appreciate that there are many synthetic derivatives of oligonucleotides.

[0056] In an embodiment of the invention, the product is a gapmer. In an embodiment of the invention the 5' and 3' wings of the gapmer comprise or consist of 2'-MOE modified nucleotides. In an embodiment of the invention the gap segment of the gapmer comprises or consists of nucleotides containing hydrogen at the 2' position of the sugar moiety, *i.e.* is DNA-like. In an embodiment of the invention the 5' and 3' wings of the gapmer consist of 2'MOE modified nucleotides and the gap segment of the gapmer consists of nucleotides containing hydrogen at the 2' position of the sugar moiety (*i.e.* deoxynucleotides). In an embodiment of the invention the 5' and 3' wings of the gapmer consist of 2'MOE modified nucleotides and the gap segment of the gapmer consists of nucleotides containing hydrogen at the 2' position of the sugar moiety (*i.e.* deoxynucleotides) and the linkages between all of the nucleotides are phosphorothioate linkages.

[0057] One embodiment of the invention provides a process as previously described herein, wherein the resulting product is greater than 90% pure. In a further embodiment, the product is greater than 95% pure. In a further embodiment, the product is greater than 96% pure. In a further embodiment, the product is greater than 97% pure. In a further embodiment, the product is greater than 98% pure. In a further embodiment, the product is greater than 99% pure. Purity of an oligonucleotide may be determined using any suitable method, e.g. high performance liquid chromatography (HPLC) or mass spectrometry (MS), in particular liquid chromatography-MS (LC-MS), HPLC-MS or capillary electrophoresis mass spectrometry (CEMS).

[0058] Yet another embodiment of the invention provides a process for producing double stranded oligonucleotides, wherein 2 complimentary single stranded oligonucleotides are produced by the method of any preceding embodiment and then mixed under conditions to allow annealing. In an embodiment, the product is a siRNA.

[0059] One embodiment of the invention provides an oligonucleotide produced by a process previously described herein. In an embodiment of the invention, the oligonucleotide produced is RNA. In an embodiment of the invention, the oligonucleotide produced is DNA. In an embodiment of the invention, the oligonucleotide produced comprises both RNA and DNA. In a further embodiment of the invention the oligonucleotide produced is a modified oligonucleotide. In an embodiment of the invention the oligonucleotide produced is an antisense oligonucleotide. In an embodiment of the invention the oligonucleotide produced is an aptamer. In an embodiment of the invention the oligonucleotide produced is a miRNA. In an embodiment of the invention, the product is a therapeutic oligonucleotide.

[0060] The invention herein disclosed utilises the properties of oligonucleotide binding to provide an improved process for their production. By providing a template oligonucleotide with 100% complementarity to the target sequence, and controlling the reaction conditions so that the product can be released and separated under specific conditions, a product with a high degree of purity can be obtained.

Releasing the product (or impurity) from the template, i.e. denaturing product (or impurity): template duplex, and separating the product (or impurity)

[0061] Releasing the product (or where the process includes an additional step of impurity release, any impurities) from the template requires the Watson-Crick base pairing between the template oligonucleotide strand and the product (or impurity) to be

broken. The product (or impurity) can then be separated from the template. This can occur as two separate steps, or as one combined step.

[0062] Releasing and separating the product (or impurity) can occur as one step, if the process is carried out in a column reactor. Running in a buffer that alters the pH or salt concentration, or contains a chemical agent that disrupts the base pairing (such as formamide or urea) will cause denaturation of the oligonucleotide strands, and the product (or impurity) will be eluted in the buffer.

[0063] When the process is carried out in other reaction vessels, the release and separation of the product (or impurity) can occur as a two-step process. First, the Watson-Crick base pairs are disrupted to separate the strands, and then the product (or impurity) is removed from the reaction vessel. When releasing and separating the product is carried out as a two-step process, the breaking of the Watson-Crick base pairs can be achieved by altering the buffer conditions (pH, salt) or introducing a chemical disrupting agent (formamide, urea). Alternatively, raising the temperature will also cause the dissociation of the two strands. The product (or impurities) can then be removed from the reaction vessel via methods including molecular weight based separation, charge based separation, hydrophobicity based separation, specific sequence based separation or a combination of these methods.

[0064] When the process is carried out in a continuous or semi-continuous flow reactor the release and separation of the product (or impurity) can be in either one step or two steps. For example, releasing and separating the product (or impurity) in one step could be effected by increasing the temperature to cause dissociation of the two strands and separating the released strands on the basis of molecular weight in the same part of the reactor that is used to elevate the temperature. Releasing and separating the product (or impurity) in two steps could be effected by increasing the temperature to cause dissociation of the two strands in one part of the reactor and separating the released strands on the basis of molecular weight in a different part of the reactor.

Specifically releasing and separating impurities from the template, but retaining the product on the template

[0065] Impurities arise when an incorrect nucleotide is incorporated into the oligonucleotide strand during chain extension, or when the chain extension reaction terminates early. Impurities also arise when the reaction includes the step of ligating segment oligonucleotides and one or more of the ligation steps fail to happen. The kinds of impurities which can arise are illustrated in Figure 6.

[0066] The properties of Watson-Crick base pairing can be exploited to specifically release any impurities bound to the template prior to the release of the product. Each double stranded oligonucleotide will dissociate under specific conditions, and those conditions are different for sequences which do not have 100% complementarity when compared to sequences with 100% complementarity. Determining such conditions is within the remit of a skilled person.

[0067] A common way of denaturing oligonucleotides is by raising the temperature. The temperature at which half of the base pairs are dissociated, *i.e.* when 50% of the duplex is in the single-stranded state, is called the melting temperature, T_m . The most reliable and accurate means of determining the melting temperature is empirically. However, this is cumbersome and not usually necessary. Several formulas can be used to calculate T_m values (Nucleic Acids Research 1987, 15 (13): 5069-5083; PNAS 1986, 83 (11): 3746-3750; Biopolymers 1997, 44 (3): 217-239) and numerous melting temperature calculators can be found on-line, hosted by reagent suppliers and universities. It is known that for a given oligonucleotide sequence, a variant with all phosphorothioate linkages will melt at a lower temperature than a variant with all phosphodiester linkages. Increasing the number of phosphorothioate linkages in an oligonucleotide tends to lower the T_m of the oligonucleotide for its intended target.

[0068] To specifically separate the impurities from a reaction mixture, first the melting temperature of the product:template duplex is calculated. Then the reaction vessel is heated to a first temperature, *e.g.* a temperature below the melting temperature of the product:template duplex, for example 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 degrees centigrade below the melting temperature. This will cause the denaturing of oligonucleotides which are not the product, *i.e.* are not 100% complimentary to the template, from the template. These can then be removed from the reaction vessel using one of the methods disclosed above, *e.g.* molecular weight based separation, charge based separation, hydrophobicity based separation, specific sequence based separation or a combination of these methods. Then the reaction vessel will be raised to a second, higher, temperature, *e.g.* above the calculated melting temperature, for example 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 degrees centigrade above the melting temperature, to cause the denaturing of the product from the template. The product can then be removed from the reaction vessel using one of the methods disclosed above, *e.g.* molecular weight based separation, charge based separation, hydrophobicity-based separation, specific sequence based separation or a combination of these methods.

[0069] A similar process can be used when the disrupting agent is an agent which causes a change in pH, salt concentration or a chemical disrupting agent. The disrupting agent is increased in concentration until just below the concentration at which the product would dissociate, to cause the denaturing of oligonucleotides which are not the product from the template. These impurities can then be removed from the reaction vessel using one of the methods disclosed above. The disrupting agent is then increased in concentration to above the concentration at which the product dissociates from the template. The product can then be removed from the reaction vessel using one of the methods disclosed above.

[0070] The product obtained from a process such as disclosed above has a high degree of purity without the need for further purification steps. For example, the product obtained is greater than 95% pure.

Properties of the template

[0071] The template requires a property which allows it to be retained in the reaction vessel when the product is removed, to prevent it from becoming an impurity in the product. In other words, the template has properties that allow it to be separated from the product. In one embodiment of the invention, this retention is achieved by coupling the template to a supporting material. This coupling results in a template-support complex which has a high molecular weight, and can therefore be retained in the reaction vessel when impurities and product are removed, for example by filtration. The template can be coupled to a solid support material such as polymeric beads, fibrous supports, membranes, streptavidin coated beads and cellulose. The template can also be coupled to a soluble support material such as polyethylene glycol, a soluble organic polymer, DNA, a protein, a dendrimer, a polysaccharide, an oligosaccharide and a carbohydrate.

[0072] Each support material can have multiple points where a template can be attached, and each attachment point can have multiple templates attached, e.g. in the manner shown in Figure 3.

[0073] The template may have a high molecular weight itself, without being attached to a support material, for example, it may be a molecule with multiple copies of the template, e.g. separated by a linker, in the manner shown in Figure 3.

[0074] The ability to retain the template in the reaction vessel also allows the template to be recycled for future reactions, either by being recovered or by use in a continuous or semi-continuous flow process.

Methods of separating the template from the product (or impurities)

[0075] The properties of the template, as disclosed above, allow separation of the template and product, or separation of the template bound product and impurities. Molecular weight based separation, charge based separation, hydrophobicity based separation, specific sequence based separation or a combination of these methods can be used.

[0076] In the case where the template is attached to a solid support, separation of the template from the product or separation of impurities from the product bound to the template is achieved by washing the solid support under appropriate conditions. In cases where the template is coupled to a soluble support or is itself composed of repeating template sequences, separation of template from product or separation of template bound product from impurities can be achieved by means of a molecular weight based separation. This can be achieved by using techniques such as ultra-filtration or nano-filtration where the filter material is chosen so that the larger molecule is retained by the filter and the smaller molecule passes through. In cases where a single separation step of impurity from template product complex or separation of product from template is not efficient enough, multiple sequential filtration steps can be employed to increase separation efficiency and so generate a product that meets the desired purity.

[0077] It is desirable to provide a process for separation of such oligonucleotides which is efficient and applicable on an industrial production scale. "Therapeutic oligonucleotides: The state of the art in purification technologies" Sanghvi et. al. Current Opinion in Drug Discovery (2004) Vol. 7 No. 8 reviews processes used for oligonucleotide purification.

[0078] WO-A-01/55160 discloses purification of oligonucleotides by forming imine linkages with contaminants then removing the imine-linked impurities with chromatography or other techniques. "Size Fractionation of DNA Fragments Ranging from 20 to 30000 Base Pairs by Liquid/Liquid chromatography" Muller et al. Eur. J. Biochem (1982) 128-238 discloses use of a solid column of microcrystalline cellulose on which has been deposited a PEG/dextran phase for separation of nucleotide sequences. "Separation and identification of oligonucleotides by hydrophilic interaction chromatography." Easter et. al. The Analyst (2010); 135(10) discloses separation of oligonucleotides using a variant of HPLC employing a solid silica support phase. "Fractionation of oligonucleotides of yeast soluble ribonucleic acids by countercurrent distribution" Doctor et al. Biochemistry (1965) 4(1) 49-54

discloses use of a dry solid column packed with dry DEAE-cellulose. "Oligonucleotide composition of a yeast lysine transfer ribonucleic acid" Madison et al; Biochemistry, 1974, 13(3) discloses use of solid phase chromatography for separation of nucleotide sequences.

[0079] Liquid-liquid chromatography is a known separation method. "Countercurrent Chromatography The Support-Free Liquid Stationary Phase" Billardello, B.; Berthod, A; Wilson & Wilson's Comprehensive Analytical Chemistry 38; Berthod, A., Ed.; Elsevier Science B.V.: Amsterdam (2002) pp 177-200 provides a useful general description of liquid-liquid chromatography. Various liquid-liquid chromatography techniques are known. One technique is liquid-liquid counter current chromatography (termed herein "CCC"). Another known technique is centrifugal partition chromatography (termed herein "CPC").

[0080] The above disclosed methods and those methods set out in WO 2013/030263 may be used to separate a product oligonucleotide e.g. from the template and/or an impurity.

Oligonucleotides used as starting materials

[0081] The oligonucleotides used as a starting material for the processes of the invention are herein described as being a "pool" and a definition thereof is provided above. The pool is a non-homogenous set of oligonucleotides. The oligonucleotides which form the pool will have been produced by other oligonucleotide production methods, and will therefore likely contain a high degree of impurities. Therefore when this pool of oligonucleotides is applied to a process of the invention, the ability to specifically remove impurities as described herein results in a purification step occurring.

[0082] The pool can contain oligonucleotides which are intended to be the same length as the template oligonucleotide (although will contain impurities of differing lengths, as well as incorrectly incorporated residues). The pool can also be composed of segments of the product oligonucleotides, which are joined together whilst assembled on the template. Each segment will be a nonhomogeneous set with impurities of differing lengths and incorrectly incorporated residues.

Ligases

[0083] In the process of the invention presently claimed, a ligase is used. In an embodiment of the invention, the ligase is an ATP dependent ligase. ATP dependent ligases range in size from 30 to >100kDa. In an embodiment of the invention, the ligase is an NAD dependent ligase. NAD dependent enzymes are highly homologous and are monomeric proteins of 70-80 kDa. In an embodiment of the invention, the ligase is a thermostable ligase. A thermostable ligase may be derived from a thermophilic bacteria.

[0084] In an embodiment of the invention, the ligase is a modified ligase. For example, a modified ligase includes a modified T4 DNA ligase, a modified Enterobacteria phage CC31 ligase, a modified Shigella phage Shf125875 ligase and a modified Chlorella ligase.

[0085] In an embodiment, wild-type T4 DNA ligase is modified at amino acid position 368 or amino acid position 371 of SEQ ID NO:3.

[0086] In an embodiment, the mutant ligase comprises or consists of SEQ ID NO:3 wherein the amino acid at position 368 is R or K.

[0087] In an embodiment, the mutant ligase comprises or consists of SEQ ID NO:3 wherein the amino acid at position 371 is any one of the following amino acids: L, K, Q, V, P, R.

[0088] In an embodiment, the corresponding residue(s) disclosed above in relation to T4 DNA ligase are mutated in any one of Enterobacteria phage CC31 ligase, Shigella phage Shf125875 ligase and Chlorella ligase. Conserved regions of DNA ligases are disclosed in Chem. Rev. 2006, 106, 687-699 and Nucleic Acids Research, 2000, Vol. 28, No. 21, 4051-4058. In an embodiment, the ligase is modified in a linker region.

[0089] In an embodiment of the invention, the ligase comprises or consists of SEQ ID NO: 23 or a ligase with at least 90% sequence identity thereto, excluding a wild type ligase e.g. Enterobacteria phage CC31 ligase.

[0090] In an embodiment of the invention, the ligase comprises or consists of any one of the following amino acid sequences: SEQ ID NOs: 10-28.

[0091] In an embodiment of the invention, the ligase is immobilised, e.g. on a bead.

[0092] In an aspect of the invention there is provided the use of a ligase comprising the amino acid sequence as set forth in SEQ ID NO:6 or SEQ ID NO:8 for ligating a 5' segment, containing one or more modified sugar moieties, to a 3' segment, wherein all of the sugar moieties within the 3' segment are unmodified. In an embodiment of the invention there is provided the use of a ligase comprising the amino acid sequence as set forth in SEQ ID NO:6 or SEQ ID NO:8 for ligating a 5' segment, containing one or more sugar moieties with a 2'-OMe modification, to a 3' segment, wherein all of the sugar moieties within the 3' segment are unmodified. In an embodiment, all of the sugar moieties in the 5' segment contain a 2'-OMe modification. In an embodiment, the 5' segment contains 5 sugar moieties with a 2'-OMe modification.

EXAMPLES

Abbreviations

[0093]

- OMe
O-Methyl
- MOE
O-Methoxyethyl (DNA backbone) or Methoxyethyl (RNA backbone)
- CBD
Cellulose Binding Domain
- HPLC
high performance liquid chromatography
- PBS
phosphate buffered saline
- HAA
Hexylammonium acetate
- SDS PAGE
sodium dodecyl sulphate polyacrylamide gel electrophoresis
- LCMS
liquid chromatography mass spectrometry
- PO
phosphodiester
- DTT
dithiothreitol

Example 1: Oligonucleotide (DNA) segment assembly and ligation with wild-type T4 DNA ligase

1.1 Chemical synthesis of starting and control sequences

[0094] In order to demonstrate that multiple short oligonucleotides ("segments") could be assembled in the correct order on a complementary template strand and ligated to give the desired final product ("target"), the segments, target and template sequences, as detailed in Table 1, were chemically synthesised using standard methods.

1.2 HPLC analysis

[0095] HPLC analysis was carried out using an Agilent ZORBAX Eclipse Plus XDB-C18 column (4.6 x 150 mm, 5 µm dp. Agilent P/N 993967-902) running at 0.2 ml/min while absorbance was monitored at 258 nm. The column was maintained at 60 °C. 20 µl of sample was injected and a gradient from 20-31% buffer B was run over 20 minutes before being stepped up to 80% buffer B for 5 minutes.

Buffer A: 75 ml 1 M HAA, 300 ml isopropyl alcohol, 200 ml acetonitrile, 4425 ml water

Buffer B: 650 ml isopropyl alcohol, 350 ml acetonitrile

Table 1

Name	Sequence	% HPLC purity	Amount (mg)
5' segment	5'-GGC CAA-3'	100.0	21.6
centre segment	5'-(p)ACC TCG GC-3'	96.9	58.1
3' segment	5'-(p)T TAC CT-3'	98.8	39.8
Target	5'-GGC CAA ACC TCG GCT TAC CT-3' (SEQ ID NO:1)	98.4	101.7
Biotinylated template	5'-biotin TT TAG GTA AGC CGA GGT TTG GCC-3' (SEQ ID NO:2)	96.9	130.7

1.3 Oligonucleotide assembly and ligation method with commercial T4 DNA ligase (SEQ ID NO:3)

[0096] The 5' segment, centre segment and 3' segment were assembled on the template: each segment and the template was dissolved in water at a concentration of 1 mg/ml and then mixed as follows.

5' segment	2 µl
centre segment	2 µl
3' segment	2 µl
biotinylated template	2 µl
H2O	36 µl

[0097] The combined oligonucleotide solution was incubated at 94 °C for 5 minutes and cooled to 37 °C before incubating at 37 °C for a further 5 minutes to allow the segments to anneal to the template. 2 µl (equivalent to 2 µg) T4 DNA ligase (NEB) and 4 µl of 1 x T4 DNA Ligation Buffer (NEB) were then added and the reaction (total reaction volume 50 µl) was incubated at room temperature for one hour. Following this, 40 µl of streptavidin coated magnetic beads were added and the suspension incubated at room temperature for 10 minutes to allow the biotinylated template to bind to the streptavidin beads. The streptavidin beads were washed with 2 x 100 µl PBS to remove unbound segments. The wash was analysed by HPLC. The reaction mixture was then incubated at 94 °C for 10 minutes to separate the bound ligation products (or any bound segments) from the template before being rapidly cooled on ice to 'melt' the DNA and stop reannealing of the oligonucleotides products (or segments) to the template. Analysis of the ligation reaction was then carried out by HPLC.

1.4 Oligonucleotide assembly and ligation method with in-house T4 DNA ligase bead slurry

1.4.1 Bead slurry generation

[0098] T4 ligase (SEQ ID NO:4) fused at the N-terminal to a cellulose binding domain (CBD) was produced using standard cloning, expression and extraction methods. This T4 ligase amino acid sequence differs from the commercial T4 ligase sequence (SEQ ID NO:3) in that the N-terminal methionine (M) has been replaced with glycine and serine (GS). This was done to aid the generation and expression of the CBD fusion protein. CBD-T4 ligase fusion protein was expressed in BL21 A1 cells (INVITROGEN). Supernatent was harvested and was added to 600 µl of PERLOZA 100 (PERLOZA) beads and shaken at 26 °C for 1 hour. The PERLOZA cellulose beads were then collected and washed with 2 ml buffer (50 mM Tris pH 8.0, 200 mM NaCl, 0.1% Tween 20, 10% Glycerol) followed by 5 ml PBS and were finally re-suspended in 200 µl PBS (10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl pH 7.4). In order to analyse protein expression, 15 µl of the PERLOZA bead slurry was mixed with 5 µl of SDS loading buffer and incubated at 80 °C for 10 minutes before being run on a SDS PAGE gradient gel (4 - 20%) according to a standard protocol.

1.4.2 Oligonucleotide assembly and ligation using bead slurry

[0099] For T4 ligase bound to PERLOZA beads, the assembly and ligation method in 1.3 above was modified as follows. In the initial segment mixture, 36 µl of H₂O was reduced to 8 µl H₂O. After annealing, 2 µl of commercial T4 DNA ligase was replaced by 20 µl of PERLOZA bead slurry. Prior to adding the streptavidin magnetic beads, the PERLOZA beads were spun down and the supernatant removed. The streptavidin magnetic beads were added to the supernatant and incubated at room temperature for 10 minutes to allow the biotinylated template to bind to the streptavidin beads.

1.5 Results and conclusions

[0100] Product, template and all three segment oligonucleotides were clearly resolved in the control chromatogram.

[0101] HPLC analysis of the ligase reactions showed that some unligated oligonucleotide segments remained, but commercial T4 DNA ligase (NEB) was able to catalyse ligation of the three segments to generate the desired product oligonucleotide (Figure 7). The PERLOZA bead bound T4 DNA ligase appeared to be less efficient at ligation of the oligonucleotide segments, with oligonucleotide segments appearing in both the control wash sample and the reaction sample (Figure 8). However, it is difficult to be sure whether the same amount of enzyme was added on the beads compared to the commercial enzyme and so a direct comparison of ligation efficiency was not possible.

Example 2: 2'-OMe ribose modified oligonucleotide segment assembly and ligation with wild-type T4 DNA ligase

2.1 2' OMe at each nucleotide position in every segment

[0102] In order to determine whether T4 DNA ligase was able to ligate oligonucleotide segments with modification at the 2' position of the ribose ring, oligonucleotide segments were synthesized with the same sequence as for Example 1, but the 2' position of the ribose ring was substituted with an OMe group and thymidine was replaced by uridine as shown below.

Table 2

Name	Sequence	% HPLC purity	Amount (mg)
5' segment 2'-OMe	5'-GGC CAA-3'	21	97.8
centre segment 2'-OMe	5'-(p)ACC UCG GC-3'	15.5	97.7
3' segment 2'-OMe	5'-(p)U UAC CU-3'	21.2	98.1
Target 2'-OMe	5'-GGC CAA ACC UCG GCU UAC CU-3' (SEQ ID NO:5)	88	96.9
(p) = phosphate			

[0103] Assembly, ligation and HPLC analysis were carried out using the methods of Example 1, with both commercial NEB ligase and T4 ligase CBD fusion bound to PERLOZA beads. The amount of water used in the reaction mix for the commercial T4 DNA ligase (NEB) experiment was 26 µl rather than 36 µl so that the final reaction volume was 40 µl. The amount of water used in the reaction mix for the in-house T4 DNA ligase bead slurry experiment was 23 µl and the amount of beads used was 5 µl so that the final reaction volume was also 40 µl. Control experiments using unmodified DNA as opposed to 2'-OMe DNA were run in parallel.

[0104] The results from the control experiments were in accordance with Example 1. No product was detected using HPLC for the 2'-OMe experiments indicating that T4 DNA ligase is unable to ligate fully 2'-OMe modified oligonucleotide segments regardless of whether a commercial T4 DNA ligase or in-house T4 DNA ligase CBD fusion bound to PERLOZA beads was used.

2.2 2'-OMe at each nucleotide position in a single segment

[0105] Using a 1 mg/ml solution of each oligonucleotide the reactions as detailed in Table 3 were set up.

