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# Guideline on the Development and Manufacture of Oligonucleotides

Draft

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# Guideline on the Development and Manufacture of Oligonucleotides

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# Executive summary

This guideline addresses specific aspects regarding the manufacturing process, characterisation, specifications and analytical control for synthetic oligonucleotides which are not covered in the Guideline on the Chemistry of Active Substances (EMA/454576/2016) or Chemistry of Active Substances for Veterinary Medicinal Products (EMA/CVMP/QWP/707366/2017). It also contains requirements and considerations related to conjugation, to active substance in solution, to medicinal product development, to oligonucleotide generics development, to oligonucleotide personalised medicine approaches and to clinical trial applications (human products only).

# 1. Introduction

This guideline has been prepared in accordance with the structure agreed for the quality part of the dossier for human medicinal products (Format ICH-CTD). The subheadings have been included for the sake of clarity.

# 2. Scope

The purpose of this guideline is to set out the type of information required for the development, manufacture and control of synthetic oligonucleotides (existing or new chemical entities) used in a medicinal product, in the context of obtaining a marketing authorisation. There is also a chapter on the requirements for clinical trial applications.

Synthetic oligonucleotides are fully or partially excluded from the scope of ICH Q3A/B (VICH GL10/GL11), ICH Q6A/B (VICH GL39/GL40) and ICH M7 (EMA/CVMP/SWP/377245/2016). This guideline addresses those specific aspects regarding the manufacturing process, characterisation, specifications and analytical control for synthetic oligonucleotides which are not covered in the Guideline on the Chemistry of Active Substances (EMA/454576/2016) and Chemistry of Active Substances for Veterinary Medicinal Products (EMA/CVMP/QWP/707366/2017), and is to be considered complementary to the latter guidelines. mRNA entities are out of scope of this guideline.

Whilst veterinary products are outside the scope of ICH guidelines there are no corresponding VICH guidelines on certain topics. Nevertheless, the principles outlined in these ICH guidelines may also be relevant to veterinary products to facilitate flexibility and to allow the applicant the option of using different approaches to product development.

For the veterinary products, the reference to GMP Eudralex Volume 4 will eventually be superseded by respective Implementing Acts, when they come into force.

# 3. Legal basis and relevant guidelines

This guideline should be read in conjunction with the introduction and general principles of Annex I to Directive 2001/83/EC as amended for human medicinal products and Annex II of Regulation (EU) 2019/6 as amended for veterinary medicinal products, and all other relevant EU and (V)ICH guidelines. These include, but are not limited to:

- Guideline on the Chemistry of Active Substances EMA/454576/2016 and Chemistry of Active Substances for Veterinary Medicinal Products (EMA/CVMP/QWP/707366/2017)
- EU GMP guide Part II: Basic Requirements for Active Substances used as Starting Materials

- EU GMP guide Part II, Q&A 12 on GMP requirements to be applied for the formulation of biological active substances with excipients, when described in the active substance section of a registration dossier
- ICH Q1 A-F Stability testing of new drug substances and drug products – Scientific guidelines (veterinary VICH GL3-5, GL45, GL51 and GL58)
- ICH Q2 Guideline on validation of analytical procedures (veterinary VICH GL1 and GL2)
- ICH Q3A Impurities in new drug substances CPMP/ICH/2737/99 (veterinary VICH GL10)
- ICH Q3B Impurities in new drug products CPMP/ICH/2738/99 (veterinary VICH GL11)
- ICH Q3C Guideline for residual solvents EMA/CHMP/ICH/82260/2006 (veterinary VICH GL18)
- ICH Q3D Elemental impurities EMA/CHMP/ICH/353369/2013 (veterinary Reflection paper EMA/CVMP/QWP/153641/2018)
- ICH Q6A Specifications: Test Procedure and Acceptance Criteria for New Drug Substances and New Drug Products – Chemical Substances CPMP/ICH/367/96 (veterinary VICH GL39)
- ICH Q8 Pharmaceutical development – scientific guideline EMA/CHMP/ICH/167068/2004
- ICH Q9 Quality risk management EMA/CHMP/ICH/24235/2006
- ICH Q11 Guideline on development and manufacture of drug substances (chemical entities and biotechnological/ biological entities) EMA/CHMP/ICH/425213/2011
- ICH Q13 Continuous manufacturing of drug substances and drug products EMA/CHMP/ICH/427817/2021
- ICH M7 Guideline on assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk EMA/CHMP/ICH/83812/2013 (veterinary EMA/CVMP/SWP/377245/2016)
- Investigation of Chiral Active Substances 3CC29a for human products, EMEA/CVMP/128/95 for the veterinary products
- Reflection paper on statistical methodology for the comparative assessment of quality attributes in drug development - EMA/CHMP/138502/2017
- CHMP SWP reflection paper on the assessment of the genotoxic potential of antisense oligodeoxynucleotides (EMEA/CHMP/SWP/199726/2004)
- Guideline on the sterilisation of the medicinal product, active substance, excipient and primary container (EMA/CHMP/CVMP/QWP/850374/2015)
- Guideline on the requirements to the chemical and pharmaceutical quality documentation concerning investigational medicinal products in clinical trials - EMA/CHMP/QWP/545525/2017

# 4. Active Substance

## *4.1. General Information 3.2.S.1*

### 4.1.1. Nomenclature 3.2.S.1.1

No additional requirements.

### 4.1.2. Structure 3.2.S.1.2

Letter codes may be used for the primary structure of the active substance, the used codes should include the nucleobases, the sugars (or morpholinos), and any substitutions thereof, and should reflect the nature of the linkages between sequential nucleosides (e.g. phosphodiester, phosphorothioate diester, thiophosphoramidate, phosphorodiamidate linkages). A legend should accompany the structure. For oligonucleotide chains, the 5`-terminus and the 3`-terminus of the chain should be clearly indicated. For small interfering RNA (siRNA), the structure of sense- and antisense strands should be provided, and the place of hybridisation of the complementary nucleotides of the sense and antisense strands should be indicated, as well as any un-hybridised overhangs in any of the strands. Counter-ions should be indicated. Molecular formula and molecular mass of the active substance, and for siRNA also the sense- and antisense strands are to be provided. If relevant, the secondary and tertiary structure (e.g. in case of hairpin loops or aptamers) should be visualised.

Full chemical structure of side chains and linkers is expected.

### 4.1.3. General Properties 3.2.S.1.3

Relevant general properties of the oligonucleotide in question should be listed.

In most cases, for active substances presented as powder, appearance, water content, pH of a solution of the oligonucleotide, molar absorptivity, and solubility in different media would be expected. Hygroscopicity needs to be indicated, e.g. with moisture sorption isotherms, or reference could be made to 3.2.S.3.1 where such information could be provided in more detail. Polymorphic form is generally not applicable, since most oligonucleotide powders are amorphous. Melting point is relevant for certain oligonucleotides e.g. siRNAs, aptamers.

For active substances in solution, the composition of the formulation and the pH are expected.

The counter ion needs to be indicated, if relevant, and whether it is present in a stoichiometric or non-stoichiometric ratio.

The stereochemistry of the nucleosides and of the phosphorothioate diester linkages needs to be discussed in this section (alternatively reference can be made to 3.2.S.3.1). If there are stereocenters without absolute configuration, i.e. if the active substance is a mixture of diastereoisomers, this should be mentioned. The control strategy to ensure consistency of stereochemistry (stereochemical purity of starting materials, coupling reagent and conditions, etc) needs to be explained.

## *4.2. Manufacture 3.2.S.2*

### 4.2.1. Manufacturer(s) 3.2.S.2.1

No additional requirements.

### 4.2.2. Description of Manufacturing Process and Process Controls 3.2.S.2.2

The chemical synthesis of a therapeutic oligonucleotide is typically carried out on a functionalised solid support using an automated synthesiser. In addition to solid-phase synthesis, alternative synthesis methods (e.g., liquid synthesis, enzymatic synthesis) could be used. However, at the time the guideline was written these methods were considered too premature to be included.

