
**Biotechnology — Nucleic acid
synthesis —**

**Part 1:
Requirements for the production
and quality control of synthesized
oligonucleotides**

Biotechnologie — Synthèse des acides nucléiques —

*Partie 1: Exigences relatives à la production et au contrôle qualité des
oligonucléotides synthétisés*



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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 276, *Biotechnology*.

A list of all parts in the ISO 20688 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Single stranded, linear bio-polymers made up of nucleotides which are called “synthetic oligonucleotides” or ‘oligos’ are indispensable components for biotechnology. For example, they are used as polymerase chain reaction (PCR) amplification primers, microarray, real time PCR or next generation sequencing (NGS) capture probes, and as input starting materials for the creation of entire target genes.

Control of quality in production is important in the synthesis of oligonucleotides. The quantification of the size range, concentration and contaminants is necessary to ensure that quality requirements are met for end-use applications. Considering that oligonucleotides are used in biologically active applications, their quality, particularly sequence and conformation, will affect fitness or function, for example molecular recognition of cognate binding site, chemical behaviour. The specific requirements for each end-use application can differ.

This document defines common quality attributes of synthetic oligonucleotides and addresses their quantification and assessment for end-use.

It is intended to help improve quality management and demonstrate product quality.

International, national or regional regulations or requirements can also apply to specific topics covered in this document. For example, when synthesized oligonucleotides are used as investigational drugs or pharmaceutical agents, regional regulations and/or good manufacturing practices (GMP) may need to be considered.

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Biotechnology — Nucleic acid synthesis —

Part 1:

Requirements for the production and quality control of synthesized oligonucleotides

1 Scope

This document specifies minimum requirements for the production and quality control of synthesized oligonucleotides (nominally up to 250 bases).

This document also describes general quality attributes for synthesized oligonucleotides as well as common methods for evaluating quality attributes.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

- ISO Online browsing platform: available at <https://www.iso.org/obp>
- IEC Electropedia: available at <http://www.electropedia.org/>

3.1

certified reference material CRM

reference material (3.4) characterized by a metrologically valid procedure for one or more specified properties, accompanied by an RM certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability

[SOURCE: ISO Guide 30:2015, 2.1.2, modified — Notes have been deleted.]

3.2

performance

<synthetic oligonucleotides> ability of oligonucleotides, which are synthesized for the specific intended use including biological assays, to fulfil the requirements for the specific use

Note 1 to entry: In the case of oligonucleotides synthesized as primers for a PCR, it is the ability of such synthetic oligonucleotides to function as primers.

Note 2 to entry: In the case of oligonucleotides synthesized as probes for use in DNA microarrays, real time PCR or NGS, it is the ability of such synthetic oligonucleotides to hybridize as probes with target oligonucleotide sequence.

Note 3 to entry: Performance is confirmed by testing that evaluates full functioning of oligonucleotides in their respective uses.

3.3

purity

<synthetic oligonucleotides> ratio between the amount of expected-sequence and/or length synthetic oligonucleotides and the total amount of oligonucleotides

Note 1 to entry: Purity of synthetic oligonucleotides is the ratio of absorbance peak area corresponding to synthetic oligonucleotides of expected sequence and/or lengths in comparison with total peak area of oligonucleotides. The measurement of absorbance at 260 nm (OD 260) is calculated by high performance liquid chromatography (HPLC), or capillary electrophoresis (CE). The purity is calculated with area normalization method from the result of HPLC or CE or mass spectrometer.

3.4

reference material

RM

material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process

[SOURCE: ISO Guide 30:2015, 2.1.1, modified — Notes have been deleted.]

3.5

synthetic oligonucleotides

chemically synthesized DNA (deoxyribonucleic acid) or RNA (ribonucleic acid)

Note 1 to entry: Various compounds such as modified bases, base analogues, end-labelling reagents, fluorescent compounds, etc., can be introduced into the synthetic oligonucleotides.

Note 2 to entry: In this document, a synthetic oligonucleotide is considered single stranded, linear, and in length from approximately 10 nucleotide-bases to 250 nucleotide-bases.