Table 3

Experiment 1 (No ligase control)	Experiment 2 (single 2'-OMe segment - 3')	Experiment 3 (single 2'-OMe segment - 5')	Experiment 4 (all 2'-OMe)	Experiment 5 (all unmodified)	Volume (µl)
template	template	template	template	template	2
5' segment	5' segment	2'-OMe substituted 5' segment	2'-OMe substituted 5' segment	5' segment	2
3' segment	2'-OMe substituted 3' segment	3' segment	2'-OMe substituted 3' segment	3' segment	2
Centre segment	Centre segment	Centre segment	2'-OMe substituted Centre segment	Centre segment	2
H ₂ O	H ₂ O	H ₂ O	H ₂ O	H ₂ O	up to 40 total

[0106] Assembly and ligation were carried out using the methods of Example 1 with commercial NEB ligase and in-house PERLOZA bound T4 DNA ligase.

[0107] Reactions were incubated at 94 °C for 5 minutes, followed by incubation for 5 minutes at 37 °C to allow for annealing. 4 µl of 1x NEB T4 DNA ligation buffer was added to each reaction along with 5 µl (approximately 2 µg) of in-house T4 DNA ligase or 2 µl (approximately 2 µg) commercial T4 DNA ligase (apart from Experiment 1 which was a no ligase control) and the ligation reaction was allowed to proceed for 2 hours at room temperature. Streptavidin magnetic beads were then added to each reaction and the reactions heated to 94 °C before rapid cooling on ice as described in Example 1 to separate the template from starting materials and products.

[0108] The processed reactions were split in two: half were analysed by HPLC as described for Example 1 (section 1.2). The other half of the sample was retained for mass spectrometry to confirm the HPLC results.

[0109] Ligation of unmodified oligonucleotide segments (experiment 5) proceeded as expected to produce full length product. A small amount of ligation was seen when the 5' segment was 2'-OMe substituted (Experiment 3) as shown in Figure 9, but no ligation was seen when the 3' segment was 2'-OMe substituted (Experiment 2). No significant product was seen when all three segments were 2'-OMe substituted (Experiment 4) in accordance with 2.1.

2.3 Conclusion

[0110] Wild-type T4 DNA ligase is poor at ligating 2'-OMe substituted oligonucleotide segments, but slightly less sensitive to modification of the 5' oligonucleotide segment than the 3' segment.

Example 3: 2'-OMe ribose modified oligonucleotide segment assembly and ligation with wild-type and mutant ligases

3.1 Materials

[0111] Wild-type Enterobacteria phage ligase CC31 (SEQ ID NO:6), wild-type Shigella phage Shf125875 ligase (SEQ ID NO:8), and 10 mutant T4 ligases of SEQ ID NO:10-19, each fused at the N-terminus to a CBD, were produced using standard cloning, expression and extraction methods. As disclosed in 1.4.1, in order to generate and express the CBD fusion proteins the N-terminal methionine (M) was replaced with glycine and serine (GS) in each case (e.g. SEQ ID NO:7 for Enterobacteria phage ligase CC31 and SEQ ID NO:9 for Shigella phage Shf125875 ligase).

[0112] The following oligonucleotides were synthesized by standard solid phase methods.

Table 4

Name	Sequence	% HPLC purity	Amount (mg)
5' segment *	5'-(OMe)G(OMe)G(OMe)C(OMe)C(OMe)AA-3'	99.35	24.7
centre segment	5'-(p)ACC TCG GC-3'	96.9	58.1

Name	Sequence	% HPLC purity	Amount (mg)
3'segment	5'-(p)TTA CCT-3'	97.88	29.5
Biotinylated template	5'-biotin TT TAG GTA AGC CGA GGT TTG GCC-3' (SEQ ID NO:2)	96.9	130.7
N.B. OMe indicates 2 methoxy substitution on the ribose ring (p) = phosphate * note that the first 5 nucleotides are 2'-OMe modified (GGCCA), but the final A is not			

3.2 Oligonucleotide assembly and ligation method with ligase bead slurries

3.2.1 Bead slurry generation

[0113] Ligases fused to CBD were bound to PERLOZA beads as described in 1.4 to generate a bead slurry.

3.2.2 Oligonucleotide assembly and ligation using bead slurry

[0114] Ligation reactions were prepared with the components below to a final volume of 50 µL in a 96 well plate:

2 µL	~1mg/mL 5' (2'-OMe) segment
2 µL	~1mg/mL centre segment
2 µL	~1mg/mL 3' segment
2 µL	~1mg/mL template
5 µL	NEB T4 DNA ligase buffer
22 µL	H ₂ O
15 µL	PERLOZA bound bead slurry

[0115] The reaction was incubated for 15 minutes at room temperature prior to the addition of PERLOZA bead slurry to allow segments to anneal to the template. PERLOZA bead slurry was added and the reaction incubated at room temperature for 1 hour. After the hour incubation, the solution was transferred into an ACOPREP advance 350 filter plate (PN 8082) and the filter plate was placed on top of an ABGENE superplate (Thermo Scientific, #AB-2800) and centrifuged for 10 minutes at 4,000 rpm to remove the PERLOZA bead slurry. Solutions were then analysed by HPLC using the method described in Example 1 (section 1.2).

[0116] Each oligonucleotide assembly and ligation was repeated 6 times for each ligase.

3.3 Results and conclusions

[0117] Wild-type Enterobacteria phage CC31 ligase (SEQ ID NO:6) and wild-type Shigella phage Shf125875 ligase (SEQ ID NO:8) are able to ligate a 2' OMe substituted 5' segment containing five 2'-OMe nucleobases and one deoxynucleobase to a segment containing only unmodified DNA. In addition, whilst wild-type T4 DNA ligase (SEQ ID NO:3 and 4) is poor at performing this reaction, as shown in Example 2 and reconfirmed here, a number of mutations at positions 368 and 371 confer the ability to ligate a 2'-OMe substituted 5' segment containing five 2' OMe nucleobases and one deoxynucleobase to a segment containing only unmodified DNA on the ligase (SEQ ID NO:10-19).

Example 4: 2' MOE ribose modified and 5-methyl pyrimidine modified oligonucleotide segment assembly and ligation with mutant DNA ligases

4.1 Materials

[0118] Modified oligonucleotide segments as set out in Table 5 below were synthesised by standard solid phase based methods.

Table 5

Segment	Sequence	MW	Mass (mg)	% purity
centre segment	5'-(p)dCdCdTdCdGdG-3'	2044.122	39	98.96
MOE 3'-segment	5'-(p)dCdTmTmAmCmCmT- 3'	2699.679	52	97.79
MOE 5'-segment	5'-mGmGmCmCmAdAdA-3'	2644.771	48	99.13
(p) = phosphate, mX = MOE bases, dX = DNA bases all 5-methyl pyrimidines				

[0119] Mutant DNA ligases (SEQ ID NO:20-28) based upon wild type Enterobacteria phage CC31 ligase, wild type T4 ligase and wild type Shigella phage Shf125875 ligase were each fused at the N-terminus to a cellulose binding domain (CBD), using standard cloning, expression and extraction methods. Ligases fused to CBD were bound to PERLOZA beads as described in 1.4 to generate a bead slurry. In order to release the ligases from the PERLOZA beads, 2 µl of TEV protease was added to the slurry and incubated overnight at 4 °C. The cleaved protein, now lacking the cellulose binding domain, was collected by centrifugation for 10 min at 4000 rpm.

4.2 Method

[0120] The reaction was set up as follows:

centre segment	20 µM final
MOE 3' segment	20 µM final
MOE 5' segment	20 µM final
Template	20 µM final
NEB T4 DNA ligase buffer	5 µl
mutant DNA ligase	15µl
H ₂ O	To make final reaction volume 50 µl

[0121] All components were mixed and vortexed prior to addition of DNA ligase. Reactions were incubated for 1 hour at 35 °C. After 1 hour reactions were stopped by heating at 95 °C for 5 minutes in a PCR block.

[0122] Samples were analysed by both HPLC and LCMS to confirm product identity according to the HPLC protocol used in Example 1 (section 1.2). Controls of commercial NEB T4 DNA ligase and a negative control (H₂O instead of any ligase) were included.

4.3 Results and conclusions

[0123] Figure 10 shows the HPLC traces for the control reaction and the reaction catalysed by SEQ ID NO:23 (clone A4 - mutant Enterobacteria phage CC31 ligase). The product and template co-elute using this HPLC method so product appears as an increase in the peak area of the product + template peak. In the mutant ligase trace (b) not only does the product + template peak increase but two new peaks appear at 10.3 and 11.2 minutes. These peaks correspond to the ligation of the centre segment with either the MOE 5' segment or the MOE 3' segment. Also, the input segment peaks are substantially smaller than the control in line with product and intermediate peaks increasing. The NEB commercial T4 ligase trace (a) showed a slight increase in the peak area for template + product, a small amount of intermediate ligation product along with a concomitant decrease in input oligonucleotide segments. The mutant ligase (SEQ ID NO:23), however, showed substantially greater product

+ template peak area and concomitant reduction in peak areas for input oligonucleotide segments. Thus the mutant ligase (SEQ ID NO:23) is a much more effective ligase for the 2' MOE substituted segments than commercial T4 DNA ligase. Similar improvements were shown for the other mutant ligases (SEQ ID NO:20, 21, 24-28).

Example 5: Effect of different nucleotide pairing at the ligation site

5.1 Materials

[0124] Mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) fused at the N-terminal to a cellulose binding domain (CBD) was produced using standard cloning, expression and extraction methods. Extracted CBD-mutant Enterobacteria phage CC31 ligase fusion protein was added to 25 ml of PERLOZA 100 (PERLOZA) cellulose beads and shaken at 20 °C for 1 hour. The PERLOZA beads were then collected and washed with 250 ml buffer (50 mM Tris pH 8.0, 200 mM NaCl, 0.1% Tween 20, 10% Glycerol) followed by 250 ml PBS and were finally re-suspended in 10 ml PBS (10 mM PO₄³⁻, 137 mM NaCl, 2.7 mM KCl pH 7.4). In order to analyse protein expression, 15 µl of the PERLOZA bead slurry was mixed with 5 µl of SDS loading buffer and incubated at 80 °C for 10 minutes before being run on a SDS PAGE gradient gel (4 - 20%) according to a standard protocol. For the release of the ligase from the beads, 70 µl of TEV protease was added and incubated overnight at 4 °C with shaking. Ligase was collected by washing the digested beads with 80 ml of PBS. The ligase was then concentrated down to 1.2 ml using an Amicon 30 Kd MCO filter.

[0125] The following biotinylated DNA template oligonucleotides (Table 6) and DNA segment oligonucleotides (Table 7) were synthesized by standard solid phase methods. Please note that the nucleotides in bold are the ones present at the ligation site (i.e. those nucleotides that were joined together in the ligation reaction - table 7; and those nucleotides that are complementary to those joined via the ligation reaction - table 6).

Table 6:

Template number	Sequence (nucleotides complementary to junction nucleotides are in bold)	SEQ ID NO
1	5'-biotin TTTGGTGCGAAGCAG ACT GAGGC-3'	30
2	5'-biotin TTTGGTGCGAAGCAG AGT GAGGC-3'	31
3	5'-biotin TTTGGTGCGAAGCAG ATT GAGGC-3'	32
4	5'-biotin TTTGGTGCGAAGCAG AAT GAGGC-3'	33
5	5'-biotin TTTGGTGCGAAGCAG TCT GAGGC-3'	34
6	5'-biotin TTTGGTGCGAAGCAG TGT GAGGC-3'	35
7	5'-biotin TTTGGTGCGAAGCAG TTT GAGGC-3'	36
8	5'-biotin TTTGGTGCGAAGCAG TAT GAGGC-3'	37
9	5'-biotin TTTGGTGCGAAGCAG CCT GAGGC-3'	38
10	5'-biotin TTTGGTGCGAAGCAG CGT GAGGC-3'	39
11	5'-biotin TTTGGTGCGAAGCAG CTT GAGGC-3'	40
12	5'-biotin TTTGGTGCGAAGCAG CAT GAGGC-3'	41
13	5'-biotin TTTGGTGCGAAGCAG GCT GAGGC-3'	42
14	5'-biotin TTTGGTGCGAAGCAG GGT GAGGC-3'	43
15	5'-biotin TTTGGTGCGAAGCAG GTT GAGGC-3'	44
16	5'-biotin TTTGGTGCGAAGCAG GAT GAGGC-3'	45

Table 7

Segment	Identifier	Sequence (junction nucleotides are in bold)
5'	A	5'-GCCTCAG-3'
5'	B	5'-GCCTCAC-3'
5'	C	5'-GCCTCAA-3'
5'	D	5'-GCCTCAT-3'
3'	E	5'-(p)TCTGCT-3'
3'	F	5'-(p) ACT GCT-3'
3'	G	5'-(p) CCT GCT-3'

Segment	Identifier	Sequence (junction nucleotides are in bold)
3'	H	5'-(p) GCTGCT -3'

(p) = phosphate
N.B. please note that, unlike previous examples, the ligation reactions in this example involve joining two segments together: a 5'-segment and a 3'-segment, i.e. there is no centre segment.

5.2 Method

[0126] Reactions were set up as follows:

Table 8

Template	5'-segment	3'-segment
1	A	E
2	B	E
3	C	E
4	D	E
5	A	F
6	B	F
7	C	F
8	D	F
9	A	H
10	B	H
11	C	H
12	D	H
13	A	G
14	B	G
15	C	G
16	D	G

[0127] For each 50 µL reaction:

3' segment (1mM stock, 20 µM final)	1 µl
5' segment (1mM stock, 20 µM final)	1 µl
Template (1mM stock, 20 µM final)	1 µl
NEB DNA ligase buffer (for T4 ligase)	5 µl
Mutant CC31 DNA ligase (0.45 mM stock, 90 µM final)	10 µl
H ₂ O	up to 50 µl

[0128] Each reaction mix was incubated at 35 °C both for 30 minutes and 1 hour. Each reaction was terminated by heating at 95 °C for 5 minutes. HPLC analysis was carried out.

5.3 Results and conclusions

[0129] All of the reactions produced a product peak after 1hour incubation in HPLC analysis. Accordingly, the ligation method works for all combinations of nucleotides at the junctions to be joined. Optimisation to improve product yield is possible, but was not necessary as the results were conclusive and it was clear that the reaction was working for all combinations of nucleotides at the junctions to be joined.

Example 6: Effect of different modifications at the ligation site

6.1 Materials

[0130] Mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) was produced as described in 5.1 and Chlorella virus DNA ligase (SEQ ID NO:29, commercially available as SplintR ligase, NEB) was purchased.

[0131] The following biotinylated template oligonucleotide and segment oligonucleotides (Table 9) were synthesized by standard solid phase methods.

Table 9

Name	Sequence (junction modification in bold)
Template	5'-biotin TTTAGGTAAGCCGAGGTTTGGCC-3' (SEQ ID NO:2)
5' segment (WT)	5'-GGCCAAA-3'
5' segment (Mo1)	5'-GGCCAA(OMe)A-3'
5' segment (Mo2)	5'-GGCCAA(F)A-3'
centre segment (WT)	5'-(p)CCTCGG-3'
centre segment (Mo4A)	5'-(p)(OMe)CCTCGG-3'
centre segment (Mo5A)	5'-(p)(F)CCTCGG-3'
centre segment (Mo7)	5'(p)(Me)CCTCGG- 3'
3' segment (WT)	5'-(p)CTTACCT-3'
3' segment (Mo8)	5'-(p)(Me)CTTACCT3'
OMe indicates 2' methoxy substitution on the ribose ring F indicates 2' fluoro substitution on the ribose ring All remaining sugar residues are deoxyribose residues Me indicates 5-methyl cytosines	

6.2 Method

[0132] Reactions were set up as follows:

Table 10

Reaction	5' segment	Centre segment	3' segment
1	WT	WT	WT
2	WT	Mo4A	WT
3	WT	Mo5A	WT
4	WT	Mo7	WT
5	Mo1	WT	WT
6	Mo1	Mo4A	WT
7	Mo1	Mo5A	WT
8	Mo1	Mo7	WT
9	Mo2	WT	WT
10	Mo2	Mo4A	WT
11	Mo2	Mo5A	WT
12	Mo2	Mo7	WT
13	WT	WT	Mo8
14	WT	Mo4A	Mo8
15	WT	Mo5A	Mo8
16	WT	Mo7	Mo8
17	Mo1	WT	Mo8
18	Mo1	Mo4A	Mo8

Reaction	5' segment	Centre segment	3' segment
19	Mo1	Mo5A	Mo8
20	Mo1	Mo7	Mo8
21	Mo2	WT	Mo8
22	Mo2	Mo4A	Mo8
23	Mo2	Mo5A	Mo8
24	Mo2	Mo7	Mo8

[0133] For each 50 µL reaction:

3'-segment (1mM, stock, 20 µM final)	1 µl
centre segment (1mM, stock, 20 µM final)	1 µl
5'-segment (1 mM, stock, 20 µM final)	1 µl
Template (1mM, stock, 20 µM final)	1 µl
H ₂ O	up to 50 µl

[0134] For Mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23)

DNA ligase buffer (50 mM Tris-HCl, 1 mM DTT)	5 µl
Mutant CC31 DNA ligase (0.45 mM)	10 µl
MnCl ₂ (50 mM)	5 µl
ATP (10 mM)	10 µl

[0135] Whereas for Chlorella virus DNA ligase (SEQ ID NO:29, commercially available as SplintR ligase, NEB)

NEB DNA ligase buffer (for Chlorella)	5 µl
Chlorella virus DNA ligase	2 µl

[0136] Each reaction mix was incubated at 20 °C 1 hour. Each reaction was terminated by heating at 95 °C for 10 minutes. HPLC analysis was carried out using the method of Example 1.

6.3 Results and conclusions

[0137] All of the reactions produced a product peak in HPLC analysis. Accordingly, the ligation method works for all combinations of modifications tested at the junctions to be joined. Optimisation to improve product yield is possible, but was not necessary as the results were conclusive and it was clear that the reaction was working for all combinations of modifications tested at the junctions to be joined.

Example 7: Ability to use different numbers of segments to build larger oligonucleotides

7.1 Materials

[0138] Mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) was produced as described in 5.1.

[0139] The following biotinylated template DNA oligonucleotides and DNA segment oligonucleotides (Table 11) were synthesized by standard solid phase methods.

Table 11

Name	Sequence
Template	5'-biotin TTTGGTGCGAAGCAGAAGGTAAGCCGAGGTTTGGCC-3' (SEQ ID NO:47)
5' segment (1)	5'-GGCCAAA-3'
centre segment (2)	5'-(p)CCTCGG-3'
centre segment/ 3'segment (5)	5'-(p)TCTGCT-3'
centre segment (3)	5'-(p)CTTACCT-3'
3' segment (4)	5'-(p)TCGCACC-3'
(p) = phosphate,	

7.2 Method

[0140] Reactions were set up as follows:

Table 12

5' segment	Centre segment(s)	3' segment	Total number of segments
1	2 and 3	5	4
1	2, 3 and 5	4	5

[0141] Reactions were run in phosphate buffered saline, pH = 7.04 in a total volume of 100 µl and set up as follows:-

Template (20 µM final)

Each segment (20 µM final)

MgCl₂ (10 mM final)

ATP (100 µM final)

Mutant CC31 DNA ligase (25 µM final)

[0142] Each reaction was incubated at 28 °C overnight before being terminated by heating at 94 °C for 1 minute. Products were analysed by HPLC mass spec.

7.3 Results and conclusions

[0143] The reaction using 4 segments produced a fully ligated product of 27 base pairs in length. The reaction using 5 segments produced a product of 33 base pairs in length. In both cases the observed mass of the product was in concordance with that expected for the desired sequence. In conclusion, it is clearly possible to assemble multiple segments to generate oligonucleotides of the desired length and sequence as defined by the appropriate complementary template sequence.

Example 8: Assembly and ligation of 5-10-5 segments to form a gapmer, wherein the 5' and the 3'segments comprise (i) 2'-OMe ribose sugar modifications, (ii) phosphorothioate linkages or (iii) 2'-OMe ribose sugar modifications and phosphorothioate linkages; and wherein the central segment is unmodified DNA

8.1 Materials

[0144] Mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) was produced as described in 5.1 the following biotinylated template DNA oligonucleotide and segment oligonucleotides (Table 13) were synthesized by standard solid phase methods.

Table 13

Name	Sequence
Template	5'-biotin TTTGGTGCGAAGCAGACTGAGGC-3' (SEQ ID NO:30)
3' segment (3OMe)	5'-(p)(OMe)G(OMe)C(OMe)C(OMe)T(OMe)C-3'
3' segment (3PS+OMe)	5'-(p)(OMe)G*(OMe)C*(OMe)C*(OMe)T*(OMe)C-3'
3' segment (3PS)	5'-(p)G*C*C*T*C-3'
centre segment (D)	5'-(p)AGTCTGCTTC-3'
5' segment (5OMe)	5'-(OMe)G(OMe)C(OMe)A(OMe)C(OMe)C-3'
5' segment (5PS+OMe)	5'-(OMe)G*(OMe)C*(OMe)A*(OMe)C*(OMe)C-3'
5' segment (5PS)	5'-G*C*A*C*C-3'
OMe indicates 2' methoxy substitution on the ribose ring All remaining sugar residues are deoxyribose residues *phosphorothioate	

8.2 Method

[0145] Reactions were set up as follows:

Table 14

Reaction	3' segment	Centre segment(s)	5' segment
1	3PS	D	5PS
2	3OMe	D	5OMe
3	3PS+OMe	D	5PS+OMe

[0146] Each of reactions 1, 2 and 3 were set up in 100 µl final volume in phosphate buffered saline with the following components:-

3' segment	20 µM final
Centre segment	20 µM final
5' segment	20 µM final
Template	20 µM final
MgCl ₂	10 mM final
ATP	50 µM final
Enzyme	25 µM final

[0147] Each reaction mix was incubated at 20 °C overnight. Each reaction was terminated by heating at 95 °C for 10 minutes. HPLC mass spec analysis was carried out.

8.3 Results and conclusions

[0148] Product oligonucleotide corresponding to the successful ligation of all three fragments was produced in all three reactions.

[0149] Accordingly, mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) is able to ligate 3 segments together to form a 'gapmer' where the 5' and 3''wings' have a phosphorothioate backbone, whereas the central region has a phosphodiester backbone, and all the sugar residues in the gapmer are deoxyribose residues. Enterobacteria phage CC31 ligase (SEQ ID NO:23) is also able to ligate 3 segments together to form a 'gapmer' where the 5' and 3' 'wings' have 2'-methoxyribose (2'-OMe) residues, whereas the central region has deoxyribose residues, and all of the linkages are phosphodiester linkages. Finally, Enterobacteria phage CC31 ligase (SEQ ID NO:23) is able to ligate 3 segments together to form a 'gapmer' where the 5' and 3' 'wings' have the combined modifications (a phosphorothioate backbone and 2'-methoxyribose residues), whereas the central region has deoxyribose residues and phosphodiester linkages.

Example 9: Assembly and ligation of segments comprising Locked Nucleic Acids (LNA)

9.1 Materials

[0150] Mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) was produced as described in 5.1. A mutant Staphylococcus aureus NAD dependent ligase (NAD-14) was produced as described in 13.1

[0151] The following biotinylated template DNA oligonucleotide and segment oligonucleotides (Table 15) were synthesized by standard solid phase methods.

Table 15

Name	Sequence
Template	5'-biotin TTTGGTGCGAAGCAGACTGAGGC-3' (SEQ ID NO:30)
5'-segment	5'- GCCTCAG-3'
LNA 5'-segment (oligo 1)	5'-GCCTCA(LNA)G-3'
Centre segment	5'-(p)TCTGCT-3'
LNA centre segment (oligo 2)	5'-(p) (LNA)TCTGCT-3'
(p) = phosphate LNA = locked nucleic acid	

9.2 Method

[0152] Reactions were set up as follows:

Reaction volume 100 µl

Template	20 µM final
Enzyme	25 µM final
All oligonucleotide segments	20 µM final

[0153] Reactions were set up varying enzyme (mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) or NAD-14), divalent cation (Mg²⁺ or Mn²⁺) and combinations of oligonucleotide segments as set out in Table 16.

