NOVEL METHODS FOR THE PURIFICATION OF OLIGONUCLEOTIDES

FIELD OF THE INVENTION

The invention relates to novel methods for the purification of oligonucleotides, including single-stranded oligonucleotides, such as those used in therapy.

BACKGROUND TO THE INVENTION

Oligonucleotides are usually purified after synthesis by column chromatography. As solid supported synthesis is currently the usual method for commercial scale oligonucleotide synthesis, the capacity of the chromatography system is matched to the scale of the synthesis. This means that commercial oligonucleotide chromatography is operated in the 5 to 10 kg range. Increasing the scale of chromatography is possible to reach batch sizes of around 20 kg. However, there is a finite limit to how much the capacity of column chromatography can be increased due to limitations on column size, flow rates, homogeneity of column packing, resin compression, etc.

As oligonucleotide synthesis technology moves toward larger scale solid supported methods or, more likely, liquid phase synthesis, the size of a batch of crude oligonucleotide synthesized will exceed the capacity of a chromatography based purification method. Thus, any process gains made from increased synthesis batch size will be lost because of the scale limitation imposed by chromatography. For example, it may be possible to synthesize 100 kg of crude oligonucleotide in a single batch using liquid phase synthesis but this would then need to be split onto 5 batches for purification if there is a 20 kg limit on column chromatography. The gains in increased scale of synthesis would then be offset by the limit on chromatography batch size.

Column chromatography generates many fractions each with a slightly different composition of product and impurities. To obtain a final product that meets specification, each of these fractions needs to be analysed and a decision made on whether to add the fraction to the product pool or discard it. This creates a significant analytical burden and is very time consuming.

Additionally, chromatography of oligonucleotides has been identified as one of the biggest challenges for the sustainable manufacture of oligonucleotides due to the large volumes of waste generated by the chromatography process. This is particularly notable where reversed-phase chromatography purification is employed.

There is, therefore, a need to move away from chromatography-based methods for the purification of oligonucleotides to new methods of purification that are scalable, simpler, less labour intensive and more sustainable.

SUMMARY OF THE INVENTION

The invention provides a method of purifying a product, wherein the product is a single-stranded oligonucleotide, comprising:

(a) obtaining a solution comprising (i) an impurity:template complex and (ii) a product:template complex, wherein the template is an oligonucleotide comprising a sequence complementary to the product,

(b) subjecting the solution to ultrafiltration/diafiltration (UF/DF) to form a first retentate comprising the product:template complex and a first permeate comprising the impurity, wherein the UF/DF is carried out using a first buffer solution at a pH of about 9-13,

(c) subjecting the first retentate of step (b) to UF/DF to form a second retentate comprising the template and a second permeate comprising the product, wherein the UF/DF is carried out using a second buffer solution at a pH greater than the pH of the first buffer solution.

The invention also provides a single stranded oligonucleotide product obtained by such methods of purification.

The present invention disclosed herein provides the following advantages as compared to chromatography-based methods for the purification of oligonucleotides:

- Ultrafiltration is scalable from grams to hundreds of kgs, and even tons, without any fundamental constraint to the technology,
- The method is fundamentally simpler than chromatography involving no solid support and control of the process via simple stepwise change of buffer composition,
- Reduction in the number of fractions collected down to even just a single product fraction reducing the need for time consuming analysis of multiple fractions,
- Improved purity of the desired oligonucleotide product, and
- Improved sustainability as simple aqueous buffers are used, and these are potentially recyclable.

DESCRIPTION OF FIGURES

Figure 1 is a scheme showing a tangential flow filtration (TFF) system.

Figure 2 shows HPLC analysis of retentate during pH screening diafiltrations using K3PO4 buffers.

Figure 3 shows HPLC analysis of retentate during pH screening diafiltrations using Na3PO4 buffers.

Figure 4 shows HPLC analysis of retentate during diafiltration with 200 mM K3PO4, pH 12.5.

Figure 5 shows HPLC analysis of permeation of 13-mer intermediate during pH 11 diafiltration with 200 mM or 300 mM K3PO4.

Figure 6 shows HPLC analysis of 20-mer product and hub-template during further purification in Experimental 2D (-8 to 0 diavolumes being concentration, and 0 to 14 diavolumes being diafiltration to recover 20-mer).

Figure 7 is a scheme showing UF/DF purification process of a single-stranded oligonucleotide (20-mer).

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Unless otherwise explained, all technical and scientific terms used herein have the same meaning as commonly understood by one skilled in the art to which this disclosure belongs. The singular terms "a," "an," and "the" include plural referents unless context clearly indicates otherwise. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. The term "plurality" refers to two or more. The term "at least one" refers to one or more.

Additionally, numerical limitations given with respect to concentrations or levels of a substance, such as solution component concentrations or ratios thereof, and reaction conditions such as temperatures, pressures, and cycle times are intended to be approximate. Unless specified otherwise, where a numerical range is provided, it is inclusive, *i.e.*, the endpoints are included.

"About" as used herein when referring to a measurable value such as an amount and the like, is meant to encompass variations of ±20% or ±10%, including ±5%, ±1 %, and ±0.1 % from the specified value, as such variations are appropriate to perform the disclosed methods.

"Purification" or "purifying" herein means the process of removing components, *e.g.* impurities, from a solution, the presence of which is not desired. In other words, purification means increasing the degree of purity of the desired oligonucleotide product. Purification is a relative term and does not require that all traces of the undesirable component be removed from the composition. Thus, the term "purified" does not require absolute purity; rather, it is intended as a relative term. A preparation of purified oligonucleotide product can be purified such that the desired oligonucleotide product, represents at least 50% of the total oligonucleotide content of the preparation. In certain embodiments, a purified oligonucleotide product, will represent at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, or at least 95% or more of the total oligonucleotide content of the preparation.

"Ultrafiltration" or "UF" refers to the process of separating substances by passing a solution through a semi-permeable membrane or filter of a specified molecular weight cut off (MWCO) or a specific pore size diameter, wherein larger molecular weight molecules are retained in the retentate, while lower molecular weight molecules pass through the membrane. These lower molecular weight

molecules may be a product and/or impurity (*e.g.* shortmers or intermediates). For the purification methods disclosed herein, during ultrafiltration, the template may be in a form allowing it to be substantially retained in the retentate, while the product and/or impurity may be substantially in the permeate. "Permeate" refers to the fraction of the solution that passes through the membrane during ultrafiltration. "Retentate" refers to the fraction of the solution that does not pass through the membrane during ultrafiltration. Suitable types of UF apparatuses are known to those in the art and can be selected based on various factors, *e.g.*, the molecular weight of the product to be filtered, the amount and size of the components of the solution to be filtered, and the volume of the solution to be filtered.

"Ultrafiltration/Diafiltration" or "UF/DF" refers to an ultrafiltration process whereby the separation is combined with a step of adding a diafiltration medium to the retentate. For example, UF/DF allows a solution to be concentrated first and then its buffer system to be exchanged. Additional additives can be introduced, for example to adjust pH and/or ionic strength. A distinction may be made between two basic types of UF/DF: the variable volume UF/DF (sometimes referred to as "discontinuous" UF/DF in the art) and the constant volume UF/DF (sometimes referred to as "continuous" UF/DF in the art).

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 present disclosure may be conjugated to another chemical moiety, *e.g.* a targeting moiety containing N-Acetylgalactosamine (GalNAc) or multiples thereof (GalNAc clusters).

An oligonucleotide may be entirely composed of nucleotide residues as found in nature (*i.e.* "natural nucleotides" or "naturally occurring nucleotides") or may contain at least one modified nucleotide, or at least one linkage between nucleotides that has been modified. Examples of naturally occurring nucleotides include deoxyadenosine monophosphate, deoxycytidine monophosphate, deoxyguanosine monophosphate, deoxythymidine monophosphate, deoxyuridine monophosphate, adenosine monophosphate, cytidine monophosphate, guanosine monophosphate, thymidine monophosphate and uridine monophosphate. A modified nucleotide is not a naturally occurring nucleotide (*i.e.* it is a non-natural nucleotide). Modified nucleotides may comprise modified backbones, sugars, and/or nucleobases. It is acknowledged that certain modifications occur sporadically in nature, *i.e.* in naturally occurring nucleotides, such as 2'-OMe or C5 pyrimidine modifications, however, in the present disclosure these are considered modified nucleotides.

As used herein, the term "therapeutic oligonucleotide" means an oligonucleotide that has a therapeutic application, *e.g.* in the prevention or treatment of a condition or disease in a human or

animal. 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).

As used herein, the term "template" means an oligonucleotide that comprises a sequence complementary to the product as described herein. The product may have a desired sequence and the template comprises a sequence which is complementary to the desired sequence. The template can comprise a sequence which is complementary to the sequence of the product. The template can consist of a sequence that is complementary to the sequence of the product. The template can have a longer sequence compared to the product sequence. Unless otherwise specified, as used herein, the term "complementary" means 100% complementary along the entire length of the product.

As used herein, the term "product" means the desired single-stranded oligonucleotide to be purified by the methods disclosed in the present disclosure.

As used herein, the term "complex" in relation to oligonucleotides means two or more oligonucleotides that are bound to each other in a sequence specific manner. It is known that binding may occur despite some nucleotide mismatch. A complex may be formed by annealing two or more oligonucleotides. "Annealing" means the pairing of an oligonucleotide with another oligonucleotide by hydrogen bonding to form a double-stranded oligonucleotide. Specific conditions may be required for annealing to occur. A complex may be formed by hybridisation of two or more oligonucleotides. An impurity:template complex means one or more impurity bound to a template. An impurity:template complex may include one or more shortmers bound to a template. An impurity:template complex may include an intermediate bound to a template. An impurity:template complex may include an intermediate partially bound to a template (for example, where the intermediate comprises an end shortmer or segment that does not bind to the template). An impurity:template complex may include one or more intermediates and one or more shortmers bound to a template. An impurity:template complex may include an oligonucleotide bound to a template, wherein the oligonucleotide is shorter than the product. An impurity:template complex may include an oligonucleotide bound to a template, wherein the oligonucleotide has the same length as the product but comprises a mismatch(es). A product:template complex means a product bound to a template.

As used herein, the term "denaturing" in relation to a complex refers to the process where the two or more bound oligonucleotides become dissociated. Denaturing occurs as a result of changing the conditions and is sometimes referred to herein as separating the oligonucleotides. Such separation can be done for example, by raising the temperature, changing the pH, or changing the salt concentration of the solution, or a combination of these changes. Denaturing a "complex" results in a oligonucleotide which could be a product or impurity being "released" from the template.

As used herein, the term "impurity" or "impurities" means the oligonucleotides that do not have the sequence of the desired product. Where a product is produced by sequential addition of

single nucleotides, e.g. solid phase synthesis, impurities may arise when the chain extension reaction terminates early. These impurities may include oligonucleotides that are shorter than the product (for example 1, 2, 3, 4, 5 or more nucleotide residues shorter). Where the production process for the product includes a step whereby linkages are formed between shortmers or segments, an intermediate may be produced if one or more of the linkages between shortmers or segments fail to form. The intermediate may comprise two or more shortmers or segments. The impurity may include a shortmer. The impurity may include an adenylated shortmer. The impurity may include an intermediate. Impurities also include oligonucleotides where incorrect nucleotides have been incorporated, resulting in a mismatch. Therefore, the impurity may be the same length as the product, but have incorrect sequence compared to the product.

As used herein, the term "shortmer" or "segment" is a smaller portion of a longer oligonucleotide, in particular a smaller portion of a product. For a given product, when all of its corresponding shortmers are ligated together, the product is formed.

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).

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 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.

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.

As used herein, the term "support material" means a chemical compound or material that is linked to one or more templates, which increases the molecular weight of the resulting template-

containing structure, thereby allowing the template to be retained in the retentate during an ultrafiltration process. The resulting template-containing structure may be referred to as "hub-template."

STATEMENT OF THE INVENTION

The disclosure provides a method of purifying a product, wherein the product is a single-stranded oligonucleotide, comprising:

(a) obtaining a solution comprising (i) an impurity:template complex and (ii) a product:template complex, wherein the template is an oligonucleotide comprising a sequence complementary to the product,

(b) subjecting the solution to ultrafiltration/diafiltration (UF/DF) to form a first retentate comprising the product:template complex and a first permeate comprising the impurity, wherein the UF/DF is carried out using a first buffer solution at a pH of about 9-13,

(c) subjecting the first retentate of step (b) to UF/DF to form a second retentate comprising the template and a second permeate comprising the product, wherein the UF/DF is carried out using a second buffer solution at a pH greater than the pH of the first buffer solution.

Such a method may be used to purify a product, *e.g.* single-stranded oligonucleotide produced by chemical synthesis via phosphoramidite chemistry, *e.g.* solid phase chemical synthesis via phosphoramidite chemistry, from impurities. Such a method may be used to purify a product, *e.g.* single-stranded oligonucleotide synthesized by a ligation reaction catalysed by an enzyme, *e.g.* synthesized according to the methods described in WO 2018/011067 and WO 2019/121500, from impurities. Releasing the impurity from the impurity:template complex requires denaturation of the impurity:template complex. The impurity can then be separated from the template. Releasing the product from the product:template complex requires denaturation of the product:template complex. The product can then be separated from the template. Denaturation of the complexes can be achieved by altering the pH of the buffer solution.

The properties of Watson-Crick base pairing can be exploited to specifically release impurity from the template prior to the release of the product from the template. The conditions required for impurity:template complex denaturation can be different from the conditions required for product:template complex denaturation. The differentiating denaturation conditions are caused by the difference in the strength of binding; *e.g.* the binding of the product to the template is stronger than the binding of the impurity to the template. The incorrect nucleotides and/or the shorter length of the impurity reduces the binding strength to the template. The pH may be adjusted to cause only denaturation of the impurity:template complex but not that of the product:template complex. The

impurity can be separated. Then the pH may be increased to cause denaturation of the product:template complex. The product can be separated.