4 Design and selection of suitable oligonucleotides that are fit for purpose

The quality and consistency requirements of synthetic oligonucleotides depend on their intended uses. For example, the quality requirements of primer and probe in a polymerase chain reaction (PCR), or in a microarray or NGS are significantly different.

In general, users are responsible for specifying quality requirements of the oligonucleotides for their specific application.

A quality grade list can be provided by the producer from which the user can select the grade that fits the intended use, for example “genomic grade” or “antisense grade” (see [5.2](#)).

Robust quality management and testing, together with a common understanding of quality attributes can provide consistent information to allow:

- users to appropriately select oligonucleotides for the intended use;
- users and producers to better communicate and agree on specification for custom oligonucleotides.

5 General quality management requirements

5.1 General requirements

The producer of synthetic oligonucleotides (hereinafter referred to as “producer”) shall establish and implement a system in which the following processes are described and documented:

- a) order receipt;
- b) oligonucleotide production;
- c) quality control.

A quality policy and quality objectives shall be determined for the order receipt process. The quality requirements for users are likely to vary depending on their intended use, the design of their experimental method, and the potential for results that affect repeatability and reproducibility. Based on the quality policy, quality objectives, and grades of synthetic oligonucleotides, when applicable, that have been clearly stated in the process of order forms, the producer should monitor whether the production is performed in accordance with appropriate processes by such means as process checklists (see [Annex A](#) for an example). In addition, necessary actions should be taken for the production processes in order to achieve the planned results by measurements and analyses of the qualities of synthetic oligonucleotides that are produced.

5.2 Oligonucleotide grading

To reduce excessive customization of the quality requirement, grading of oligonucleotide quality can be considered. When the producer documents the grading of oligonucleotide quality, the grades should be determined based on the intended use. The purification methods should be chosen and determined to fit each grade; see [7.3](#).

5.3 Control of documents

The producer shall have a procedure(s) ensuring the control of documented information including records required by this document and shall ensure that unintended use of any obsolete document is prevented. When the documented information including records is retained in electronic media, the producer shall ensure the control of those electronic media.

5.4 Quality management system

The producer shall adopt a quality management system. The quality management system shall establish necessary procedures and ensure the execution of producing control based on the established procedures. The quality management system shall regularly check whether the production of synthetic oligonucleotides based on this document is performed correctly.

NOTE For labelled oligonucleotides, additional quality indicators can be considered according to the characteristics of the label, for example strength and wavelength of fluorescence.

5.5 Personnel and training

The producer shall ensure that personnel are competent for performing the procedures specified in this document and shall properly supervise the personnel. The producer shall provide appropriate training and assessment of the competence to the personnel.

5.6 Safety control

The producer shall establish a safety program considering applicable requirements in order to ensure the safety of the personnel performing oligonucleotide synthesis and purification as specified by this document.

6 Resource management

The producer shall ensure the suitable condition of the facilities as well as area for the production of oligonucleotides. The producer shall maintain equipment and instruments to produce synthetic oligonucleotides. The producer shall properly control raw material (including reagents, pure water and ancillary materials) that can potentially affect the quality of synthetic oligonucleotides.

An example of equipment and instrumentation suitable for production of synthetic oligonucleotides is listed in [Annex B](#).

7 Requirements for production process

7.1 General

The production of a single sequence synthetic oligonucleotide generally consists of the following processes:

- a) synthesis;
- b) purification;
- c) quality control inspection;
- d) drying, when applicable;
- e) formulation.

These processes shall be performed with regularly maintained instruments, for example, pipettes or dryers.

NOTE 1 Several production processes are used to produce complex oligonucleotide pools. For example, several oligonucleotides are combined to produce complex oligonucleotide pools. Alternatively, a complex oligonucleotide pool is produced via array-based synthesis. Appropriate quality control measures can be selected and implemented based on the production method.

NOTE 2 General methods to determine quality attributes are not necessarily appropriate for array-based synthesis.

NOTE 3 In some cases, single sequence oligonucleotides are synthesized with wobble bases for specific applications requiring differential annealing. In this case, bases are transversed or transitioned at a specific site.