Table 16

Enzyme	SEQ ID NO:23	SEQ ID NO:23	NAD-14	NAD-14
Divalent cation	10 mM MgCl ₂	10 mM MnCl ₂	10 mM MgCl ₂	10 mM MnCl ₂
Cofactor	100 µM ATP	100 µM ATP	100 µM NAD	100 µM NAD
Buffer	PBS, pH = 7.04	PBS, pH = 7.04	50 mM KH ₂ PO ₄ , pH 7.5	50 mM KH ₂ PO ₄ , pH 7.5
5' segment + Centre segment	Product	Product	Product	Product
Oligo 1 + Centre segment	Product	Product	Product	Product
5' segment + Oligo 2	Product	Product	Product	Product
Oligo 1 + oligo 2	No product	No product	No product	No product

[0154] Each reaction mix was incubated at 28 °C overnight. Each reaction was terminated by heating at 94 °C for 1 minute. HPLC mass spec. analysis was carried out.

9.3 Results and conclusions

[0155] Product oligonucleotide was produced in control reaction reactions (unmodified oligonucleotides only) and where a single locked nucleic acid was included in one segment at the ligation junction regardless of whether it was on the 3' or 5' side of the junction. When locked nucleic acids were included at both sides (oligo 1 + oligo 2) no product was detected. The data was similar for both enzymes and regardless of whether Mg²⁺ or Mn²⁺ were used.

[0156] Enzyme mutations and/or selection screens could be carried out to identify an enzyme capable of ligating segments with a locked nucleic acid at both the 3' and 5' side of the junction.

Example 10: Assembly and ligation of three segments (7-6-7) to form a gapmer wherein the 5' and the 3' segments comprise 2' MOE ribose sugar modifications and all linkages are phosphorothioate linkages, using a variant of Enterobacteria phage CC31 ligase in the presence of Mg²⁺ or Mn²⁺

10.1 Phosphorothioate bond formation

[0157] In order to determine whether a mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) was able to ligate modified oligonucleotide segments with a phosphorothioate backbone, 2' MOE ribose sugar modifications and 5-methylated pyrimidine bases, reactions were performed using the oligonucleotide segments shown in table 15. Reactions were performed in the presence of Mg²⁺ and Mn²⁺ ions.

10.2 Materials

[0158] Oligonucleotides were chemically synthesised using standard methods as shown below:

Table 17

Name	Sequence
5' segment 2'-MOE PS	5'-mG*mG*mC*mC*mA*dA*dA-3'
centre segment PS	5'-(p)*dC*dC*dT*dC*dG*dG-3'
3' segment 2'-MOE PS	5'-(p)*dC*dT*mU*mA*mC*mC*mU-3'
Biotinylated template	5'-biotin dT dT dT dA dG dG dT dA dA dG dC dC dG dA dG dG dT dT dT dG dG dC dC- 3' (SEQ ID NO:2)
(p)* = 5-posporotioate, * = phosphorothioate linkage, mX = MOE bases, dX = DNA bases all segments and product have 5-methyl pyrimidines (with the exception of the template) mT and m (Me)U are considered to be equivalent N.B. the target 2'MOE PS molecule produced by ligation of the segments in table 17, when hybridised to the biotinylated template shown in table 17, is: 5'-mG*mG*mC*mC*mA*dA*dA*dC*dC*dT*dC*dG*dG*dC*dT*mU*mA*mC*mC*mU-3' (SEQ ID NO:1)	

[0159] Purified mutant Enterobacteria phage CC31 ligase (SEQ ID NO:23) was prepared as described in example 5.1. HPLC analysis was carried out.

10.3 Oligonucleotide assembly and ligation method with Enterobacteria phage CC31 ligase variant (SEP ID NO:23)

[0160] Reactions were prepared as follows:

MgCl₂ reaction

10 x T4 DNA ligase buffer (NEB)*	5 ul
template	20 µM final concentration
5' segment 2' MOE PS	20 µM final concentration
centre segment PS	20 µM final concentration
3' segment 2' MOE PS	20 µM final concentration

ligase (24.3 µM)	10 µl
water	made up to 50 µL
*1 x buffer contains 50 mM Tris-HCl, 10 mM MgCl ₂ , 1 mM ATP, 10 mM DTT, pH 7.5	
MnCl ₂ reaction	
10 x ligase buffer*	5 µl
ATP (10 mM)	5 µl
MnCl ₂ (50 mM)	5 µl
template	20 µM final concentration
5' segment 2' MOE PS	20 µM final concentration
centre segment PS	20 µM final concentration
3' segment 2' MOE PS	20 µM final concentration
ligase (24.3 µM)	10 µl
water	made up to 50 mL
*1 x buffer contains 50 mM Tris-HCl, 10 mM DTT, pH 7.5	

[0161] The final reactions contained 20 µM of each segment and template, 5 mM MgCl₂ or 5 mM MnCl₂, 1mM ATP, 50 mM Tris-HCl, 10 mM DTT, pH 7.5 and 4.9 µM ligase. Additional reactions were prepared containing no enzyme and served as a negative control. Reactions were incubated for 16 hours at 25 °C and then quenched by heating to 95 °C for 5 minutes. Precipitated proteins were cleared by centrifugation and samples were analysed by HPLC.

10.4 Results and Conclusion

[0162] Product, template and segment oligonucleotides were clearly resolved in the control chromatogram and no ligation was observed. Ligase reactions performed in the presence of 5 mM MgCl₂ led to the formation of an intermediate product formed from the ligation of the 5' segment and centre segments, but no full length product was detected. Ligase reactions performed in the presence of MnCl₂ produced both full length product and intermediate (5' segment plus centre segment intermediate). Both ligase reactions showed that unligated oligonucleotide segments remained. However, optimisation of the protocol is possible in order to maximise product yield.

Example 11: Assembly and ligation of three segments (7-6-7) to form a gapmer wherein the 5' and the 3' segments comprise 2' MOE ribose sugar modifications and all linkages are phosphorothioate linkages, using wild-type Chlorella virus DNA ligase in the presence of native Mg²⁺

11.1 Materials

[0163] In order to determine whether Chlorella virus DNA ligase (SEQ ID NO:29, commercially available as SplintR ligase, NEB) was able to ligate modified oligonucleotide segments with a phosphorothioate backbone, 2' MOE ribose sugar modifications and 5-methylated pyrimidine bases reactions were performed using the oligonucleotide segments shown in example 10.2 table 17. Reactions were performed at 25 °C, 30 °C and 37 °C to investigate the effect of temperature on the enzyme activity.

11.2 Oligonucleotide assembly and ligation method with commercial chlorella virus DNA ligase (SEQ ID NO:29)

[0164] Each Oligonucleotide segment and template were dissolved in nuclease free water as detailed below:

Biotinylated template	249.6 ng/µl
5' segment 2' MOE PS	182.0 ng/µl
Centre segment PS	534.0 ng/µl
3' segment 2' MOE PS	531.0 ng/µl

[0165] Reactions were prepared as follows:

10 x buffer (NEB)*	6 µl
template	3.8 µl
5' segment 2' MOE PS	18.1 µl
centre segment PS	4.8 µl
3' segment 2' MOE PS	6.3 µl
water	15 µl
SplintR ligase (25 U/µl)	6 µl
*1 x buffer contains 50 mM Tris-HCl, 10 mM MgCl ₂ , 1 mM ATP, 10 mM DTT, pH 7.5	

[0166] The final reactions contained 20 µM of each segment and template, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT, pH 7.5 and 2.5 U/µl ligase. Reactions were incubated at 25 °C, 30 °C and 37 °C. Additional reactions were prepared containing no enzyme and served as a negative control. Following 16 hours incubation, reactions were quenched by heating to 95 °C for 10 minutes. Precipitated proteins were cleared by centrifugation and samples were analysed by HPLC.

11.3 Results and Conclusion

[0167] Product, template and segment oligonucleotides were clearly resolved in the control chromatogram and no ligation was observed. HPLC analysis of the ligase reactions showed that unligated oligonucleotide segments remained, but Chlorella virus DNA ligase was able to successfully ligate the segments. The activity of the ligase increased with increasing temperature. At 25 °C the Chlorella virus DNA ligase was able to successfully ligate the 5' segment and centre segment but no full length product was observed. At 30 °C and 37 °C, full length product was detected in addition to the intermediate formed from 5' segment and centre segment.

Example 12: Screening a panel of 15 ATP and NAD ligases for activity towards the ligation of three segments (7-6-7) to form a gapmer wherein the 5' and the 3' segments comprise 2' MOE ribose sugar modifications and all linkages are phosphorothioate linkages

12.1 Materials

[0168] Wild-type ATP and NAD dependent ligases described in table 18 and 19 were each fused at the N-terminus to a CBD. Genes were synthesised, cloned into pET28a and expressed in E.coli BL21(DE3) using standard cloning, expression and extraction methods.

Table 18

ATP dependent Ligases		
Name	Origin	SEQ ID
M1I5D1_Pbcv	Paramecium bursaria Chlorella virus NE-JV-4	SEQ ID NO:48
M1I998_Pbcv	Paramecium bursaria Chlorella virus NYs1	SEQ ID NO:49
M1HX09_Pbcv	Paramecium bursaria Chlorella virus NE-JV-1	SEQ ID NO:50
M1HUL0_Atcv	Acanthocystis turfacea Chlorella virus Canal-1	SEQ ID NO:51
M1HRK1_Atcv	Acanthocystis turfacea Chlorella virus Br0604L	SEQ ID NO:52
M1I273_Atcv	Acanthocystis turfacea Chlorella virus NE-JV-2	SEQ ID NO:53
M1I600_Atcv	Acanthocystis turfacea Chlorella virus TN603.4.2	SEQ ID NO:54
M1H4A4_Atcv	Acanthocystis turfacea Chlorella virus GM0701.1	SEQ ID NO:55
F5B464_Sphage	Synechococcus phage S-CRM01	SEQ ID NO:56
A0A0F9M1S3	marine sediment metagenome - uncharacterized protein	SEQ ID NO:57

Table 19

NAD dependent ligases		
Name	Origin	SEQ ID
MtNAD	Mycobacterium tuberculosis (strain ATCC 25618/H37Rv)	SEQ ID NO:58
EfNAD	Enterococcus faecalis (strain ATCC 700802/V583)	SEQ ID NO:59
HiNAD	Haemophilus influenzae (strain ATCC 51907/DSM 11121/ KW20/Rd)	SEQ ID NO:60
SaNAD	Staphylococcus aureus	SEQ ID NO:61
SpNAD	Streptococcus pneumoniae (strain P1031)	SEQ ID NO:62

[0169] CBD-Ligase fusions were bound to PERLOZA beads as described in 1.4 with the following modifications. CBD-ligase fusion proteins were grown from a single colony of BL21(DE3) cells (NEB) and grown in a 50 mL expression culture. The cells were harvested by centrifugation, resuspended in 5-10 mL Tris-HCl (50 mM, pH 7.5) and lysed by sonication. The lysate was cleared by centrifugation and 1 mL of PERLOZA 100 (PERLOZA) beads (50 % slurry, pre-equilibrated with 50 mM Tris-HCl pH 7.5) was added to the supernatant which was shaken at 20 °C for 1 hour. The PERLOZA cellulose beads were then collected and washed with 30 ml buffer (50 mM Tris pH 8.0, 200 mM NaCl, 0.1% Tween 20, 10% Glycerol) followed by 10 ml Tris-HCl (50 mM, pH 7.5) and were finally re-suspended in 1 mL Tris-HCl (50 mM, pH 7.5). In order to analyse protein expression, 20 µl of the PERLOZA bead slurry was mixed with 20 µl of SDS loading buffer and incubated at 95 °C for 5 minutes before being run on a SDS PAGE gradient gel (4 - 20%) according to a standard protocol.

12.2 2' MOE and phosphorothioate modified oligonucleotide assembly and ligation method

[0170] Modified oligonucleotide segments with a phosphorothioate backbone, 2' MOE ribose sugar modifications and 5-methylated pyrimidine bases shown in example 10.2 table 17 were used. Each oligonucleotide segment and template was dissolved in nuclease free water as detailed below:

Biotinylated template	1500 ng/µl
5' segment 2' MOE PS	1008 ng/µl
Centre segment PS	725 ng/µl
3' segment 2' MOE PS	1112 ng/µl

[0171] ATP Assay mix was prepared as follows:

template	85.6 µl
5' segment 2' MOE PS	43.5 µl
centre segment PS	46.8 µl
3' segment 2' MOE PS	40.1 µl
DTT 1M	8 µl
MgCL2 1M	4 µl
ATP (50 mM)	16 µl
Tris 0.5 M	80 µl
water	476 µl

[0172] NAD Assay mix was prepared as follows:

template	85.6 µl
5' segment 2' MOE PS	43.5 µl
centre segment PS	46.8 µl
3' segment 2' MOE PS	40.1 µl
DTT 1M	8 µl
MgCL2 1M	4 µl
NAD (50 mM)	1.6 µl
Tris 0.5 M	80 µl

water	490.4 µl
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[0173] Each immobilized protein (40 µl, 50 % PERLOZA bead slurry) was pipetted into a PCR tube. The beads were pelleted by centrifugation and the supernatant was removed by pipetting. Assay mix (40 µl) was added to each reaction (The final reactions contained 20 µM of each segment and template, 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP or 100 µM NAD, 10 mM DTT, pH 7.5, and 40 µl of ligase on PERLOZA beads). A reaction containing no protein served as a negative control. Reactions were incubated for 18 hours at 30 °C and then quenched by heating to 95 °C for 10 minutes. Precipitated proteins were cleared by centrifugation and samples were analysed by HPLC.

12.3 Results and Conclusion

[0174] Product, template and segment oligonucleotides were clearly resolved in the control chromatogram and no ligation was observed. HPLC analysis of the ligase reactions showed that all proteins catalyzed the successful ligation of the 5' segment and centre segment to form an intermediate product, but only some ligases catalyzed the ligation of all three segments to yield the full length product as described in table 20. The NAD dependent ligase from *Staphylococcus aureus* (SaNAD, SEQ ID NO:61) yielded the most full length product. Optimisation to improve product yield is possible and within the skilled person's skill set.

Table 20

Gene name	SED ID NO	Conversion (%)*	
		intermediate	product
M1I5D1_Pbcv	48	5.0	0.8
M1I998_Pbcv	49	5.1	0.0
M1HX09_Pbcv	50	14.4	6.6
M1HUL0_Atcv	51	9.5	2.0
M1HRK1_Atcv	52	2.8	9.4
M1I273_Atcv	53	12.0	6.4
M1I600_Atcv	54	8.8	5.1
M1H4A4_Atcv	55	2.1	0.0
F5B464_Sphage	56	2.1	0.0
A0A0F9M1S3_ms_metagenome	57	1.4	0.0
MtNAD	58	7.1	0.0
EfNAD	59	19.1	0.0
HiNAD	60	0.3	0.0
SaNAD	61	10.5	11.8
SpNAD	62	19.0	0.0
* Conversion was calculated from the HPLC peak area relative to the template which is not consumed in the reaction and serves as an internal standard. Conversion=product area/ (template + product area)*100			

Example 13: Semi-continuous ligation reaction

13.1 Materials

[0175] A mutant *Staphylococcus aureus* ligase (NAD-14) fused at the N-terminal to a CBD was produced using standard cloning, expression and extraction methods. CBD-NAD-14 mutant ligase was then bound to PERLOZA beads: 50 ml of protein lysate was added to 7.5 ml PERLOZA beads, incubated at room temperature for 1 hour and then the beads were collected in a glass column (BioRad Econo-Column 10cm length, 2.5cm diameter #7372512). The beads were washed with 200 ml Buffer Y (50mM Tris8, 500mM NaCl, 0.1% Tween 20, 10% Glycerol), then with 200 ml Buffer Z (50mM Tris8, 200mM NaCl, 0.1% Tween 20, 10% Glycerol) and 200ml PBS. The estimated concentration of mutant NAD-14 ligase on the beads was 69 µM of ligase per ml of beads.

[0176] The following template DNA oligonucleotide and segment oligonucleotides (Table 21) were synthesized by standard solid phase methods.

Table 21

Segment	Sequence	MW	% HPLC purity
Template 3	5' TTTGGTGCGAAGCAGACTGAGGC-3' (SEQ ID NO:30)		
centre segment	5'-(p)dTdCdTdGdCdT-3'	1865.4	97.5
MOE 3'-segment	5'-(p)dTdCmGmCmAmCmC-3'	2546.7	98.0
MOE 5'-segment	5'-mGmCmCmUmCdAdG-3'	2492.7	98.3
(p) = phosphate, mX = MOE bases, dX = DNA bases all 5-methyl pyrimidines all linkages are phosphodiester linkages			

[0177] A "tri-template hub" (approximately 24 kDa) comprising a support material referred to as the "hub" and three template sequences was produced (Figure 11). Each copy of the template was covalently attached, at its own individual attachment point, to the "hub". The "tri-template hub" molecule is a higher molecular weight than the target (product) oligonucleotide (100% complementary to the template sequence), thereby allowing it to be retained when the impurities and products are separated from the reaction mixture. It should be noted that in this particular case the template sequence was SEQ ID NO:30 and three copies were attached to the hub. In the following example, a tri-template hub is also produced, but with a different template sequence. Accordingly, as the template sequence varies between examples, so too does the tri-template hub.

[0178] The following reaction mix (total volume 5 ml) was prepared:

250 µl	1 M KH ₂ PO ₄ , pH 7.5	(50 mM final)
108 µl	0.07011 M centre segment	(1.5 mM final)
137 µl	0.05481 M 3'-segment	(1.5 mM final)
168 µl	0.04461 M 5'-segment	(1.5 mM final)
750 µl	0.00387M Hub (Template)	(0.55 mM final)
350 µl	50mM NAD ⁺	(3.5 mM final)
1000 µl	50mM MgCl ₂	(10 mM final)
2237 µl	Nuclease free H ₂ O	

13.2 Methods

[0179] A semi-continuous system was set up as shown in Figure 12.

[0180] 4 ml of PERLOZA beads and immobilised mutant NAD-14 ligase were packed into a Pharmacia XK16 column (B). A water bath and peristaltic pump (C) was used to keep the temperature of the column, using the column water compartment, at 30 °C. The beads were equilibrated by running 120 ml (30x column volume) of buffer containing 50mM KH₂PO₄ at pH7.5 for 120 minutes at 1 ml/min. An AKTA explorer pump A1 (A) was used to create the flow through the Pharmacia XK16 column.

[0181] Following column equilibration, the 5 ml reaction mix (mixed well by vortexing) was loaded onto the column, collected in the reservoir tube (D) and recirculated through the column using the AKTA explorer A1 pump. The reaction mix was recirculated through the system at a flow rate of 1 ml/min in continuous circulation mode for 16 hours. Samples were collected after 30 minutes, 60 minutes, 90 minutes, 4 hours, 5 hours, 6 hours, 7 hours, 14 hours and 16 hours for HPLC analysis.

13.3 Results and conclusions

[0182]

Table 22

Sample	5'-segment (%)	Centre segment (%)	3'-segment (%)	5' + centre intermediate (%)	Product (%)
30 min	7.80	11.00	24.00	39.40	4.40
60 min	3.30	4.50	18.80	41.50	18.90
90 min	2.10	2.80	15.10	33.80	34.40
4 hr	1.80	2.40	9.60	19.20	56.80
5 hr	1.72	2.40	8.30	15.70	61.50
6 hr	1.69	2.50	8.60	15.90	69.40
7 hr	1.70	2.40	8.30	14.80	70.90
14 hr	2.00	2.70	1.60	5.40	88.10
16 hr	0.80	1.90	1.70	3.30	89.50

[0183] The percentage of each segment, intermediate and product is expressed as fractional peak area relative to the tri-template hub peak area.

[0184] In conclusion, the semi-continuous flow reaction worked and after 16 hours the reaction was almost complete.

Example 14: Separating oligonucleotides of different sizes by filtration: a) separation of a 20-mer oligonucleotide (SEQ ID NO:1) and a hub comprising three non-complementary 20-mer oligonucleotides (SEQ ID NO:30); (b) separation of a 20-mer oligonucleotide (SEQ ID NO:1) from segment 6-mer and 8-mer oligonucleotides (see Table 1) and a hub comprising three complementary 20-mer oligonucleotides (SEQ ID NO:2)

14.1 Materials

[0185] All oligonucleotides used were synthesized by standard solid phase methods.

A tri-template hub, as described in 13.1 (Figure 11), was used.

A variety of filters of varying molecular weight cut-offs and from different manufacturers were used as shown in Tables 23 and 24.

14.2 Methods

14.2.1 Dead-end filtration set-up and protocol for screening of polymeric membranes: (protocol 1)

[0186] A dead-end filtration rig was set-up as shown in Figure 13 comprising a MET dead-end filtration cell placed in a water bath sitting on a magnetic stirrer and hotplate. Pressure inside the cell was provided from a flow of nitrogen.

[0187] The coupon of membrane (14 cm²) to be tested was first cut to the appropriate size and placed in the cell. The membrane was first conditioned with HPLC grade water (200 ml) and then with PBS buffer (200 ml). The cell was then depressurised, the remaining PBS solution was removed and replaced by a solution containing oligonucleotides (40 ml of oligonucleotides in PBS at a 1 g/L concentration). The cell was placed on a hot stirrer plate and the solution was heated to the desired temperature while being stirred using magnetic agitation. Pressure was applied to the cell (aiming for approximately 3.0 bar; the actual pressure was recorded in each case). Stirring of the solution was either stopped or continued and permeate solution was collected (approximately 20 ml) and analysed by HPLC. Flux was recorded. The system was then depressurised to allow sampling and analysis by HPLC of the retentate solution. More PBS buffer (20 ml) was then added to the filtration cell and the previous procedure was repeated 3 times. The membrane was finally washed with PBS buffer.

[0188] All samples were analysed by HPLC without any dilution.

14.2.2 Cross-flow filtration set-up and protocol for screening of polymeric membranes: (protocol 2)

[0189] A cross-flow filtration rig was set-up as shown in Figure 14. The feed vessel (1) consisting of a conical flask contained the oligonucleotide solution to be purified. The solution was pumped to a cross-flow filtration cell (4) using an HPLC pump (2) while temperature within the cell was maintained using a hot plate (3). The solution within the cell was recirculated using a gear pump (6). A pressure gauge (5) enabled the pressure to be read during the experiment. Samples of the retentate solution were taken from the sampling valve (7) while the permeate solution was sampled from the permeate collection vessel (8).

[0190] The coupon of membrane to be tested was first cut to the appropriate size and placed in the cell. The system was washed with a PBS solution (100 ml). Temperature of the solution was adjusted to the desired set point. A solution containing oligonucleotide products in PBS (7.5 ml at 1 g/L) was fed into the system. PBS solution was then pumped into the system using the HPLC pump at a flow rate matching the flow rate of the permeate solution (typically 3 ml/min). Pressure was recorded using the pressure gauge. The retentate solution was sampled for HPLC analysis every 5 diafiltration volumes. The permeate solution was sampled for HPLC analysis every diafiltration volume. The experiment was stopped after 20 diafiltration volumes.