Irrespective of the manufacturing strategy, the dossier should describe relevant process characteristics and equipment in adequate detail.

**Schematic representation of the manufacturing process**

For the graphical presentations of the synthetic process(es) for oligonucleotides, it is considered acceptable to replace certain chemical structures with-letter codes in the reaction schemes to improve legibility. Letter codes should be accompanied with a legend.

**Sequential procedural narrative**

The sequential procedural narrative should describe each step in the manufacturing process. During oligonucleotide synthesis the same standardised steps are typically repeated cyclically, e.g. in Solid Phase Oligonucleotide Synthesis (SPOS) the oligonuclelotide sequence is built up on a solid support by repeated cycles of deprotection, coupling, oxidation/sulfurisation and capping steps. These standardised steps with their associated Proven Acceptable Ranges (PARs) need not be described in detail each time they are used, provided clear descriptions of the used general conditions (e.g. equivalents, reagents, solvents, reaction times, …) are given. The final cleavage and deprotection step should be described in detail, including any use of reagents, in case of which a discussion of their mutagenic potential should also be included in 3.2.S.3.2. Amounts can be described as weights/volumes or equivalents.

Splitting or combining of sub-batches/multiple cycles may be performed at different stages during manufacturing, e.g. based on equipment capacity or operational efficiency in SPOS. The quality criteria applied in the decision on splitting or pooling of sub-batches should be provided, along with an adequate justification for the selected approach. Moreover, material traceability from the synthesis steps through the final drug substance is expected and S.2.2 should contain an unambiguous definition of the commercial batch size (range). When continuous manufacturing approaches are intended, the requirements of ICH Q13 on the description of the manufacturing process should be considered.

Synthetic oligonucleotides are generally purified using chromatographic techniques, often starting from a relatively complex crude intermediate. It is recognised that the crude mixture typically contains pre- and post-, and often co-eluting, structurally related impurities. Detailed information on fraction collection, preparation of mock pools and all applied acceptance criteria during the purification steps should be provided. In the case that re-purification steps of side fractions is part of the established purification procedure, this should be addressed in the dossier as well. Appropriate measures to prevent cross-contamination due to the successive purification of different oligonucleotides using the same column should be in place, as required by GMP.

**Annealing (for siRNA)**

The annealing process is performed to assemble the two complementary single strands (sense and antisense) into the drug substance duplex. Annealing conditions (e.g. buffer composition, time, temperature) should be specified, and volumetric ratio of the single strands should be optimised in order to minimise the unhybridised excess for any single strand. If any small-scale experiments are performed at batch level in view of optimisation of the volumetric ratio of the single strands, the approach should be explained, as well as the in-process control (IPC) non-denaturing method(s) used for measurement of duplex purity, with the applied limit for the residual single strand. The volumetric ratio used in the manufacturing process should be recorded, as well as the residual single strand excess after annealing and the duplex purity, measured as IPC.

**Concentration step**

If any concentration step (e.g. evaporation under vacuum, ultrafiltration) is in place, this needs to be described including the relevant process parameters and IPCs.

**Lyophilisation**

Lyophilisation of synthetic oligonucleotides is considered common practice. Lyophilisation process parameters should be described.

**Oligonucleotide active substance in solution**

See 4.9.

**Reprocessing, recovery and rework**

The terms should be used and understood as defined in EU GMP Part II.

## 4.2.3. Control of Materials 3.2.S.2.3

**Active Substance (AS) Starting Material(s)**

The considerations for selection and justification of starting materials outlined in ICH Q11 and its associated Q&A can be applied to synthetic oligonucleotides. The name and address of all starting material manufacturers should be provided. The addition of manufacturers for the starting materials needs to be approved by a variation according to European legislation. Information, in the form of flowcharts, indicating the synthetic process(es) of all starting materials including details of reagents, solvents and catalysts used, should be provided, followed by a criticality assessment of which starting material impurities may have an impact on the impurity profile of the oligonucleotide.

Starting materials from human or animal origin should, if possible, be avoided. If used, Ph. Eur. chapter 5.2.8 on 'Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Medicinal Products,' and the 'Note for Guidance on Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products' (EMA/410/01) should be considered, and transmissible spongiform encephalopathy (TSE) safety should be addressed.

*Nucleoside phosphoramidites*

Protected nucleoside phosphoramidites (with protection of 5'-hydroxy group and heterocyclic base as relevant) are generally acceptable as starting materials in the manufacturing process of synthetic oligonucleotides. Nevertheless, a justification on the designation of starting materials needs to be provided.

For more complex nucleotide derivatives carrying modifications in the phosphate, sugar or base moiety, more detailed information regarding their manufacture (e.g., precursors and used reagents) and impurity profile is required than for established building blocks such as 5'-dimethoxytrityl (5'-DMT) protected 2'-deoxyribose 2-cyanoethyl-N,N-diisopropylaminophosphoramidites.

Quality attributes for nucleoside phosphoramidites used as starting materials for synthetic oligonucleotide generally include: appearance, identification, assay, impurities, purity, water content and residual solvents. Identity, assay, and impurity profile are typically controlled using liquid chromatography with ultraviolet (UV) and/or mass spectrometry (MS) detection. In addition, $^{31}$P-nuclear magnetic resonance (NMR) spectroscopy may be used to assess the starting material purity. The critical impurities of the nucleoside phosphoramidites which can react like the parent compound during coupling and may accumulate in the final drug substance (e.g. 3'-DMT-5'-amidite isomer) should be adequately controlled and limited in the starting material specifications. The impurity profiles of the starting materials and their potential impact on the quality of the final drug substance should be investigated during manufacturing process development. This should include a fate and purge assessment of the impurities that may be formed downstream in the manufacturing process.

The solid support preloaded with the first nucleotide of the oligonucleotide sequence (through a linker) is also considered starting material. However, the unloaded solid support itself is not considered a

starting material as it is not incorporated as a significant structural fragment into the structure of the drug substance.

For nucleoside loaded solid supports, quality attributes related to the purity and potential impurities of the loaded nucleoside are recommended. For the solid support itself, reference is made to section 'Other materials used in the manufacturing process'.

*Non-nucleotide structural moieties*

Conjugation and other derivatisations of oligonucleotides are commonly used. In these cases, sometimes complex structures are added to the oligonucleotide sequence. The classification of these materials will be handled on a case-by-case basis and early interaction (scientific advice) with the regulatory agencies is recommended. However, also for starting materials of non-nucleotide structural moieties (e.g. poly(ethylene glycol) (PEG)-chains, N-acetyl galactosamine (GalNAc) moieties, fatty acids), compliance with the requirements as laid down in ICH Q11 and its associated Questions and answers is expected and its selection as starting material should be justified. For instance, sufficient subsequent chemical transformation steps after the starting material should be performed under good manufacturing practices (GMP).

**Other materials used in the manufacturing process**

A list of all other reagents, such as solid support, solvents and chromatographic materials used in the manufacturing process of an oligonucleotide should be provided. Adequate specifications for all materials should be laid down considering their role in the process but covering as a minimum identity as well as purity and/or assay where applicable. For some specific reagents (e.g. acids used in the detritylation step) certain impurities have to be controlled to minimise the likeliness of the generation of product-related impurities. For materials used in the coupling steps residual moisture may be a critical attribute.

The solid support is a key component of the SPOS process, controlled pore glass (CPG) and polystyrene resins are most commonly used. Typical quality attributes of the resin include: appearance, identification, mesh size and loading. For CPG also the pore size and for polystyrene cross-linking and swelling volume are typical quality attributes.

## 4.2.4. Control of Critical Steps and Intermediates 3.2.S.2.4

The criticality of the manufacturing steps for oligonucleotides made by solid phase synthesis should be evaluated during development according to the principles described in ICH Q 9 – Q 11. In-process controls should be defined. The control of critical steps can be achieved by a combination of analytical tests and process controls. During SPOS critical steps could include, e.g., DMT deprotection, coupling, oxidation/ sulfurisation reaction or capping monitoring, cleavage, concentration and drying, annealing (if relevant) and lyophilisation (if relevant) steps.