In one embodiment, UF/DF of step (b) is carried out using a first buffer solution at a pH greater than or equal to 8. In one embodiment, UF/DF of step (b) is carried out using a first buffer solution at a pH greater than or equal to 9. In one embodiment, UF/DF of step (b) is carried out using a first buffer solution at a pH of about 9-13. In one embodiment, the pH of the first buffer solution is about 9-12.5, about 9-12, about 9-11.5, about 9-11, about 10-12.5, about 10-12, about 10-11.5, about 10-11 or about 10.5-11.5. In one embodiment, the pH of the first buffer solution is 9.5-12.5. In one embodiment, the pH of the first buffer solution is about 9-12.5. In one embodiment, the pH of the first buffer solution is about 9-12. In one embodiment, the pH of the first buffer solution is about 9-11.5. In one embodiment, the pH of the first buffer solution is about 9-11. In one embodiment, the pH of the first buffer solution is 9.5-11.5. In one embodiment, the pH of the first buffer solution is about 10-12.5. In one embodiment, the pH of the first buffer solution is about 10-12. In one embodiment, the pH of the first buffer solution is about 10-11.5. In one embodiment, the pH of the first buffer solution is about 10-11. In one embodiment, the pH of the first buffer solution is about 11.5-12.5. In one embodiment, the pH of the first buffer solution is about 10.5-11.5. In one embodiment, UF/DF of step (b) is carried out using a first buffer solution, wherein the pH of the first buffer solution is selected to specifically denature the impurity:template complex. In one embodiment, UF/DF is carried out using a first buffer solution, wherein the pH of the first buffer solution releases the impurity from the template. In one embodiment, UF/DF is carried out using a first buffer solution, wherein the pH of the first buffer solution does not denature the product:template complex.

In one embodiment, UF/DF of step (c) is carried out using a second buffer solution at a pH greater than the pH of the first buffer solution. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9 or about 2.0. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.4. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.5. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.6. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.7. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.8. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.9. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.0. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.1. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.2. In one embodiment, the pH of the second buffer solution is greater

than the pH of the first buffer solution by about 1.3. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.4. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.5. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.6. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.7. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.8. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 1.9. In one embodiment, the pH of the second buffer solution is greater than the pH of the first buffer solution by about 2.0.

In one embodiment, the pH of the second buffer solution is about 10-14, about 10-13.5, about 10-13, about 11-14, about 11-13.5, about 11-13, about 11-12.5, about 11-12 or about 11.5-12.5. In one embodiment, the pH of the second buffer solution is about 10-14. In one embodiment, the pH of the second buffer solution is about 10-13.5. In one embodiment, the pH of the second buffer solution is about 10-13. In one embodiment, the pH of the second buffer solution is about 10-12.5. In one embodiment, the pH of the second buffer solution is about 11-14. In one embodiment, the pH of the second buffer solution is about 11-13.5. In one embodiment, the pH of the second buffer solution is about 11-13. In one embodiment, the pH of the second buffer solution is about 11-12.5. In one embodiment, the pH of the second buffer solution is about 11-12. In one embodiment, the pH of the second buffer solution is about 11.5-12.5. In one embodiment, the pH of the second buffer solution is about 12-13.5. In one embodiment, UF/DF of step (c) is carried out using a second buffer solution, wherein the pH of the second buffer solution is selected to specifically denature the product:template complex. In one embodiment, UF/DF is carried out using a second buffer solution, wherein the pH of the second buffer solution releases the product from the template. The skilled person will appreciate that the product:template complex denatures at a higher pH compared to the impurity:template complex.

In one embodiment, the first buffer solution and/or the second buffer solution comprises CABS buffer. In one embodiment, the first buffer solution and/or the second buffer solution comprises CAPS buffer. In one embodiment, the first buffer solution and/or the second buffer solution comprises an AMP buffer. In one embodiment, the first buffer solution and/or the second buffer solution comprises a CAPSO buffer. In one embodiment, the first buffer solution and/or the second buffer solution comprises a carbonate buffer. In one embodiment, the first buffer solution and/or the second buffer solution comprises a bicarbonate buffer. In one embodiment, the first buffer solution and/or the second buffer solution comprises a phosphate buffer. Depending on the pH required to denature the impurity:template complex and/or to denature the product:template complex, an appropriate buffer may be selected, respectively.

The skilled person knows that a buffer solution is a solution where the pH does not change significantly on dilution or if an acid or base is added at constant temperature. Buffer solutions resist pH change because of a chemical equilibrium between the acid and its conjugate base.

In the case of phosphate buffers, phosphoric acid (H_3PO_4) is a triprotic acid which undergoes a stepwise dissociation to form $H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}.

Phosphate acts best as a buffer in the regions of pH near its three pK's: pK_1 = 2.12, pK_2 = 7.21; and pK_3 = 12.44. The "Henderson Hasselbalch Equation" is a convenient form to use in buffer calculations, particularly when the pH is within one unit above or one unit below the pK. Taking tribasic phosphate buffers as an example, at pH 12.44 ± 1 (pH 11.44 – 13.44), the ratio of the two predominant species in the buffer, HPO_4^{2-} and PO_4^{3-} can be calculated.

The skilled person understands that a buffer with the required pH can be prepared via multiple ways.

For example, the buffer solution may be prepared from 300 mM tripotassium phosphate and titrated to the desired pH with an acid or a base. The resulting buffer solution at the desired pH may comprise other phosphate species in addition to tripotassium phosphate, e.g. dipotassium phosphate. As used herein, the term "300 mM tripotassium phosphate buffer" with a specified pH refers to a buffer solution prepared by adding an acid (e.g. H_3PO_4) to 300 mM tripostassium phosphate until the specified pH is reached. Tripotassium phosphate is also known as tribasic potassium phosphate. Trisodium phosphate is also known as tribasic sodium phosphate.

Alternatively, the same buffer solution may be prepared by mixing in water the two predetermined phosphate salts, *i.e.* dipotassium phosphate and tripotassium phosphate, in the amounts calculated from Henderson Hasselbalch Equation, to achieve the specified pH.

In one embodiment, the first buffer solution and/or second buffer solution comprises a phosphate buffer. In one embodiment, the first buffer solution comprises a phosphate buffer. In one embodiment, the second buffer solution comprises a phosphate buffer. In one embodiment, the first buffer solution and/or second buffer solution comprises potassium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises sodium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises dipotassium phosphate, tripotassium phosphate, disodium phosphate, and/or trisodium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises dipotassium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises tripotassium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises disodium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises trisodium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises tripotassium phosphate or trisodium phosphate. In one embodiment, the first buffer solution comprises sodium phosphate and the second buffer solution also comprises sodium phosphate. In one embodiment, the first buffer solution comprises potassium phosphate and the

second buffer solution also comprises potassium phosphate. In one embodiment, the first buffer solution comprises trisodium phosphate and the second buffer solution also comprises trisodium phosphate. In one embodiment, the first buffer solution comprises tripotassium phosphate and the second buffer solution also comprises tripotassium phosphate. In one embodiment, the first buffer solution comprises potassium phosphate and the second buffer solution comprises sodium phosphate. In one embodiment, the first buffer solution comprises tripotassium phosphate and the second buffer solution comprises trisodium phosphate.

In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 1000 mM, about 100 to about 800 mM, about 100 to about 600 mM, about 100 to about 500 mM, about 100 to about 450 mM, about 100 to about 400 mM, about 100 to about 350 mM, about 150 to about 300 mM, about 150 to about 350 mM, about 200 to about 500 mM, about 200 to about 400 mM, about 200 to about 350 mM, about 200 to about 300 mM, about 250 to about 500 mM, about 250 to about 400 mM, about 250 to about 350 mM phosphate solution.

In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 1000 mM, about 100 to about 800 mM, about 100 to about 600 mM, about 100 to about 500 mM, about 100 to about 450 mM, about 100 to 400 mM, about 100 to 350 mM, about 150 to 300 mM, about 150 to about 350 mM, about 200 to about 500 mM, about 200 to about 400 mM, about 200 to about 350 mM, about 200 to about 300 mM, about 250 to about 500 mM, about 250 to about 400 mM, about 250 to about 350 mM trisodium phosphate.

In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 500 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 450 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 400 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 150 to about 350 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 500 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from 200 to about 450 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 400 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 350 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 300 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 250 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 300 mM trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 350 mM

trisodium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 400 mM trisodium phosphate.

In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 1000 mM, about 100 to about 800 mM, about 100 to about 600 mM, about 100 to about 500 mM, about 100 to about 450 mM, about 100 to about 400 mM, about 100 to about 350 mM, about 150 to about 300 mM, about 150 to about 350 mM, about 200 to about 500 mM, about 200 to about 400 mM, about 200 to about 350 mM, about 200 to about 300 mM, about 250 to about 500 mM, about 250 to about 400 mM, about 250 to about 350 mM tripotassium phosphate.

In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 500 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 450 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 100 to about 400 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 150 to about 350 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 500 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 450 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 400 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 350 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 to about 300 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 200 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 250 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 300 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 350 mM tripotassium phosphate. In one embodiment, the first buffer solution and/or the second buffer solution is derived from about 400 mM tripotassium phosphate.

In one embodiment, the first buffer solution is derived from about 100 to about 500 mM tripotassium phosphate and the second buffer solution is derived from about 100 to about 500 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 100 to about 500 mM trisodium phosphate and the second buffer solution is derived from about 100 to about 500 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 100 to about 500 mM tripotassium phosphate and the second buffer solution is derived from about 100 to about 500 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 500 mM tripotassium phosphate and the second buffer solution is derived from about

200 to about 500 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 500 mM trisodium phosphate and the second buffer solution is derived from about 200 to about 500 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 500 mM tripotassium phosphate and the second buffer solution is derived from about 200 to about 500 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 400 mM tripotassium phosphate and the second buffer solution is derived from about 200 to about 400 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 400 mM trisodium phosphate and the second buffer solution comprises about 200 to about 400 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 400 mM tripotassium phosphate and the second buffer solution is derived from about 200 to about 400 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 300 mM tripotassium phosphate and the second buffer solution is derived from about 200 to about 300 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 300 mM trisodium phosphate and the second buffer solution is derived from about 200 to about 300 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 to about 300 mM tripotassium phosphate and the second buffer solution is derived from about 200 to about 300 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 mM tripotassium phosphate and the second buffer solution is derived from about 200 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 200 mM trisodium phosphate and the second buffer solution is derived from about 200 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 200 mM tripotassium phosphate and the second buffer solution comprises about 200 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 300 mM tripotassium phosphate and the second buffer solution is derived from about 300 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 300 mM trisodium phosphate and the second buffer solution is derived from about 300 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 300 mM tripotassium phosphate and the second buffer solution is derived from about 300 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 400 mM tripotassium phosphate and the second buffer solution is derived from about 400 mM tripotassium phosphate. In one embodiment, the first buffer solution is derived from about 400 mM trisodium phosphate and the second buffer solution is derived from about 400 mM trisodium phosphate. In one embodiment, the first buffer solution is derived from about 400 mM tripotassium phosphate and the second buffer solution is derived from about 400 mM trisodium phosphate.

In one embodiment, the first buffer solution comprises potassium phosphate at a pH of about 10-12. In one embodiment, the second buffer solution comprises potassium phosphate at a pH of about 11-13. In one embodiment, the second buffer solution comprises sodium phosphate at a pH of

about 11-13. In one embodiment, the first buffer solution is derived from about 200 mM to about 400 mM tripotassium phosphate, at a pH of about 10-12. In one embodiment, the first buffer solution is derived from about 200 mM to about 300 mM tripotassium phosphate, at a pH of about 10.5-11.5. In one embodiment, the second buffer solution is derived from about 200 mM to about 400 mM trisodium phosphate, at a pH of about 11-13. In one embodiment, the second buffer solution is derived from about 200 mM to about 400 mM tripotassium phosphate, at a pH of about 11-13. In one embodiment, the second buffer solution is derived from about 200 to about 300 mM trisodium phosphate, at a pH of about 11.5-12.5. In one embodiment, the second buffer solution is derived from about 200 to about 300 mM tripotassium phosphate, at a pH of about 11.5-12.5. In one embodiment, the second buffer solution is derived from about 200 to about 300 mM trisodium phosphate, at a pH of about 12. In one embodiment, the second buffer solution is derived from about 200 to about 300 mM tripotassium phosphate, at a pH of about 12.

In one embodiment, the first buffer solution and/or second buffer solution comprises disodium phosphate and trisodium phosphate. In one embodiment, the first buffer solution and/or second buffer solution comprises dipotassium phosphate and tripotassium phosphate. Predetermined ratios of disodium phosphate to trisodium phosphate and dipotassium phosphate to tripotassium phosphate may be used to achieve a desired pH.

At a basic pH, the oligonucleotide, *e.g.* the impurity and/or the product, may become negatively charged and the membrane for UF/DF, *e.g.* membrane comprising polyethersulfone, may have a negative surface charge. The negative charges may lead to repulsion of the oligonucleotide at the surface of the membrane. This may lead to no permeation or reduced permeation of the oligonucleotide, *e.g.* the impurity or the product, through the membrane. Without wishing to be bound by any theory, it is believed that ions precent at a certain concentration would reduce the electrostatic interaction between the oligonucleotide and the membrane surface and thus increase permeation of the oligonucleotide. Both ionic strength and conductivity of a solution relate to the concentration of the ions present in the solution, so either parameter can be used to determine whether a buffer solution can provide an adequate permeate flux during ultrafiltration.

In one embodiment, the buffer of the first buffer solution and/or second buffer solution provides both (1) the desired pH for denaturing the relevant complexes and (2) the desired ionic strength for maintaining an adequate permeate flux during ultrafiltration. In one embodiment, the phosphate buffer provides the desired pH and ionic strength of the first buffer solution and/or second buffer solution. In one embodiment, the dipotassium phosphate and tripotassium phosphate provide the desired pH and ionic strength of the first buffer solution and/or second buffer solution. In one embodiment, the disodium phosphate and trisodium phosphate provide the desired pH and ionic strength of the first and/or second buffer solution.

In some embodiments, the buffer alone may not achieve the desired ionic strength to maintain an adequate permeate flux during ultrafiltration. Accordingly, in one embodiment, the first buffer

solution and/or second buffer solution further comprises an ionic strength adjustor. The ionic strength adjustor may be a salt. The ionic strength adjustor may be a metal salt. The ionic strength adjustor may be a sodium salt. The ionic strength adjustor may be a potassium salt. The ionic strength adjustor may be NaCl. The ionic strength adjustor may be KCl.

In one embodiment, the first buffer solution and/or the second buffer solution comprises about 200 mM to about 1500 mM, about 300 mM to about 1500 mM, about 300 mM to about 1200 mM or about 600 mM to about 900 mM cations, optionally monovalent cations. In one embodiment, the first buffer solution and/or the second buffer solution comprises about 200 mM to about 1500 mM, about 300 mM to about 1500 mM, about 300 mM to about 1200 mM or about 600 mM to about 900 mM K^+ ions. In one embodiment, the first buffer solution and/or the second buffer solution comprises 200 mM to about 1500 mM, about 300 mM to about 1500 mM, about 300 mM to about 1200 mM or about 600 mM to about 900 mM Na^+ ions. In one embodiment, the first buffer solution and/or the second buffer solution comprises about 300 mM to about 1200 mM K^+ ions. In one embodiment, the first buffer solution and/or the second buffer solution comprises about 300 mM to about 1200 mM Na^+ ions. In one embodiment, the first buffer solution and/or the second buffer solution comprises about 300 mM to about 900 mM K^+ ions. In one embodiment, the first buffer solution and/or the second buffer solution comprises about 300 mM to about 900 mM Na^+ ions. The concentration of the ions described here refers to the total concentration of the particular ions in the buffer solution, *e.g.*, derived from both the buffer and an ionic strength adjustor.