7.2 Oligonucleotide synthesis

A quality control inspection shall be performed by using the adequately calibrated and maintained measuring instruments.

Oligonucleotide synthesizers shall be regularly maintained and controlled. Operators shall be qualified according to documented procedures. The records related to the operations shall be retained.

7.3 Purification

Appropriate purification equipment and methods shall be selected based on risks to the oligonucleotide design specifications and intended use as agreed upon with the user.

Purification options include: reverse HPLC (C8 to C18), anion exchange HPLC (SAX, WAX), polyacrylamide gel electrophoresis (PAGE), oligonucleotide purification cartridge (OPC), high purity salt free (HPSF) purification and direct precipitation (desalting). The purification method can be selected according to the intended use of oligonucleotides. The record of used method shall be retained.

Purification operation shall be performed by qualified personnel. The records related to the operations shall be retained.

7.4 Quality control inspection

The quality control inspection shall be performed by using the adequately calibrated and maintained measuring instruments. Measurement operation shall be performed by qualified personnel in accordance with documented procedures. In addition, operation record shall be retained. The quality control process shall be conducted to establish identity, purity, impurity, quantity, and other important attributes of the oligonucleotide with respect to its intended use.

7.5 Drying

Drying can be performed with centrifugal evaporation, freeze drying or air drying equipment.

These operations shall be performed by qualified personnel in accordance with documented procedures, and the operation record shall be retained.

7.6 Formulation

These operations shall be performed by qualified personnel in accordance with documented procedures, and the operation record shall be retained.

8 Requirements for quality control process

8.1 General

The quality control process shall be conducted to establish identity, purity, impurity, quantify, and other important attributes of (an) oligonucleotide(s) with respect to its (their) intended use.

Appropriate method(s) shall be selected and validated for establishing the identity and quality of oligonucleotides.

The measurement shall be performed by qualified personnel.

8.2 Validation and verification of analytical methods

Analytical methods used to perform the quality control and to determine the quality attribute(s) shall be validated and verified. Instrumentation should be calibrated by utilizing certified reference materials.

NOTE An example of accuracy control of measurement instruments by using certified/standard reference materials is described in [Annex E](#).

8.3 Identity and purity

8.3.1 Identity of the base sequence

The base sequence can be determined by appropriate methods, for example electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) as ion source, time of flight (ToF), ion trap or quadrupole mass spectrometer (MS) as mass analyser, or sequencing.

NOTE 1 An example for the confirmation of base sequences using MS is described in [Annex D](#).

NOTE 2 MS can be used to determine the sequence for short oligonucleotides while there are limitations on accurate analysis for sequence determination depending on the length of synthetic oligonucleotides.

The determined identity of the base sequence shall be documented.

8.3.2 Purity

The purity of a given oligonucleotide for a specific intended use should be determined and agreed upon between the producer and user.

EXAMPLE The purity of a synthetic oligonucleotide can be determined as the ratio between the amount of intended oligonucleotide and the total amount of oligonucleotides. The intended oligonucleotide(s) is (are) the one(s) with the "expected-length" and/or the correct sequence.

Methods to determine the purity can include one or a combination of the following methods:

- a) analytical reverse-phase or ion exchange high performance liquid chromatography;
- b) capillary or slab gel electrophoresis with validation of relative linearity response;
- c) MS (for example ESI or MALDI as ion source, ToF, ion trap or quadrupole MS as mass analyser);
- d) sequencing.

NOTE Synthetic oligonucleotides are complex molecules and will produce different results based on the oligonucleotide design and production process.

The determined purity shall be documented.

8.3.3 Impurity

In some applications, it is important to determine the impurity.

The acceptable degree or range of impurity of a given oligonucleotide for a specific intended use should be determined and agreed upon between the producer and user. In general, impurity is the quantity of oligonucleotide that interferes with the intended use. The degree of impurity shall be determined using an appropriate method.

NOTE Impurities include but are not limited to organic solvent, mono-nucleotides, short nucleic acids and nucleic acids with incorrect sequences.