During oligonucleotide purification by preparative chromatography, individually collected fractions are usually combined into a pool of fractions. The pooling strategy should be defined and acceptance criteria for the purity of individual fractions and the main pool should be stated. These criteria for purity usually include overall purity and criteria for individual impurities. In case secondary purification is proposed in the manufacturing process, adequate requirements for side-fractions that are allowed to undergo such purification, and the conditions thereof, should be defined. It should be stated which fractions are discarded. Filtration and lyophilisation steps should also be adequately controlled.

Intermediates as defined in the manufacturing process are tested before use in the next stage of the manufacturing process. The methods used for IPC and/or intermediate testing should be described and confirmation of analytical method validation provided where applicable.

The EU GMP Part II definition of intermediate should be considered, i.e., "A material produced during steps of the processing of an API that undergoes further molecular change or purification before it becomes an API. Intermediates may or may not be isolated." For intermediates, justified specifications should be presented. Only intermediates complying with specifications may finally be pooled.

For double stranded oligonucleotides, specifications for the single strand intermediates prior to annealing, consisting of at least identity, purity and impurities, a description of the analytical methods used, and relevant analytical method validation data should be provided. Results from forced degradation studies on the single strands should be provided to demonstrate the stability indicating nature of the analytical methods. The holding time– and storage conditions for single strand intermediates should be supported by stability data.

## 4.2.5. Process Validation and/or Evaluation 3.2.S.2.5

No additional requirements apply for synthetic oligonucleotides than for other synthetic substances (i.e. process validation data would normally not be expected in the dossier), except for oligonucleotide active substance in solution: see 4.9. Re-use of preparative columns should be appropriately validated.

## 4.2.6. Manufacturing Process Development 3.2.S.2.6

In order to support the development of a manufacturing process that produces an active substance of suitable quality, a risk-based approach as outlined in ICH Q8 Pharmaceutical development, ICH Q9 Quality risk management, and ICH Q11 Guideline on development and manufacture of drug substances (chemical entities and biotechnological/biological entities) is expected to be applied throughout the process development.

Risk assessments should be performed to determine the criticality of individual quality attributes on the overall quality of the active substance and resulting finished product. These risk assessments should support the overall control strategy.

Process risk assessments and process characterisation studies should be carried out to identify the impact of process parameters on the quality attributes, including an assessment of the potential impact of process limits and failures on quality and/or process consistency, and the identification of appropriate risk mitigation actions where relevant.

The process risk assessments, together with the knowledge gained from process characterisation and development studies, should be used to establish the final set of process controls for active substance manufacturing. This includes the classification of the IPCs and tests as critical or non-critical.

It is acknowledged that there are general aspects of oligonucleotide synthesis where prior knowledge and manufacturing experience may be extrapolated between different processes. Also, the starting materials and their properties are mostly well-known. If justified, manufacturers may make reference to prior knowledge for general aspects of the manufacturing process (e.g. choice of solid support or coupling reagents). However, it is expected that substance specific aspects such as reaction times, temperatures and molar equivalents are addressed in the development section. If in-house knowledge from other products is referred to, the data and source should be identified as appropriate and differentiated from product-specific data. A discussion of how this data is to be used should be integrated with the relevant product-specific data to provide an overall understanding of product development and control. The use of prior knowledge should always be explained and justified in the dossier, with a focus on the context and relevance of the prior knowledge to the current assessment. If prior knowledge from scientific papers is quoted, copies of the paper should be provided as appropriate.

## *4.3. Characterisation 3.2.S.3*

### 4.3.1. Elucidation of Structure and other Characteristics 3.2.S.3.1

The structure of the oligonucleotide should be confirmed by analytical data, this includes the primary, secondary, and tertiary structure where relevant. Mass spectrometry is a suitable analytical tool for the structure elucidation of oligonucleotides. Variants of the MS technique can be used to determine the molecular mass of an oligonucleotide and to confirm its nucleotide sequence. Typical representative spectra and interpretation of the fragmentation data, including assignments and tables with theoretical and observed mass values, should be provided.

Elemental analysis (e.g. by combustion analysis, or inductively coupled plasma optical emission spectroscopy (ICP-OES) for phosphorous and sodium content) may be used in view of structure confirmation.

NMR experiments are recommended to be part of the characterisation studies, to elucidate the nucleobase, sugar and backbone compositions, identity, and connectivity of nucleotides. One- and two-dimensional techniques should be used to assign the structure by means of $^{1}H$, $^{13}C$ and $^{31}P$ NMR data where relevant. $^{31}P$ NMR typically provides information on primary and secondary structure of phosphorothioate diester and/or phosphodiester backbones. In case of fluorination (e.g. 2-fluoro substitution of the riboses), also $^{19}F$ NMR can be used.

Nucleoside stereochemistry, including stereochemical purity of the phosphoramidite starting materials originating from their nucleoside precursors, and potential anomerisation propensity during chain elongation of the oligonucleotide synthesis, needs to be discussed.

For oligonucleotides with phosphorothioate diester internucleotide linkages, the phosphorus atoms of the phosphorothioate diester internucleotide linkages are chiral. The stereochemistry of the internucleotide linkages is determined during the coupling reaction, and delivers a mixture of $2^n$ diastereomers (with n the number of phoshorothioate linkages). If separation of these $2^n$ diastereomers is not practically possible, an estimation of the diastereoisomeric distribution (ratio of isomers), and the reproducibility thereof, is expected.

Phosphodiester internucleotide linkages are non-chiral.

The impact of the stereochemistry on the biological/pharmacological activity should be discussed (see Investigation of Chiral Active Substances 3CC29a for human products or EMEA/CVMP/128/95 for veterinary products).

Ultraviolet, circular dichroism (CD) and infrared (IR) spectroscopy are part of the standard characterisation program. Additional information on the secondary structure can be gathered from these techniques.

Usually, no biological assay is required for the characterisation or routine release of antisense oligonucleotides or siRNA, which exert their function by annealing onto a complementary strand.

Aptamers exert their biological activity by selectively binding to a target molecule. The biological activity of aptamers depends heavily on their 3-dimensional structure. Therefore, characterisation of this attribute (e.g. binding to biological target) is expected. Also, the secondary structure (the presence of guanine residues could lead to the formation of G-quadruplexes) should be investigated and results provided.

In case of conjugated molecules, characterisation tests on the conjugated and non-conjugated moieties are expected. Specificity of conjugation needs to be demonstrated, and the secondary structure of conjugated versus unconjugated oligonucleotide should be compared unless otherwise justified.

In case of siRNA, characterisation tests on the sense strand, the antisense strand and the duplex are expected.

**Evidence of chemical structure**

The information will normally include such evidence as:

List of characterisation techniques used for oligonucleotides (example table):

| Test | Analytical technique |
|---|---|
| Molecular mass | LC-MS, MALDI-TOF MS, ESI-TOF MS |
| Sequence analysis | LC-MS/MS of intact molecule<br>LC-MS of enzymatically treated material<br>Failure sequence analysis of the crude active substance with e.g. IP-HPLC-TOF-MS |
| Identity of potential counter ions | FAAS (flame atomic absorption spectroscopy), ICP-OES for sodium counter ion |
| Extinction coefficient | UV spectroscopy |
| Spectral characterisation | Circular dichroism (CD) spectroscopy<br>FT-IR spectroscopy<br>$^{31}$P NMR, $^{19}$F NMR, $^{13}$C NMR, $^{1}$H NMR<br>Imino $^{1}$H NMR* |
| Melting temperature | Thermal dependent UV absorbance |
| Identification as duplex* | Non-denaturing IPRP HPLC UV |
| Thermodynamic transitions | Differential scanning calorimetry (DSC)<br>Thermogravimetric analysis (TGA) |
| Tertiary structure** | Near UV CD spectroscopy |
| Biological characterisation** | Cell-based and other biological assays |

* for siRNA duplexes

** for aptamers or for oligonucleotides conjugated to moieties with 3-dimensional structure (e.g. antibody-oligonucleotide conjugates)

**Physico-chemical characteristics**

Physicochemical characterisation of the drug substance could include solubility and hygroscopicity studies, determination of the isoelectric point (pI) and thermogravimetric studies as e.g. differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). The morphology may be examined by powder X-ray diffraction (PXRD) and/or DSC.