In one embodiment, the first buffer solution and/or second buffer solution has a conductivity greater than or equal to about 20 mS/cm. In one embodiment, the first buffer solution and/or second buffer solution has a conductivity greater than or equal to about 25 mS/cm. In one embodiment, the first buffer solution and/or second buffer solution has a conductivity greater than or equal to about 30 mS/cm. In one embodiment, the first buffer solution and/or second buffer solution has a conductivity greater than or equal to about 40 mS/cm. In one embodiment, the first buffer solution and/or second buffer solution has a conductivity of about 20 mS/cm to about 150 mS/cm, about 20 mS/cm to about 120 mS/cm, about 20 mS/cm to about 100 mS/cm, about 20 mS/cm to about 80 mS/cm, about 20 mS/cm to about 60 mS/cm, about 25 mS/cm to about 150 mS/cm, about 25 mS/cm to about 120 mS/cm, about 25 mS/cm to about 100 mS/cm, about 25 mS/cm to about 80 mS/cm, about 25 mS/cm to about 60 mS/cm, about 30 mS/cm to about 100 mS/cm, about 30 mS/cm to about 80 mS/cm, about 30 mS/cm to about 70 mS/cm, about 30 mS/cm to about 60 mS/cm, about 30 mS/cm to about 50 mS/cm, about 30 mS/cm to about 40 mS/cm or about 35 mS/cm to about 45 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 120 mS/cm, about 25 mS/cm to about 100 mS/cm, about 25 mS/cm to about 80 mS/cm, about 25 mS/cm to about 60 mS/cm, about 30 mS/cm to about 100 mS/cm, about 30 mS/cm to about 60 mS/cm, about 30 mS/cm to about 50 mS/cm, or about 35 mS/cm to about 45 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution

has a conductivity of about 25 mS/cm to about 120 mS/cm, about 25 mS/cm to about 100 mS/cm, or about 25 mS/cm to about 80 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 60 mS/cm, about 30 mS/cm to about 50 mS/cm, or about 35 mS/cm to about 45 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 120 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 100 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 30 mS/cm to about 120 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 30 mS/cm to about 100 mS/cm. In one embodiment, the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 80 mS/cm. In one embodiment, first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 60 mS/cm. In one embodiment, first buffer solution and/or the second buffer solution has a conductivity of about 30 mS/cm to about 60 mS/cm.

In one embodiment, the template has an additional property that allows the template to be retained in the retentate. In one embodiment, the template is present in a form that allows the template to be retained in the retentate. In one embodiment, the template is present in a form that allows the template to be retained in the second retentate. In one embodiment, the template is present in a form that allows the template to be retained in the second retentate of step (c) of the disclosed methods. It is understood that if the template is present in a form that allows the template to be retained in the second retentate then the template in a complex, *e.g.* impurity:template complex or product:template complex, will also be retained in the retentate. In one embodiment, the product:template complex is retained in the first retentate of step (b) of the disclosed method. In one embodiment, the template is present in a form having a molecular weight greater than the molecular weight cut off (MWCO) of a membrane. In one embodiment, the template is present in a form where multiple, repeated copies of the template are linked, and may or may not be separated by a linker sequence. In one embodiment, the template is attached to a support material. In one embodiment, the template together with the attached support material has a molecular weight greater than the molecular weight cut off (MWCO) of a membrane. In one embodiment, the template together with the attached support material has a molecular weight greater than the molecular weight cut off (MWCO) of a membrane allowing the template to be retained in the retentate.

The support material may be a soluble support material. The soluble support material may be selected from the group consisting of: polyethylene glycol, a soluble organic polymer, linker sequence, RNA, DNA, a protein, a dendrimer, a polysaccharide, an oligosaccharide, and a carbohydrate. The support material may be polyethylene glycol (PEG). The support material may be an insoluble support material. The support material may be a solid support material. The solid support material may be selected from the group consisting of: a glass bead, a polymeric bead, a fibrous support, a membrane,

a streptavidin coated bead and cellulose. The solid support material may be a streptavidin coated bead. The solid support material may be part of the reaction vessel itself, *e.g.* a reaction wall.

In one embodiment, one or more copies of the template are attached to the support material. In one embodiment, 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. The multiple repeated copies of the template may be direct repeats, *e.g.* they are not separated by a linker. The support material can have multiple attachment points where a template can be attached. The support material is also referred to as a "hub." Each attachment point may itself have multiple, repeated copies of the template attached in a continuous manner. The multiple repeated copies of the template may be separated by a linker. The multiple repeated copies of the template may be direct repeats, *e.g.* they are not separated by a linker. In one embodiment, multiple copies of the template are attached to the support material at multiple attachment points. In one embodiment, two or more copies of template are attached to the support material *e.g.* at multiple attachment points. In one embodiment, two, three, four, five, six, seven, eight, night or ten copies of template are attached to the support material *e.g.* at multiple attachment points. In one embodiment, three copies of template are attached to the support material *e.g.* at multiple attachment points. In one embodiment, four copies of template are attached to the support material *e.g.* at multiple attachment points. In one embodiment, five copies of template are attached to the support material *e.g.* at multiple attachment points. In one embodiment, six copies of template are attached to the support material *e.g.* at multiple attachment points. In one embodiment, six copies of template are attached to a support material, wherein the support material is a "hub" comprising polyethylene glycol (PEG).

In one embodiment, the template is recycled for future use. In one embodiment, the template is recovered from the second retentate of step (c) of the method and recycled for future use.

In one embodiment, the UF/DF is carried out using a membrane. In one embodiment, the UF/DF in step (b) and/or (c) is carried out using a membrane. The membrane may have a molecular weight cut off (MWCO) that allows the impurity and product to pass through. The membrane may have a molecular weight cut off (MWCO) that prevents the template from passing through. The membrane may have a molecular weight cut off (MWCO) greater than the molecular weight of the impurity and product, and less than the molecular weight of the template when in a form allowing the template to be retained in the retentate *e.g.* when the template is attached to a support material. The membrane may have a molecular weight cut off (MWCO) of about 10 to about 100 kDa, about 10 to about 80 kDa, about 10 to about 50 kDa, about 10 to about 40 kDa, about 20 to about 80 kDa, about 20 to about 50 kDa, about 20 to about 40 kDa, about 30 to about 50 kDa, about 25 to about 35 kDa or about 20 to about 30 kDa. The membrane may have a molecular weight cut off (MWCO) of about 10 to about 40 kDa, about 20 to about 40 kDa or about 20 to about 30 kDa. In one embodiment, the membrane has a about 10 to about 40 kDa. In one embodiment, the membrane has a molecular

weight cut off (MWCO) of about 20 to about 40 kDa. In one embodiment, the membrane has a molecular weight cut off (MWCO) of about 20 to about 30 kDa. In one embodiment, the membrane has a molecular weight cut off (MWCO) of about 25 to about 35 kDa. In one embodiment, the membrane has a molecular weight cut off (MWCO) of about 30 kDa.

The second membrane may have a molecular weight cut off (MWCO) that prevents the product from passing through during filtration. Such membrane may be used to replace/remove the buffer in a solution of the product. For example, such membrane may be used when subjecting the second permeate of step (c) to UF/DF to form a third retentate comprising the product and substantially free of the second buffer. In one embodiment, the membrane has a molecular weight cut off (MWCO) of about 1 to about 10 kDa, about 1 to about 7 kDa, about 1 to about 5 kDa, about 2 to about 5 kDa or about 2 to about 4 kDa. In one embodiment, the membrane has a molecular weight cut off (MWCO) of about 2 to about 5 kDa. In one embodiment, the membrane has a molecular weight cut off (MWCO) of about 3 kDa.

In one embodiment, the membrane comprises polysulfone, polypropylene, cellulose acetate, polyactic acid, nitrocellulose, mix cellulose ester cellulose nitrate, regenerated cellulose, polyethersulfone (PES), polyamide, cellulose derivative, polyvinylidene fluoride, polytetra-fluoroethylene, or polycarbonate track etched membranes. In one embodiment, the membrane is part of a filtration element comprising a hollow fiber, tubular, spiral-wound, cassette, plate, or frame membrane type. The membrane may comprise polyethersulfone. Commercially available membranes that can be used are manufactured by various vendors such as Millipore Corporation (Billerica, Mass.), Pall Corporation (East Hills, N.Y.), GE Healthcare Sciences (Piscataway, N.J.), and Sartorius Corporation (Goettingen, Germany).

In one embodiment, the product is synthesised by a ligation reaction catalysed by an enzyme. In one embodiment, the product is synthesised by enzymatic ligation of shortmers. In a further embodiment the enzymatic ligation is carried out by a ligase. The product may be synthesised by a process comprising the following steps:

i) providing a template oligonucleotide (I) complementary to the sequence of the product;

ii) providing a pool of oligonucleotides (II) containing shortmers;

iii) contacting (I) and (II) in conditions to allow annealing; and

iv) joining the shortmers by enzymatic ligation with a ligase to form the product.

In one embodiment, the shortmers are 3 to 20 nucleotides long. In one embodiment, the shortmers are 3 to 15 nucleotides long. In one embodiment, the shortmers are 5 to 10 nucleotides long. In one embodiment, the shortmers are 5 to 8 nucleotides long. In one embodiment, the shortmers are 5, 6, 7 or 8 nucleotides long. In a particular embodiment, there are three shortmers: a 5′ shortmer that is 7 nucleotides long, a central shortmer that is 6 nucleotides long and a 3′ shortmer that is 7 nucleotides long, which when ligated together form a product that is 20 nucleotides long (a

"20-mer"). In a particular embodiment, there are three shortmers: a 5' shortmer that is 6 nucleotides long, a central shortmer that is 8 nucleotides long and a 3' shortmer 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 shortmers: a 5' shortmer that is 5 nucleotides long, a central shortmer that is 10 nucleotides long and a 3' shortmer that is 5 nucleotides long, which when ligated together form a product that is 20 nucleotides long (a "20-mer"). In a particular embodiment, there are four shortmers: a 5' shortmer that is 5 nucleotides long, a 5'-central shortmer that is 5 nucleotides long, a central-3' shortmer that is 5 nucleotides long, and a 3' shortmer that is 5 nucleotides long, which when ligated together form a product that is 20 nucleotides long (a "20-mer").

In one embodiment the disclosed method is for purifying a product, wherein the product is 10 to 200 nucleotides long. In one embodiment, the product is 10 to 150 nucleotides long. In one embodiment, the product is 10 to 100 nucleotides long. In one embodiment, the product is 10 to 80, 10 to 70, 10 to 60, 10 to 50, 10 to 40 or 10 to 30 nucleotides long. In one embodiment, the product is 10 to 50 nucleotides long. In one embodiment, the product is 10 to 40 nucleotides long. In one embodiment, the product is 10 to 35 nucleotides long. In one embodiment, the product is 10 to 30 nucleotides long. In a further embodiment of the invention the product is 15 to 35 nucleotides long. In a further embodiment of the invention the product is 15 to 30 nucleotides long. In one embodiment, the product is 20 to 30 nucleotides long. In one embodiment, the product is 20 to 35 nucleotides long. In one embodiment, the product is 20 to 32 nucleotides long. In an embodiment of the invention the product is 20, 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". In an embodiment of the invention the product is 31 nucleotides long, a "31-mer". In an embodiment of the invention the product is 32 nucleotides long, a "32-mer".

In an embodiment of the invention, the method for purifying a therapeutic product.

A product in accordance with the present invention may have at least one backbone modification and/or at least one sugar modification and/or at least one base modification.

One embodiment of the invention provides a method as disclosed herein, wherein the product contains at least one modified nucleotide residue. In one embodiment, the at least one modified nucleotide comprises modification of the sugar moiety, modification of the nucleobase and/or

modification of the backbone. In a further embodiment, the modification is at the 2′ position of the sugar moiety.

The product 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'-0-propargyl; and their derivatives.

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.

Other sugar modifications include "bridged" or "bicylic" 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), altriol 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.

Products 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.

In an embodiment of the invention, the modified product 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.

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; N^2-cyclopentylguanine (cPent-G), N^2-cyclopentyl-2-aminopurine (cPent-AP), and N^2-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).

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.

The product 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.

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

In an embodiment of the invention, the oligonucleotide comprises one or more modifications selected from the group consisting of: 2′-Fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), 2′-amino, 5-methyl pyrimidine, 7-deazaguanosine, abasic nucleotide, phosphorothioate (PS), phosphoramidate (PA) and phosphorodiamidate. In an embodiment of the invention, the oligonucleotide comprises one or more modifications selected from the group consisting of: 2′-Fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), 5-methyl pyrimidine, and phosphorothioate (PS). In an embodiment of the invention, the oligonucleotide comprises one or more modifications selected from the group consisting of: 2′-Fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), 5-methyl cytosine, 5-methyl uracil and phosphorothioate (PS).

In an embodiment of the invention, the oligonucleotide comprises a combination of two or more modifications as disclosed above. In an embodiment of the invention, the oligonucleotide comprises two or more modifications selected from the group consisting of: 2′-Fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), 2′-amino, 5-methyl pyrimidine, 7-deazaguanosine, abasic nucleotide, phosphorothioate (PS), phosphoramidate (PA) and phosphorodiamidate. In an embodiment of the invention, the oligonucleotide comprises two or more modifications selected from the group consisting of: 2′-Fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), 5-methyl pyrimidine, and phosphorothioate (PS). In an embodiment of the invention, the oligonucleotide comprises two or more modifications selected from the group consisting of: 2′-Fluoro (2′-F), 2′-O-methyl (2′-OMe), 2′-O-methoxyethyl (2′-MOE), 5-methyl cytosine, 5-methyl uracil and phosphorothioate (PS).

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.

In one embodiment, the product is a sense strand of a double-stranded oligonucleotide. In one embodiment, the product is an anti-sense strand of a double-stranded oligonucleotide. The purified sense strand and the purified anti-sense strand may form a double stranded oligonucleotide *e.g.* an siRNA.