[0191] In the case of the experiment using the Snyder membrane having a 5 kDa molecular weight cut off (lot number 120915R2) and SEQ ID NO:46 and SEQ ID NO:30 the above methodology was modified as follows. The coupon of membrane to be tested was first cut to the appropriate size and placed in the cell. The system was washed with a Potassium phosphate solution (100 ml, 50mM, pH 7.5). Temperature of the solution was adjusted to the desired set point. A solution containing oligonucleotide products in potassium phosphate (approximately 1 g/L) was added to ethylenediaminetetraacetic acid (EDTA) (230µL of a 500mM solution). The solution was then fed into the system. Potassium phosphate buffer was then pumped into the system using the HPLC pump at a flow rate matching the flow rate of the permeate solution (typically 4 ml/min). Pressure was recorded using the pressure gauge. The retentate solution was sampled for HPLC analysis every 5 diafiltration volumes. The permeate solution was sampled for HPLC analysis every diafiltration volume. The experiment was stopped after 15 diafiltration volumes

14.3 Results

[0192]

Table 23: results for the dead-end filtration experiments following protocol 1 (14.2.1)

Stirring (Yes or No)	Membrane	MWCO (kDa)	Lot number	SEQ ID NO	Temp. (°C)	Rejection (%)	
						Product	Tri-template hub
Yes	NADIR	10	226162	1 and 30	60	38	96
Yes	NADIR	10	226162	1 and 2	60	90	98
					80	40	43
Yes	NADIR	10	226162	1 and 2	60	83	92
					65	77	84
					70	38	41
					75	12	20
Yes	NADIR	5	226825	1 and 2	60	97	95
					65	99	93
					70	97	92
					75	90	95
No	NADIR	5	226825	1 and 2	75	66	96
No	NADIR	10	226162	1 and 2	75	80	80
No	Snyder	5	120915R2	1 and 2	75	97	98
No	Osmonics	5	622806PT	1 and 2	75	99	98
No	Osmonics	10	622806PW	1 and 2	75	86	95
MWCO = molecular weight cut-off							

[0193] In the experiment using the 10 kDa MWCO NADIR membrane at 60 °C, clear separation between the product sequence (SEQ ID NO:1) and the non-complementary tri-template hub (comprising SEQ ID NO:30) was demonstrated. Figure 15 shows a) a chromatogram of the retentate solution, which remained in the filtration cell and contained mainly tri-template hub, after two diafiltration volumes; and b) a chromatogram of the permeate, solution enriched in the product, after two diafiltration volumes.

Table 24: results for the cross-flow filtration experiments (following protocol 2) (14.2.2)

Membrane	MWCO (kDa)	Lot number	SEQ ID NO	Temp. (°C)	Pressure	Diafiltration volume	Rejection (%)			
							Segment 1	Segment 2	Segment 3	Product
Osmonics	10	622806PW	1 and 2	75	3.1	0	N/A	N/A	N/A	100
						5	N/A	N/A	N/A	64
						10	N/A	N/A	N/A	40
						15	N/A	N/A	N/A	12
Snyder	5	120915R2	1 and 2	75	3.1	0	N/A	N/A	N/A	100
				82		5	N/A	N/A	N/A	88
				75		10	N/A	N/A	N/A	98
				75		15	N/A	N/A	N/A	98
				75		20	N/A	N/A	N/A	96
Snyder	5	120915R2	1 and 2	85	3.1	0	N/A	N/A	N/A	99
						5	N/A	N/A	N/A	63
						10	N/A	N/A	N/A	79
						15	N/A	N/A	N/A	100
						20	N/A	N/A	N/A	100
Snyder	5	120915R2	1 and 2+ segments from table 1	50	3.0	0	100	100	100	100
						5	39	39	40	100
						10	82	86	86	100
						15	77	81	79	100
						20	*	*	*	100
Snyder	5	120915R2	1 and 2	80	3.1	0	N/A	N/A	N/A	96
						5	N/A	N/A	N/A	22
						10	N/A	N/A	N/A	10
						20	N/A	N/A	N/A	38
Snyder	5	120915R2	46 and 30	80	3.4	0	N/A	N/A	N/A	98
						5	N/A	N/A	N/A	83
						10	N/A	N/A	N/A	68
						15	N/A	N/A	N/A	72
MWCO = molecular weight cut-off										
* concentration of solutes too low preventing meaningful analysis										

[0194] In the experiment using the 5 kDa MWCO Snyder membrane at 50 °C and 3.0 bar pressure, clear separation between the segment sequences (see table 1) and the complementary tri-template hub (comprising SEQ ID NO:2) and product (SEQ ID NO:1) was demonstrated. Figure 16 shows a) a chromatogram of the retentate solution, which contained mainly tri-template hub and product, after 20 diafiltration volumes; and b) a chromatogram of the permeate, which contained mainly segment oligonucleotides, after 20 diafiltration volumes.

[0195] In the experiment using the 5 kDa MWCO Snyder membrane at 80 °C and 3.1 bar pressure, clear separation between the complementary tri-template hub (comprising SEQ ID NO: 2) and product (SEQ ID NO:1) was demonstrated. Figure 17 shows a) a chromatogram of the retentate solution, which contained tri-template hub only, after 20 diafiltration volumes; and b) a chromatogram of the permeate solution, which contained the product only, after 2 diafiltration volumes.

14.4 Conclusions

[0196] Oligonucleotides of different lengths and molecular weights can be separated using filtration. As shown above, the type of membrane and the conditions, such as temperature, affect the level of separation. For a given set of oligonucleotides of different lengths/molecular weights, suitable membranes and conditions can be selected to allow the required separation. For

example, we have demonstrated that segment oligonucleotides (shortmers of 6 and 8 nucleotides in length), as outlined in table 1, can be separated from the product oligonucleotide (20-mer oligonucleotide having SEQ ID NO:1) and tri-template hub (comprising 3 x 20-mer of SEQ ID NO:2 attached to a solid support), and the product oligonucleotide and tri-template hub can, in turn, be separated from each other.

Overall conclusions

[0197] We have shown that it is possible to synthesize oligonucleotides, including oligonucleotides with a range of therapeutically relevant chemical modifications, in solution by assembling short oligonucleotide segments on a complementary template, ligating the segments together and separating the product oligonucleotide from both impurities and its complementary template in an efficient process that is scalable and suitable for large scale therapeutic oligonucleotide manufacture.

[0198] By synthesizing oligonucleotides in solution we have avoided the scale up constraints imposed by solid phase methods. In using the inherent properties of DNA to recognise complementary sequences specifically and bind complementary sequences with an affinity that reflects both the fidelity of the complementary sequence and the length of the complementary sequence we have been able to produce oligonucleotides of high purity without the need for chromatography. This both improves the efficiency of the production process and the scalability of the process. By recovering the template in an unchanged state during the separation process we are able to reuse the template for further rounds of synthesis and so have avoided the economic consequences of having to make one equivalent of template for every equivalent of product oligonucleotide formed.

[0199] Finally, although wild type ligases are known to ligate normal DNA effectively, we have shown that modifications to DNA result in decreased ligation efficiency and multiple modifications to the DNA are additive in their effect on decreasing the efficiency of ligation which can, in some cases render the DNA ligase completely ineffective. We have shown that by appropriate mutation and evolution of DNA ligases, ligation efficiency can be restored and appropriately modified DNA ligases are effective catalysts for synthesizing oligonucleotides which contain multiple modifications.

SEQUENCE LISTING

SEQ ID NO	Sequence Identifier
1	Example 1 desired product oligonucleotide sequence ("target")
2	Example 1-4, 6, 10 and 11 template oligonucleotide sequence
3	Wild type T4 DNA ligase protein sequence
4	Wild type T4 DNA ligase protein sequence (when fused to CBD)
5	Example 2 target sequence
6	Wild-type Enterobacteria phage CC31 DNA ligase protein sequence
7	Wild-type Enterobacteria phage CC31 DNA ligase protein sequence (when fused to CBD)
8	Wild-type Shigella phage Shf125875 DNA ligase protein sequence
9	Wild-type Shigella phage Shf125875 DNA ligase protein sequence (when fused to CBD)
10	Mutant ligase (T4 backbone) protein sequence
11	Mutant ligase (T4 backbone) protein sequence
12	Mutant ligase (T4 backbone) protein sequence
13	Mutant ligase (T4 backbone) protein sequence
14	Mutant ligase (T4 backbone) protein sequence
15	Mutant ligase (T4 backbone) protein sequence
16	Mutant ligase (T4 backbone) protein sequence
17	Mutant ligase (T4 backbone) protein sequence
18	Mutant ligase (T4 backbone) protein sequence
19	Mutant ligase (T4 backbone) protein sequence
20	Mutant ligase (T4 backbone) protein sequence
21	Mutant ligase (T4 backbone) protein sequence
22	Mutant ligase (T4 backbone) protein sequence
23	Mutant ligase (Enterobacteria phage CC31 backbone - clone A4) protein sequence
24	Mutant ligase (Enterobacteria phage CC31 backbone) protein sequence
25	Mutant ligase (Enterobacteria phage CC31 backbone) protein sequence

SEQ ID NO	Sequence Identifier
26	Mutant ligase (Enterobacteria phage CC31 backbone) protein sequence
27	Mutant ligase (Enterobacteria phage CC31 backbone) protein sequence
28	Mutant ligase (Shigella phage Shf125875 backbone) protein sequence
29	Wild-type Chlorella ligase protein sequence
30	Example 5, 8 and 9 template oligonucleotide sequence
31	Example 5 template oligonucleotide sequence
32	Example 5 template oligonucleotide sequence
33	Example 5 template oligonucleotide sequence
34	Example 5 template oligonucleotide sequence
35	Example 5 template oligonucleotide sequence
36	Example 5 template oligonucleotide sequence
37	Example 5 template oligonucleotide sequence
38	Example 5 template oligonucleotide sequence
39	Example 5 template oligonucleotide sequence
40	Example 5 template oligonucleotide sequence
41	Example 5 template oligonucleotide sequence
42	Example 5 template oligonucleotide sequence
43	Example 5 template oligonucleotide sequence
44	Example 5 template oligonucleotide sequence
45	Example 5 template oligonucleotide sequence
46	Example 14 "20 mer" oligonucleotide sequence
47	Example 7 template oligonucleotide sequence
48	Paramecium bursaria Chlorella virus NE-JV-4 ligase
49	Paramecium bursaria Chlorella virus NYs1 ligase
50	Paramecium bursaria Chlorella virus NE-JV-1 ligase
51	Acanthocystis turfacea Chlorella virus Canal-1 ligase
52	Acanthocystis turfacea Chlorella virus Br0604L ligase
53	Acanthocystis turfacea Chlorella virus NE-JV-2 ligase
54	Acanthocystis turfacea Chlorella virus TN603.4.2 ligase
55	Acanthocystis turfacea Chlorella virus GM0701.1 ligase
56	Synechococcus phage S-CRM01 ligase
57	marine sediment metagenome ligase
58	Mycobacterium tuberculosis (strain ATCC 25618/H37Rv) ligase
59	Enterococcus faecalis (strain ATCC 700802/V583) ligase
60	Haemophilus influenzae (strain ATCC 51907/DSM 11121/ KW20/Rd) ligase
61	Staphylococcus aureus ligase
62	Streptococcus pneumoniae (strain P1031) ligase

SEQ ID NO:1
GGCCAAACCTCGGCTTACCT

SEQ ID NO:2
TTTAGGTAAGCCGAGGTTTGGCC
SEQ ID NO:3

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVYRLTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDIEFTLA
TRKLTGNAAIEELTGYITDGGKDDVEVLRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGLDLLKEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVNNLDEAKVIYKKYIDQGLEGIIKKNIDGLWENARSKNLYKFKEVIDVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTRIMENQNYIYGKILECECNGWLKSDGRTDYV

KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:4

GSILKILNEIASIGSTKQKQAILEKNKDNELLKRVRITYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTL
ATRKLTGNAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIK
FPFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKK
EPEGLDFLDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDV
RFSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGILKNIDGLWENARSKNLYKFKEVIDVDLKIVGI
YPHRKDPTKAGGFILESECGIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDY
VKLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:5

GGC CAA ACC UCG GCU UAC CU

SEQ ID NO:6

MILDIINEIASIGSTKEKEAIIRRHKDNELLKRVRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKR
VTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFPFA
QLKADGARAFAEVRGDELDDVKILSRAGNEYLGDLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPLD
DIFGDLPELSKAKEFKEESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFALE
LMVQGYSQMILIENHIVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKEVITIDLRIVDIYEHKQ
PGKAGGFYLESECGLIKVKAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLFLPI
AIKMRRDKDVANTFADIWGDFFHEVTGL

SEQ ID NO:7

GSILDIINEIASIGSTKEKEAIIRRHKDNELLKRVRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKR
VVTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFPF
AQLKADGARAFAEVRGDELDDVKILSRAGNEYLGDLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPL

DDIFGDLPELSKAKEFKEESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFRA
LELMVQGYSQMILIENHIVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKEVITIDLRIVDIYEH
SKQPGKAGGFYLESECGLIKVKAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLF
LPIAIKMRRDKDVANTFADIWGDFFHEVTGL

SEQ ID NO:8

MILDILNQIAAIGSTKTKQEILKKNKDNKLLERVYRLTYARGIQYYIKKWPGPGERSQAYGELLEDDMLDFIEFTLA
TRKLTGNAAIKELMGYIADGKPDDVEVLRRVMMRDLEVGASVSIANKVWPGLIQLQPQMLASAYDEKLITKNIK
WPFAQLKADGARCFAEVRDDGVQFFSRAGNEYHGLTLLADELMENTKEARERHPNGVLIDGELVYHSFDIKKA
VSSGNDLSFLFGDNEESEEVQVADRSTSNGLANKSLQGTISPKEAEGMVLQAWDYVPLDEVYSDGKIKGQKYDV
RFAALENMAEGFKRIEPIENQLVHNLDEAKVVYKKYVDQGLEGIILKNRDSYWENKRSKNLIKFEVIDIALEVVG
YYEHSKDPNKLGGVELVSRCRITTTDCSGGFKDTTHKTVDGVKVLIPLDERHDLDRERLMAEAREGKLIGRIADC
ECNGWVHSGREGTVGIFLPIIKGFRFDKTEADSFEDVFGPWSQTGL

SEQ ID NO:9

GSILDILNQIAAIGSTKTKQEILKKNKDNKLLERVYRLTYARGIQYYIKKWPGPGERSQAYGELLEDDMLDFIEFTL
ATRKLTGNAAIKELMGYIADGKPDDVEVLRRVMMRDLEVGASVSIANKVWPGLIQLQPQMLASAYDEKLITKNIK
WPFAQLKADGARCFAEVRDDGVQFFSRAGNEYHGLTLLADELMENTKEARERHPNGVLIDGELVYHSFDIKKA
VSSGNDLSFLFGDNEESEEVQVADRSTSNGLANKSLQGTISPKEAEGMVLQAWDYVPLDEVYSDGKIKGQKYDV
RFAALENMAEGFKRIEPIENQLVHNLDEAKVVYKKYVDQGLEGIILKNRDSYWENKRSKNLIKFEVIDIALEVVG
YYEHSKDPNKLGGVELVSRCRITTTDCSGGFKDTTHKTVDGVKVLIPLDERHDLDRERLMAEAREGKLIGRIADC
ECNGWVHSGREGTVGIFLPIIKGFRFDKTEADSFEDVFGPWSQTGL

SEQ ID NO:10

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRITYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIK
PFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKK
EPEGLDFLDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIILKNIDGLWENARSKNLYKFKRVIDVDLKIVGI
YPHRKDPTKAGGFILESECGIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:11

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRITYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA

TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKGVIDVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:12

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRILTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKKVIDVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:13

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRILTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEVILVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:14

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRILTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEVIKVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:15

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRILTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEVIQVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:16

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRILTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR

FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEVIVVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:17

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVRILTYSRGLQYYIKKWPKGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGDLLEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVWNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEVIRVDLKIVGIY

PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:18

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVYRLTYSRGLQYYIKKWPKPGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGLDLLKEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEAIDVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:19

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVYRLTYSRGLQYYIKKWPKPGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGLDLLKEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKEAIDVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:20

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVYRLTYSRGLQYYIKKWPKPGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGLDLLKEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKRVIWDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:21

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVYRLTYSRGLQYYIKKWPKPGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGLDLLKEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKKVIEVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:22

MILKILNEIASIGSTKQKQAILEKNKDNELLKRVYRLTYSRGLQYYIKKWPKPGIATQSFGMLTLTDMLDFIEFTLA
TRKLTGNAAIEELTGYITDGKKDDVEVLRRVMMRDLECGASVSIANKVWPGLIPEQPQMLASSYDEKGINKNIKF
PAFAQLKADGARCFAEVRGDELDDVRLLSRAGNEYLGLDLLKEELIKMTAEARQIHPEGVLIDGELVYHEQVKKE
PEGLDFLFDAYPENSKAKEFAEVAESRTASNGIANKSLKGTISEKEAQCMKFQVWDYVPLVEIYSLPAFRLKYDVR
FSKLEQMTSGYDKVILIENQVNNLDEAKVIYKKYIDQGLEGIIILKNIDGLWENARSKNLYKFKKVIHVDLKIVGIY
PHRKDPTKAGGFILESECGKIKVNAGSGLKDKAGVKSHELDRTTRIMENQNYIYGKILECECNGWLKSDGRTDYV
KLFLPIAIRLREDKTKANTFEDVFGDFHEVTGL

SEQ ID NO:23

MILDIINEIASIGSTKEKEAIRRHKDNELLKRVFRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKR
VTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFP
QKADGARAFAEVRGDELDDVKILSRAGNEYLGLDLLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPLD
DIFGDLPELSKAKEFKEESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFR
ALELMVQGYQMILIHVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKRVIWDLRIVDIYEH
SKQPGKAGGFYLESEGLIKVAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLFL
PIAIKMRRDKDVANTFADIWGDFFHEVTGL

SEQ ID NO:24

MILDIINEIASIGSTKEKEAIRRHKDNELLKRVFRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKR

VTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFPAPA
QLKADGARAFAEVRGDELDDVKILSRAGNEYLGLDLLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPLD
DIFGDLPELSKAKEFKESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFALE
LMVQGYSQMILIENHIVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKKVIKIDLRIVDIYEHSKQ
PGKAGGFYLESECGLIKVKAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLFLPI
AIKMRRDKDVANTFADIWGDFHEVTGL

SEQ ID NO:25

MILDIINEIASIGSTKEKEAIIRRHKDNELLKRVRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKRV
VTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFPAPA
QLKADGARAFAEVRGDELDDVKILSRAGNEYLGLDLLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPLD
DIFGDLPELSKAKEFKESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFALE
LMVQGYSQMILIENHIVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKGVIKIDLRIVDIYEHSKQ
PGKAGGFYLESECGLIKVKAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLFLPI
AIKMRRDKDVANTFADIWGDFHEVTGL

SEQ ID NO:26

MILDIINEIASIGSTKEKEAIIRRHKDNELLKRVRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKRV
VTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFPAPA
QLKADGARAFAEVRGDELDDVKILSRAGNEYLGLDLLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPLD
DIFGDLPELSKAKEFKESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFALE
LMVQGYSQMILIENHIVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKGVIKIDLRIVDIYEHSKQ
PGKAGGFYLESECGLIKVKAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLFLPI
AIKMRRDKDVANTFADIWGDFHEVTGL

SEQ ID NO:27

MILDIINEIASIGSTKEKEAIIRRHKDNELLKRVRMTYDGKLQYYIKKWDTRPKGDIHLTLEDMLYLLEEKLAKRV
VTGNAAKEKLEIALSQTSDADAEVVKVLLRDLRCGASRSIANKVWKNLIPEQPQMLASSYDEKGIEKNIKFPAPA
QLKADGARAFAEVRGDELDDVKILSRAGNEYLGLDLLKQQLIEMTKEARERHPGGVMIDGELVYHASTLPAGPLD
DIFGDLPELSKAKEFKESRTMSNGLANKSLKGTISAKEAAGMKFQVWDYVPLDVVYSEGKQSGFAYDVRFALE
LMVQGYSQMILIENHIVHNLDEAKVIYRKYVDEGLEGIILKNIGAFWENTRSKNLYKFKRVIKIDLRIVDIYEHSKQ
PGKAGGFYLESECGLIKVKAGSGLKDKPGKDAHELDRTRIWENKNDYIGGVLESECNGWLAAEGRTDYVKLFLPI
AIKMRRDKDVANTFADIWGDFHEVTGL

SEQ ID NO:28

MILDILNQIAAIGSTKTKQEILKKNKDNKLLERVYRLTYARGIQYYIKKWPGPGERSQAYGELLEDDMLDFIEFTLA
TRKLTGNAAIKELMGYIADGKPDDVEVLRVMMRDLEVGASVSIANKVWPGLIQLQPQMLASAYDEKLITKNIK
WPAPAQLKADGARCFAEVRDDGVQFFSRAGNEYHGLTLLADELMENTKEARERHPNGVLIDGELVYHSFDIKKA
VSSGNDLSFLFGDNEESEEVQVADRSTSNGLANKSLQGTISPKEAEGMVLQAWDYVPLDEVYSDGKIKGQKYDV
RFAALENMAEGFKRIEPIENQLVHNLDEAKVVYKKYVDQGLEGIILKNRDSYWENKRSKNLIKFKRVIVIALEVVG
YYEHSKDPNKLGGVELVSRCRITTDGSGFKDTTHKTVDGVKVLIPLDERHDLDRERLMAEAREGKLIGRIADC
ECNGWVHSGREGTVGIFLPIIKGFRFDKTEADSFEDVFGPWSQTGL

SEQ ID NO:29

MAITKPLLAATLENIEDVQFPCLATPKIDGIRSVKQTQMLSRTFKPIRNSVMNRLLTELLPEGSDGEISIEGATFQD
TTSAVMTGHKMYNAKFSYYWFDYVTDPLKKYIDRVEDMKNYITVHPHILEHAQVKIIPVEINNITELLQYER
DVLSKGFEGVMIRKPDGKYKFGIRSTLKEGILLKMKQFKDAEATIISMTALFKNTNTKTKDNFGYSKRSTHKSQKV
EEDVMGSIEVDYDGVVFSIGTGFDADQRRDFWQNKESYIGKMVKFKYFEMGSKDCPRFPVFIGIRHEEDR

SEQ ID NO:30

TTTGGTGCGAAGCAGACTGAGGC

SEQ ID NO:31

TTTGGTGCGAAGCAGAGTGAGGC

SEQ ID NO:32

TTTGGTGCGAAGCAGATTGAGGC

SEQ ID NO:33

TTTGGTGCGAAGCAGAATGAGGC

SEQ ID NO:34

TTTGGTGCGAAGCAGTCTGAGGC

SEQ ID NO:35

TTTGGTGCGAAGCAGTGTGAGGC

SEQ ID NO:36

TTTGGTGCGAAGCAGTTTGAGGC

SEQ ID NO:37

TTTGGTGCGAAGCAGTATGAGGC

SEQ ID NO:38

TTTGGTGCGAAGCAGCCTGAGGC

SEQ ID NO:39

TTTGGTGCGAAGCAGCGTGAGGC

SEQ ID NO:40

TTTGGTGCGAAGCAGCTTGAGGC

SEQ ID NO:41

TTTGGTGCGAAGCAGCATGAGGC

SEQ ID NO:42

TTTGGTGCGAAGCAGGCTGAGGC

SEQ ID NO:43

TTTGGTGCGAAGCAGGGTGAGGC

SEQ ID NO:44

TTTGGTGCGAAGCAGGTTGAGGC

SEQ ID NO:45

TTTGGTGCGAAGCAGGATGAGGC

SEQ ID NO:46

GCCUCAGTCTGCTTCGCACC

SEQ ID NO:47

TTTGGTGCGAAGCAGAAGGTAAGCCGAGGTTTGGCC

SEQ ID NO:48

MAITKPLLAATLENIEDVQFPCLATPKIDGIRSVKQTQMLSRTFKPIRNSVMNRLLTELLPEGSDGEISIEGATFQD

TTSAVMTGHKMYNAKFSYYWFDYVTDPLKKYSRVEDMKNYITAHPHILDHEQVKIIPVEINNITELLQYE

RDVLSKGFEGVMIRKPDGKYKFGRSTLKEGILLKMKQFKDAEATIISMTALFKNTNTKTKDNFGYSKRSTHKNGK

VEEDVMGSIEVDYDGVVFSIGTGFDADQRRDFWQNKESYIGKMKVKYFEMGSKDCPRFPVFIGIRHEEDH

SEQ ID NO:49

MTIAKPLLAATLENLDDVKFPCLVTPKIDGIRSLKQQHMLSRFTFKPIRNSVMNKLLSELLPEGADGEICIEDSTFQA

TTSAVMTGHKVYDEKFSYYWFDYVDDPLKSYTDRVNDMKKYVDDHPPHILEHEQVKIIPVEINNIDELSQYE

RDVLAKGFEGVMIRRPDGKYKFGRSTLKEGILLKMKQFKDAEATIISMSPRLKNTNAKSKDNLGYSKRSTHKSGK

VEEETMGSIEVDYDGVVFSIGTGFDDEQRKHFWENKDSYIGKLLKFYFEMGSKDAPRFPVFIGIRHEEDC

SEQ ID NO:50

MTAIQKPLLAASFKKLTVADVYPVFATPKLDGIRALKIDGAFVSRTFKPIRNRAIADALQDLLPNGSDGEILSGST

FQDASSAVMTAKAGIGANTIFYWFDYVKDDPNKPYLDRMTDMENYLKERPEILNDDRIKIVPLIPKKIETKDELD

TFEKICLDQGFEGVMIRSGAGKYKFGRSTEKEGILIKIKQFEDDEAVVIGFTPMQTNTNDKSMNELGDMKRSSHK

DGKVNLDTLGALEVDWNGITFSIGTGFDHALRDKLWSEKDKLIGKIVKFYFAQGVKTAPRFPVFIGFRDPDDM

SEQ ID NO:51

MAIQKPLLAASLKMSVGDLTFPVFATPKLDGIRALKVGGTIVSRFTFKPVRNSAISEVLASILPDGSDGEILSGKTF

QESTSTVMTADAGLGSGTMFFWFDYVKDDPNKGYLDRIADMKSFDRHPEILKDKRVTIVPLFPKKIDTTEELHE

FEKWCLDQGFEGVMVRNAGGKYKFGRSTEKEQILVKIKQFEDDEAVVIGVSALQNTNTNDKKLNQLGEMRRTSH

QDQKVELEMLGALDVDWNGIRFSIGTGFDTRVDLWKRREGVIGKIVKFYFSQGIKTAPRFPVFLGFRDKDD
M

SEQ ID NO:52

MAIQKPLLAASLKKLSVDDLTFPVYATPKLDGIRALKIDGTLVSRTFKPIRNTTISKVLTSLLPDGS DGEILSGKTFQ
DSTSTVMSADAGIGSGTTFWFDYVKDDPNKG YLDRIADIKKFIDCRPEILKDSRVIIVPLFPKKIDTAEELNVFEK
WCLDQGFEGVMVRNAGGKYKFRSTEKEQILVKIKQFEDDEAVVIGVSALQTNNTNDKKVNELGEMRRTSHQDG
KVDLDMLGALDVDWNGIRFGIGTGFDKDTREDLWKRRDSIIGKIVKFYFSQGVKTAPRFPVFLGFRDKNDM