## 4.3.2. Impurities 3.2.S.3.2

Purity is an important critical quality attribute (CQA) for oligonucleotides. Impurities are often categorised as either product-related impurities or non-oligonucleotide impurities. Product-related impurities contain structural elements of the oligonucleotide sequence. Non-oligonucleotide impurities include process reagents and their potential by-products, residual solvents, elemental impurities and potential mutagenic impurities.

Product-related impurities

Product-related impurities may originate from different sources:

- starting materials;
- formation during the manufacturing process;
- resulting from degradation during the manufacturing process or during storage.

The levels of certain product-related impurities may be the result of combined contributions from these sources.

Applicants are expected to make significant efforts to characterise product-related impurities that are present or likely to be present, based on in-depth knowledge and understanding of the synthesis, impurity profile of the starting materials and fate of these impurities, and potential degradation pathways of intermediates and active substance. Prior knowledge and literature may help the applicant in the characterisation exercise. It is acknowledged, that such exercise can be challenging due to the relatively high molecular weights of such impurities, and due to the presence of mixtures of closely related impurities.

Related substances resulting from starting materials

Reactive impurities present in starting material building blocks may be incorporated in the sequence during the assembly of the oligonucleotide and as a consequence are usually persistent and end up in the final active substance at a level depending on their impurity level in the starting material building block and the incorporation frequency of that building block in the oligonucleotide chain. Such impurities can include e.g. structures due to a modification at a single sugar, or at a single base, or a positional isomer of one of the phosphoramidite building blocks. Unlike conventional synthesis of small-molecule chemicals, downstream processing and purification steps are usually unable to purge them. Narrow acceptance limits should be set for reactive and critical starting material impurities for each starting material used in the manufacture of the oligonucleotide.

Related substances formed during the manufacturing process

Related substances designated as process impurities may be a result of undesired or incomplete reactions during synthesis or cleavage.

*Modified internucleotide linkages*

Phosphate diester (PO) impurities in phosphorothioate (PS) diester oligonucleotides are structurally related impurities to the parent oligonucleotide by replacement of one or more phosphorothioate diester linkage by a phosphate diester linkage, whereby any one of the phosphorothioate diester

linkages may be substituted, usually resulting in a mixture of different components. These substitutions can be due to e.g. suboptimal oxidation or capping conditions, or inadequately controlled phosphorus deprotection reaction, or use of insufficiently aged oxidiser solution.

*Stereoisomers*

Nucleoside stereochemistry, including stereochemical purity of the phosphoramidite starting materials originating from their nucleoside precursors, needs to be discussed, as well as the product-related substances resulting from these.

*Deletion sequences and truncated sequences*

Deletion sequences are oligonucleotide impurities with one or several building blocks missing. They can be formed e.g. under conditions of incomplete detritylation, sulfurisation or oxidation. Their formation is often prevented by systematic acetylation to cap unreacted coupling sites. The acetylation procedure results in acetylated oligonucleotide fragments (truncated sequences), the majority of which are removed by the further downstream processing.

*Insertion sequences*

If a particular building block is coupled more than once during a coupling step, an insertion sequence is generated. Insertion sequences may occur e.g. as the result of premature deprotection during prolonged coupling reactions or due to suboptimal concentration of activating agent.

*Oligonucleotides with base modifications*

Abasic oligonucleotides can be formed e.g. when detritylation, capping or cleavage steps are suboptimally controlled. Acetylation of the bases can occur when the capping step is suboptimally controlled. Cyanoethylthymine (CNET) impurities have a substitution (acrylonitrile adduct) of the heterocyclic base due to suboptimal control of the phosphorous deprotection step.

*Cross-linked oligonucleotides*

These structures can be formed e.g. by condensation of the parent oligonucleotide with a shorter oligonucleotide that contains an abasic site, or by condensation of two growing oligonucleotide chains.

*Residual single stranded oligonucleotides in double stranded oligonucleotides*

For double stranded oligonucleotides, impurity characterisation is required both at the level of the individual single strand intermediates, before annealing, and also on the double strand active substance. The latter is performed under non-denaturing conditions, as to quantify residual single strands in the double strand active substance. Also the potential formation of impurities/degradants resulting from the annealing step should be discussed. The impurity control strategy should consist of three sets of impurity specifications, based on a combination of denaturing and non-denaturing chromatographic methods: sense strand intermediate, antisense strand intermediate, and final drug substance. The control of single strand intermediates plays a critical role in the impurity control strategy, due to the better analytical separation capacity at this level compared to the final drug substance.

*Aggregates*

Aggregation propensity should be investigated.

<u>Related substances resulting from degradation during the manufacturing process or during storage</u>

Degradation products of oligonucleotides may also occur as process impurities. Their content may increase during storage. Generally, the following pathways can contribute to the formation of degradation products of oligonucleotides:

- oxidation (PO impurity of PS oligonucleotides);
- thermal stress;
- acidic stress;
- basic stress;
- hydrolysis;
- condensation;
- photolysis.

Possible routes of degradation should be discussed - see section 3.2.S.7.1.

Analytical methods

Suitable analytical methods (with appropriate limits of detection (LOD) and limits of quantitation (LOQ)) used to detect the likely impurities considered above, or other related impurities, the exact identities of which may be unknown, should be developed and described. These methods should be state of the art, and - to the extent possible - capable of resolving impurities from the parent oligonucleotide and from each other. It is acknowledged that full resolution of all individual product-related impurities is usually not technically achievable with a single method due to the complex mixture of structure-related impurities, many of them with about the same mass. In that case, efforts are expected to improve the separation, such as a complementary detection method (e.g. an MS detector in-line with an UV spectrophotometric detector of an ion-pair reversed-phase high performance liquid chromatography (IP-RP-HPLC)), or addition of an orthogonal analytical method. If despite these efforts there are still unresolved active substance impurities, these should be characterised and controlled as groups, e.g. on basis on structural class, ideally in a way that the reported results for this structural class are reflective of the success or failure of a particular synthesis step or control strategy or degradation pathway.

Copies of relevant chromatograms should be provided. A summary should be given on the nature and levels of the actual impurities detected in the batch samples of the material.

As mentioned above, double stranded oligonucleotides should be analysed both under denaturing (e.g. anion exchange (AX)-HPLC) and non-denaturing (e.g. size-exclusion (SE)-HPLC) conditions.

**Reporting, identification and qualification thresholds, and their relation to specification setting for product-related impurities of single-strand molecules**

For product-related impurities, the reporting threshold depends on the lower limit of quantification of the analytical method used to measure these impurities, which in turn depends on the size and complexity of the molecule structure.

The identification threshold establishes the level above which there is a requirement to identify and characterise impurities of unknown structure. It is standard practice in drug substance and drug product specifications to set the limit on unspecified impurities equal to this value. In general, an identification threshold of 1.0% is accepted.

The qualification threshold establishes the level above which there is a requirement to qualify impurities of known structure. In general, a qualification threshold of 1.5% is accepted. Mutagenic impurities are excluded from this approach and should be assessed as per ICH M7 (EMA/CVMP/SWP/377245/2016). All specification limits for specified impurities should be supported by batch analysis data, and those above the qualification threshold should be supported by qualification data.

**Qualification requirements for product-related impurities**

Product-related impurities can be divided into 4 separate classes, as mentioned below.

Class I consists of impurities that are also major metabolites, with structure and sequence the same as the parent, e.g. impurities lacking one or more nucleotides from the 3' or 5' end of the parent oligonucleotide, single-stranded impurity of double-stranded parent oligonucleotide. Class II consists of impurities that contain only structural elements found in naturally occurring nucleic acids, e.g. phosphate diester impurity of phosphorothioate diester oligonucleotide. Class I and class II impurities do not require further qualification, even if present above the qualification threshold.