In one embodiment, the solution of step (a) comprises about 1 to about 100 mg/ml, about 5 to about 100 mg/ml, about 5 to about 80 mg/ml, about 5 to about 60 mg/ml, about 10 to about 80 mg/ml about 10 to about 60 mg/ml, about 20 to about 60 mg/ml, about 30 to about 60 mg/ml, about 35 to about 55 mg/ml, about 30 to about 50 mg/ml, about 40 to about 50 mg/ml or about 35 to about 45 mg/ml template. For the avoidance of doubt, the weight of template does not include that of the linker or support material that the template is attached to. In one embodiment, the solution of step (a) comprises about 5 to about 60 mg/ml, about 10 to about 60 mg/ml, about 20 to about 60 mg/ml, about 30 to about 60 mg/ml, about 35 to about 55 mg/ml, or about 35 to about 45 mg/ml template. In one embodiment, the solution of step (a) comprises about 20 to about 60 mg/ml template. In one embodiment, the solution of step (a) comprises about 30 to about 60 mg/ml template. In one embodiment, the solution of step (a) comprises about 40 to about 50 mg/ml template. In one embodiment, the solution of step (a) comprises about 40 mg/ml template. The concentration of

template in the solution of step (a) may be based on the starting concentration of template in the ligation reaction catalysed by an enzyme. In one embodiment, the solution of step (a) comprises about 4 to about 120 mg/ml, about 5 to about 120 mg/ml, about 10 to about 120 mg/ml, about 20 to about 120 mg/ml, about 40 to about 120 mg/ml, about 60 to about 120 mg/ml, about 70 to about 110 mg/ml, or about 70 to about 90 mg/ml total oligonucleotide. The total oligonucleotide concentration may be an estimate based on doubling the template concentration.

In one embodiment, the solution of step (a) comprises about 0.5 to about 10 mM, about 0.5 to about 9 mM, about 0.5 to about 8 mM, about 0.5 to about 7 mM, about 0.5 to about 6 mM, about 0.5 to about 5 mM, about 1 to about 10 mM, about 1 to about 9 mM, about 1 to about 8 mM, about 1 to about 7 mM, about 1 to about 6 mM, about 2 to about 10 mM, about 2 to about 9 mM, about 2 to about 8 mM, about 2 to about 7 mM, about 2 to about 6 mM, or about 2 to about 5 mM, about 3 to about 10 mM, about 3 to about 9 mM, about 3 to about 8 mM, about 3 to about 7 mM, about 3 to about 6 mM, or about 3 to about 5 mM template. In one embodiment, the solution of step (a) comprises about 2 to about 8 mM template. In one embodiment, the solution of step (a) comprises about 2 to about 7 mM template. In one embodiment, the solution of step (a) comprises about 3 to about 7 mM template. In one embodiment, the solution of step (a) comprises about 2 to about 6 mM template. In one embodiment, the solution of step (a) comprises about 3 to about 6 mM template.

In one embodiment, the method is carried out at room temperature. In one embodiment, the UF/DF steps are carried out at room temperature. In one embodiment, the UF/DF of step (b) and/or step (c) is carried out at room temperature. In one embodiment, the UF/DF of step (b) and/or step (c) is carried out at about 20 to about 30 degrees Celsius, about 22 to about 28 degrees Celsius, about 24 to about 28 degrees Celsius or about 25 to about 27 degrees Celsius. In one embodiment, the UF/DF of step (b) and/or step (c) is carried out at about 25 to about 27 degrees Celsius. One advantage of using such temperatures is that these temperatures have little to no impact on oligonucleotide stability and/or degradation.

In one embodiment, the UF/DF of step (b) and/or step (c) is carried below about 70 degrees Celsius, below about 60 degrees Celsius or below about 50 degrees Celsius. In one embodiment, the UF/DF of step (b) and/or step (c) is carried out below about 50 degrees Celsius. In one embodiment, the UF/DF of step (b) and/or step (c) is carried out at about 20 to 50 degrees Celsius or about 20 to 49 degrees Celsius. In one embodiment, the pH may additionally be combined with an increase in temperature to effect the release of the impurity and/or the product from the template. For example, the pH of the first and/or second buffer solution may be 12 and the UF/DF is carried out at 30-40 degrees Celsius.

In one embodiment, the UF/DF is carried out with a diavolume of at least 3, or at least 4, or at least 5. In one embodiment, the UF/DF is carried out with a diavolume from 3 to 30, or from 3 to 30, or from 3 to 20, or from 10 to 20, or from 10 to 12, or from 3 to 10, or from 5 to 10, or from 5 to 8.

In one embodiment, the maximum transmembrane pressure (TMP) is 5 barg, 4 barg, 3 barg, or 2 barg. In one embodiment, the maximum transmembrane pressure (TMP) is 4 barg.

In one embodiment, the UF/DF is carried out using a tangential flow filtration system. The method may comprise additional steps. In one embodiment, the method further comprises a step to replace the buffer solution with water. This may be known as desalting. In one embodiment, the method further comprises step (d) subjecting the second permeate of step (c) to UF/DF to form a third retentate comprising the product and substantially free of the second buffer solution, wherein the UF/DF is carried out using water and a membrane having a molecular weight cut off (MWCO) of about 1 kDa to about 5 kDa. The method may further comprise subjecting the third retentate of step (d) to lyophilization to form a lyophilized composition comprising the product.

In one embodiment, the method further comprises a step to recover the template. The recovered template may be recycled for future use. In one embodiment, the method further comprises step (e) subjecting the second retentate of step (c) to UF/DF to form a fourth retentate comprising the template, wherein the UF/DF is carried out using a third buffer solution a pH of about 7-8. The third buffer solution may comprise tris.HCL. The third buffer solution may comprise 100 mM tris.HCl.

In one embodiment, the method further comprises a step to increase purity of the product and/or remove residual template. In one embodiment, the method further comprises a step of subjecting the second permeate of step (c) to UF/DF, wherein the UF/DF is carried out using the second buffer solution.

In one embodiment, wherein the product is synthesised by a ligation reaction catalysed by an enzyme, the solution of step (a) is prepared comprising the steps:

(a1) removing the enzyme from the ligation reaction by filtration to provide a filtrate;

(a2) treating the filtrate with EDTA to provide a mixture; and

(a3) concentrating the mixture of step (a2) to provide the solution of step (a).

In one embodiment, the steps of the method are carried out in sequential order. In one embodiment, step (a) is carried out followed by step (b) and then step (b) is carried out followed by step (c). In one embodiment, steps (a1)-(a3) are carried out followed by step (b) and then step (b) is carried out followed by step (c).

In one embodiment, step (d) and step (e) are carried out sequentially. In one embodiment, step (d) and step (e) are carried out in parallel. In one embodiment, step (e) is carried out before step (d), but after step (c).

In one embodiment, the method is carried out using a continuous or semi-continuous flow process.

In an embodiment of the invention, the process is for large scale purification of a product, in particular a therapeutic single-stranded oligonucleotide. In the context of the present invention large scale purification of oligonucleotides means purification at a scale greater than or equal to 1 litre, *e.g.*

the process is carried out on a 1 L or larger solution of step (a). Alternatively or in addition, in the context of the present invention large scale purification of oligonucleotides means purification at gram scale of product, in particular the purification of greater than or equal to 2 grams, greater than or equal to 5 grams, or greater than or equal to 10 grams of product. In an embodiment of the invention, the amount of oligonucleotide product purified is at gram or kilogram scale or greater. In an embodiment of the invention, the amount of oligonucleotide product purified is at gram scale. In an embodiment of the invention the amount of product purified 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 purified 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 purified 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 purified is 1 kg or more. In an embodiment of the invention, the amount of oligonucleotide product purified 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 purified 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.

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

In an embodiment of the invention, oligonucleotide purification takes place at a scale greater than or equal to: 2, 3, 4, 5, 6, 7, 8, 9, 10 litres, *e.g.* on a 2, 3, 4, 5, 6, 7, 8, 9 or 10 L solution of step (a). In an embodiment of the invention, oligonucleotide purification 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.* on a 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 70, 75, 80, 85, 90, 95, 100 L solution. In an embodiment of the invention, oligonucleotide purification 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.* on a 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 L solution.

In an embodiment of the invention, the method is carried out at a volume of 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 for the solution of step (a).

In an embodiment of the invention, the method is carried out at a volume of between about 5 and about 10,000 L, between about 10 and about 5,000 L, between about 20 and about 2,000 L, or between 50 and 1,000 L for the solution of step (a).

One embodiment of the invention provides a method as previously described herein, wherein the purified product is greater than or equal to about 80% pure. The purified product refers to the product in the second permeate of step (c) or the product in any subsequent steps, and the purity is measured by HPLC (%area). Purity may be assessed as a proportion of total oligonucleotide. In a further embodiment, the product is greater than or equal to about 90% pure. In a further embodiment, the product is greater than or equal to about 95% pure. In a further embodiment, the product is greater than or equal to about 96% pure. In a further embodiment, the product is greater than or equal to about 97% pure. In a further embodiment, the product is greater than or equal to about 98% pure. In a further embodiment, the product is greater than or equal to about 99% pure. In a further embodiment, the product is greater than or equal to 99%, 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8% or 99.9% pure. In one embodiment, the product in the second permeate of step (c) has a purity of at least: about 80%, about 90%, about 95%, about 96%, about 97%, about 98% or about 99%.

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

The present invention includes, but is not limited to, the following clauses/embodiments:

1. A method of purifying a product, wherein the product is a single-stranded oligonucleotide, comprising:
    (a) obtaining a solution comprising (i) an impurity:template complex and (ii) a product:template complex, wherein the template is an oligonucleotide comprising a sequence complementary to the product,
    (b) subjecting the solution to ultrafiltration/diafiltration (UF/DF) to form a first retentate comprising the product:template complex and a first permeate comprising the impurity, wherein the UF/DF is carried out using a first buffer solution at a pH of about 9-13,
    (c) subjecting the first retentate of step (b) to UF/DF to form a second retentate comprising the template and a second permeate comprising the product, wherein the UF/DF is carried out using a second buffer solution at a pH greater than the pH of the first buffer solution.

2. A method of purifying a product, wherein the product is a single-stranded oligonucleotide, comprising:

(b) subjecting a solution comprising (i) an impurity:template complex and (ii) a product:template complex to ultrafiltration/diafiltration (UF/DF) to form a first retentate comprising the product:template complex and a first permeate comprising the impurity, wherein the UF/DF is carried out using a first buffer solution at a pH of about 9-13, and wherein the template is an oligonucleotide comprising a sequence complementary to the product,

(c) subjecting the first retentate of step (b) to UF/DF to form a second retentate comprising the template and a second permeate comprising the product, wherein the UF/DF is carried out using a second buffer solution at a pH greater than the pH of the first buffer solution.

3. The method according to clause 1 or clause 2, wherein the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9 or about 2.0.

4. The method according to any one of the preceding clauses, wherein the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5.

5. The method according to any one of the preceding clauses, wherein the pH of the first buffer solution is about 9-12.5, about 9-12, about 9-11.5, about 9-11, about 10-12.5, about 10-12, about 10-11.5, about 10-11 or about 10.5-11.5.

6. The method according to any one of the preceding clauses, wherein the pH of the first buffer solution is about 10-12.

7. The method according to any one of the preceding clauses, wherein the pH of the second buffer solution is about 10-14, about 10-13.5, about 10-13, about 11-14, about 11-13.5, about 11-13, about 11-12.5, about 11-12 or about 11.5-12.5.

8. The method according to any one of the preceding clauses, wherein the pH of the second buffer solution is about 11-13.

9. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 120 mS/cm, about 25 mS/cm to about 100 mS/cm, about 25 mS/cm to about 80 mS/cm, about 25 mS/cm to about 60 mS/cm, about 30 mS/cm to about 100 mS/cm, or about 30 mS/cm to about 80 mS/cm.

10. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 100 mS/cm.

11. The method according any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution has a conductivity of about 30 mS/cm to about 80 mS/cm.

12. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises a phosphate buffer.

13. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises potassium phosphate, optionally dipotassium phosphate and/or tripotassium phosphate.

14. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises tripotassium phosphate.

15. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution is derived from about 100 to 500 mM, about 100 to 400 mM, about 150 to 350 mM, 200 mM to about 400 mM, about 200 to 300 mM tripotassium phosphate.

16. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises sodium phosphate, optionally disodium and/or trisodium phosphate.

17. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises trisodium phosphate.

18. The method according to any one of the preceding clauses, wherein first buffer solution and/or the second buffer solution is derived from about 100 to 500 mM, about 100 to 400 mM, about 150 to 350 mM, 200 mM to about 400 mM, about 200 to 300 mM trisodium phosphate.

19. The method according to any one of the preceding clauses, wherein the first buffer solution comprises potassium phosphate and the second buffer solution comprises sodium phosphate.

20. The method according to any one of the preceding clauses, wherein the first buffer solution comprises potassium phosphate at a pH of about 10-12.

21. The method according to any one of the preceding clauses, wherein the second buffer solution comprises sodium phosphate at a pH of about 11-13.

22. The method according to any one of the preceding clauses, wherein the first buffer solution comprises tripotassium phosphate and the second buffer solution comprises trisodium phosphate.

23. The method according to any one of the preceding clauses, wherein the first buffer solution is derived from about 200 mM to about 400 mM tripotassium phosphate, at a pH of about 10-12.

24. The method according to any one of the preceding clauses, wherein the second buffer solution is derived from about 200 mM to about 400 mM trisodium phosphate, at a pH of about 11-13.

25. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution further comprises an ionic strength adjustor.

26. The method according to clause 25, wherein the ionic strength adjustor comprises a cation, optionally sodium ion or potassium ion,

27. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises about 200 mM to about 1500 mM, about 200 mM to about 1200 mM, about 300 mM to about 1500 mM, about 300 mM to about 1200 mM, about 400 mM to about 800 mM, or about 600 mM to about 900 mM cation, optionally sodium ion or potassium ion.

28. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises about 300 mM to about 1200 mM sodium ion.

29. The method according to any one of the preceding clauses, wherein the first buffer solution and/or the second buffer solution comprises about 300 mM to about 1200 mM potassium ion.

30. The method according to any one of the preceding clauses, wherein the template is present in a form that allows the template to be retained in the second retentate.

31. The method according to any one of the preceding clauses, wherein the template is attached to a support material.

32. The method according to any one of the preceding clauses, wherein multiple copies of the template are attached to a hub.

33. The method according to any one of the preceding clauses, wherein the UF/DF of step (b) and/or step (c) is carried out using a membrane having a molecular weight cut off (MWCO) of about 5 to about 50 kDa, about 10 to about 40 kDa, about 20 to about 40 kDa or about 20 to about 30 kDa.

34. The method according to any one of the preceding clauses, wherein the UF/DF of step (b) and/or step (c) is carried out using a membrane having a molecular weight cut off (MWCO) of about 20 to about 40 kDa, optionally about 30 kDa.