Methods to determine impurity can include one or a combination of the following methods:

- a) analytical reverse-phase or ion exchange high performance liquid chromatography;
- b) capillary or slab gel electrophoresis with validation of relative linearity response;
- c) MS (ToF or ESI);
- d) sequencing.

The determined impurity shall be documented.

8.4 Quantity

8.4.1 General

Sufficient quantity of synthetic oligonucleotides shall be provided for the intended use as agreed in advance.

The producer should confirm that the synthesized amount fulfils the user's requirements.

The agreed quantity of synthetic oligonucleotides shall be documented.

8.4.2 Concentration

The oligonucleotide concentration measurement can be used as a part of quality control during production or product testing (check). The optical density (OD) can be used to measure the concentration of oligonucleotides present in a solution. In general, the optical density at 260 nm is used.

The molar concentration is calculated based on the molar extinction coefficient and the OD. [Formula \(C.1\)](#) and the parameters to calculate the molar extinction coefficient are shown in [Annex C](#).

As different assumptions are used in the calculation of molar extinction coefficient, the molar extinction coefficient data, including the used assumption, shall be documented (see Reference [Z]).

Other methods such as HPLC can also be used to measure concentration after demonstrating the correlation with the optical density, when applicable, by using oligonucleotide RM or CRM.

The measured concentration shall be documented.

8.4.3 Mass

The mass shall be documented in nanograms (ng) or convertible SI traceable units.

The mass is calculated by [Formula \(1\)](#):

$$m = OD_{260} \times V_f \times C \times D \quad (1)$$

where

- m is the mass, in ng;
- OD_{260} is the absorbance at 260 nm;
- V_f is the fluid volume, in ml;
- C is the conversion factor, in ng/ml;
- D is the dilution factor.

8.5 Molar mass and/or base length

The molar mass and/or base length of synthesized oligonucleotides shall be determined and documented. The molar mass can be determined directly using mass spectrometry (MS) (including ESI or MALDI as ion source, ToF, ion trap or quadrupole MS as mass analyser), or indirectly via electrophoresis with a controlled slab or capillary electrophoresis instruments.

A confirmation operation shall be performed by qualified personnel, and the operation record shall be retained.

MS is a method to determine the total atomic mass of a molecule. When using MS, the molar mass of synthetic oligonucleotides can be calculated according to [Formula \(2\)](#):

$$\begin{aligned} M = & [(\#dA \times 313,20) + (\#dC \times 289,17) + (\#dG \times 329,19) + (\#dT \times 304,18) \\ & + (\#dU \times 290,16) + (\#I \times 314,18) + (\#rA \times 329,19) + (\#rC \times 305,17) \\ & + (\#rG \times 345,19) + (\#rU \times 306,15)] + (\#rI \times 330,18) - 62] + 1 \end{aligned} \quad (2)$$

where

- M is the molar mass in g/mol;
- $\#dA$ is the number of adenine (deoxyribonucleic acid);
- $\#dC$ is the number of cytosine (deoxyribonucleic acid);
- $\#dG$ is the number of guanine (deoxyribonucleic acid);
- $\#dT$ is the number of thymine (deoxyribonucleic acid);
- $\#dU$ is the number of uracil (deoxyribonucleic acid);
- $\#I$ is the number of inosine (deoxyribonucleic acid);
- $\#rA$ is the number of adenine (ribonucleic acid);

#rC is the number of cytosine (ribonucleic acid);

#rG is the number of guanine (ribonucleic acid);

#rU is the number of uracil (ribonucleic acid);

#rI is the number of inosine (ribonucleic acid).

NOTE 1 See References [8], [9], [10], [11], [12] as well as C.1 and Table C.1 for further information.

NOTE 2 Formula (2) can be used to calculate the molar mass based on 3' terminal modification of phosphoric acid.

When a different formula is used, this should be documented.

The molar mass of modified oligonucleotides can be calculated by using the molar mass value of modification compounds shown as examples in C.2.

8.6 Melting temperature (T_m)

The T_m value can be calculated by Formula (F.1) shown in Annex F. The T_m value shall be documented when calculated.