Class III consists of impurities that are sequence variants of the parent oligonucleotide such as n-1 or n+1 impurities with nucleotide(s) lacking or nucleotide(s) added within the chain (not at the 3' or 5' end of the parent oligonucleotide). These can be difficult to distinguish from each other, and therefore they are often in first instance identified and quantified as a group of impurities containing n-1 or n+1 sequences. When the level of these impurities as a group is below the 1.5% qualification threshold, no further characterisation of individual impurities, and no further qualification as a group is required. However, if the level of the impurities as a group is above 1.5%, the sequences need to be identified separately, and for those individual sequences exceeding the 1.5% threshold, qualification is required.

Class IV consists of impurities that contain structural elements not found in the parent oligonucleotide or in naturally occurring nucleic acids (e.g. abasic impurities). These require qualification if they are present above the 1.5% qualification threshold. It is preferable to rely on optimisation of manufacturing processes to minimise impurities rather than to establish a preclinical testing program for their qualification. If qualification is required, in silico and/or in vitro approaches may be considered. If appropriate, the CHMP SWP reflection paper on the assessment of the genotoxic potential of antisense oligodeoxynucleotides (EMEA/CHMP/SWP/199726/2004) should be considered.

Oligonucleotides themselves and product-related impurities are not within the scope of ICH M7 / EMA/CVMP/SWP/377245/2016.

Process-related non-oligonucleotide impurities

Non-oligonucleotide impurities include process reagents, by-products, residual solvents, elemental impurities, ligands, and protecting groups. The solid phase synthesis process requires extensive washing of the solid support with solvents. Reagents and solvents used for the coupling steps are washed with incremental quantities of a suitable solvent. Nevertheless, for all reagents and solvents used in the manufacturing process, the depletion should be addressed in the dossier by either data and/or risk analysis. The use of purge arguments in oligonucleotide control strategies can be considered if adequately justified. Any residuals of reagents and/or solvents should either comply with ICH M7 / EMA/CVMP/SWP/377245/2016 (if mutagenic), or – if not mutagenic - ICH Q3A/VICH GL10 or ICH Q3C/VICH GL18 thresholds, as relevant, or (in absence of ICH Q3C/VICH GL18 thresholds), be toxicologically qualified.

## *4.4. Control of the Active Substance 3.2.S.4*

### 4.4.1. Specification 3.2.S.4.1

Synthetic oligonucleotides are out of the scope of ICH Q6A, but the principles outlined in ICH Q6A could be taken into account when setting the specifications (resp. VICH GL39 for veterinary products).

Oligonucleotides are normally used for manufacture of sterile products, therefore, acceptance criteria for bioburden and endotoxins are expected. Typical quality attributes in the specification are as follows (non-exhaustive list):

- appearance (+ appearance of solution if relevant);
- identification of the oligonucleotide:
    - identification by mass;
    - identification by sequence analysis (the order in which the nucleotides are arranged in the oligonucleotide chain)
    - identification by retention time
- assay/content;
- counter-ion identity and content;
- purity and impurities (total impurities; individual or groups of impurities (unspecified/unidentified impurities));
- pH of solution;
- water content;
- residual solvents;
- elemental impurities (depending on ICH Q3D or EMA/CVMP/QWP/153641/2018 risk assessment);
- bacterial endotoxins;
- microbiology.

For double-stranded oligonucleotides, the purity should be tested both with a non-denaturing method (to allow measurement of single strand residues) and a denaturing method.

For aptamers a test on biological activity should be included in the active substance or finished product specification.

## 4.4.2. Analytical Procedures 3.2.S.4.2

**Analytical development**

For parameters such as counter ion, water content, residual solvents, bacterial endotoxins and microbiology, Ph. Eur. methods or suitable in-house methods may be employed.

Specific analytical procedures to control the identity, purity and assay of the oligonucleotide should be developed:

*Identification*

The evidence of chemical structure should be discussed under Section 3.2.S.3.1.

The identity of the oligonucleotide should be confirmed for each batch with a combination of analytical procedures, such as, identification by mass (e.g. electrospray ionisation mass spectrometry, ion-pair HPLC with ultraviolet and mass spectrometry detection, high resolution mass spectrometry or similar), and identification by sequence analysis (e.g. electrospray ionisation mass spectrometry, ion-pair high performance liquid chromatography-time-of-flight mass spectrometry, duplex melting temperature,

high resolution mass spectrometry or similar). Furthermore, identification and quantification of the counter-ion is needed.

In the case of double-stranded oligonucleotides the identity of the duplex and the identity of the single strands should be demonstrated. A combination of non-denaturing and denaturing chromatographical methods in combination with mass spectrometry is generally performed. As an orthogonal method, measurement of the melting point by UV is recommended.

For aptamers sequence confirmation might be challenging especially due to the lengths and when conjugated e.g. to PEG. A combination of tests should be developed to unambiguously demonstrate identity.

*Purity*

Oligonucleotides are excluded from the scope of ICH Guideline Q3A/VICH GL10, ''Impurities in New Drug Substances'', but the principles outlined in this guideline can be applied to list the oligonucleotide-related impurities in the specification (i.e. each specified identified impurity, each specified unidentified impurity, any unspecified impurity with an acceptance criterion of not more than (≤) the identification threshold and total impurities).

Generally, it is not possible to resolve all oligonucleotide-related impurities by traditional chromatographic methods and a high number of impurities co-elutes with the main peak. The use of analytical methods that combine chromatography with different detectors (e.g. with UV detection and mass spectrometry) could improve the separation, identification and quantification of impurities.

Due to the complexity of the molecule and the nature of the synthetic process, the impurity profile of oligonucleotides will result not only in impurities that are single entities but groups of highly similar compounds of equal sequence length. Therefore, it may be accepted to report specified impurities as groups, mixtures or classes. As an example for a phosphorothioate antisense oligonucleotide potential groups amongst others might be: Full length $(P{=}O)_1$, Total n-1, Total n+1, Total abasic, CNET, Dithioate/Thioate, Early and Late eluting impurities.

Grouping by chemical classes or by retention time might be feasible and should be adequately justified.

Impurity characterisation data are a pre-requisite to justify such an approach.

In the case of double-stranded oligonucleotides a combination of non-denaturing and denaturing analytical methods should be employed. Orthogonal analytical methods (e.g. AX-HPLC and IP-RP)-HPLC might be used. Different types of impurities are measured by these orthogonal methods and clearly the limits for the same groups when measured with different technique may differ. The amount of each single strand present in the duplex as an impurity should be determined and specified.

The thresholds proposed for these oligonucleotide-related impurities can be supported, but not limited, with published literature. The limits applied for each identified/unidentified impurity or groups of impurities should be supported with i) toxicological data to confirm the safety of the thresholds proposed, ii) batch and stability data, iii) analytical control strategies and iv) adequate process understanding of the origin and fate of these impurities that can impact drug substance CQAs.

*Assay*

The assay and how it is calculated should be clearly defined. The assay might be determined by UV or by a weight-based assay against a standard of known purity and concentration using HPLC with detection by UV absorption. Calculation may also include the use of an extinction coefficient.

Oligonucleotides as lyophilised powder often contain high levels of water that can be expected to increase during storage. Therefore, assay should be expressed in terms of the anhydrous substance

unless otherwise justified. The use of other correction factors as e.g. purity and counter-ion should be stated and justified.

*Changes of the analytical methods during development*

During development of the oligonucleotide, changes – major or minor – on the analytical procedures could be introduced. The changes performed during development should be discussed.

### 4.4.3. Validation of Analytical Procedures 3.2.S.4.3

The analytical procedures used for the control of the drug substance should be fully validated. In general, the validation of analytical tests concerning the active substance should be performed according to the requirements of the current Guidelines (ICH Q2, VICH GL1 and GL2). Chromatograms and, where relevant, MS data, showing that the separation of oligonucleotide-related impurities from the drug substance peak is sufficient to allow peak integration should be presented.