35. The method according to clauses 33 or 34, wherein the membrane comprises polyethersulfone.

36. The method according to any one of the preceding clauses, wherein the product comprises about 10-50, about 10-40, about 10-35, about 13-30, about 15-35, about 15-30, about 15-25, about 20-32, or about 20-23 nucleotides.

37. The method according to any one of the preceding clauses, wherein the product comprises about 10-35 nucleotides, optionally about 15-35 nucleotides.

38. The method according to any one of the preceding clauses, wherein the solution of step (a) comprises about 5 to about 60 mg/ml, about 10 to about 60 mg/ml, about 20 to about 60 mg/ml, about 30 to about 60 mg/ml, about 35 to about 55 mg/ml, or about 35 to about 45 mg/ml template.

39. The method according to any one of the preceding clauses, wherein the solution of step (a) comprises about 30 to about 60 mg/ml template, optionally about 35 to about 55 mg/ml template.

40. The method according to any one of the preceding clauses, wherein the solution of step (a) comprises about 0.5 to about 8 mM, about 0.5 to about 7 mM, about 0.5 to about 6 mM, about 1 to about 8 mM, about 1 to about 7 mM, about 1 to about 6 mM, about 2 to about 8 mM, about 2 to about 7 mM, about 2 to about 6 mM, about 2 to about 5 mM, about 3 to about 7 mM, about 3 to about 6 mM, or about 3 to about 5 mM template

41. The method according to any one of the preceding clauses, wherein the solution of step (a) comprises about 2 to about 7 mM template, optionally about 3 to about 6 mM template.

42. The method according to any one of the preceding clauses, wherein the impurity is a shortmer or an intermediate.

43. The method according to any one of the preceding clauses, wherein UF/DF of step (b) and/or step (c) is carried out about 20 to about 30 degrees Celsius, about 22 to about 28 degrees Celsius, about 24 to about 28 degrees Celsius or about 25 to about 27 degrees Celsius.

44. The method according to any one of the preceding clauses, further comprising:

(d) subjecting the second permeate of step (c) to UF/DF to form a third retentate comprising the product and substantially free of the second buffer solution, wherein the UF/DF is carried out using water and a membrane having a molecular weight cut off (MWCO) of about 1 kDa to about 5 kDa.

45. The method according to clause 44, further comprising subjecting the third retentate of step (d) to lyophilization to form a lyophilized composition comprising the product.

46. The method according to any one of the preceding clauses, further comprising:

(e) subjecting the second retentate of step (c) to UF/DF to form a fourth retentate comprising the template, wherein the UF/DF is carried out using a third buffer solution at a pH of about 7-8.

47. The method according to any one of the preceding clauses, wherein the template is recycled for future use.

48. The method according to any one of the preceding clauses, wherein the product is synthesized by a ligation reaction catalysed by an enzyme.

49. The method according to clause 48, wherein the solution of step (a) is prepared comprising the steps:

(a1) removing the enzyme from the ligation reaction by filtration to provide a filtrate;

(a2) treating the filtrate with EDTA to provide a mixture; and

(a3) concentrating the mixture of step (a2) to provide the solution.

50. The method according to any one of the preceding clauses, wherein the UF/DF is carried out at a crossflow rate of about 2 to about 20 L/min/m2, about 2 to about 10 L/min/m2 or about 5 to about 7 L/min/m2.

51. The method according to any one of the preceding clauses, wherein the product in the second permeate of step (c) has a purity of at least: about 80%, about 90% about 95%, about 96%, about 97%, about 98% or about 99%.

52. The method according to any one of the preceding clauses, wherein the method is carried out at gram scale or kilogram scale or greater.

53. The method according to any one of the preceding clauses, wherein the product comprises at least one backbone modification and/or at least one sugar modification and/or at least one base modification.

54. A single-stranded oligonucleotide product obtained by the method according to any one of the preceding clauses.

55. The single-stranded oligonucleotide product according to clause 54, wherein the single-stranded oligonucleotide product is a therapeutic oligonucleotide.

56. The single-stranded oligonucleotide product according to clause 54 or clause 55, wherein the single-stranded oligonucleotide product is a gapmer.

57. A single stranded oligonucleotide product according to clause 54, wherein the single-stranded oligonucleotide product forms part of a double-stranded oligonucleotide.

58. A single stranded oligonucleotide product according to clause 57, wherein the double-stranded oligonucleotide is an siRNA.

EXAMPLES

The exemplary methods (Examples 1 to 8) described below used illustrative oligonucleotide products, shortmers, and templates, of which the sequences are specified in Tables 1-3 below.

Table 1. Single stranded antisense oligonucleotide, OLIGO-1

Compounds	**Sequence**	**SEQ ID NO**
Product, 5′ to 3′ (OLIGO-1)	g*c*a*g*a**G*G*T*G*A*A*G*C*G*A** a*g*u*g*c	SEQ ID NO: 1
5′ Segment (Shortmer-1)	g*c*a*g*a**G*G**	SEQ ID NO: 2
Center segment (Shortmer-2)	*T*G*A*A*G*C**	SEQ ID NO: 3
3′ Segment (Shortmer-3)	*G*A** a*g*u*g*c	SEQ ID NO: 4
Template-1, 5′ to 3′	Aminohexyl-*T-T-T-G-C-A-C-T-T-C-G-C-T-T-C-A-C-C-T-C-T-G-C*T*T*T*	SEQ ID NO: 5

Chemical modification legends for Table 1:

Lowercased letters: nucleotides having 2′-methoxyethyl (2′-MOE) substituted ribose sugars;

Italicized uppercased letters: nucleotides having 2′-deoxy ribose sugars;

- : phosphodiester linkage;

* : phosphorothioate linkage;

All cytidines (c, *C*) are 5-methyl substituted;

Uridine (*u*) is 5-methyl substituted.

Table 2. Double stranded siRNA - antisense strand, OLIGO-2

Compounds	Sequence	SEQ ID NO
Product, 5′ to 3′ (OLIGO-2)	mU*fC*mA*fU-mC-fU-mA-mU-mC-mA-mG-fA-mC-fU-mU-fC-mU-fU-mA-fC*mG	SEQ ID NO: 6
5′ Segment (Shortmer-4)	mU*fC*mA*fU-mC-fU-mA	SEQ ID NO: 7
Center segment (Shortmer-5)	mU-mC-mA-mG-fA-mC-fU	SEQ ID NO: 8
3′ Segment (Shortmer-6)	mU-fC-mU-fU-mA-fC*mG	SEQ ID NO: 9
Template-2, 5′ to 3′	Aminohexyl-T-T-T-C-G-T-A-A-G-A-A-G-T-C-T-G-A-T-A-G-A-T-G-A*T*T*T	SEQ ID NO: 10

Table 3. Double stranded siRNA - sense strand, OLIGO-3

Compounds	Sequence	SEQ ID NO
Product, 5′ to 3′ (OLIGO-3)	GalNAc*invAB*mC-mG-mU-mA-mA-mG-mA-mA-fG-mU-fC-mU-fG-mA-mU-mA-mG-mA-mU-mG-mA*invAb	SEQ ID NO: 11
5′ Segment (Shortmer-7)	GalNAc*invAb*mC-mG-mU-mA-mA-mG-mA	SEQ ID NO: 12
Center segment (Shortmer-8)	mA-fG-mU-fC-mU-fG-mA	SEQ ID NO: 13
3′ Segment (Shortmer-9)	mU-mA-mG-mA-mU-mG-mA*invAb	SEQ ID NO: 14
Template-3, 5′ to 3′	Aminohexyl-T-T-T-T-C-A-T-C-T-A-T-C-A-G-A-C-T-T-C-T-T-A-C-G*T*T*T	SEQ ID NO: 15

Chemical modification legends for Tables 2 and 3:

mX: 2′-O-methyl modified nucleotide;

fX: 2′-fluoro modified nucleotide;

invAb: inverted abasic;

- : phosphodiester linkage;

* : phosphorothioate linkage;

GalNAc: N-Acetylgalactosamine-containing targeting moiety.

Example 1: Determining optimal pHs for separation of OLIG-1, shortmers (6-mer/7-mer), and intermediates (13-mers)

A Tangential Flow Filtration (TFF) system (Figure 1) was used for purification of oligonucleotides. The feed vessel (1) contained the oligonucleotide solution to be purified, and the solution was pumped into a flat sheet membrane cassette (2) by the feed pump (3) and back to the feed vessel via a pressure control valve (4). Permeate was removed by the permeate pump (5) while a further transfer pump (6) added liquid to keep the recirculating volume constant. Samples of the retentate solution were taken from the feed vessel.

Experiment 1A

An experiment was carried out using the input reaction mixture of composition shown in Table 4 below.

Table 4: Input composition

Oligo	**HPLC area%**
Shortmer-1	12.6
Shortmer-3	6.4
Shortmer-3, adenylated	0.9
Shortmer-2	8.6
Shortmer-2, adenylated	2.5
Intermediate 1 (13-mer)	1.5
Intermediate 2 (13-mer)	0
OLIGO-1 (20-mer, Product)	20.1
hub-template	25.2

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and prepared by washing with a potassium phosphate solution (100 mM, pH 7). To the solution of oligonucleotides in 100 mM Tris.HCl, pH 7.6 (ca. 11 g/L total oligo, retentate concentration ca. 5.5 g/L hub-template) was added a solution of tetrapotassium EDTA, 1.25 molar equivalents of EDTA relative to the magnesium quantity in the input mixture. The solution was then fed into the system and recirculated through the membrane cassette at 26 degC. Tripotassium phosphate buffers at 200 mM concentration and increasing pH (9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5 and 13.2) were pumped into the system at flow rates matching the permeate flow rate. The retentate was sampled for analysis every 2 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. See Figure 2. The pH of the buffer was increased every 10 diafiltration volumes.

Experiment 1B

An experiment was carried out using the input reaction mixture of composition shown in Table 5 below.

Table 5: Input composition

Oligo	HPLC area%
Shortmer-1	4.4
Shortmer-3	6.4
Shortmer-3, adenylated	1.2
Shortmer-2	9.3
Shortmer-2, adenylated	1.1
Intermediate 1 (13-mer)	7.5
Intermediate 2 (13-mer)	0
OLIGO-1 (20-mer, Product)	31.5
hub-template	37.4

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and prepared by washing with a potassium phosphate solution (100 mM, pH 7). To the solution of oligonucleotides in 100 mM Tris.HCl, pH 7.6 (ca. 9.2 g/L total oligo, retentate concentration ca. 4.6 g/L hub-template) was added a solution of tetrapotassium EDTA, 1.25 molar equivalents of EDTA relative to the magnesium quantity in the input mixture. The solution was then fed into the system and recirculated through the membrane cassette at 26 degC. Trisodium phosphate buffers at 300 mM concentration and increasing pH (9.0, 9.5, 10.0, 10.5, 11.0, 11.5, 12.0) were pumped into the system at flow rates matching the permeate flow rate. The retentate was sampled for analysis every 2 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. See Figure 3. The pH of the buffer was increased every 10 diafiltration volumes.

Using both tripotassium phosphate and trisodium phosphate the annealed shortmers (6-mer and 7-mers), intermediates (13-mers) and OLIGO-1 (20-mer) could be cleanly separated from the hub-template, which was rejected by the membrane and recovered in the retentate. In addition, the annealed shortmers and intermediates were removed from the retentate before the 20-mer product, providing a means of effecting a purification of the 20-mer product. A chromatogram of the permeate solution shows that it contains 20-mer product, with no hub-template and a reduced level of the 13-mer compared to the input oligonucleotide solution.

This experiment demonstrated that oligonucleotides of different lengths and molecular weights can be separated using ultrafiltration at a low temperature (ca. 25 degC). As shown above, the pH of the buffer used during ultrafiltration affects the level of separation. For example, oligonucleotides of 6, 7 and 13 nucleotides in length can be separated from the product (OLIGO-1, 20-mer) and six-template hub (comprising 6 x 26-mer attached to a core) by diafiltration with a pH 11 buffer, and the product and hub-template can, in turn, be separated from each other by diafiltrarion with a pH 12 buffer. Oligonucleotides of different sequences may require different conditions with respect to buffer strength and pH, which can be determined by pH screening experiments as described herein.

Example 2: Determining optimal ionic strength for permeation of OLIG-1, shortmers (6-mer/7-mer), and intermediates (13-mers)

Experiment 2A

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and prepared by washing with 100 mM Tris.HCl, pH 7.6. Input oligo composition was as shown in Table 4 above. To the solution of oligonucleotides in 100 mM Tris.HCl, pH 7.6 (ca. 14 g/L total oligo, retentate concentration ca. 7 g/L hub-template) was added a solution of tetrapotassium EDTA, 1.25 molar equivalents of EDTA relative to the magnesium quantity in the input mixture. The solution was then fed into the system and recirculated through the membrane cassette at 26 degC. Sodium hydroxide solutions at increasing concentration (50 mM, 75 mM, 100 mM) were pumped into the system at flow rates matching the permeate flow rate. The retentate was sampled for analysis once a stable retentate pH and conductivity was achieved, and the UV absorbance of the permeate was monitored using the TFF system instrumentation. Comparison of the retentate pH and conductivity for sodium hydroxide diafiltration with the HPLC analysis of the retentate shows that despite the pH being >12 throughout, there was no significant permeation of oligonucleotides through the membrane with the conductivity ranging from about 18 to 24 mS/cm.

Experiment 2B

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and prepared by washing with 100 mM Tris.HCl, pH 7.6. Input oligo composition was as shown in Table 4 above. To the solution of oligonucleotides in 100 mM Tris.HCl, pH 7.6 (ca. 12 g/L total oligo, retentate concentration ca. 6 g/L hub-template) was added a solution of tetrapotassium EDTA, 1.25 molar equivalents of EDTA relative to the magnesium quantity in the input mixture. The solution was then fed into the system and recirculated through the membrane cassette at 26 degC. Phosphate buffer (400 mM KH2PO4/K2HPO4, pH 8) was pumped into the system at flow rates matching the permeate flow rate. The retentate was sampled for analysis after 8.5 diafiltration volumes and the UV

absorbance of the permeate was monitored using the TFF system instrumentation. The diafiltration buffer was then switched to tribasic potassium phosphate (200 mM, pH 12.5) and the retentate sampled for analysis every 1 diafiltration volume while the UV absorbance of the permeate was monitored using the TFF system instrumentation. In this experiment, no oligonucleotide permeation was observed at pH 8 despite the high ionic strength (ca. 700-800 mM K+) having been previously demonstrated to permeate un-annealed single strand oligonucleotides. When the diafiltration buffer was switched to tribasic potassium phosphate (200 mM, pH 12.5), however, all of the 6-mer, 7-mer, 13-mer and 20-mer species permeated the membrane. See Figure 4.