SEQ ID NO:53

MAIQKPLLAASLKKLSVDDLTFPVYATPKLDGIRALKIDGTIVSRTFKPIRNTTISNVLSLLPDGS DGEILSGKTF
QDSTSTVMSADAGIGSGTTFWFDYVKDDPDKGYLDRIADMKKFVDSHPEILKDRRVTVPLIPKKIDTVEELNV
FEQWCLDQGFEGVMVRNAGGKYKFRSTEKEQILVKIKQFEDDEAVVIGVSALQTNVNDKKMNELGDMRRTSH
KDGKIDLEMLGALDVEWNGIRFGIGTGFDKDTREDLWKRRDSIIGKVVKFYFSQGIKTAPRFPVFLGFRDEND
M

SEQ ID NO:54

MAIQKPLLAASLKKMSVDNLTFPVYATPKLDGIRALKIDGTLVSRTFKPIRNTTISKVLASLLPDGS DGEILSGKTF
QDSTSTVMTTDAGIGSDTTFFWFDYVKDDPDKGYLDRIADMKTFVDQHPEILKDSCVTIVPLFPKKIDTPEELHV
FEKWCLDQGFEGVMVRTAGGKYKFRSTEKEQILVKIKQFEDDEAVVIGVSALQTNNTNDKKLNQLGEMRRTSH
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DM

SEQ ID NO:55

MAIQKPLLAASLKKMSVDDLTFPVYTTPKLDGIRALKIDGTLVSRTFKPVRNSAISEVLASLLPDGS DGEILSGKTF
QDSTSTVMTTDAGIGSDTTFFWFDYVKDDPNKG YLDRIADMKTFIDQHPEMLKDNHVTIVPLIPKKIDTVEELNI
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SEQ ID NO:56

MLAGNFDPKKAKFPYCATPKIDGIRFLMVNGRALSRTFKPIRNEYIQKLLSKHLPDGIDGELTCGDTFQSSTS AIM
RIAGEPDFKAWIFDYVDPDSTSILPFIERFDQISDIYNGPIPFKHQVLGQSILYNIDDLNRYEEACLNEG YEGVML
RDPYGTYKFRSSTNEGILLKVKRFEDA EATVIRIDEKMSNQNIAEKDNFGRTKRSSCLDGMVPMETT GALFVRN
SDGLEFSIGSGLNDEM RDEIWKNKSSYIGKLVKYKFPQGVKDLPRHPVFLGFRDPDDM

SEQ ID NO:57

MDAHELMKLN EYAERQNQKQKKQITKPM LAASLKDITQLDYSKGYLATQKLDGIRALMIDGKLVSRTFKPIRNN
HIREMLEDVL PDGADGEIVCPGAFQATSSGVM SANGEPEFIYMFYVKDDITKEYWRR TQDMVQW LINQGPT
RTPGLSKL KLLVPTLIK NYDHLKTYETECIDKGFE GVILRTPDSPYKCGRSTAKQEWLLKLRFADDEAVVIGFTEK
MHNDNEATKDKFGHTVRSSHKENKR PAGTLGSLIVRDIKTEIEFEIGTGFDDEL RQKIWDARPEWDGLCVKYKH
FAISGVKEKPRFPSFIGVRDVEDM

SEQ ID NO:58

MSSPDADQTAPEVLRQWQALAEVREHQFRYYVRDAPIISDAEFDELLRRLEALEEQHPELRTPDSPTQLVGGA
GFATDFEPVDHLERMLS LDNAFTADELA AWAGRIHA EVGDAAHYLCELKIDGVALSLVYREGRLTRASTRGDGR
TGEDVTLNARTIADVPERLTPGDDYPVPEVLEVRGEVFFRLDDFQALNASLVEEGKAPFANPRNSAAGSLRQKDP
AVTARRRLRMICHGLGHVEGFRPATLHQAYLALRAWGLPVSEHTTLATDLAGVRERIDYWGEHRHEVDHEIDG
VVVKVDEVALQRR LGSTSRA PRWAIAYKYPPEEAQT KLDIRVNVGRTGRITPFAFMTPVKVAGSTVGQATLHN
ASEIKRKGV LIGDTVIRKAGDVIPEVLGPVVELRDG SEREFIMPTTCPECGSPLAPEKEGDADIRCPNARGCPGQ
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VPILDEDGFRRL LADGPASRT

SEQ ID NO:59

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YTVA DFGPMKAKTQFEALEELSAIGFRTNPERQLCQSIDEVWAYIEEYHEKRSTLPYEIDGIVIKVNEFALQDELG

FTVKAPRWAIAYKFPPEEAETVVEDIEWTIGRTGVTPTAVMAPVRVAGTTVSRASLHNADFIQMKDIRLNDHVI
IYKAGDIIPEVAQVLVEKRAADSQPYEMPTHCPICHSELVHLDEEVALRCINPKCPAQIKEGLNHFVSRNAMNIDG
LGPRVLAQMYDKGLVKDVADLYFLTEEQLMTLDKIKEKSANNIYTAIQGSKENSVERLIFGLGIRHVGAKAAKILA
EHFGDLPTLSRATAEEIVALDSIGETIADSVVTYFENEEVHELMAELEKAQVNLTYKGLRTEQLAEVESPFKDKTV
VLTGKLAQYTREEAKEKIEENLGGKVTGSVSKKTDIVVAGEDAGSKLTKAESLGVTWVNEQEMVDALDASHF

SEQ ID NO:60

MTNIQTQLDNLRKTLRQYEYEHVLDNPSVPDSEYDRLFHQKALELEHPEFLTSDSPTQRVGAKPLSGFSQIRH
EIPMLSLDNAFSDAEFNAFVKRIEDRLILLPKPLTFCCEPKLDGLAVSILYVNGELTQAATRGDGTTGEDITANIRT
IRNVPLQLLTDNPPARLEVRGEVFMPHAGFERLNKYALEHNEKTFANPRNAAAGSLRQLDPNITSKRPLVLNAYGI
GIAEGVDLPTTHYARLQWLKSIGIPVNPEIRLCNGADEVLGFYRDIQNKRSSLGVDIDGTVLKINDIALQNELGFI
SKAPRWAIAYKFPAQEELTLNDVEFQVGRTGAITPVAKLEPVFVAGVTVSNATLHNGDEIERLNIAIGDTVWIRR
AGDVIPQIIGVLHERRPDNAKPIIFPTNCPVCDSQIIRIEGEAVARCTGGLFCAAQRKEALKHFVSRKAMDIDGVG
GKLIEQLVDRELIHTPADLFKLDLTTLTRLERMGAKSAENALNSLENASTTLARFIFALGIREVGEATALNLANHF
KTLDAKLDANLEELQQVPDVGEVWANRIFIFWREAHNVAVVEDLIAQGVHWETVEVKEASENLFKDKTVVLTGT
LTQMGRNEAKALLQQLGAKVSGSVSSKTDVFIAGDAAGSKLAKAQELNITVLTEEEFLAQITR

SEQ ID NO:61

MADLSSRVNELHDLLNQYSYEYYVEDNPSVPDSEYDKLLHELIKIEEEHPEYKTVDSPTVRVGGEAQASFNKVNH
DTPMLSLGNAFNEDDLRFKFDQRIREQIGNVEYMCELKIDGLAVSLKYVDGYFVQGLTRGDGTTGEDITENLKTIH
AIPLKMKPEPLNVEVRGEAYMPRRSFLRLNEEKEKNDEQLFANPRNAAAGSLRQLDSKLTAKRKLSVFIYSVNDFT
DFNARSQSEALDELDKLGFTTNKNRARNVNIDGVLEYIEKWTSQRESLPYDIDGIVIKVNDLDQQDEMGTQKS
PRWAIAYKFPAEEVVTKLLDIELSIGRTGVTPTAILEPVKVAGTTVSRASLHNEDLIHDRDIRIGDSVVVKAGDI
IPEVRSIPERRPEDAVTYHMPTHCPCSGHELVRIEGEVALRCINPKCQAQLVEGLIHFVSRQAMNIDGLGTKIIQ
QLYQSELIKDVADIFYLTEEDLLPLDRMGQKKVDNLLAAIQKAKDNSLENLLFGLGIRHLGVKASQVLAEKYETID
RLLTVTEAELVEIHDIGDKVAQSVVTYLENEDIRALIQLKDKKHVNMIYKGIKTSDIEGHPFSGKTIVLTGKLHQ
MTRNEASKWLASQGAKVTSSVTKNTDVVIAGEDAGSKLTKAQSLGIEIWTEQQFVDKQNELNS

SEQ ID NO:62

MNKRMINELVALLNRYATEYYTSDNPSVSDSEYDRLYRELVELETAYPEQVLADSPTHRVGKVLDGFKEYSHQY
PLYSLQDAFSREELDAFDARVRKEVAHPTYICELKIDGLSISLTYEKGILVAGVTRGDGSGIGENITENLKRVKDIPL
TLPEELDITVRGECYMPRASFDQVNQARQENGEPEFANPRNAAAGTLRQLDTAVVAKRNLATFLYQEASPSTRD
SQEKGLKYLEQLGFVVNPKRILAENIDEIWNFIQEVGQERENLPYDIDGVVIVNDLASQEELGFTVKAPKWAVA
YKFPAEEKEAQLLSVDWTVGRTGVTPTANLTPVQLAGTTVSRATLHNVDYIAEKDIRKDDTVIVYKAGDIIPAV
LRVVESKRVSSEKLDIPTNCPSCNSDLLHFEDEVALRCINPRCPAQIMEGLIHFASRDAMNITGLGPSIVEKLFAN
LVKDVADIYRLQEEDFLLLEGVKEKSAAKLYQAIQASKENSAEKLLFGLGIRHVGSKASQLLLQYFHSIENLYQADS
EEVASIESLGGVIAKSLQTYFATEGSEILLRELKETGVNLDYKGQTVVADAALSGLTWLTGKLERLKRSEAKSLE
SLGAKVTGSVSKKTDLVVVGADAGSKLQKAQELGIQVRDEAWLES

REFERENCES CITED IN THE DESCRIPTION

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P A T E N T K R A V

1. Fremgangsmåde til at fremstille et enkeltstrengt oligonukleotidprodukt som har mindst én modificeret nukleotidrest, hvor modifikationen er valgt fra gruppen bestående af modifikation ved 2'-positionen af sukkerdelen, modifikation af nukleobasen og modifikation af hovedkæden, og hvor produktet fremstilles i gram- eller kilogram-skala, eller større, og/eller fremgangsmåden udføres i en 1 L-reaktor eller større reaktor, omfattende:

- a) at tilvejebringe et skabelonnukleotid (I) som er komplementært til sekvensen af produktet, hvilken skabelon har egenskaber som tillader at det kan blive adskilt fra produktet;
- 10 b) at tilvejebringe en pulje af oligonukleotider (II) som indeholder oligonukleotider som er segmenter af produktsekvensen, hvor mindst ét segment indeholder mindst én modificeret nukleotidrest og hvor modifikationen er valgt fra gruppen bestående af modifikation ved 2'-positionen af sukkerdelen, modifikation af nukleobasen og modifikation af hovedkæden;
- 15 c) at bringe (I) og (II) i kontakt med hinanden i betingelser som tillader hybridisering;
- d) at forbinde segmentoligonukleotiderne ved enzymatisk ligering med en ligase for at danne produktet;
- e) at ændre betingelserne for at adskille eventuelle urenheder, omfattende at denaturere den hybridiserede skabelon og urenhedsoligonukleotidstreng og at adskille urenhederne;
- 20 f) at ændre betingelserne for at adskille produktet, omfattende at denaturere den hybridiserede skabelon og produktoligonukleotidstreng og at adskille produktet; og
- g) at genbruge skabelonen til anvendelse i fremtidige reaktioner.

25 2. Fremgangsmåde ifølge krav 1, hvorved denatureringen resulterer fra en temperaturstigning, ændring af pH eller ændring af saltkoncentrationen i en bufferopløsning.

3. Fremgangsmåde ifølge krav 1 eller 2, indbefattende to trin med at øge temperaturen: i) for at denaturere eventuelle hybridiserede urenheder og ii) for at denaturere hybridiseret produkt.

30 4. Fremgangsmåde ifølge et hvilket som helst af krav 1-3, hvor segmenterne er 3 til 15 nukleotider lange.

5. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor produktet er 10 til 200 nukleotider langt, eventuelt 20 til 30 nukleotider langt, eventuelt 20 til 25 nukleotider langt.

35 6. Fremgangsmåde ifølge krav 5, hvor produktet er 20 nukleotider langt, hvilket produkt omfatter tre segmentoligonukleotider:

(i) et 5'-segment som er 7 nukleotider langt, et centralsegment som er 6 nukleotider langt og et 3'-segment som er 7 nukleotider langt;

40 (ii) et 5'-segment som er 6 nukleotider langt, et centralsegment som er 8 nukleotider langt og et 3'-segment som er 6 nukleotider langt; eller

(iii) et 5'-segment som er 5 nukleotider langt, et centralsegment som er 10 nukleotider langt og et 3'-segment som er 5 nukleotider langt.

7. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor egenskaben som tillader at skabelonen kan blive adskilt fra produktet er at skabelonen er fastgjort
5 til et støttemateriale.

8. Fremgangsmåde ifølge krav 7, hvor støttematerialet er et opløseligt støttemateriale, eventuelt hvor støttematerialet er valgt fra gruppen bestående af polyethylenglycol, en opløselig organisk polymer, DNA, et protein, en dendrimer, et polysakkarid, et oligosakkarid og et kulhydrat.

10 **9.** Fremgangsmåde ifølge krav 7, hvor støttematerialet er et uopløseligt støttemateriale, eventuelt hvor støttematerialet er valgt fra gruppen bestående af: en glaskugle, en polymerkugle, en fibrøs støtte, en membran, en streptavidin-coatet kugle, cellulose eller er del af selve reaktionsbeholderen, f.eks. en reaktionsvæg.

10. Fremgangsmåde ifølge et hvilket som helst af krav 7-9, hvor multiple, gentagede
15 kopier af skabelonen fastgøres på en kontinuerlig måde via et enkelt fastgørelsespunkt til støttematerialet.

11. Fremgangsmåde ifølge et hvilket som helst af krav 1-10, hvor egenskaben som tillader at skabelonen kan blive adskilt fra produktet er molekylvægten af skabelonen.

20 **12.** Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor reaktionen udføres ved anvendelse af en kontinuerlig eller semikontinuerlig strømningsfremgangsmåde.

13. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor modifikationen er ved 2'-positionen af sukkerdelen og er valgt fra gruppen bestående af 2'-F, 2'-OMe, 2'-MOE og 2'-amino, eller hvor oligonukleotidet omfatter en PMO, en LNA, en PNA, en
25 BNA eller en SPIEGELMER.

14. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor modifikationen er i nukleobasen og er valgt fra gruppen bestående af en 5-methylpyrimidin, en 7-deazaguanosin og et abasisk nukleotid.

30 **15.** Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor modifikationen er i hovedkæden og er valgt fra gruppen bestående af phosphorthioat, phosphoramidat og phosphordiamidat.

16. Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor det resulterende produkt er mindst 90% rent, eventuelt hvor produktet er mindst 95% rent, eventuelt hvor produktet er mindst 98% rent.

35 **17.** Fremgangsmåde til at fremstille et dobbeltstrenget oligonukleotidprodukt, hvor 2 komplementære enkeltstrengede oligonukleotider fremstilles ved fremgangsmåden ifølge et hvilket som helst af de foregående krav og derefter blandes under betingelser som tillader hybridisering.

40 **18.** Fremgangsmåde ifølge et hvilket som helst af de foregående krav, hvor fremgangsmåden er til at fremstille et terapeutisk oligonukleotid.