### 4.4.4. Batch Analyses 3.2.S.4.4

This section should summarise the batch analysis data for the oligonucleotide batches used for non-clinical and clinical studies and for the batches used to support the quality of the drug substance.

As recommended in other guidelines, presentation of this information in tabular form is recommended for improved clarity. Apart from the analytical determination, for each batch the following information should be provided: date of manufacture, batch number, batch size (in terms of mass or molarity), scale (laboratory/pilot/commercial), route of synthesis (commercial or not), place of manufacture, and use of batches.

Specifications often evolve during development, from the early stages to the final commercial version. The differences in the results obtained in the batches used in earlier development and pilot/commercial batches should be explained and justified.

The improvement in the analytical methods during development of the oligonucleotide could lead to the observation of new impurities or groups of impurities in pilot/commercial batches. In those cases, comparison of the batch analysis data should be performed, and the need for qualification of these new (group of) impurity/ies should be discussed.

### 4.4.5. Justification of Specification 3.2.S.4.5

The proposed specification should be supported with batch data from non-clinical, clinical studies and batches used to support the quality of the drug substance combined with an adequate understanding of the manufacturing process and factors that could affect the CQAs of the oligonucleotide.

For the identity test, specifications are required based on at least two complementary techniques (see 3.2.S.4.2), one of them being a sequence confirmation test.

For parameters such as residual solvents, elemental impurities, bacterial endotoxins and microbiology, the limits should be justified in line with applicable EU/(V)ICH Guidelines, Ph. Eur. Or European legislation.

The specifications for oligonucleotide-related impurities, including the qualification approach, should be justified as indicated in 3.2.S.3.2 and 3.2.S.4.2.

## *4.5. Reference Standards or Materials 3.2.S.5*

Oligonucleotides are often very hygroscopic powders, therefore appropriate precautions against moisture uptake by the reference standard during storage and during analysis should be taken when relevant.

The origin of the reference standards should be briefly indicated (e.g. batch synthesised according to the commercial process). If a 2-tiered system is used (primary reference standard and working reference standard) the preparation and qualification strategy should be briefly explained, and the characterisation results obtained for the reference standard batches, the approach to periodically requalify the reference standards, as well as the approach that will be followed to qualify future batches of reference standards, including the measures that will be taken to prevent drift in oligonucleotide content, should be presented.

For double stranded oligonucleotides, reference standards are expected for the sense strand, the antisense strand, and the active substance itself.

If a complementary strand is used for the purpose of an identity test (e.g. melting temperature) of a single strand oligonucleotide, it should be described.

## *4.6. Container Closure System 3.2.S.6*

The container closure system should be suitable, considering the substance properties, storage conditions and use: e.g. for hygroscopic powders, appropriate desiccant should be included. Alternatively, storage under inert atmosphere could be considered.

## *4.7. Stability 3.2.S.7*

### 4.7.1. Stability Summary and Conclusions 3.2.S.7.1

The principles outlined in EMA's and (V)ICH scientific guidelines on the stability of drug substances should be followed with regards to aspects such as the types of studies conducted, protocols used, selection of batches, container closure system and storage conditions.

The choice of test conditions applied during stability storage (temperature and humidity) should be justified. Forced degradation studies are needed to obtain a comprehensive overview of the degradation pathways of the drug substance; these data might be especially important for the development of the drug product.

The potential degradation pathways of the oligonucleotide should be discussed considering the backbone- and sugar-modifications. The most common degradation impurities result from oxidation, deamination and depurination/depyrimidation (abasic impurities); suitable analytical procedures should be developed enabling their detection and quantification. Furthermore, particular attention should be paid to those degradation impurities (e.g. abasic impurities) which may be a concern for safety.

For hygroscopic powders, it is expected that water content should be part of the stability protocols.

Aggregation may also occur for oligonucleotides and should be investigated.

The retest period or shelf-life (for active substance in solution) and storage conditions should be justified following EMA's and (V)ICH scientific guidelines on the stability of active substances.

Variability in stability testing results should be avoided by establishing appropriate handling procedures during analytical testing.

### 4.7.2. Post-approval Stability Protocol and Stability Commitment 3.2.S.7.2

General principles outlined in EMA's and (V)ICH scientific guidelines should be followed.

### 4.7.3. Stability Data 3.2.S.7.3

The results of the stability studies, including forced degradation studies and stress conditions, should be presented in an appropriate tabular or graphical format. The information given in this section on the batches used during stability should be, at least, the batch number, manufacturing site, manufacturing process (commercial or not), container closure system and batch size (laboratory/pilot/commercial). Cross-reference to the detailed information on the stability batches to other sections of Section S.4 may also be made.

Complete information about the analytical procedures used during stability, if different from those described in 3.2.S.4 should be presented in this section, along with the validation of the analytical method/s used for stability testing.

## *4.8. Conjugation*

Conjugation has emerged as a popular mechanism to alter or enhance the properties of oligonucleotide drug candidates. Conjugation to GalNAc has been used to improve delivery of therapeutic oligonucleotides to hepatocytes. Poly(ethylene glycol) is commonly used to improve the properties of aptamers. Other types of conjugation to e.g. monoclonal antibodies or synthetic peptides are under development.

However, there is added complexity with respect to the characterisation and control of these conjugates. The control of the unconjugated oligonucleotide which is usually classified as an intermediate is essential. Adequate specifications and control methods should be established for these intermediates. In cases where no intermediate is isolated these approaches should be justified and an adequate control strategy should be developed (see also 4.2).

The underlying conjugation chemistry should be described in the manufacturing process development section. Conjugatable versus non-conjugatable impurities should be identified by means of a risk analysis and the incorporation into the target molecule should be investigated. Purging of process-related impurities from the conjugation process should be investigated.

An additional quality attribute for conjugated oligonucleotides is the amount of the free unconjugated oligonucleotide and the free form of the conjugate moiety (e.g. free PEG/linker). Di-PEGylation or multi-PEGylation (or other conjugation moieties) may also occur and should be adequately controlled.

The choice of the starting material of the conjugation component needs to be justified according to ICH Q11, and the corresponding 'Questions & Answers'. It has to be assured that all steps of the intermediate synthesis starting from the defined starting material are performed under GMP. Consequently, e.g. the activation of the suitable PEG starting material is considered a part of the manufacturing process and an activated PEG derivative (e.g. in the form of an N-hydroxysuccinimide (NHS) ester*)* may not be suitable as starting material and is considered to be an intermediate itself.

Full information on the manufacturing of the conjugated moiety and the linker (if applicable) should be provided in Section 3.2.S.2.2 of Module 3, including flowchart, process description with all process steps, raw materials and manufacturing process controls.

In numerous development programmes, polymers or other conjugation moieties are coupled to the oligonucleotide via a chemical linker. The points mentioned above are also applicable for such chemical linkers, especially for the selection of suitable starting materials and the control of the impurity profile.

The critical attributes should be evaluated and a justification for the specification attributes should be provided. The basic principles of ICH M7/ and EMA/CVMP/SWP/377245/2016 regarding a mutagenic impurities assessment should be considered for chemical linkers and conjugation moieties.

In many cases, the conjugation moiety and the linkers are manufactured by a different manufacturer than the synthetic oligonucleotide. In the case of multiple suppliers of the conjugation moiety and/or linker, for each supplier separate documentation is expected, and a compiled specification for the conjugation moiety should be elaborated by the manufacturer of the oligonucleotide-conjugate.

Oligonucleotide-conjugated material from all suppliers of the conjugation moiety and/or linker should be manufactured and batch analysis and stability data should be generated.

It is recommended to consider the legal framework for cases where a New Active Substance status is claimed and an unconjugated or differently conjugated product is already approved.

Conjugation-specific aspects regarding the summary of product characteristics (SmPC) and labelling may be discussed with the Competent Authorities prior to submission.