Experiment 2C

Two large-scale experiments were carried out using the input reaction mixture of compositions as shown in Table 6.

Table 6: Input compositions

Oligo	**2C-1 HPLC area%**	**2C-2 HPLC area%**
Shortmer-1	5.3	5.7
Shortmer-3	2.7	3.1
Shortmer-3, adenylated	0.5	0.4
Shortmer-2	4.7	4.8
Shortmer-2, adenylated	0.5	0.6
Intermediate 1 (13-mer)	5.3	3.7
Intermediate 2 (13-mer)	0	0
OLIGO-1 (Product, 20-mer)	35.7	40.1
Impurity N+1	0.3	0.3
Impurity RT~14.57	0.1	0
Impurity RT~18.14	0.2	0.2
Impurity 4-arm	1.8	1.7
hub-template	42.6	39.1

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette (Pall cassette) and prepared by washing with 100mM Tris.HCl, pH 7.6. To the solution of oligonucleotides, ca. 80 g in 4.5 L of 100 mM Tris.HCl, pH 7.6 was added a solution of tetrapotassium EDTA, 1.25 molar equivalents of EDTA relative to the magnesium quantity in the input mixture. A portion of the solution, ca. 0.9 L was then fed into the system and recirculated through the membrane cassette at 26 degC. The remaining oligo solution was pumped into the system at flow rates matching the permeate flow rate

until all of the solution was consumed. At that point the retentate concentration reached ca. 80 g/L total oligo, with ca. 40 g/L hub-template. The diafiltration buffer was then switched to tribasic potassium phosphate (200 mM or 300 mM, pH 11.1) and the retentate sampled for analysis every 2 diafiltration volumes while the UV absorbance of the permeate was monitored using the TFF system instrumentation. Once analysis showed that the 13-mer intermediate had been reduced to <0.2%area by HPLC in the retentate (typically after 8-12 diavolumes), the diafiltration buffer was then switched to tribasic sodium phosphate (200 mM or 300 mM, pH 12.0). The retentate was sampled for analysis every 2 diafiltration volumes while the UV absorbance of the permeate was monitored using the TFF system instrumentation. Once analysis showed that the 20-mer product had been reduced to <10%area by HPLC in the retentate (typically after 4-14 diavolumes), the diafiltration buffer was then switched to 100 mM Tris.HCl, pH 7.6 and diafiltration continued until the retentate pH was ca. 7.8 and the hub-template could be recovered for re-use. The two large scale experiments demonstrated that the 6-mer, 7-mer and 13-mer can be permeated selectively to achieve separation from the 20-mer product. The yield and purity of the two experiments are shown below in Table 7.

Table 7

Experiment		**2C-1**	**2C-2**
Yield	Total mass	24.56g	27.53g
	(assay)	(84.0%)	(84.6%)
	Mass API	20.6g	23.3g
	Molar % yield**	62.2%	76.5%
HPLC area%	OLIGO-1	98.63	98.72
	13mer	0.20	<LOQ
	M+419	0.66	0.44
	hub-template	0.51	0.84
hub-template (H-T)	Input	38.9g	35.7g
	Recovery (%)	35.7g (92%)	33.5g (94%)

**molar % yield based on hub-template charge = (mol API isolated / 6) / mol H-T

Also, increasing the ionic strength of the buffer by increasing the concentration while maintaining the same pH in turn increased the rate of permeation of the 13-mer intermediate . See Figure 5.

The UF/DF purification process was also conducted using a Merck membrane (Merck Pellicon 3) instead of the Pall membrane cassette, and the process behaved identically using the Merck membrane as when using the Pall membrane cassette.

Experiment 2D

The 20-mer product (OLIGO-1) obtained from Experiment 2C-2 was further processed to remove traces of the hub-template. 20-mer Product (3g) was dissolved in trisodium phosphate buffer (2250 mL, 300 mM, pH 12.0) to form a solution of ca. 1.1 g/L oligonucleotide. The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and prepared by washing with 300 mM trisodium phosphate, pH 12. A portion of the solution, ca. 0.25 L was then fed into the system and recirculated through the membrane cassette at 26 degC. The remaining oligo solution was pumped into the system at flow rates matching the permeate flow rate until all of the solution was consumed. A diafiltration was then performed, with 300 mM trisodium phosphate, pH 12 pumped into the system at flow rates matching the permeate flow rate and the UV absorbance of the permeate was monitored using the TFF system instrumentation. Diafiltration was continued until the permeate UV signal showed a low level of oligonucleotide permeation. The retention of the hub-template versus the permeability of the 20-mer is shown by the HPLC analysis of the retentate. See Figure 6. The diafiltration buffer was switched to 100 mM Tris.HCl, pH 7.6 and diafiltration continued until the retentate pH was ca. 7.8 and the hub-template could be recovered for re-use. It was observed that as the conductivity was reduced the UV signal showing permeation of OLIGO-1 also quickly decreased.

The permeates from the ultrafiltration and the pH 12 diafiltration were then combined and concentrated by ultrafiltration using a 3 kDa MWCO membrane, then desalted by diafiltration with purified water. The resulting solution of the 20-mer product is then lyophilised.

As shown in Table 7 above the Input material contained ca. 0.84%area of hub-template by HPLC, and re-filtration through the 30 kDa MWCO membrane removed this completely to give the 20-mer product with an HPLC purity of 99.4%area. See Table 8.

Table 8: Input vs output purity for reprocessed OLIGO-1

Experiment		**2C-2**	**2D**
HPLC area%	OLIGO-1	98.72	99.4
	13mer	<LOQ	Not detected
	M+419	0.44	0.5
	hub-template	0.84	Not detected

Example 3: Purification of crude OLIGO-1 prepared by solid phase oligonucleotide synthesis

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and prepared by washing with 100 mM Tris.HCl, pH 7.6. A solution of 20-mer (OLIGO-1) prepared by solid phase oligo synthesis, total oligo content 25.9 mg/mL in 14%wt aqueous ammonium hydroxide was mixed with a

solution of hub-template, total oligo content 35 mg/mL such that a 1:1 ratio of 20-mer product to template was formed. The solution was then fed into the TFF system and recirculated through the membrane cassette at 26 degC. A first diafiltration was then performed, with 100 mM Tris.HCl pH 7.6 pumped into the system at flow rates matching the permeate flow rate until 8 diafiltration volumes had been collected. The retentate was sampled for analysis every 2 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. A second diafiltration was then performed, with 300 mM tripotassium phosphate, pH 11.1 pumped into the system at flow rates matching the permeate flow rate until 10 diafiltration volumes had been collected. The retentate was sampled for analysis every 2 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. A third diafiltration was then performed, with 300 mM tripotassium phosphate, pH 12.0 pumped into the system at flow rates matching the permeate flow rate until 10 diafiltration volumes had been collected. The retentate was sampled for analysis every 2 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. At the end of the experiment, the diafiltration buffer was replaced with purified water, and the retentate was desalted to obtain a solution of the hub-template in water, which was lyophilised. The permeates from the pH 12 diafiltration were then combined and concentrated by ultrafiltration using a 3 kDa MWCO membrane, then desalted by diafiltration with purified water. The resulting solution of 20-mer product was then lyophilised.

The first diafiltration with 100 mM Tris.HCl pH 7.6 did not appear to remove any oligonucleotides from the retentate; the permeate UV signal showed no upward inflection, and there was no change in the HPLC analysis.

When the diafiltration buffer was switched to 300 mM tripotassium phosphate, pH 11.1, the permeate UV signal showed some removal of oligo, and this was confirmed by loss of early eluting impurities from the retentate.

Compared to the input oligonucleotide solution, there was an increase in 20-mer area% in the isolated product of approximately 4% (from 87.8% to 91.9%), due to the complete removal of some early eluting impurities and the reduction of other impurities.

Example 4: pH and buffer screening for purification of OLIGO-3

Cytia Akta Crossflow was set up as shown in Figure 1. The feed vessel contained the oligonucleotide solution to be purified. The solution was pumped through to the membrane. Temperature throughout was not controlled and allowed to be ambient temperature (ca. 25-27°C). Transmembrane pressure, conductivity and UV were measured. Samples of the retentate and permeate were taken at regular intervals to track progress of filtration. Samples of the retentate were taken from the retentate vessel and samples of the permeate were taken from the line to the permeate collection vessel.

The reaction mixture containing OLIGO-3 (product) was treated with 75 mM solution of tetrapotassium EDTA (1.25 equiv relative to $MgCl_2$ charge in the enzymatic ligation reaction) to sequester magnesium ions from the enzyme. The resulting solution (109 ml = 1 diavolume) is added to the membrane system feed vessel.

The 30 kDa membrane was fitted in the membrane holder after being stored in 0.1M NaOH. The system was flushed first with deionised water until conductivity was seen to be decreasing and then using 200 mM tribasic potassium phosphate, pH adjusted to 8.5. A solution containing oligonucleotide products in 100 mM tris pH 7.6 was fed into the system. The solution was pumped into the system at a feed flow rate of 150 ml/min. Transmembrane pressure was set to 2barg. The retentate solution was sampled for HPLC analysis at regular intervals. A variety of pHs were screened from 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0. The pH was adjusted based on HPLC results. It was seen that intermediates were not permeating the membrane until pH 11.0 When screened at pH 11 again, product was seen to be permeating the membrane and so the pH dropped to 10.7. See the tables below.

The filtration was also completed at 300 mM tribasic potassium phosphate, pH adjusted to 11.1. Product permeated the membrane as well as intermediates.

Filtration to separate the product from the hub-template was run using 200 mM tribasic sodium phosphate, pH adjusted to 12.0. The retentate solution and permeate solution were sampled for HPLC at regular intervals. The experiment was stopped after 25 diavolumes. See Table 10.

Table 9.

pH/conc. (mM)	Diavol. (cum.)	% retention						
		Short mer-7	Short mer-8	Short mer-9	5' interme diate	3' interme diate	Product	Hub-Template
11.0/200	0	100%	100%	100%	100%	100%	100%	100%
11.0/200	2	0%	0%	0%	0%	0%	77.53%	77.32%
11.0/200	4	0%	0%	0%	0%	0%	68.88%	67.54%
11.0/200	6	0%	0%	0%	0%	0%	63.43%	67.82%
11.0/200	10	0%	0%	0%	0%	0%	64.28%	82.35%
11.0/200	15	0%	0%	0%	0%	0%	38.81%	69.88%
pH/conc. (mM)	Diavol. (cum.)	% retention						
		Short mer-7	Short mer-8	Short mer-9	5' interme diate	3' interme diate	Product	Hub-Template
10.7/200	0	100%	100%	100%	100%	100%	100%	100%

10.7/200	2	100%	95.96 %	100%	100%	0%	70.09%	77.60%
10.7/200	4	0%	0%	0%	25.23%	0%	72.80%	84.82%

Table 10.

pH/conc. (mM)	Diavolumes	% retention	
		Product	Hub-Template
12.0/200	0	77%	85%
12.0/200	2	71%	82%
12.0/200	4	51%	74%
12.0/200	6	44%	76%
12.0/200	8	26%	89%
12.0/200	10	21%	80%
12.0/200	12	22%	70%
12.0/200	14	14%	74%
12.0/200	20	4%	73%
12.0/200	25	3%	71%

Table 11.

pH/conc. (mM)	Diavolumes	% retention		
		5' Intermediate	Product	Hub-Template
11.0/300	0	100%	100%	100%
11.0/300	5	0%	44.26%	62.43%
11.0/300	10	0%	21.10%	100%
11.0/300	15	0%	4.6%%	52.12%

In the experiment using 200 mM tribasic potassium phosphate, pH 10.7, it could be seen that there was good separation of the shortmer and intermediates as well as good retention of the product and hub-template. The separation of product from template was successful using 200 mM tribasic sodium phosphate, pH 12.0. See Table 10.

Oligonucleotides of different lengths and molecular weights can be separated using ultrafiltration. As shown above, the conditions such as buffer strength and pH affect the level of separation. It was demonstrated that segment oligonucleotides (shortmers of 7 nucleotides in length) and 14-mer intermediates can be separated from the product (OLIGO-3, 21-mer oligonucleotide) and hub-template (comprising 6 x 27-mer attached to a solid support), and the product and hub-template can, in turn, be separated from each other with the proper buffer strength and pH.

Example 5: pH and buffer screening for purification of OLIGO-2

Input Solution Reaction Mixture

Oligonucleotide solution reaction mixture (300 mg scale synthesis of OLIGO-2) was a 40 mL reaction solution with 437 mg of hub-template and an estimated 272.70 mg of product 21-mer oligonucleotide (OLIGO-2).

Analysis of the input material is shown in Table 12 below with the area% of the identified oligo component peaks. ~4% shortmers remaining and 4% intermediates.

Table 12.

Oligo compounds	Product	Shortmer-4	Shortmer-5 and Shortmer-6	5' Intermediate	3' Intermediate
HPLC area%	86.93	3.18	0.61	3.5	0.41

The reaction mixture was treated with 75 mM solution of tetrapotassium EDTA (1.25 equiv relative to $MgCl_2$ charge in the enzymatic ligation reaction) to sequester magnesium ions.

Diafiltration 1

The TFF system was fitted with a 30 kDa MWCO PES membrane cassette and prepared by washing with 250 mM tripotassium phosphate solution pH10.5.

150 mL of oligonucleotide input solution (~6 mg/mL total oligo, ~2.9 mg/mL hub-template) was poured into the reservoir. A flow rate of 150 mL/min was applied (~8 L/min/m2) to homogenise feed solution, once homogenised a transmembrane pressure of 2 bar was applied.

250 mM tribasic potassium phosphate diafiltration buffer was used, the buffer pH was increased in 0.1 increments from 10.5 to 10.8 to identify the pH conditions for permeation of the main impurities, followed by the 21-mer product. Constant retentate volume was retained throughout the diafiltration.

The retentate solution was sampled and analysed at regular intervals, retentate volume remained constant throughout, results are shown in Table 1. Each buffer condition was maintained for a number of diavolumes as defined in Table 14. UV absorbance of the permeate was monitored using the TFF system instrumentation.

Diafiltration 2

Retentate volume, TMP, flow rate and membrane were maintained from Diafiltration 1. The buffer was switched to 200 mM tribasic sodium phosphate pH 12.0 and continued for 6 diavolumes. The sampling interval and results are shown in Table 13.