8.7 Report

A synthesis report for synthetic oligonucleotides shall include, but is not limited to, the following:

- a) the product identifier;
- b) the product lot numbers:
 - process of production and quality control shall be able to be traced back;
- c) the user ordering synthesis;
- d) the purification method;
- e) the oligonucleotide sequence:
 - description shall be in the form of IUPAC code, examples are shown in Annex C;
- f) the molar mass in g/mol and/or the numbers of bases (base length);

NOTE 1 See Formula (2). In principle, molar mass for oligonucleotides moieties do not include modifiers which include linkers. A phosphate group at the 5' end is assumed not to exist.

- g) the formula used to calculate the molar mass;
- h) the OD unit;

NOTE 2 OD is a widely used expression of oligonucleotide quantity.

NOTE 3 See 8.4.3.

- i) the molar extinction coefficient data including the used assumption;

NOTE 4 See Formula (C.1).

- j) the total number of moles;

NOTE 5 This value can be calculated on the basis of the absorbance indices shown in Table C.1, and Formula (C.1) described in Annex C.

- k) the melting temperature (T_m);

- l) the guanine-cytosine (GC)-content;
- m) the recommended condition (for example a specific buffer including TE buffer) for resuspending the synthetic oligonucleotides;
- n) the expiration date:
 - expiration date should be determined from the empirical data — estimates of shelf life can be applied based on results from similar oligonucleotide stability studies;

NOTE 6 As an example, typical shelf life is two years for dried products stored at 4 °C, and one year for products in solution stored at –20 °C.
- o) the storage condition:
 - storage condition should be determined from the empirical data — storage condition can be applied based on results from similar oligonucleotide stability studies;

NOTE 7 In principle, 4 °C for dried products, and –20 °C for products in solution whereas a different temperature range can be used based on the agreement between the user and producer.
- p) the shipping conditions.

8.8 Verification of corrective action and improvements

The quality management system shall take action to eliminate the causes of issues including nonconformities and customer complaints against products. Appropriate actions should be considered when opportunities for improvement of products are recognized. The quality management system shall ensure that the records on corrective actions or improvements are retained. The quality management system shall regularly review the status of corrective actions and improvements.

9 Additional requirements for synthetic oligoribonucleotides (RNA)

Requirements for the quality management system described in [Clauses 4](#) to [8](#) shall also be applied to synthetic RNA.

In addition, because of RNA's susceptibility to degradation by catabolic enzymes, the following additional requirements apply:

- a) Sterilized nuclease-free apparatuses, such as containers or tips, shall be used.
- b) Pure water or buffer solutions used for the production shall be made from a controlled water purification system. The electrical conductivity of water made from adopted purification system shall be checked regularly. Reagents shall be prepared in appropriate facilities under controlled circumstances in order to avoid contamination of nucleases. In addition, for dried products, warning statements shall encourage prompt use of reagents after opening a sealed vessel. Materials should be transported in a dry form. When products are transported and stored in solution, they shall be kept in a frozen state during transport and stored at –80 °C.

Annex A (informative)

Process checklist

[Table A.1](#) provides an example of checklist that can be used for the conformity assessment of synthetic oligonucleotides.

Table A.1 — Process checklist

Process	Requirement	Conformity				Comments
		Con-forming	Non-con-forming	Not applicable	Documents and sections	
Order-receiving process	product number					
	customer information					
	sequence information					
	modification, etc.					
	purification method					
	date of receipt of orders/ due date					
	person in charge of receipt of orders					
	yield					
Production process	equipment control					
	synthesis					
	person in charge of synthesis					
	purification					
	person in charge of purification					
	yield measurement					
	person in charge of yield measurement					
Quality control process	quality control					
	facility control					
	person in charge of quality control					
	report writing					
	person in charge of report writing					
Shipping process	person in charge of shipping					

Table A.1 (continued)

Process	Requirement	Conformity				Comments
		Con-forming	Non-con-forming	Not applicable	Documents and sections	
General	quality manager					
	training / qualification					
	legal requirement					
	corrective action					
	document control					
	record control					

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Annex B (informative)

List of equipment and devices and their control standards

An example of the equipment and device list and their control standards is shown in [Table B.1](#) [13][14].