Monoclonal antibodies are manufactured by recombinant technologies and therefore considered biological medicinal products. Consequently, the oligonucleotide conjugated to a monoclonal antibody is considered as a biological medicinal product as well. The impact of specific regulatory requirements and GMP aspects should be considered.

## *4.9. Active Substance in Solution*

Synthetic oligonucleotides used for the manufacture of medicinal products should comply with the definition of active substances as per the framework of EU Regulation. They are typically isolated as solids (lyophilised powders), however, mixtures of oligonucleotides with excipients in solution can be submitted as part of the manufacture of the active substance in 3.2.S. This situation is not uncommon for biological or biotechnological products, where the active substance (bulk material) may also contain excipients including other components such as buffers (ICH Q6B/VICH GL40), and it is acknowledged that synthetic oligonucleotides are often purified in aqueous buffers (by chromatography or ultrafiltration/diafiltration) in a similar manner to biological substances.

Such an approach will have to be justified by the applicant based on acceptable stability for the solution and prior knowledge of the same class of oligonucleotides. In absence of prior knowledge, and/or in case of doubt, applicants are advised to apply for scientific advice on this aspect.

For the synthetic steps of the manufacturing process of oligonucleotide molecules, Eudralex Volume 4 EU GMP Part II should apply.

Similarly to biological formulated active substances, from the purification/downstream process some principles and aspects of the GMP guidelines as laid down in Directive 2003/94/EC (Directive 91/412/EEC for veterinary products) and interpreted in Eudralex Volume 4 Part I and Part III, including technical annexes should apply, such as a control strategy taking into account the intended use of the active substance. Such control strategy should aim to protect the intermediate or active substance from contamination (particularly of a microbiological nature) and from loss of quality. Since active substance in solution will in most cases be processed into sterile medicinal product, the bioburden limits and other requirements from EMA/CHMP/CVMP/QWP/850374/2015 apply.

The control strategy and full process validation data (including evidence of adequate implementation thereof) will have to be provided in the marketing application.

Buffer components present in the final composition of the active substance in solution which is used directly for further processing into the finished product will have to be managed as excipients (GMP

Part I., 4.14) and comply with Ph. Eur. if applicable. The water used in the last processing steps should be water for injections (WFI) in case of a parenteral product. Sections 3.2.S.2.3, 3.2.P.1 and 3.2.P.4 (or parts 2.A.1 and 2.C.2 for veterinary products) of the marketing application should contain the quality standard of these components.

Also, in analogy with biological or biotechnological products, the concept of shelf-life (instead of retest period) will have to apply to oligonucleotide active substance in solution. The principles laid down in the 'Guideline on Start of Shelf-life of the Finished Dosage Form' are also not applicable for oligonucleotide active substances in solution.

For the same reasons, the concept of Active Substance Master Files (ASMF), and of Certificate of Suitability (CEP) (in case of active substances for which a Ph. Eur. monograph exists) as laid down in Directive 2001/83/EC and Regulation (EU) 2019/6 as amended, cannot be applied in the context of medicinal products manufactured from an oligonucleotide active substance in solution, this since the quality requires not only a combination of physico-chemical and microbiological testing, but also extensive knowledge of the production process and its (microbiological) control strategy. The applicant for a medicinal product manufactured from an oligonucleotide active substance in solution could therefore not comply with the requirement to 'take responsibility for the medicinal product' without having full and transparent access to these quality-related data. The use of an ASMF would prevent such access, and should therefore not be allowed for oligonucleotide active substances in solution.

In case an oligonucleotide active substance as a lyophilised powder, and an oligonucleotide active substance in solution are included in the same submission, this should be adequately addressed not only in the respective separate active substance parts (such as the S.4.1 `Specification` section), but also in the related drug product parts (such as the P.2.1 `Components of the drug product`section).

# 5. Medicinal Product Considerations

The quality target product profile (QTPP) relates to quality, safety and efficacy, considering e.g. the route of administration, dosage form, bioavailability, strength and stability of a medicinal product containing an oligonucleotide as active substance.

(V)ICH Guidelines ICH Q3B (VICH GL11) and ICH Q6A (VICH GL39) are not or only partly applicable to oligonucleotides. The thresholds for impurities as discussed above in 3.2.S.4.1 are also applicable to the resulting medicinal products. Limits should be set for degradants formed during manufacturing or storage of the finished product, considering these thresholds and the maximal daily dose. Limits should be justified on a case-by-case basis considering the batch analysis history and qualification data.

Oligonucleotides are included in the scope of ICH Q3D 'Guideline for Elemental Impurities' (Reflection paper EMA/CVMP/QWP/153641/2018 for veterinary products), thus the requirements laid down in this guideline are applicable for medicinal products containing oligonucleotides as active substances. Also, the risk considerations and requirements for nitrosamine impurities are applicable to oligonucleotide active substances that are used in finished products for human use.

Potential interactions of the oligonucleotide with the excipients present in the formulation and leachables that could result from manufacturing materials and packaging materials such as stoppers should be evaluated during pharmaceutical development.

If the mode of action is based on the primary structure and the content (quantity) of the oligonucleotide only, no potency assay is needed for release and stability testing of the finished product. Applicants are encouraged to give more details on the possible (absence of) higher-order structure, e.g. based on near UV CD spectroscopy or other techniques, as well as computation investigations when feasible. Additionally, experiments on the higher-order structure stability

characteristics in the formulation), with techniques such as CD or others are recommended as characterisation data, to justify the omission of such analysis in the routine control strategy.

Furthermore, where relevant, formulation development should address the aggregation propensity and the nature of the aggregates formed, especially under stress conditions including terminal sterilisation (see below).

Most of the medicinal product formulations containing oligonucleotide as active substance are for parenteral use. The principles for the choice of sterilisation process for finished products and containers are presented in the form of decision trees in the 'Guideline on the Sterilisation of the Medicinal Product, Active Substance, Excipient and Primary Container' are also relevant for single-stranded oligonucleotides. Terminal sterilisation provides the highest sterility assurance level, thus this should be the method of choice unless demonstrated unsuitable.

A combination of sterile filtration, pre-sterilised container closure system and aseptic processing is only acceptable if the applicant demonstrates by data that the use of a terminal steam sterilisation process under the least stressful conditions ($F_0 \geq 8$ minutes) causes significant degradation. In case of moderate degradation, exceeding the qualification threshold is not a valid argument in itself to reject terminal sterilisation. Formulation optimisation efforts (e.g. pH, buffer system, osmolality), and choice of container closure system should be made during pharmaceutical development in view of enabling terminal sterilisation. For siRNA and aptamers for which the higher-order structure will be impacted by heat sterilisation, a theoretical rationale and/or reference to relevant literature is sufficient to justify aseptic processing, and product-specific experimental data are not expected.

If oligonucleotide drug products in development show moderate degradation towards heat stress, feasibility of terminal sterilisation should be addressed from early-development onwards. At that point, assay loss and increase in impurities/degradations products at levels that would not be observed with aseptic processing, may still be qualified in toxicological and pivotal clinical studies, including those impurities that exceed the qualification threshold. Such studies should address the physicochemical properties, biological activity, and if relevant the immunogenicity risk of the product after terminal sterilisation. All of this with due consideration of the potential issues that may occur during formulation development (e.g. pH and buffering range) and further upscaling towards the commercial-scale terminal sterilisation process. To this extent, timely availability of stability indicating analytical methods is a pre-requisite. If needed complementary/orthogonal methods should be established to detect and quantify difficult-to-detect-impurities.

Thresholds for oligonucleotide–related impurities as defined above in 3.2.S.4.1, also apply to finished products: for single strand oligonucleotides related impurities should be reported above the LOQ of the analytical method, identified above 1.0% and qualified above 1.5%. If aggregation/oligomerisation occurs during finished product manufacture and/or storage, aggregates/higher order structures should be included in the finished product release and stability specification, unless otherwise justified.

Manufacturing processes should take into account any special characteristics such as hygroscopicity of (lyophilised) active substance, as well as any temperature and/or light sensitivity of the active substance, as relevant.