Once ~90% permeation of the product (21-mer oligonucleotide) was observed, addition of sodium phosphate buffer was halted and switched to deionised water to desalt the retentate for accurate quantification of remaining oligo species.

Table 13 – Diafiltration 1 and 2 Retentate HPLC Results

Diafiltration Buffer	Diavolumes	Remaining Peak Area as a % of Starting Retentate			
		Total Shortmer Impurities	Total Intermediate Impurities	21-mer Product	Hub-Template
K3PO4 250mM pH10.5	0	100.0%	100.0%	100.0%	100.0%
	2	0.0%	101.1%	100.6%	101.4%
	4	0.0%	93.4%	99.0%	103.4%
	6	0.0%	78.0%	100.9%	105.7%
K3PO4 250mM pH10.6	2	0.0%	75.7%	103.0%	108.4%
	4	0.0%	59.3%	97.7%	103.9%
	10	0.0%	44.3%	104.2%	110.9%
K3PO4 250mM pH10.7	2	0.0%	42.7%	97.6%	106.7%
	4	0.0%	29.2%	92.3%	104.7%
	10	0.0%	13.3%	87.2%	110.0%
K3PO4 250mM pH10.8	2	0.0%	9.5%	72.1%	101.4%
	6	0.0%	0.0%	59.4%	103.8%
Na3PO4 200mM pH12.0	6	0.0%	0.0%	23.5%	91.0%
	10	0.0%	0.0%	9.4%	106.4%

Diafiltration 3

(A) - *Concentration and desalting of 200 mM sodium phosphate pH 12.0 permeate (from Diafiltration 2)*

1 kDa MWCO PES (0.1m^2 surface area) membrane was fitted. The system and cartridge were prepared by washing with tribasic sodium phosphate 200 mM pH12.0 buffer. A flow rate of 450 mL/min was applied (4.5 L/min/m^2) with a transmembrane pressure of 2 bar. The pH 12.0 permeate containing the product (21-mer oligonucleotide) was concentrated and then desalted with deionised water for accurate quantification of total oligonucleotides and impurity analysis (see Table 14).

(B) *- Concentration and desalting of 250 mM potassium phosphate pH 10.8 permeate (from Diafiltration 1)*

Significant loss of the product (21-mer oligonucleotide) was observed to the pH 10.8 permeate. To fully quantify the loss, the permeate was concentrated and desalted for accurate quantification of total oligonucleotides and impurity analysis (see Table 14). The same process steps/conditions as in (A) above were followed.

Table 14 - Purity Analysis and Yield

Step	Sample	21-mer Product Purity (%area)	Total Impurities Excluding Hub-Template (% area)	Total Impurities (% area)	21-mer Product Mass (mg)	Yield of 21-mer product %
Diafiltration 3 (B)	Desalted concentrated pH 10.8 permeate	92.1	6.20	7.49	60.92	22.32%
Diafiltration 3 (A)	Desalted concentrated pH 12.0 permeate	90.6	3.60	8.99	127.14	46.57%

Table 13 data shows that using 250mM pH 10.6 – 10.7 tripotassium phosphate, the annealed segment 7-mers and intermediate 14-mers can be separated from the hub-template. The majority of 21-mer product and a hub-template remain annealed and rejected by the membrane. At pH10.8 250 mM tripotassium phosphate, there is significant permeation of the 21-mer product.

200 mM tribasic sodium phosphate efficiently separated the remaining annealed 21-mer product and drove its permeation through the membrane, this was quantified by SoloVPE and purity analysis:

Collectively the pH10.8 and pH12.0 permeates contain 68.89% yield of 21-mer product with >90% purity. ~9.4% (remaining peak area) of the 21-mer product remains in the retentate from Diafiltration 2 with the hub-template.

It was demonstrated that segment oligonucleotides (shortmers of 7 nucleotides in length) and 14-mer intermediates can be separated from the product oligonucleotide (OLIGO-2, 21-mer oligonucleotide) and hub-template (comprising 6 x 27-mer attached to a solid support), and the product and hub-template can, in turn, be separated from each other with the proper buffer strength and pH.

Example 6: Purification of single stranded antisense oligonucleotide (OLIGO-1)

Ultrafiltration/diafiltration (UF/DF) experiments were carried out using an ÄKTA crossflow tangential flow filtration (TFF) system (Cytiva), and Pall Centramate T-Series cassettes, with Omega PES membranes – molecular weight cut-off (MWCO) is specified below. The TFF system was operated within the manufacturer specified ranges for the membrane cassettes; crossflow rate 5-7 L/min/m2, maximum transmembrane pressure (TMP) 4 barg. pH and conductivity of permeate were measured using the instruments integrated into the ÄKTA crossflow system. pH and conductivity of retentate were measured using a Mettler-Toledo SevenCompact Duo S213 pH and conductivity meter.

HPLC Method: Column: Waters XBridge peptide BEH C18 300A, 3.5um, 2.1 mm x 150 mm; Column temperature: 50 degC; Mobile phase A: 5mM TBuAA and 1uM EDTA in 90:10 %v/v Water:Acetonitrile; Mobile phase B: 5mM TBuAA and 1uM EDTA in 20:80 %v/v Water:Acetonitrile; Flow rate: 0.5 mL/min; Gradient: Time (minutes)/MPB %; 0/35, 18/71, 25/95, 25.1/35, 35/35; UV detection wavelength: 260 nm; Injection volume: 7 uL.

The product (OLIGO-1) solution from an enzymatic ligation reaction, ca. 4.5 L containing approximately 40 g of 6-mer, 7-mer, 13-mer (intermediate) and 20-mer (product) oligonucleotides annealed to approximately 40 g of hub (1 equiv., 1 wt) comprising six 26-mer template oligonucleotides (hub-template) in 100 mM tris.HCl at pH 7.6, was filtered to remove the enzyme.

Step 1: The combined filtrate was mixed with a 75 mM solution of tetrapotassium EDTA (1.25 equiv relative to $MgCl_2$ charge in the enzymatic ligation reaction) to sequester magnesium ions. A portion of the resulting EDTA treated filtrate (ca. 1 L, this volume = 1 diavolume, which determines hub-template concentration in retentate, target 40 mg/mL) was added to the TFF system and the remaining EDTA treated filtrate was then concentrated at ca. 25-27°C by ultrafiltration using a 30 kDa MWCO polyether sulfone (PES) membrane until the volume is ca. 0.9-1.0 L, so the total oligo concentration in the retentate reaches approximately 80 g/L.

Step 2: A first diafiltration was then performed at 25-27°C with 300 mM tripotassium phosphate, pH 11.1. Diafiltration was continued for 10 diavolumes, and the retentate is analysed by HPLC at 2 diavolume intervals to track the clearance of 6-mer, 7-mer and 13-mer. The operation was stopped when the level of 13-mer in the retentate was reduced to less than 0.2%area by HPLC. Approximately 10-20 diavolumes, or 10-20 L of the buffer was required.

Step 3: A second diafiltration was performed on the permeate from the first diafiltration at 25-27°C, with 300 mM tribasic sodium phosphate, pH 12.0. Diafiltration was continued for 4 diavolumes, and the retentate is analysed by HPLC at 2 diavolume intervals to track the clearance of product. The operation was stopped when the level of product in the retentate was reduced to approximately 8-12%area by HPLC. Approximately 4-14 diavolumes, or 4-14 L of the buffer was required.

Step 4: A third diafiltration was performed on the retentate from the second diafiltration at 25-27°C, with 100 mM tris.HCl buffer at pH 7.6. Diafiltration was continued for about 6 diavolumes and until the pH of the retentate fell to 7.6-7.8. The retentate solution containing the hub-template was concentrated to about 0.5 L.

Step 5: Optionally, the permeate from the second diafiltration can be further purified to remove residual hub-template. A portion of the permeate from the second diafiltration (0.9-1.0 L, this volume = 1 diacvolume) was added to the TFF system, and the remaining permeate was concentrated at ca. 25-27°C by ultrafiltration using a 30 kDa MWCO polyether sulfone (PES) membrane. The product passed through the membrane during this operation, leaving residual hub-template in the retentate. A fourth diafiltration with 300 mM tribasic sodium phosphate, pH 12.0 was performed to recover the product from the retentate. Diafiltration was continued for 10-12 diavolumes and the operation was stopped when the product concentration in the retentate was below 1 mg/mL.

Step 6: A portion of the combined permeates from the ultrafiltration and diafiltration of Step 5 (0.9-1.0 L, this volume = 1 diavolume, which determines product concentration in the retentate) was added to the TFF system, and the remaining combined permeates were concentrated at ca. 25-27°C by ultrafiltration using a 3 kDa MWCO polyether sulfone (PES) membrane until the product concentration in the retentate reached 25-50 mg/mL. A diafiltration was then performed with purified water to remove trisodium phosphate from the retentate. Diafiltration was continued until the permeate conductivity was below 0.05 mS/cm. Approximately 14-24 diavolumes was required. The product solution was concentrated until the retentate volume reached approximately 0.5 L (the product concentration was approximately 40-50 mg/mL by optical density (OD)).

A schematic summary of the purification process of the 20-mer product is shown in **Figure 7**. The resulting product solution was obtained as the sodium salt in water (HPLC purity >99%area) and the yield was 65-75% based on the input of starting materials (6-mer/7-mers) of the enzymatic ligation reaction.

Example 7 Purification of single stranded sense oligonucleotide (OLIGO-3)

The product (OLIGO-3) solution from the enzymatic ligation reactions, 22 ml reaction volume, diluted to 100 ml using 100 mM tris pH 7.6, containing approximately ca. 198 mg of 7-mer, 14-mer (intermediates) and 21-mer (product) oligonucleotides annealed to ca. 212 mg hub comprising six 27-mer template oligonucleotides (hub-template) in 100 mM tris.HCl at pH 7.6 was filtered to remove the enzyme.

Step 1: The combined filtrate from the enzyme removal was treated with 75 mM solution of tetrapotassium EDTA (1.25 equiv relative to $MgCl_2$ charge in the enzymatic ligation reaction) to sequester magnesium ions from the enzyme. The resulting solution (109 ml = 1 diavolume) was added to the membrane system feed vessel.

Step 2: A first diafiltration was performed at ca. 25-27°C using a 30 kDa molecular weight cut off (MWCO) polyether sulfone (PES) membrane using 200 mM tribasic potassium phosphate,

adjusted to pH 10.7 using phosphoric acid. Diafiltration is continued until the level of 7-mer and 14-mer in the retentate have been reduced to < 1%area by HPLC. Analysis was completed by HPLC taking samples at ca. 2 diavolume intervals to track impurity clearance. Approximately 8-12 diavolumes was required.

Step 3: A second diafiltration was performed on the permeate from the first diafiltration at ca. 25-27°C with 200 mM tribasic sodium phosphate, adjusted to pH 12.0 using phosphoric acid. Diafiltration was continued for 10-15 diavolumes and the retentate was analysed by taking samples at 2 diavolume intervals to track the clearance of product. The operation was stopped when the level of product left was reduced to approximately 2-12%area by HPLC. Approximately, 10-15 diavolumes, or 1000 ml – 1500 ml of the buffer was required.

Step 4: A third diafiltration was performed on the retentate from the second diafiltration at ca. 25-27°C with purified water. Diafiltration was continued until the conductivity of the retentate fell to < 0.05 mS/cm. The retentate containing hub-template was retained for re-use in the ligation reaction, 100 ml.

Step 5: Optionally, the permeate from the second diafiltration can be further purified to remove residual hub-template. A portion of the permeate from the second diafiltration (100 ml, this volume = 1 diavolume) was added to the TFF system, and the remaining permeate was concentrated at ca. 25-27°C by ultrafiltration using a 30 kDa MWCO polyether sulfone (PES) membrane. The product passed through the membrane during this operation, leaving residual hub-template in the retentate. A fourth diafiltration with 200 mM tribasic sodium phosphate, pH 12.0 was performed to recover the product from the retentate. Diafiltration was continued for 10-15 diavolumes and the operation was stopped when the product concentration in the retentate was below 1 mg/mL.

Step 6:

A portion of the combined permeates from the ultrafiltration and diafiltration of Step 5 (100 ml, this volume = 1 diavolume, which determines product concentration in the retentate) was added to the TFF system, and the remaining combined permeates were concentrated at ca. 25-27°C by ultrafiltration using a 1 kDa MWCO polyether sulfone (PES) membrane until the retentate volume in the retentate reached 1-2 mg/ml. A diafiltration was then performed with purified water to remove tribasic sodium phosphate from the retentate. Diafiltration was continued until the permeate conductivity was below 0.05 mS/cm. Approximately 29-38 diavolumes was required. The product solution was concentrated until the retentate volume reached 1 vol (the product concentration was approximately 1.7 mg/mL by OD).

The resulting product solution was obtained as the sodium salt in water (HPLC purity >84%area) and the yield was 63% based on the input of starting materials (7-mers) of the enzymatic ligation reaction.

Example 8 Purification of single stranded antisense oligonucleotide (OLIGO-2)

The product (OLIGO-2) solution from the enzymatic ligation reaction, ~40 ml reaction volume, contained approximately ca. 273 mg of 7-mer, 14-mer (intermediates) and 21-mer (product) oligonucleotides annealed to ca. 437 mg hub-template (1 equiv., 1wt) comprising six 27-mer template oligonucleotides and a hub core, in 100 mM tris-HCl pH7.6 buffer. The solution was centrifuged to remove the enzyme.

Step 1: The filtrate from the enzyme removal was treated with 75 mM solution of tetrapotassium EDTA (1.25 equiv relative to $MgCl_2$ charge in the enzymatic ligation reaction) to sequester magnesium ions from the enzyme. The resulting solution was diluted with 100 mM tris pH 7.6 volume of 80mL was added to the membrane system feed vessel. Along with necessary washes the final volume was (150 ml = 1 diavolume).

Step 2: A first diafiltration, was performed at ca. 25-27°C using a 30 kDa molecular weight cut off (MWCO) polyether sulfone (PES) membrane using 250 mM tribasic potassium phosphate, adjusted to pH 10.7 using phosphoric acid. Diafiltration was continued until the level of 7-mer and 14-mer in the retentate have been reduced to < 1%area by HPLC. Analysis was completed by HPLC taking samples at ca. 2 diavolume intervals to track impurity clearance. Approximately 8-12 diavolumes were required.

Step 3: A second diafiltration was performed at ca. 25-27°C with 200 mM tribasic sodium phosphate, adjusted to pH 12.0 using phosphoric acid. Diafiltration was continued for 10-15 diavolumes and the retentate was analysed by taking samples at 2 diavolume intervals to track the clearance of product. The operation was stopped when the level of product left was reduced to approximately 5-15%area by HPLC. Approximately, 10-15 diavolumes, or 1500ml – 2250 ml of the buffer was required.