19. Fremgangsmåde ifølge et hvilket som helst af kravene 1-16 eller 18, hvor oligonukleotidproduktet er en gapmer.

DRAWINGS

Figure 1

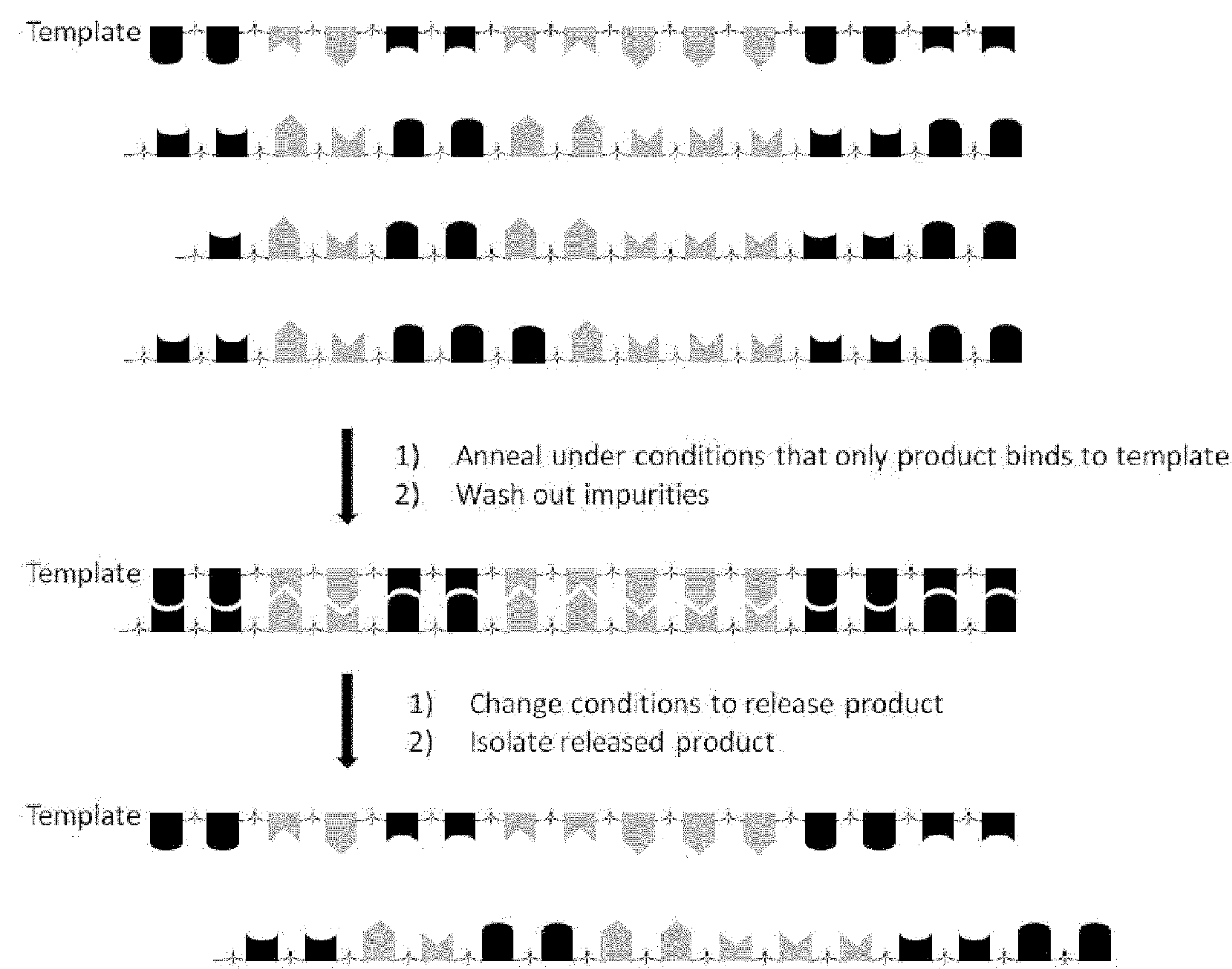


Figure 2

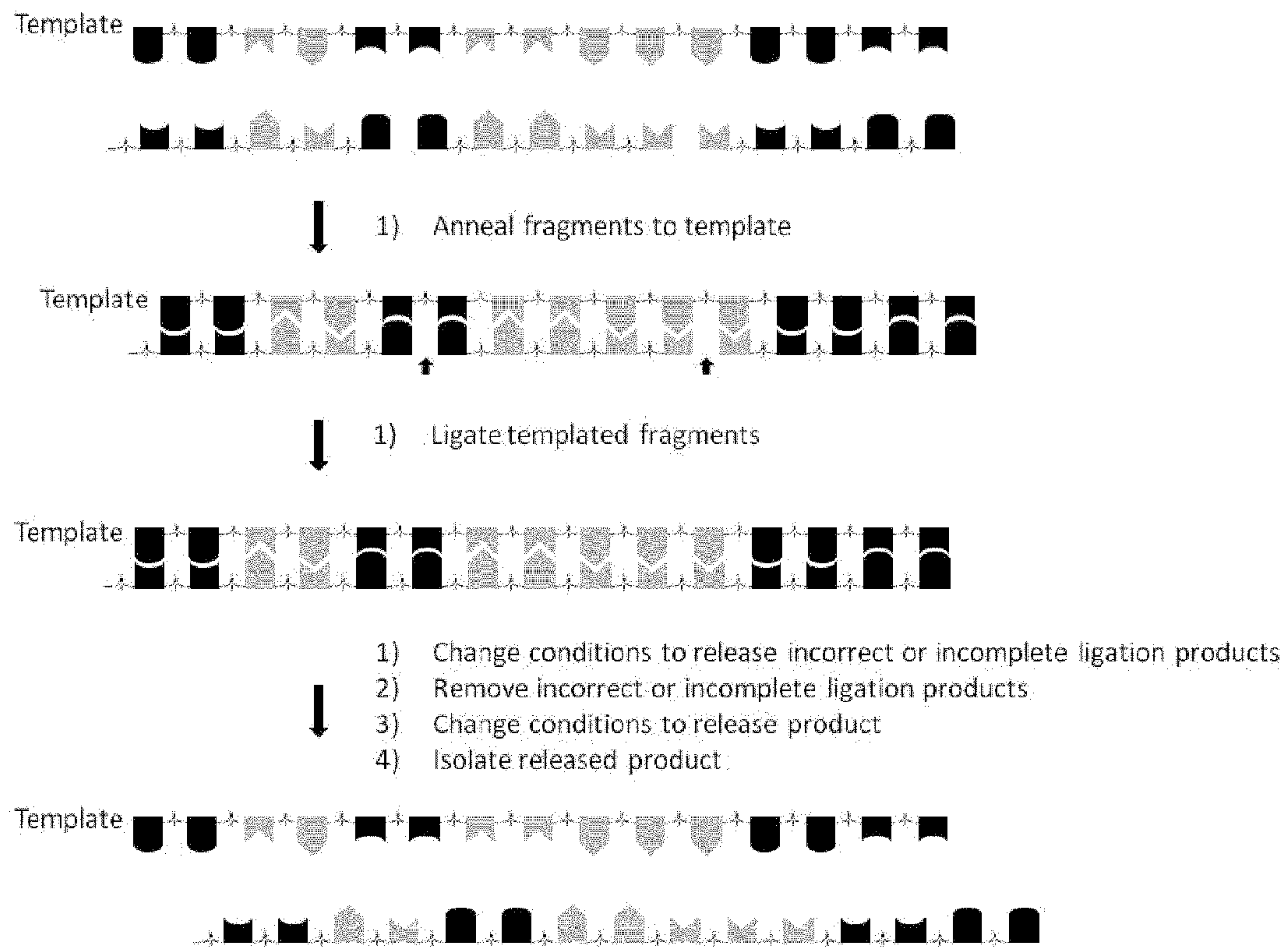


Figure 3

Single Template



Repeating Template

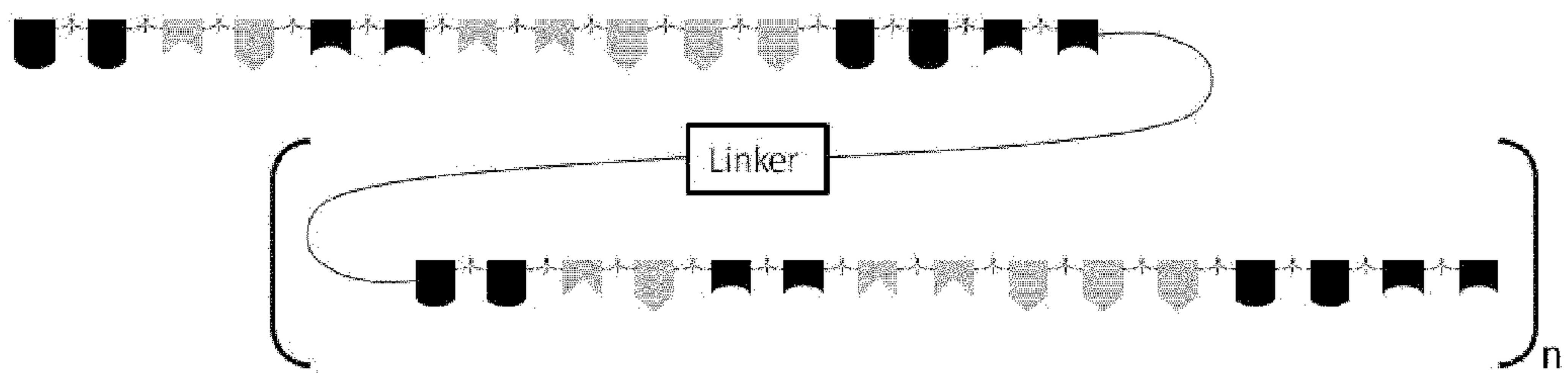


Figure 4

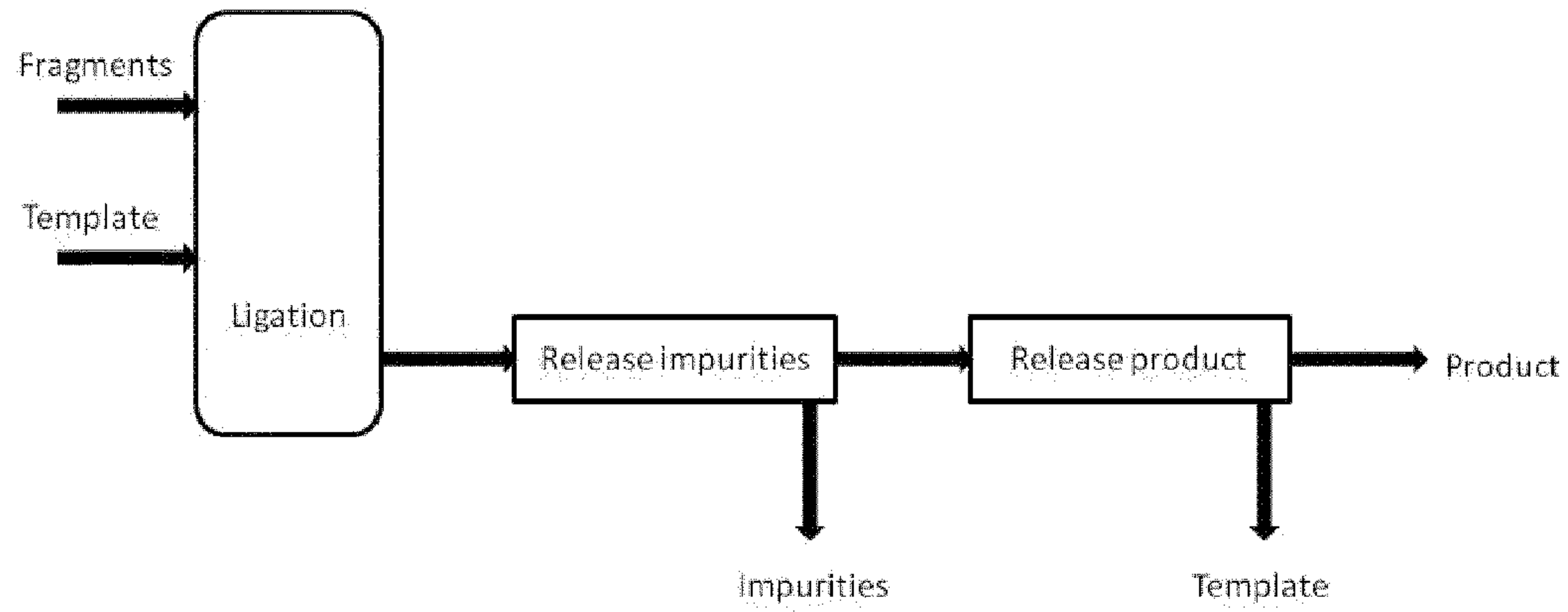
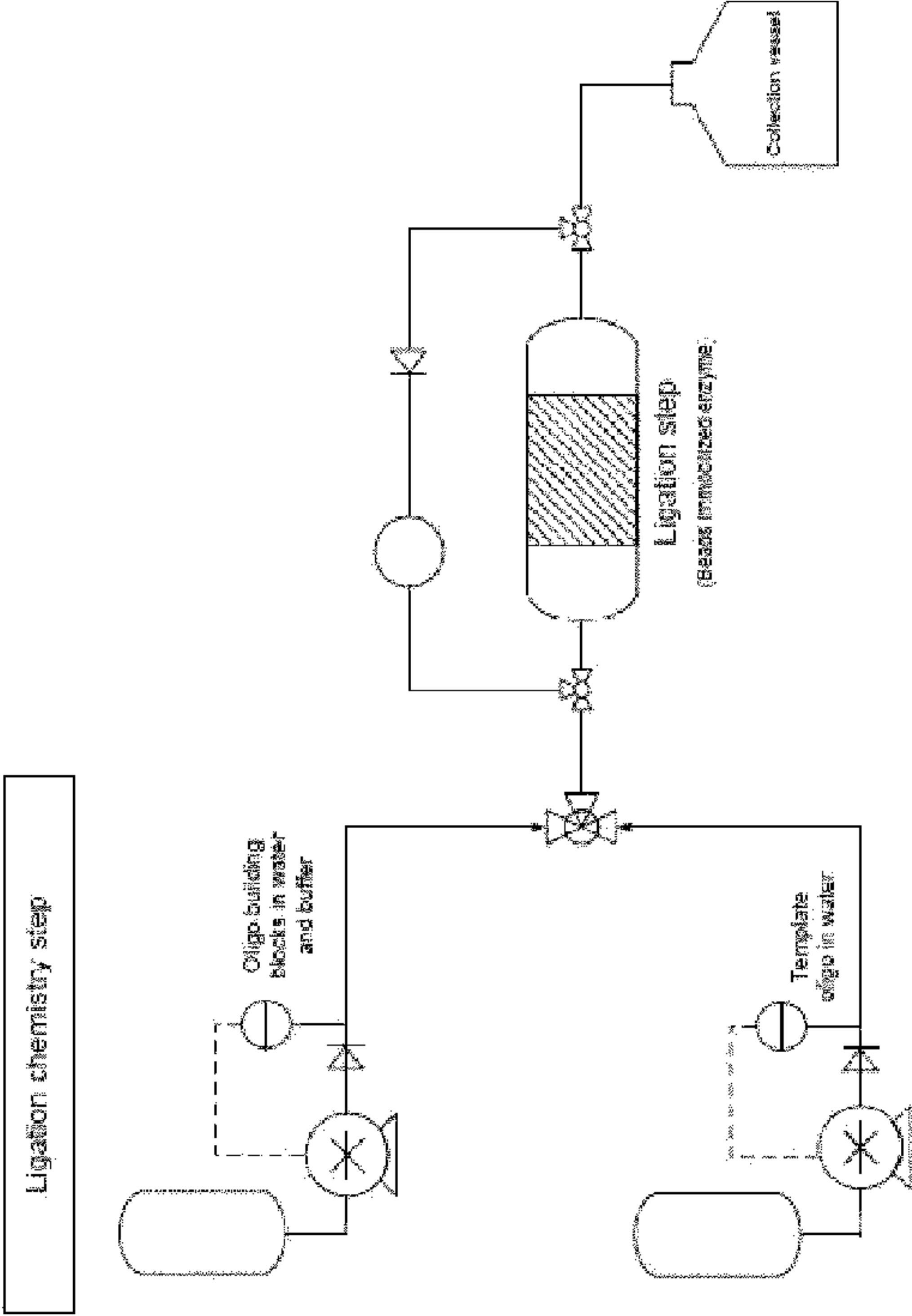
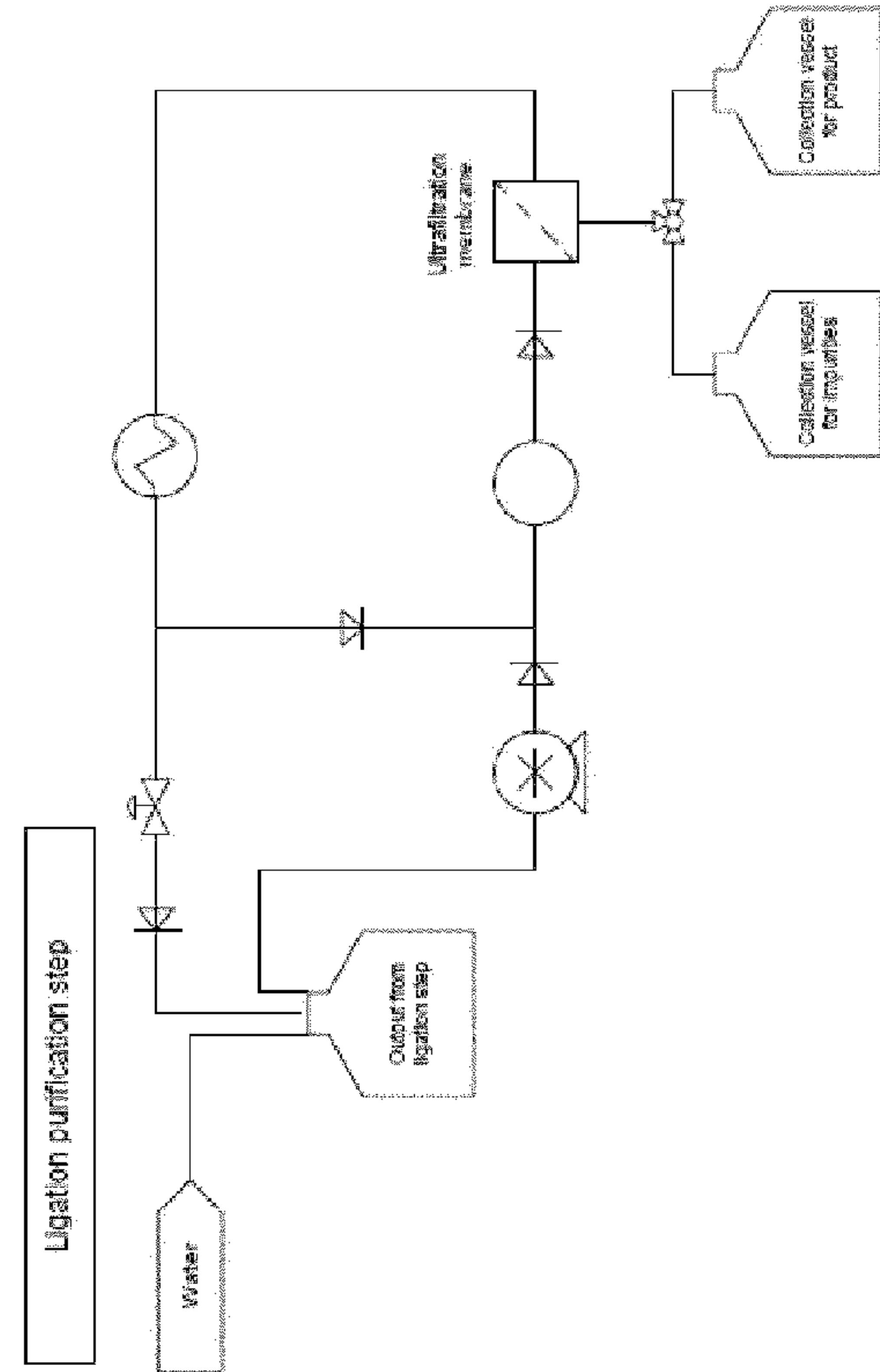


Figure 5

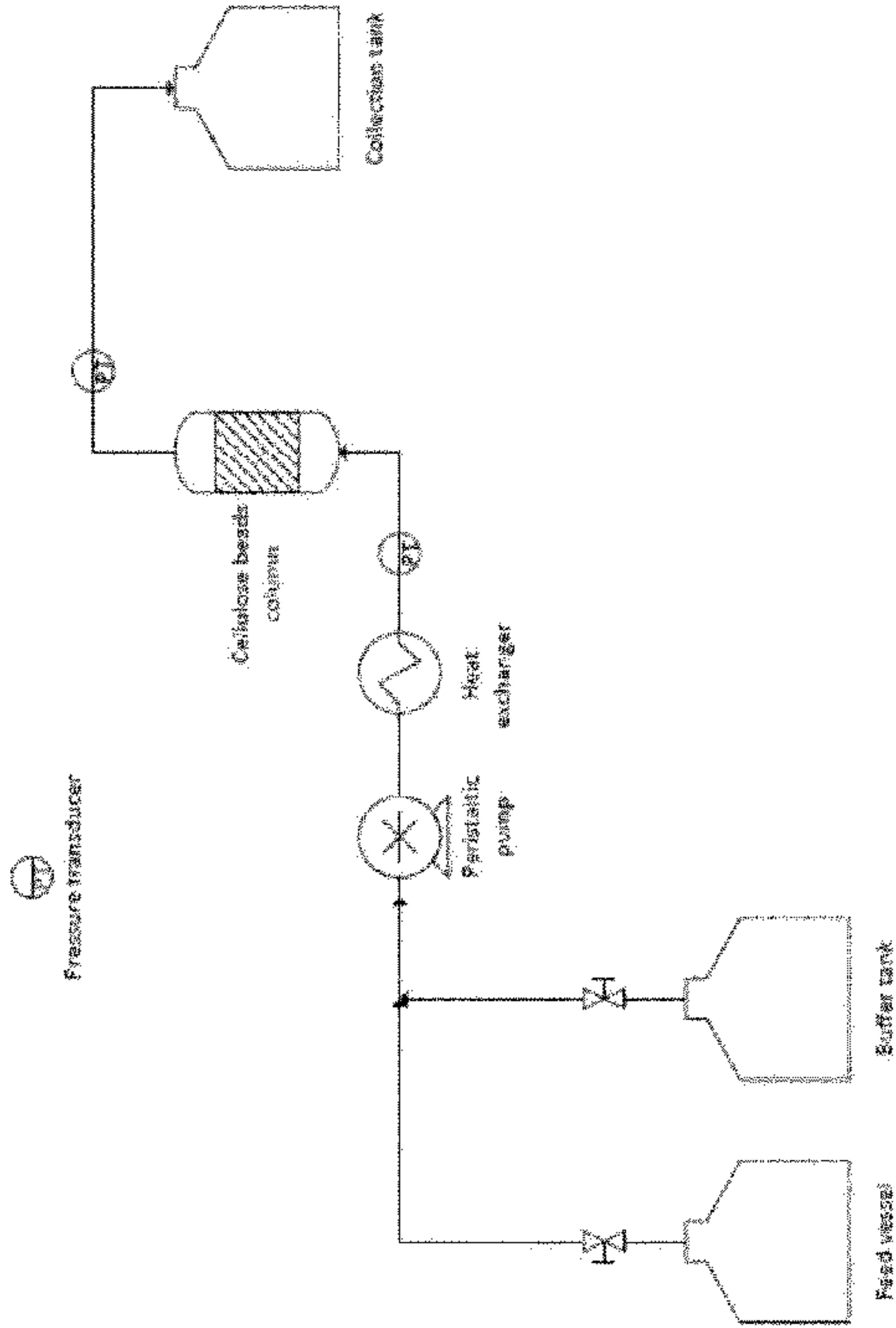
(a)



(b)



(c)



(d)

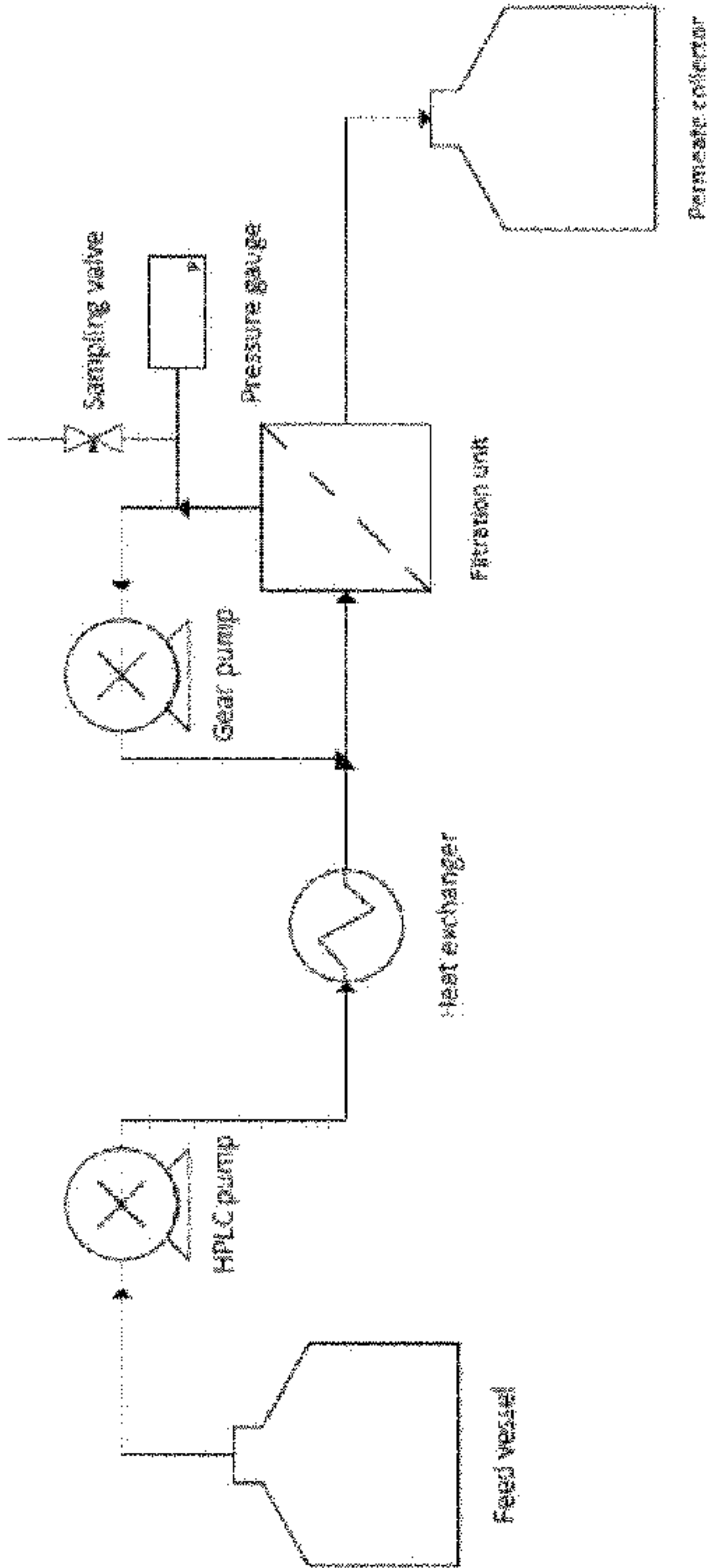
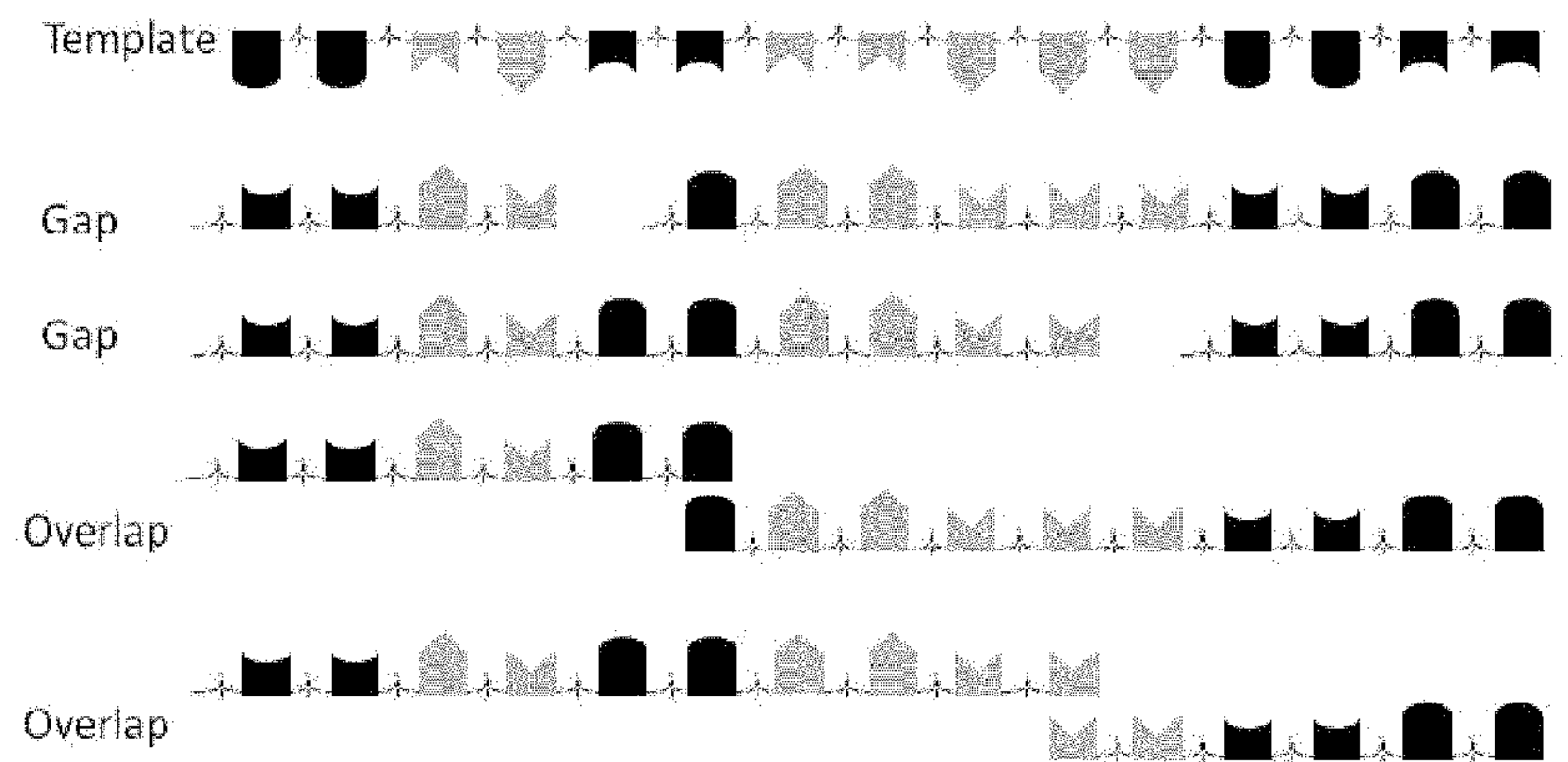