If correction factors are applied during dispensing (e.g. based on assay, purity, moisture content, residual solvent content, and/or salt content of active substance) to achieve a specific declared (labelled) amount of active in the formulation, these have to be described and justified in the dossier.

The label claim strategy should be conclusively described and justified, including (where relevant) calculation of active substance assay, any correction factors applied during dispensing, any in-process controls for assay adjustment during drug product manufacturing, and assay calculation for release- and stability testing. Any changes in label claim strategy during development have to be described in

detail and justified , to ensure that the dose definition used in clinical trial(s) can be bridged unequivocally to the proposed commercial product with label claim as per the SmPC/labelling.

Additional characteristics for complex finished product dosage forms should be considered on a case-by-case basis.

For aptamers a test on biological activity should be included in the active substance or finished product specification.

# 6. Generics Development (human products only)

In general, for the development of generic medicinal products the requirements as described in the 'Guideline on bioequivalence (CPMP/EWP/QWP/1401/98 Rev.1/ Corr**)' are applicable to synthetic oligonucleotides.

In the case of complex formulations (e.g. lipid nanoparticles) additional clinical and/or non-clinical studies will be needed and the basic principles as described in the reflection paper on 'Data requirements for intravenous liposomal products developed with reference to an innovator liposomal product' should be considered.

Analytical comparability testing using a broad panel of characterisation tests as described above and relevant for the specific oligonucleotide class forms the basis of comparability demonstration.

In the case of oligonucleotides containing a phosphorothioate linkage additional investigations regarding the diastereomeric composition should be performed. Due to the stereochemistry at the phosphorus chiral center of the phosphorothioate linkage, these active substances contain many different diastereomers. Suitable state of the art analytical methods should be employed to characterise stereochemistry.

It is the responsibility of the applicant to demonstrate that the purity methods are suitable to cover the complete impurity profile of the oligonucleotide or whether additional purity testing with additional supplementary methods is necessary. When differences in the impurity profiles with the reference product are observed it should be demonstrated that impurities not present in the reference product are qualified and do not raise any safety concerns. Impurities above 1.0% should be identified and above 1.5% qualified.

Comparative forced degradation studies are also recommended and the suitability of the analytical purity methods to fully characterise the impurity profiles of both products should be demonstrated.

The analytical methods used in the comparability exercise should be suitable, sufficiently qualified and/or validated and sensitive to detect potential differences between both products. In the case that statistical models are used to demonstrate comparability between the generic product and the reference product they should be adequately described and justified. Batches preferably from the commercial process should be used for the side-by-side analyses. The number of batches used in the comparability studies should be adequately justified (Reference to: 'Reflection paper on statistical methodology for the comparative assessment of quality attributes in drug development' - EMA/CHMP/138502/2017).

For medicinal products where European product-specific guidance on the demonstration of the bioequivalence has been published the generic product should comply with the quality requirements described therein e.g. for comparability studies. Additionally, the principles of EMA's Quality Working Party (QWP) Questions and Answers on assessment of quality of finished products containing known active substances applies.

# 7. Requirements for Clinical Trial Applications (human products only)

The requirements for oligonucleotides intended to be used in clinical studies evolve depending on the stage of development, with increasing expectations going towards Phase 3 and approaching the marketing authorisation application (MAA). The main focus should be on the safety of the oligonucleotide, especially in the early stages of development.

Oligonucleotides are predominantly manufactured by solid phase supported synthesis hence prior knowledge could be leveraged from development of similar oligonucleotides; nevertheless, details regarding the specific solid phase process (type of solid support, activator, oxidation/sulfurisation and capping agents used) and purification stages will be expected. Lyophilisation process parameters – where applicable – are expected in late clinical development.

With regard to starting materials of the active substance, which in most cases will be suitably protected phosphoramidites, it is expected that - from an early-stage - particular attention will be paid to certain impurities in these starting materials known to be critical. Specifications for starting materials should be provided in the investigational medicinal product dossier (IMPD), however setting of limits for certain impurities based on criticality assessment is only expected for later development.

Similar expectations also apply to the crude oligonucleotide after deprotection and cleavage from the solid support and to the ensuing critical purification steps (e.g. chromatography, ultrafiltration).

For double-stranded oligonucleotides specifications for the single-strand intermediates should be provided.

The changes introduced during manufacturing process development should be described in terms of potential impact on the quality of oligonucleotide; particular attention should be paid to differences in levels of critical impurities compared to pre-clinical batches.

The sequence of the oligonucleotides should be fully characterised; in certain cases, e.g. aptamers, higher order structures should also be studied. Also, potential for aggregation should be investigated.

Product-related impurities should be identified and/or qualified in the course of development. Given the notoriously complex impurity profiles of synthetic oligonucleotides, use of orthogonal analytical procedures such as AX-HPLC & RP-IP-HPLC (or a procedure combining orthogonal principles such as HPLC-UV-MS) is strongly encouraged from an early stage. Furthermore, for double stranded oligonucleotides, use of both a denaturing and non-denaturing analytical procedure will be expected.

For aptamers, a test for biological activity should be included in the specifications for the investigational medicinal product prior to initiation of phase I studies, based on an appropriate, reliable and qualified method.

With regard to the stability studies to be conducted on the synthetic oligonucleotides, it is essential that stability-indicating analytical procedures are employed and that the relevant storage conditions are explored taking into account the nature of the oligonucleotide in question. Determination of retest period/shelf-life and storage conditions may refer to prior knowledge gained from other oligonucleotide molecules with similar chemistry and similar manufacturing processes if justified and supported by data.

# 8. N-of-1 / Personalised Medicines (human products only)

With the development of antisense oligonucleotides over decades and the increasing number of identified unique severely debilitating or life-threatening diseases affecting only 1 person in the world

(N-of-1) oligonucleotides are being developed to treat diseases that are caused by well-identified mutations in single genes.

If more than a few patients may be candidates for targeted treatment of unmet medical need with the oligonucleotide, then the oligonucleotide is no longer considered individualised. For the development of these products, guidance is provided in the 'Toolbox guidance on scientific elements and regulatory tools to support quality data packages for PRIME and certain marketing authorisation applications targeting an unmet medical need'.

It should be demonstrated that the applied methods to confirm identity will be able to discriminate between all sequences used in the development programme.

For both, active substance and finished drug product, suitable state-of-the-art analytical purity methods with stability indicating properties should be developed. It is not recommended to employ only one single method as e.g. IEX or SEC with limited relevance.

Generic impurity methods e.g. published in the literature may be used initially. Analytical methods should be periodically re-assessed during development and be updated as necessary when additional knowledge is gained.

Testing of overall purity is mandatory; however, characterisation of product-related impurities may be limited compared to products which are not used for only one patient.

No full stability programme considering the requirements of ICH Q1 stability guidelines for the N-of-1 treatment approach is needed. It is however expected that initially for each oligonucleotide intended to be used in a clinical trial some stability data will be generated. At later timepoints when more comprehensive data are available alternative approaches based on prior knowledge may be feasible. The overall stability programme should be justified sufficiently.

In case stability data will be generated only for the active substance it needs to be justified that these data are also relevant for the drug product considering the intended storage conditions and the proposed shelf-life.

There are several options combining forced degradation / stress test studies with accelerated stability studies in such a proposal.

Omission of stability studies for active substance and drug product only based on public information available for approved medicinal products, without access to the actual data, is not acceptable.

Regarding formulation of N-of-1 oligonucleotides, the choice of formulations similar to already approved products are recommended. Limited pharmaceutical development of the drug product manufacturing might be appropriate.

A microbiology control strategy should be developed, and sterility of the product applied by parenteral application has to be ensured.

Risk assessments may be sufficient to omit testing of certain impurities as e.g. elemental impurities.

Regarding GMP aspects a discussion with the competent GMP Supervisory Authority for the manufacturing site(s) is recommended. This may also cover aspects regarding the re-use of materials as resins or ultra-/diafiltration cassette and the potential risk of carry-over.