Step 4: A third diafiltration was performed at ca. 25-27°C with deionised water to desalt the hub-template in the retentate. Diafiltration is continued until the conductivity is <0.05 mS/cm. The retentate containing hub-template is retained for re-use.

Step 5: A portion of the Step 3 permeate (100 ml, this volume = 1 diavolume, which determines product concentration in the retentate) was added to the TFF system, and the remaining permeate was concentrated at ca. 25-27°C by ultrafiltration using a 1 kDa MWCO polyether sulfone (PES) membrane until the product in the retentate reached 1-2 mg/ml. A diafiltration was then performed with purified water to remove tribasic sodium phosphate from the retentate. Diafiltration was continued until the permeate conductivity was below 0.05 mS/cm. Approximately 29-38 diavolumes were required. The product solution was concentrated until the retentate volume reached 1 vol (the product concentration was approximately 1.88 mg/mL by OD).

The resulting product solution was obtained as the sodium salt in water (HPLC purity >90%area) and the yield was 68.9% based on the input of starting materials (7-mers) of the enzymatic ligation reaction.

Example 9: Determining optimal ionic strength for permeation of OLIGO-4 shortmers (6-mer/ 7-mer), and intermediates (25-mer/26-mer)

Example 9 used an illustrative oligonucleotide product, shortmers, and template of which the sequences are specified in Table 15 below.

Table 15. Single stranded antisense oligonucleotide, OLIGO-4

Compounds	Sequence	SEQ ID NO
Product, 5′ to 3′ (OLIGO-4)	g*c*a*g*a*_G*G*T*G*A*A*G*C*T*G*A*A*G*C*T*G*A*A*G*C G*A*_a*g*u*g*c	SEQ ID NO: 16
5′ Segment (Shortmer-1)	g*c*a*g*a*_G*G*_	SEQ ID NO: 2
Center segment (Shortmer-2); OLIGO-4 has three center segments	_T*G*A*A*G*C*_	SEQ ID NO: 3
3′ Segment (Shortmer-3)	_G*A*_a*g*u*g*c	SEQ ID NO: 4
Template-4, 5′ to 3′	Aminohexyl-_T-T-T-G-C-A-C-T-T-C-G-C-T-T-C-A-G-C-T-T-C-A-G-C-T-T-C-A-C-C-T-C-T-G-C*T*T*T_	SEQ ID NO: 17

Chemical modification legends for Table 15:

Lowercased letters: nucleotides having 2′-methoxyethyl (2′-MOE) substituted ribose sugars;

Italicized uppercased letters: nucleotides having 2′-deoxy ribose sugars;

- : phosphodiester linkage;

* : phosphorothioate linkage;

All cytidines (c, *C*) are 5-methyl substituted;

Uridine (*u*) is 5-methyl substituted.

Experiment 9A

An experiment was carried out using the input reaction mixture of composition shown in Table 16 below.

Table 16: Input composition

Oligo	**HPLC area%**
'5'-shortmer' (7-mer)	23.0
'center-shortmer' (6-mer)	23.3
'3'-shortmer' (7-mer)	12.2
Intermediate (25-mer)	1.6
Intermediate (26-mer)	Not detectable
Product (32-mer; OLIGO-4)	16.3
Hub-template	23.6

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and conditioned by washing with 200 mM tribasic potassium phosphate, pH 11.21. Input oligo composition was 4.39 mg/mL total oligo, retentate concentration 1.04 mg/ml hub-template. The oligo solution was fed into the system and recirculated through the membrane cassette at 25°C. Tribasic potassium phosphate pH 11.21 buffer solutions at increasing concentration (200 mM, 250 mM, 300 mM) were pumped into the system at flow rates matching the permeate flow rate. The retentate was sampled for analysis every 3 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. The ionic strength of the buffer was increased every 12 diavolumes. It was observed that the shortmer impurities were completely cleared after 6 diavolumes of diafiltration with the lower ionic strength buffer (200 mM tribasic potassium phosphate, pH 11.21) while the removal of the intermediate required a greater ionic strength buffer (300 mM tribasic potassium phosphate, pH 11.21). Once analysis showed that the 25-mer intermediate ('5'-shortmer plus 'center-shortmer' plus 'center-shortmer' plus 'center-shortmer') had been reduced to <0.5%area by HPLC in the retentate (typically after 12 diavolumes), the diafiltration buffer was then switched to a higher pH tribasic potassium phosphate buffer (300 mM, pH 11.88). The retentate was sampled for analysis every 2 or 3 diafiltration volumes while the UV absorbance was monitored using the TFF system instrumentation. Once analysis showed that the 32-mer OLIGO-4 product had been reduced to <2% area by HPLC in the retentate (typically after 12-18 diavolumes), the diafiltration buffer was switched to purified water to desalt the hub-template. The purified water was pumped into the system at flow

rates matching the permeate flow rate until the retentate conductivity was 43.7 µS/cm. The hub-template was collected from the TFF system and stored at -5°C. The permeate that contained the 32-mer OLIGO-4 collected from the diafiltration process was concentrated by ultrafiltration using a 3 kDa MWCO membrane to 0.79 mg/mL and desalted by pumping purified water into the system at flow rates matching the permeate flow rate until the retentate conductivity was 41.9 mS/cm. The full-length product was collected from the TFF system and stored at -5°C.

The experiment demonstrated that the 6-mer, 7-mers and 25-mer can be permeated selectively to achieve separation from the 32-mer OLIGO-4 product. The purity obtained for this experiment is shown below (Table 17).

Table 17: product composition

HPLC area%	'5'-shortmer' (7-mer)	Not detectable
	'center-shortmer' (6-mer)	Not detectable
	'3'-shortmer' (7-mer)	Not detectable
	Intermediate (25-mer)	0.38
	Intermediate (26-mer)	Not detectable
	Product (32-mer)	92.41
	hub-template	2.28

Experiment 9B

An experiment was carried out using the input reaction mixture of composition shown in Table 18 below.

Table 18: Input composition

Oligo	**HPLC area%**
'5'-shortmer' (7-mer)	6.0
'centre-shortmer' (6-mer)	13.1
'3'-shortmer' (7-mer)	11.8
Intermediate (25-mer)	10.3
Intermediate (26-mer)	1.3
Product (32-mer; OLIGO-4)	25.1

Hub-template	32.3

The TFF system was fitted with a 30 kDa MWCO, PES membrane cassette and conditioned by washing with 300 mM tribasic potassium phosphate, pH 11.31. Input oligo composition was kept at 4.39 mg/mL mL total oligo, retentate concentration 1.42 mg/ml hub-template. The oligo solution was fed into the system and recirculated through the membrane cassette at room temperature (25°C). Tribasic potassium phosphate buffer, 300 mM, pH 11.31 was pumped into the system at flow rates matching the permeate flow rate. The retentate was sampled for analysis every 3 diafiltration volumes and the UV absorbance of the permeate was monitored using the TFF system instrumentation. It was observed that the shortmer impurities were completely cleared after 3 diavolumes of diafiltration. Once analysis showed that the 25-mer ('5'-shortmer plus 'center-shortmer' plus 'center-shortmer' plus 'center-shortmer') and 26-mer ('5'-shortmer plus 'center-shortmer' plus 'center-shortmer' plus '3'-shortmer) intermediates had been reduced to <1.0%area by HPLC in the retentate (typically after 44 diavolumes), the diafiltration buffer was then switched to tribasic potassium phosphate buffer (300 mM, pH 11.95). The retentate was sampled for analysis every 3 or 4 diafiltration volumes while the UV absorbance was monitored using the TFF system instrumentation. Once analysis showed that the 32-mer OLIGO-4 product had been reduced to <15% area by HPLC in the retentate (typically after 48 diavolumes), the diafiltration buffer was switched to purified water to desalt the hub-template. The purified water was pumped into the system at flow rates matching the permeate flow rate until the retentate conductivity was 31.7 mS/cm. The hub-template was collected from the TFF system and stored at -5 °C. The permeate that contained the 32-mer OLIGO-4 was concentrated by ultrafiltration using a 3 kDa MWCO membrane to 0.69 mg/mL and desalted by pumping purified water into the system at flow rates matching the permeate flow rate until the retentate conductivity was 20.8 mS/cm. The 32-mer OLIGO-4 was collected from the TFF system and stored at -5°C.

The experiment demonstrated that the 6-mer, 7-mers, 25-mer and 26-mer can be permeated selectively to achieve separation from the 32-mer OLIGO-4 product. The purity obtained for this experiment is shown below (Table 19).

Table 19: Product composition

HPLC area%	'5'-shortmer' (7-mer)	Not detectable
	'center-shortmer' (6-mer)	Not detectable
	'3'-shortmer' (7-mer)	Not detectable
	Product (32-mer)	86.24
	Intermediate (25-mer)	1.00
	Intermediate (26-mer)	1.09

	hub-template	11.67

CLAIMS

1. A method of purifying a product, wherein the product is a single-stranded oligonucleotide, comprising:
   (a) obtaining a solution comprising (i) an impurity:template complex and (ii) a product:template complex, wherein the template is an oligonucleotide comprising a sequence complementary to the product,
   (b) subjecting the solution to ultrafiltration/diafiltration (UF/DF) to form a first retentate comprising the product:template complex and a first permeate comprising the impurity, wherein the UF/DF is carried out using a first buffer solution at a pH of about 9-13,
   (c) subjecting the first retentate of step (b) to UF/DF to form a second retentate comprising the template and a second permeate comprising the product, wherein the UF/DF is carried out using a second buffer solution at a pH greater than the pH of the first buffer solution.

2. The method according to claim 1, wherein the pH of the second buffer solution is greater than the pH of the first buffer solution by about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9 or about 2.0.

3. The method according to claim 1 or 2, wherein the pH of the first buffer solution is about 9-12.5, about 9-12, about 9-11.5, about 9-11, about 10-12.5, about 10-12, about 10-11.5, about 10-11 or about 10.5-11.5.

4. The method according to any one of claims 1 to 3, wherein the pH of the second buffer solution is about 10-14, about 10-13.5, about 10-13, about 11-14, about 11-13.5, about 11-13, about 11-12.5, about 11-12 or about 11.5-12.5.

5. The method according to any one of the preceding claims, wherein the first buffer solution and/or the second buffer solution has a conductivity of about 25 mS/cm to about 120 mS/cm, about 25 mS/cm to about 100 mS/cm, about 25 mS/cm to about 80 mS/cm, about 25 mS/cm to about 60 mS/cm, about 30 mS/cm to about 100 mS/cm, or about 30 mS/cm to about 80 mS/cm.

6. The method according to any one of the preceding claims, wherein the first buffer solution and/or the second buffer solution comprises a phosphate buffer.

7. The method according to any one of the preceding claims, wherein the first buffer solution and/or the second buffer solution comprises potassium phosphate.

8. The method according to any one of the preceding claims, wherein the first buffer solution and/or the second buffer solution comprises sodium phosphate.

9. The method according to any one of the preceding claims, wherein the first buffer solution comprises potassium phosphate and the second buffer solution comprises sodium phosphate.

10. The method according to any one of the preceding claims, wherein the first buffer solution and/or the second buffer solution further comprises an ionic strength adjustor.

11. The method according to claim 10, wherein the ionic strength adjustor is a cation, optionally sodium ion or potassium ion.

12. The method according to any one of the preceding claims, wherein the first buffer solution and/or the second buffer solution comprises about 200 mM to about 1500 mM, about 200 mM to about 1200 mM, about 300 mM to about 1500 mM, about 300 mM to about 1200 mM, about 400 mM to about 800 mM, or about 600 mM to about 900 mM cation, optionally sodium ion or potassium ion.

13. The method according to any one of the preceding claims, wherein the template is present in a form that allows the template to be retained in the second retentate.

14. The method according to any one of the preceding claims, wherein the template is attached to a support material.

15. The method according to any one of the preceding claims, wherein the UF/DF of step (b) and/or step (c) is carried out using a membrane having a molecular weight cut off (MWCO) of about 5 to about 50 kDa, about 10 to about 40 kDa, about 20 to about 40 kDa or about 20 to about 30 kDa.

16. The method according to claim 15, wherein the membrane comprises polyethersulfone.

17. The method according to any one of the preceding claims, wherein the product comprises about 10-50, about 10-40, about 10-35, about 13-30, about 15-35, about 15-30, about 15-25, about 20-32, or about 20-23 nucleotides.

18. The method according to any one of the preceding claims, wherein the solution of step (a) comprises about 5 to about 60 mg/ml, about 10 to about 60 mg/ml, about 20 to about 60 mg/ml, about 30 to about 60 mg/ml, about 35 to about 55 mg/ml, or about 35 to about 45 mg/ml template.

19. The method according to any one of the preceding claims, wherein the impurity is a shortmer or an intermediate.

20. The method according to any one of the preceding claims, wherein UF/DF of step (b) and/or step (c) is carried out about 20 to about 30 degrees Celsius, about 22 to about 28 degrees Celsius, about 24 to about 28 degrees Celsius or about 25 to about 27 degrees Celsius.

21. The method according to any one of the preceding claims, further comprising:

(d) subjecting the second permeate of step (c) to UF/DF to form a third retentate comprising the product and substantially free of the second buffer solution, wherein the UF/DF is carried out using water and a membrane having a molecular weight cut off (MWCO) of about 1 kDa to about 5 kDa.

22. The method according to claim 21, further comprising subjecting the third retentate of step (d) to lyophilization to form a lyophilized composition comprising the product.

23. The method according to any one of the preceding claims, further comprising:

(e) subjecting the second retentate of step (c) to UF/DF to form a fourth retentate comprising the template, wherein the UF/DF is carried out using a third buffer solution at a pH of about 7-8.

24. The method according to any one of the preceding claims, wherein the product is synthesized by a ligation reaction catalysed by an enzyme.

25. The method according to claim 24, wherein the solution of step (a) is prepared comprising the steps:

(a1) removing the enzyme from the ligation reaction by filtration to provide a filtrate;

(a2) treating the filtrate with EDTA to provide a mixture; and

(a3) concentrating the mixture of step (a2) to provide the solution of step (a).

26. The method according to any one of the preceding claims, wherein the UF/DF is carried out at a crossflow rate of about 2 to about 20 L/min/m2, about 2 to about 10 L/min/m2 or about 5 to about 7 L/min/m2.

27. The method according to any one of the preceding claims, wherein the product in the second permeate of step (c) has a purity of at least: about 80%, about 90% about 95%, about 96%, about 97%, about 98% or about 99%.

28. A single-stranded oligonucleotide product obtained by the method according to any one of the preceding claims.