Figure 6

Gaps or overlaps arising from
missing or additional nucleotides



Impurities arising from missing
or additional nucleotides

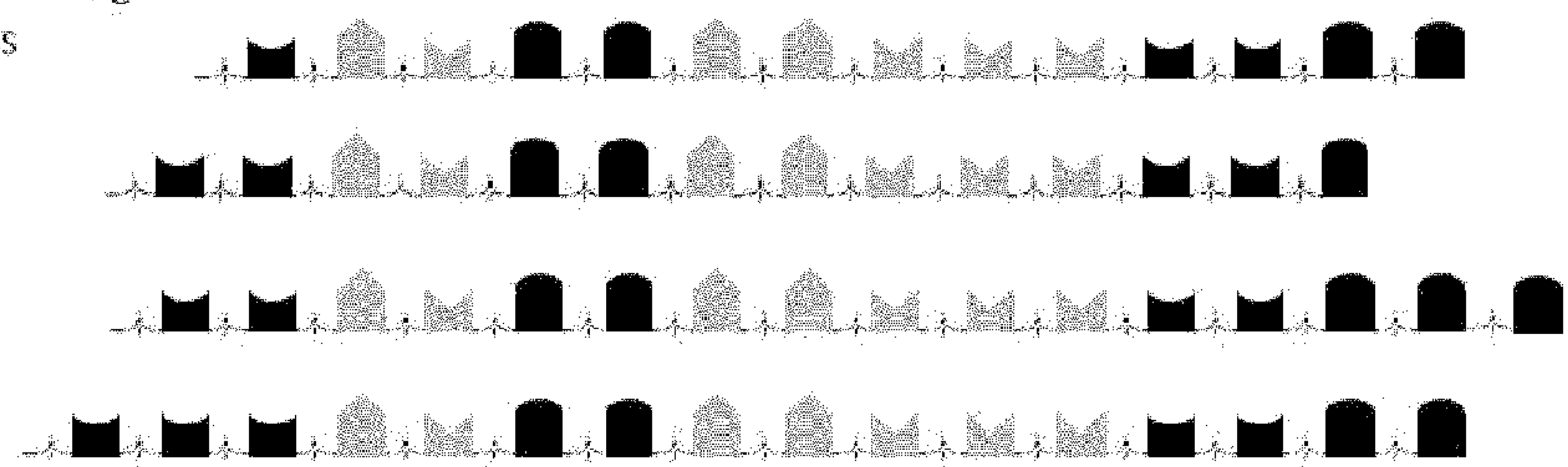


Figure 7

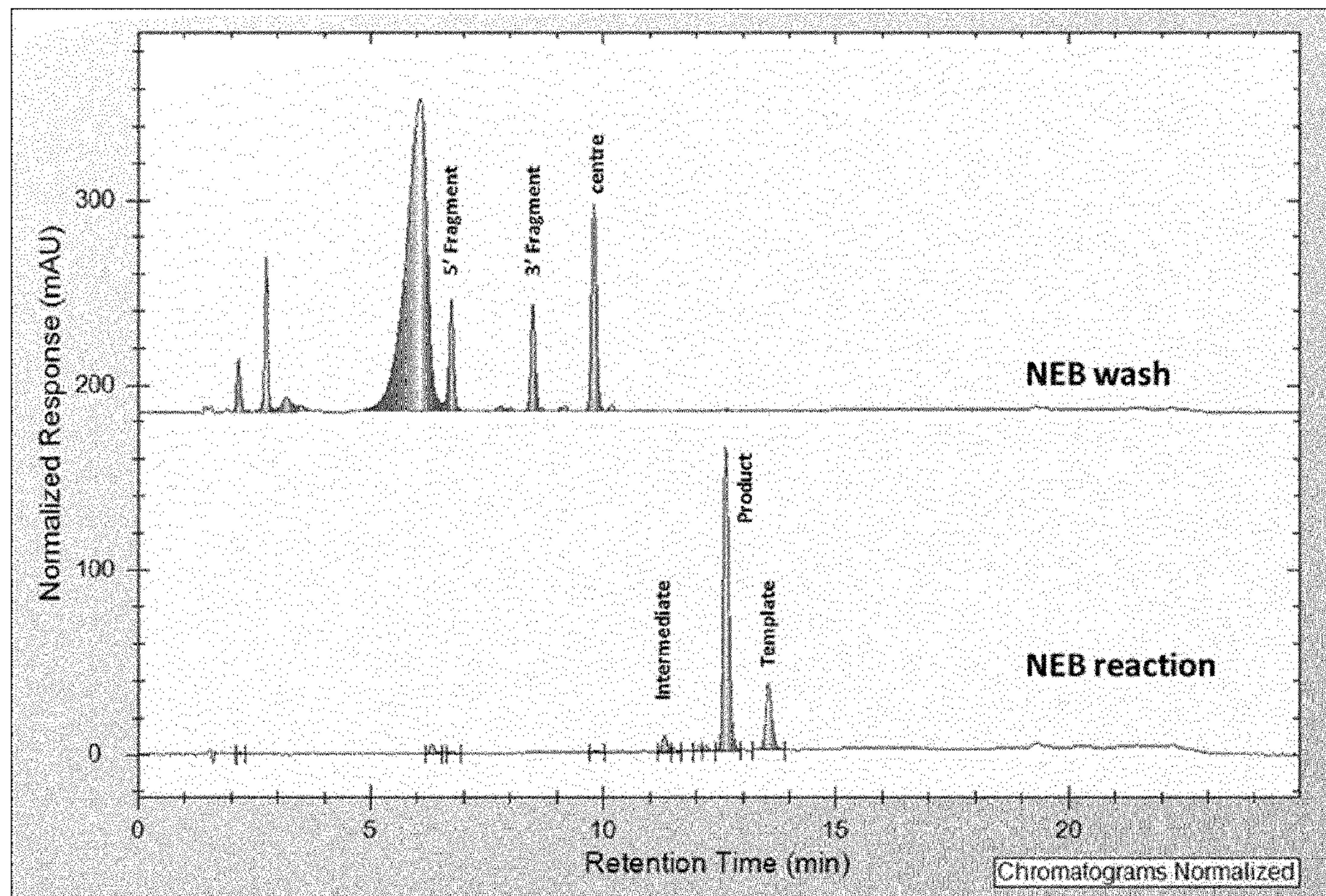


Figure 8

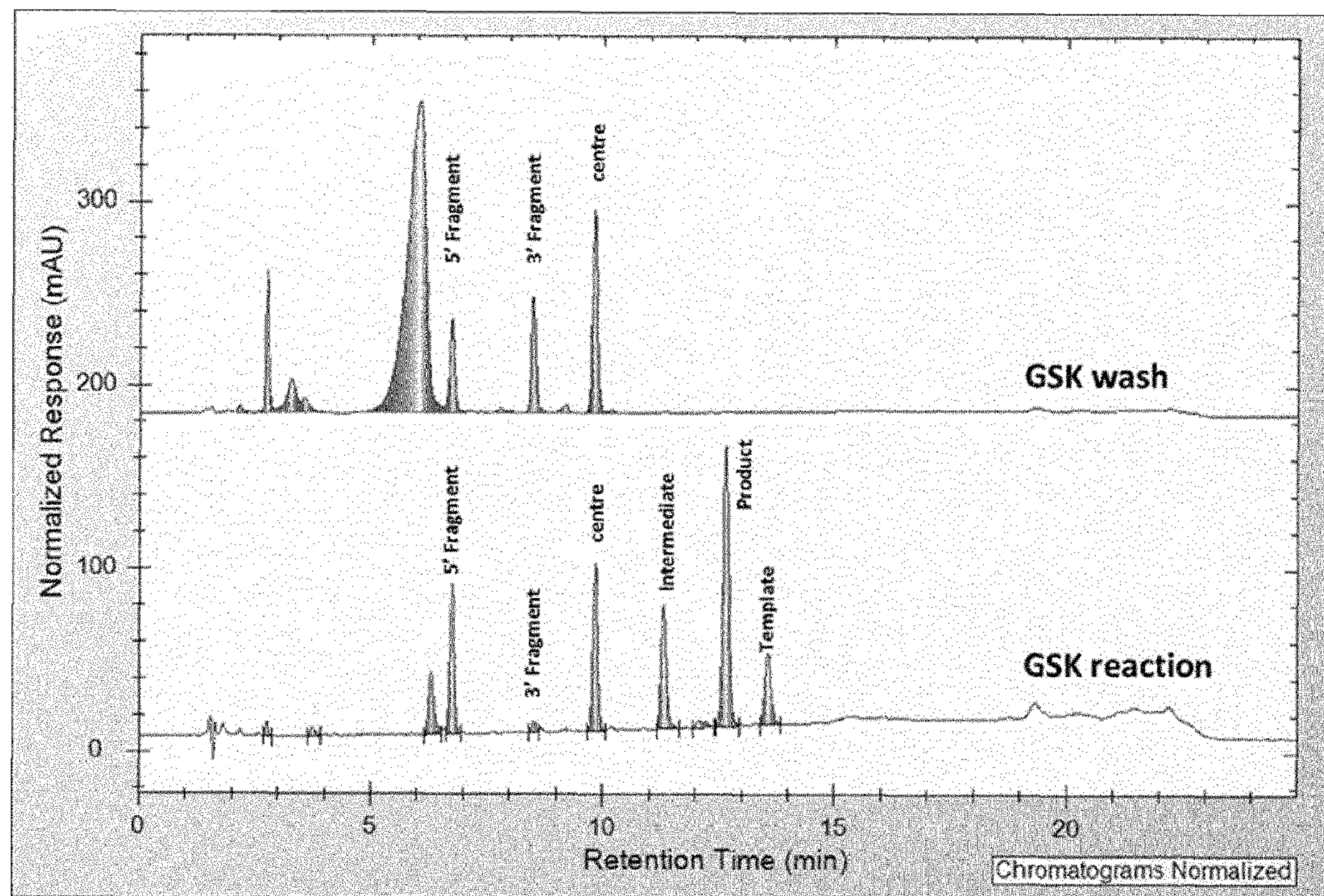
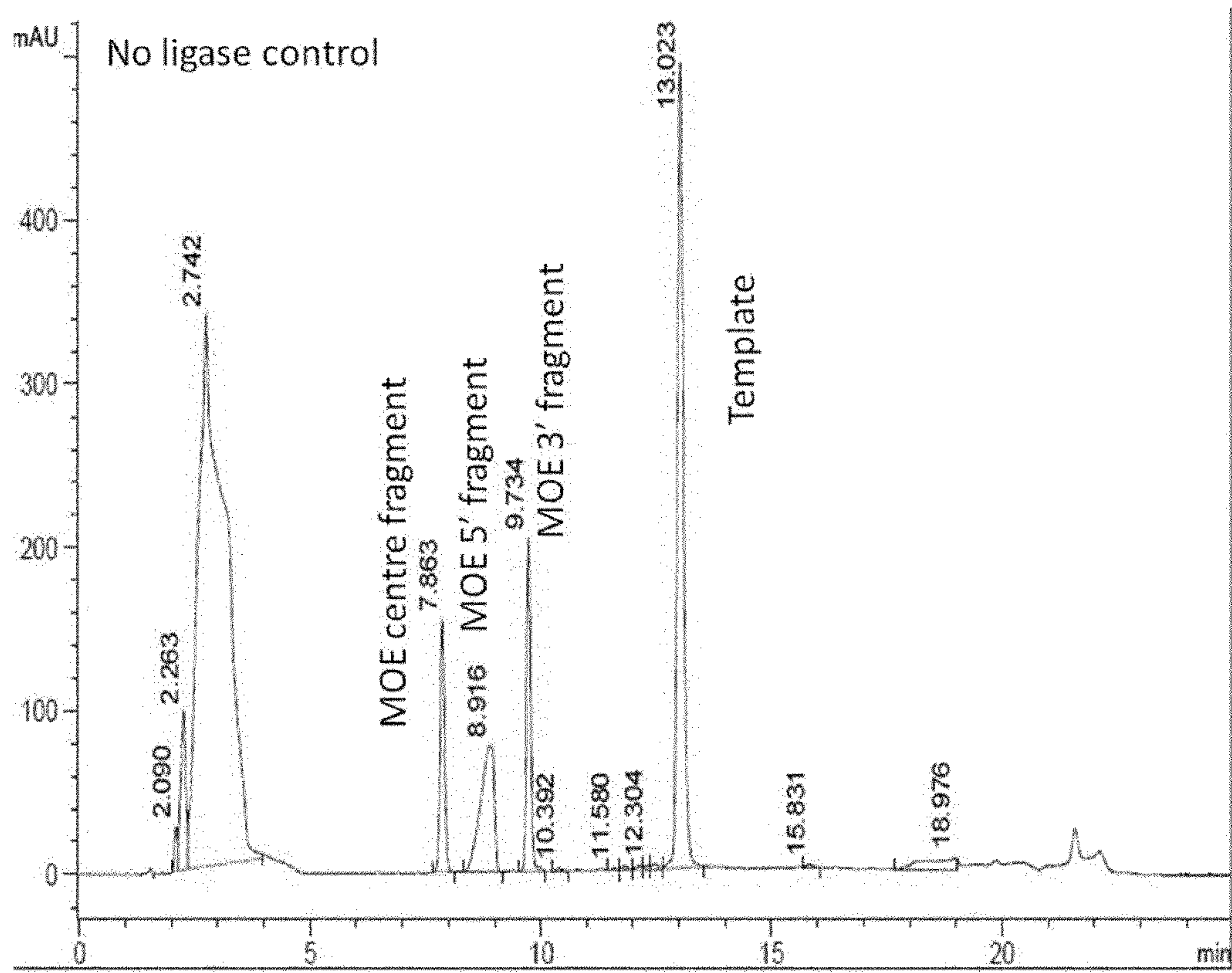


Figure 10

(a)



(b)

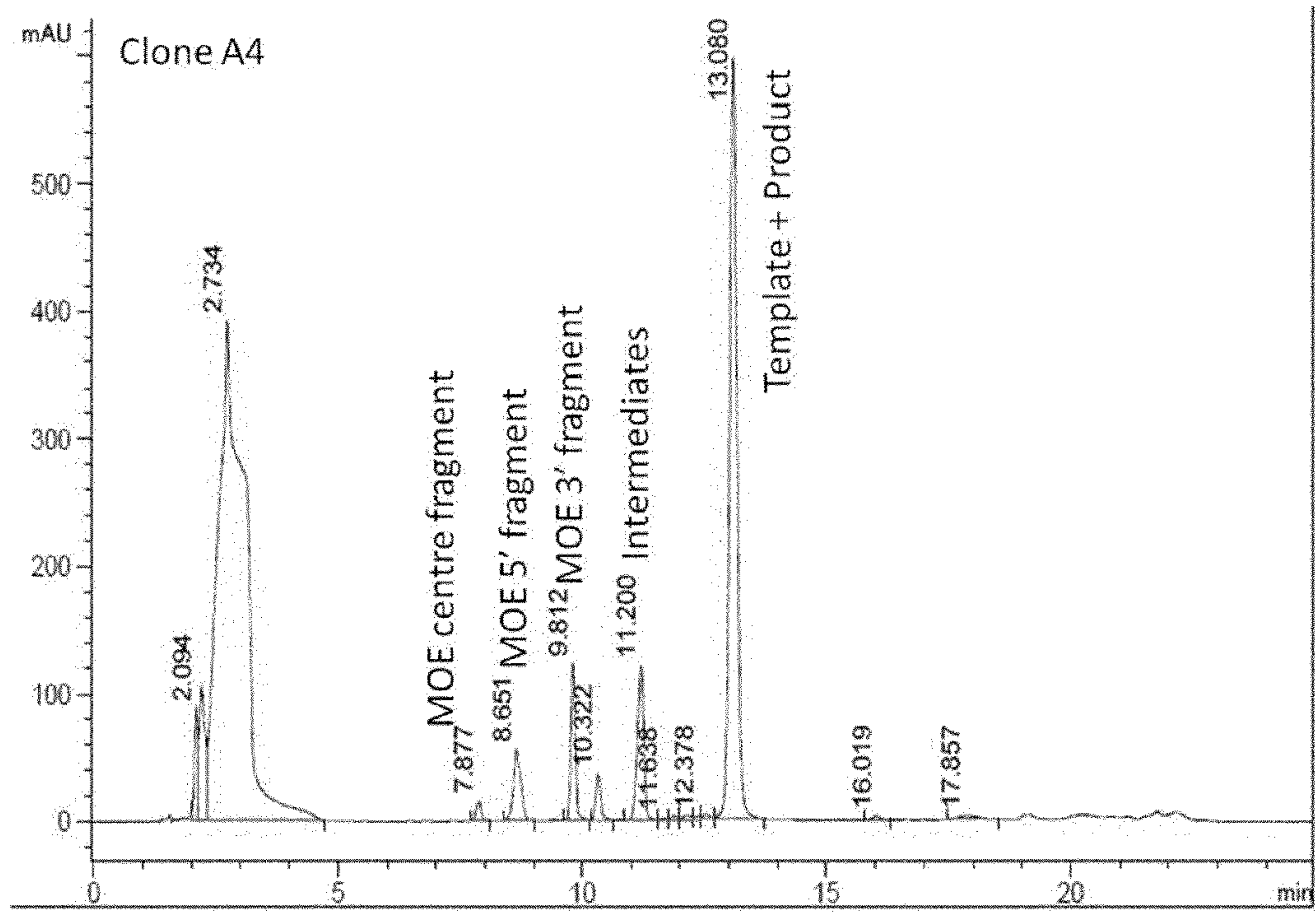


Figure 11

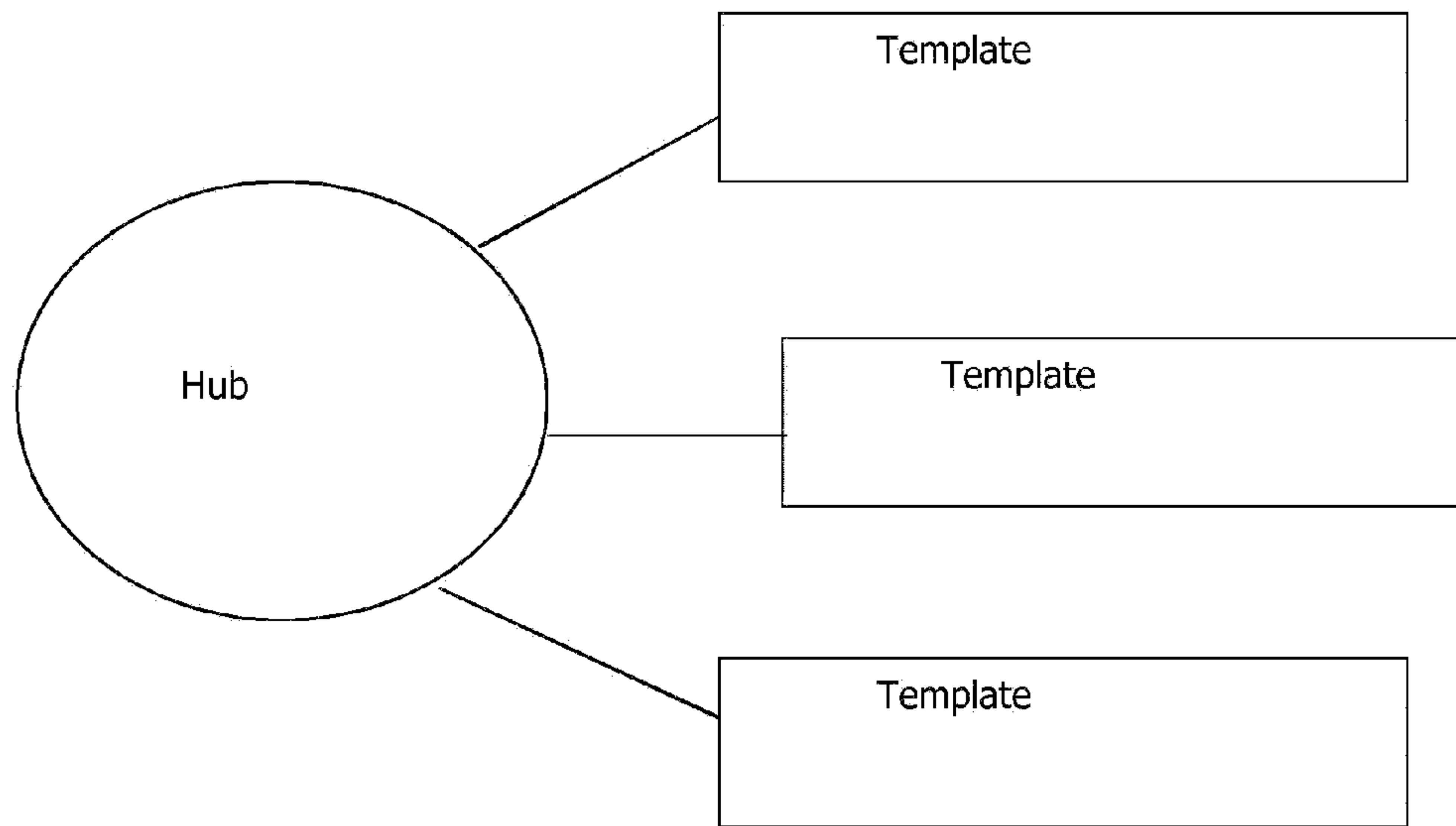


Figure 12

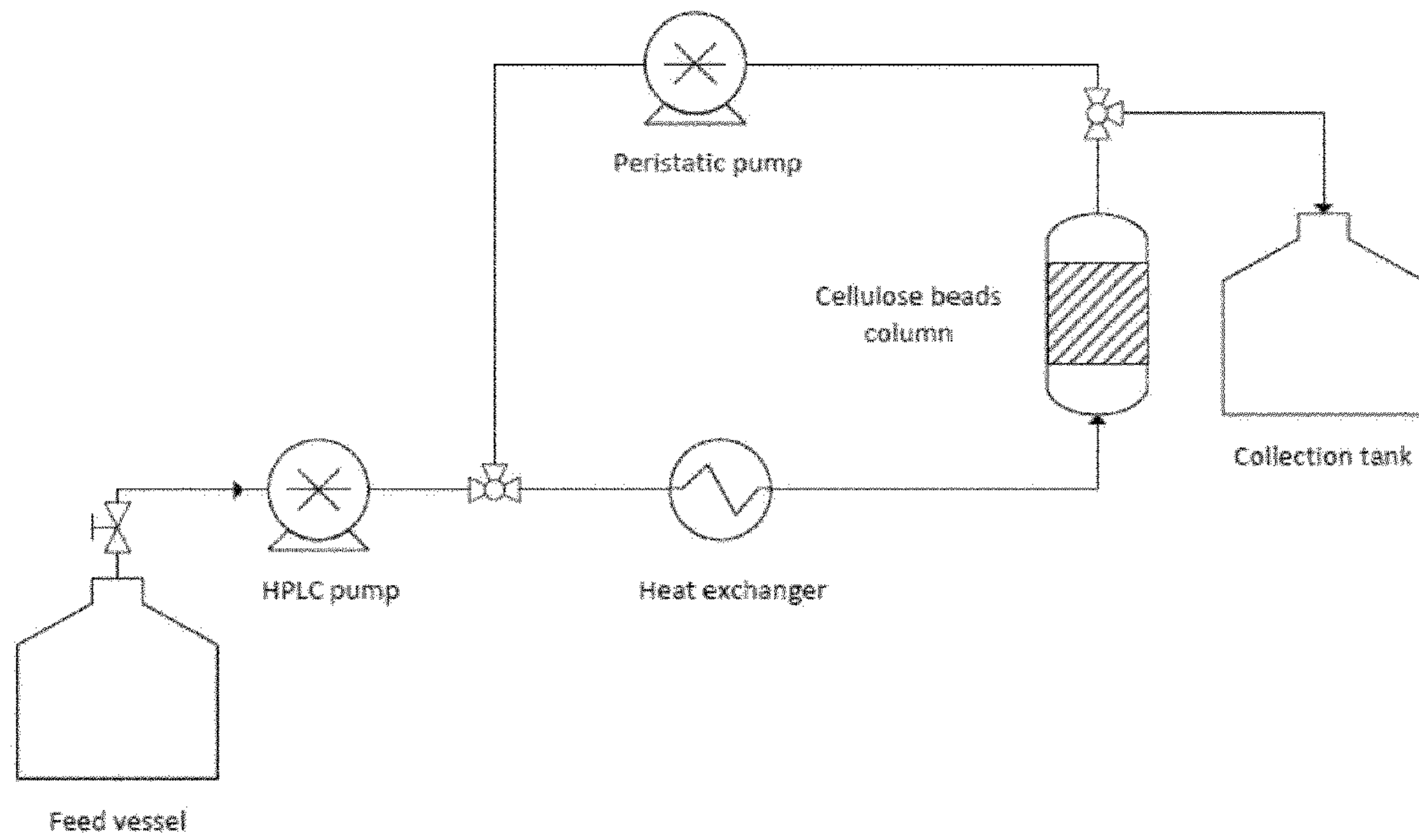


Figure 13

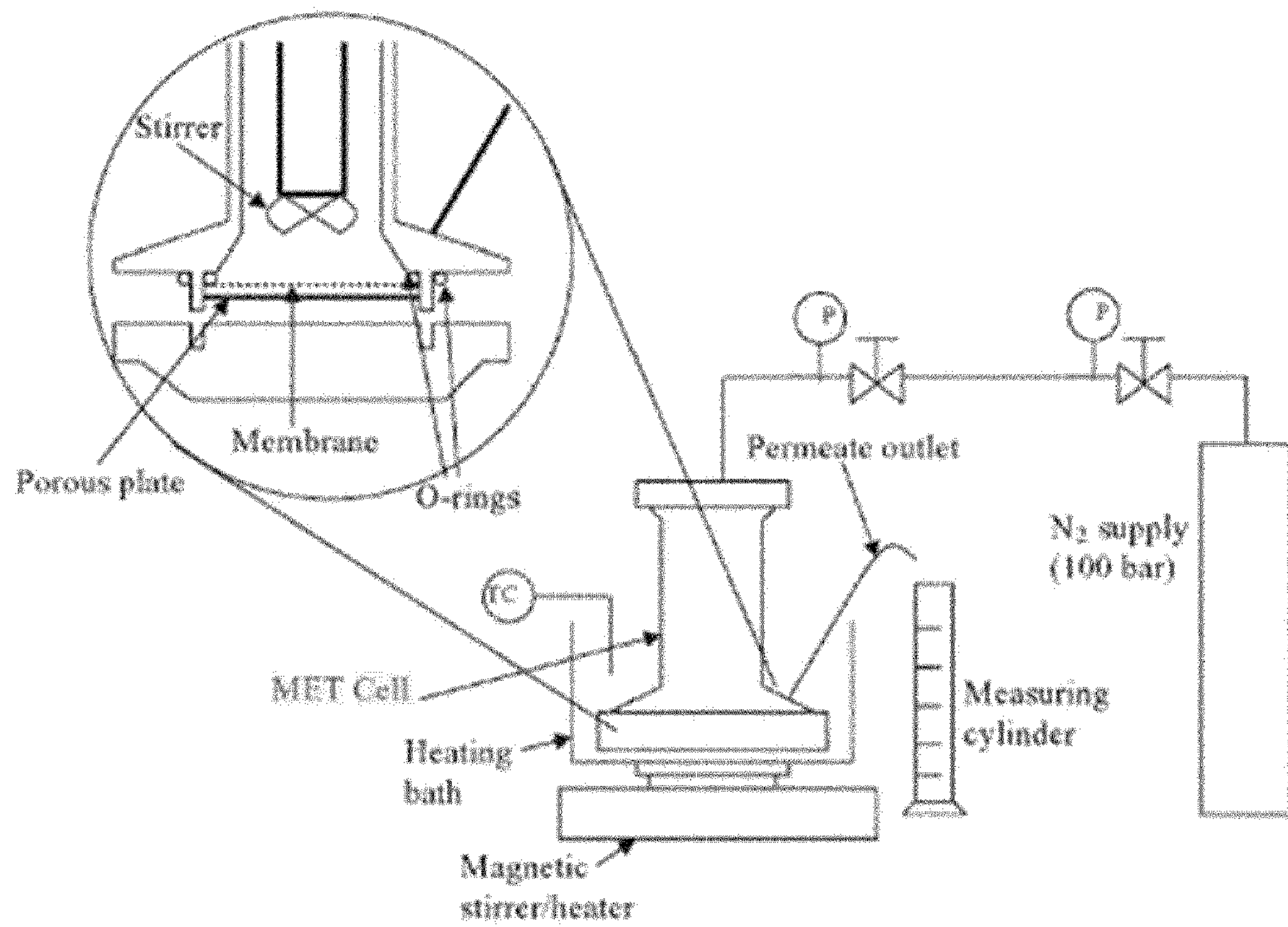


Figure 14

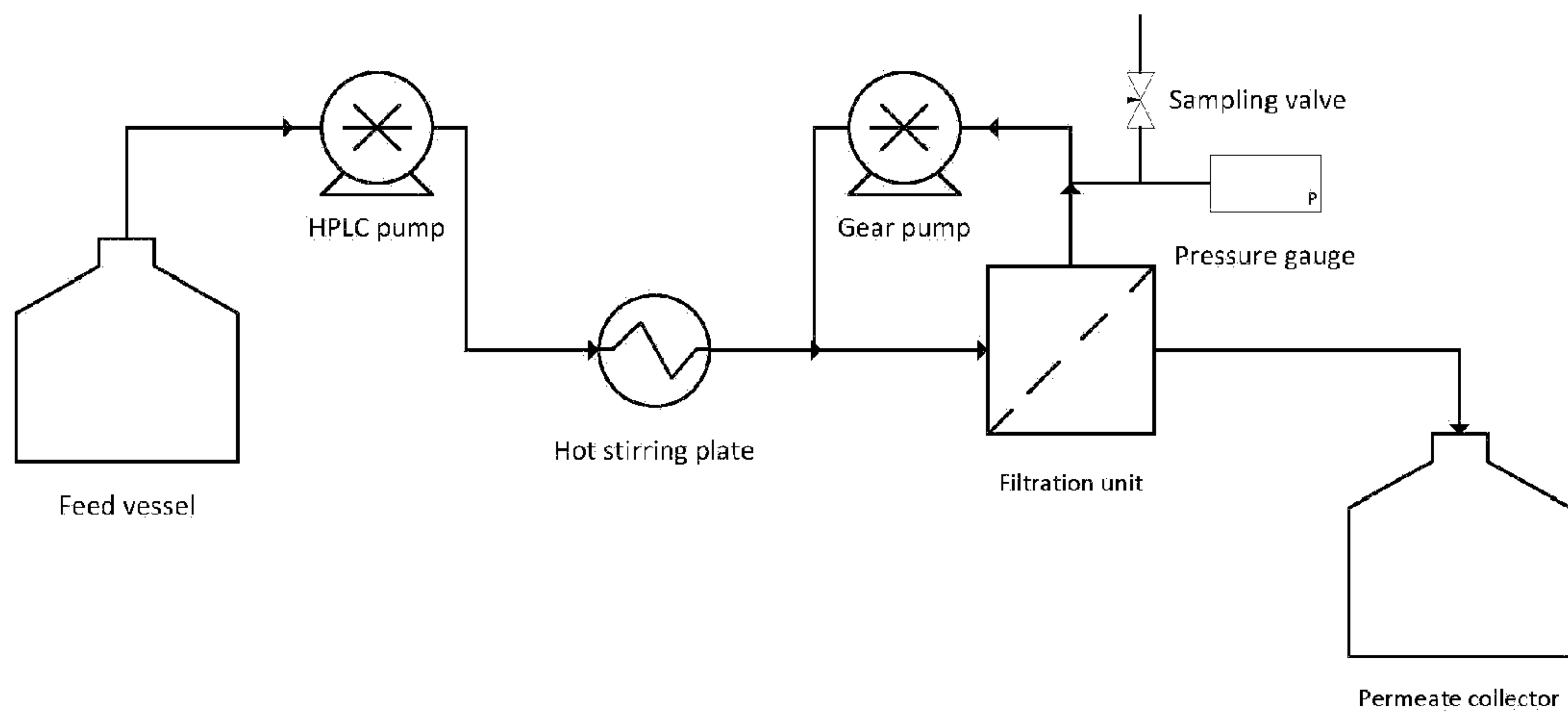
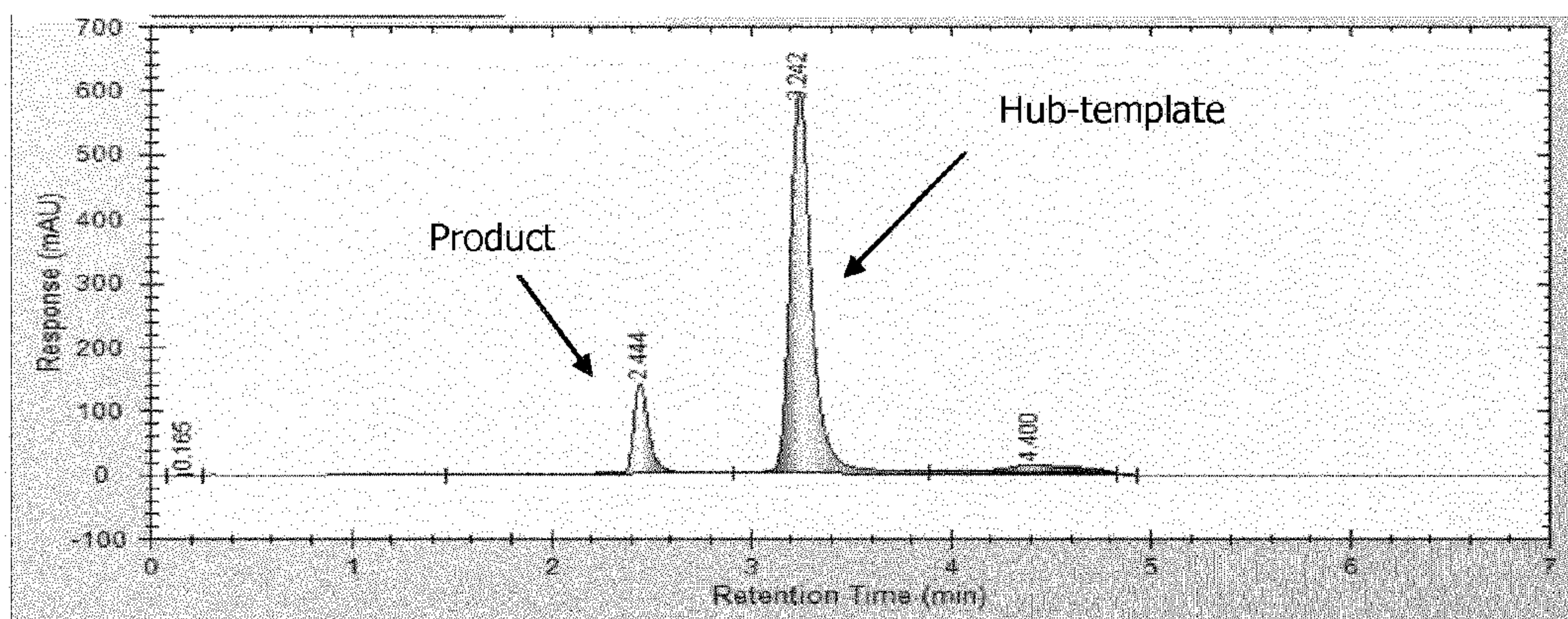


Figure 15

(a)



(b)

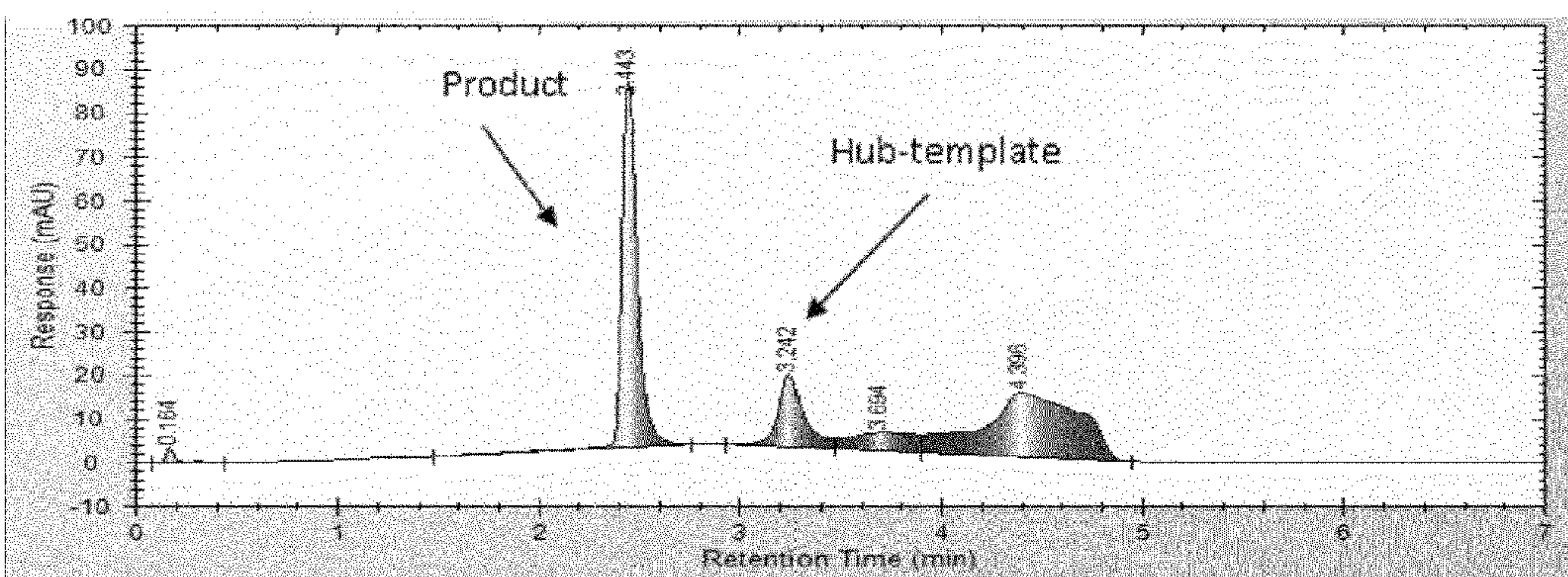
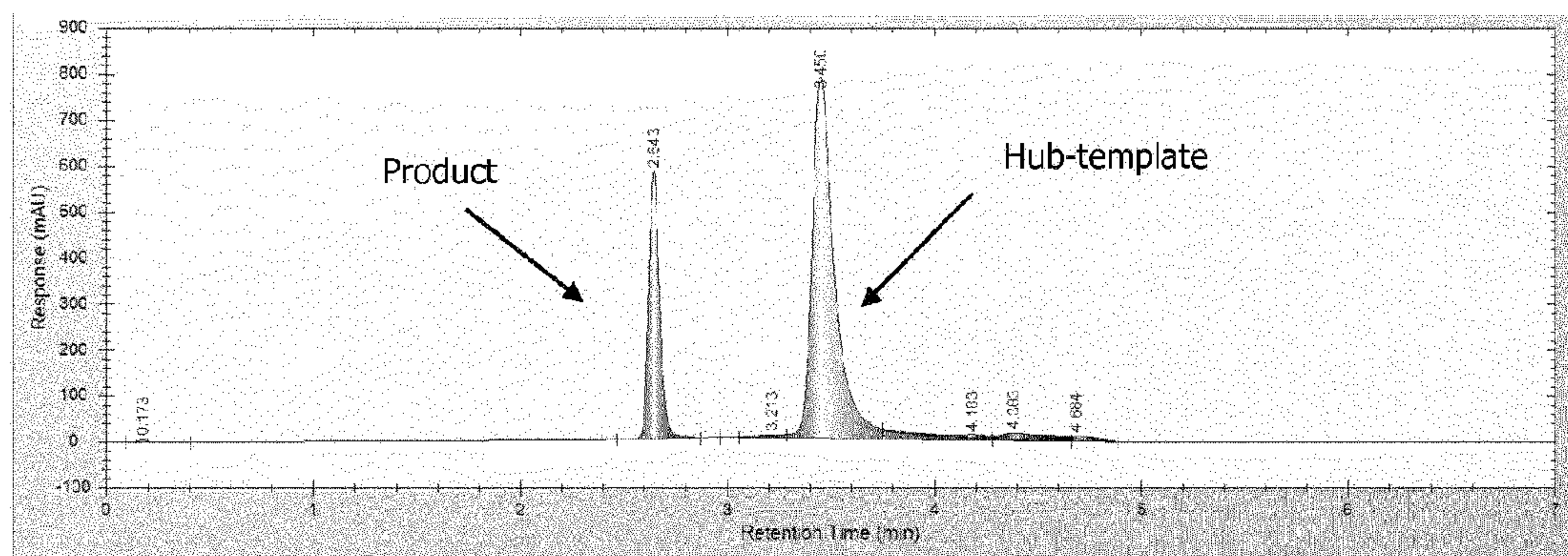


Figure 16

(a)



(b)

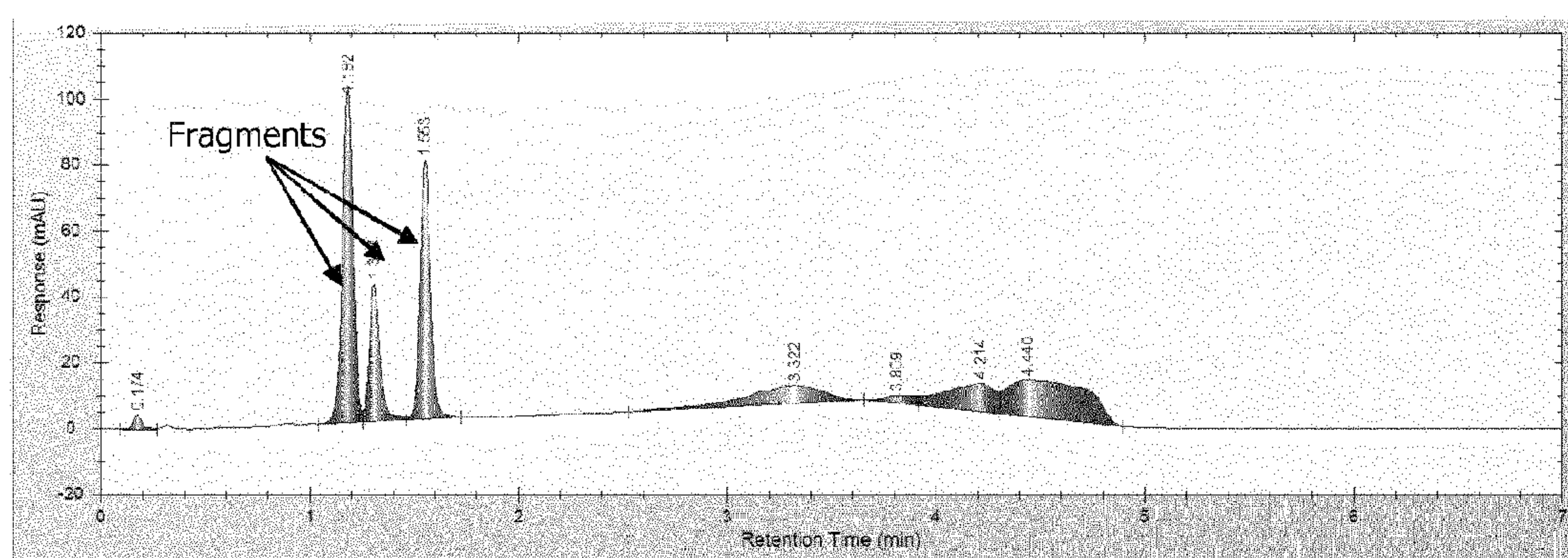
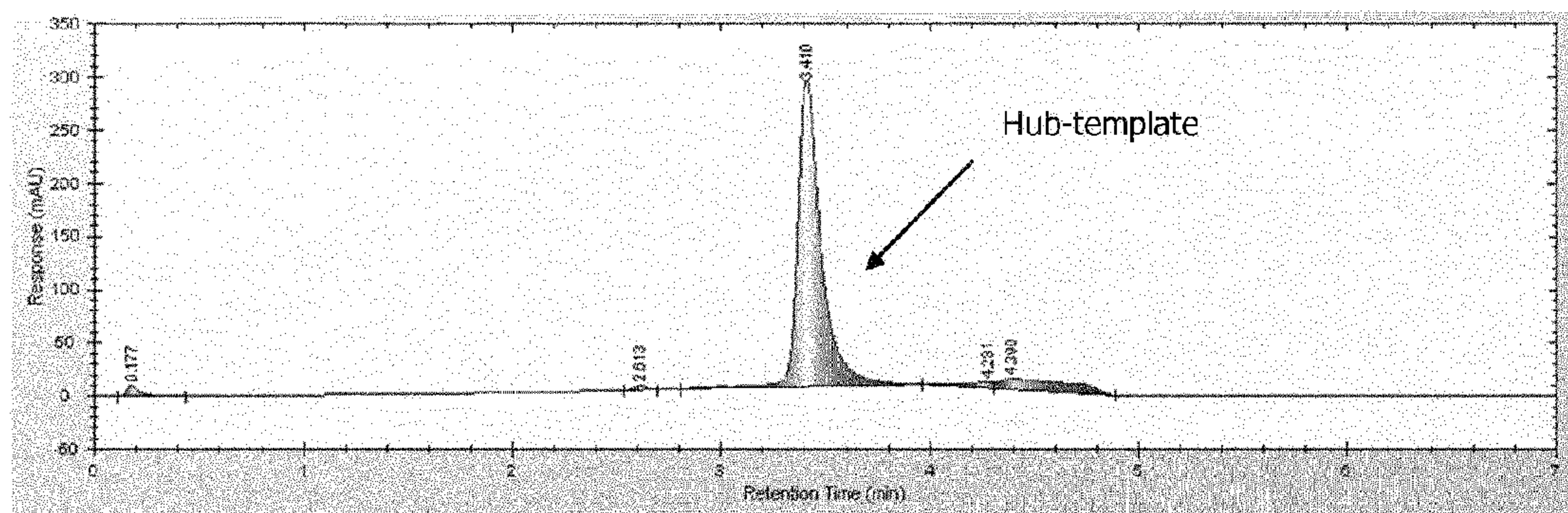


Figure 17

(a)



(b)