Table B.1 — List of equipment and devices and their control standards

Types of equipment	Specifications	Intended use	Requirements	Inspection frequency
Temperature-controlled equipment (incubator, refrigerator, freezer, etc.)	—	Storage of reagents	Temperature stability and homogeneity.	At the time of installation, every two years, and after repair.
			Temperature check.	Daily
Pure water production system	—	Quality control	Electric conductivity check.	Weekly
pH meter	—	Reagent preparation	Adjustment using at least two kinds of traceable buffer solutions of proper quality.	Daily or at the point of use
Weighing device	—	Reagent preparation	Confirmation of zero point and read check using standard weight, or implementation of built-in performance check.	Daily or at the point of use
Pipettor or pipette	—	Dispensing of reagents	Calibration and check for accuracy of pipetting amount.	Periodically ^a
Centrifuge machine	—	Centrifugation	Operate normally without abnormal noise.	At the point of use
Oligonucleotide synthesizer	Devices that can perform synthesis of oligonucleotides by β -cyanoethyl phosphoramidite Method1), 2)	Oligonucleotide synthesis	Check for valves and flow volume of reagents.	Periodically ^a
Automated pipetting system	Automated pipetting robots, etc.	Dispensing of reagents or synthetic oligonucleotides, etc.	Check for accuracy of pipetting amount.	Periodically ^a

^a Inspection frequencies are defined for ensuring their fitness for purpose, by considering the normal way of use and its frequency (see e.g. ISO 9001 or standards related to the quality management system of the organization).

^b For example an optical glass filter, which is designed for calibration of spectrophotometers.

Table B.1 (continued)

Types of equipment	Specifications	Intended use	Requirements	Inspection frequency
High performance liquid chromatography system	Devices that can fit appropriate columns and are equipped with a detector which can measure absorbance wavelength from 254 nm to 260 nm, with a recorder which can sum up peak area if needed, and with a fraction collector.	Oligonucleotide purification	Built-in performance check and check for efficacy of columns.	Periodically ^a
Electrophoresis equipment	Devices that can perform polyacrylamide or agarose gel electrophoresis under native and denaturing conditions, and detecting devices such as UV lamps.	Confirmation of base lengths	Ability for separation and detection of appropriate molar mass markers.	Periodically ^a
Capillary electrophoresis system	Devices that can separate synthetic oligonucleotides under native or denaturing conditions and are equipped with detectors and recorders that can sum up peak area.	Confirmation of base lengths	Ability for separation and detection of appropriate molar mass markers.	Periodically ^a
Dryer	Devices that are usable for purpose of drying synthetic oligonucleotide solutions.	Drying of synthetic oligonucleotides	Check for drying time.	At the point of use
Spectrophotometer	Single beam or double beam spectrophotometers.	Yield measurement of synthetic oligo-nucleotides	Built-in performance check.	Periodically ^a
		Yield measurement of synthetic oligo-nucleotides	Calibration by use of optical filter ^b for calibration.	Periodically ^a
^a Inspection frequencies are defined for ensuring their fitness for purpose, by considering the normal way of use and its frequency (see e.g. ISO 9001 or standards related to the quality management system of the organization).				
^b For example an optical glass filter, which is designed for calibration of spectrophotometers.				

Annex C (informative)

Calculation of molar mass and molar numbers

C.1 Information required for calculating molar mass and molar extinction coefficient

Information required for calculating the molar mass (see 8.5) and the molar extinction coefficient are shown in Tables C.1 and C.2. The information given here is a non-exhaustive list of common examples, especially regarding the labelled forms.

The molar number of synthetic oligonucleotides can be calculated with the absorbance (expressed by the OD value) divided by the molar extinction coefficient.

The molar extinction coefficient at pH 7,0 and 260 nm can be calculated according to Formula (C.1):

$$\begin{aligned} \varepsilon = & (\#dA \times 15,2) + (\#dC \times 7,4) + (\#dG \times 11,8) + (\#dT \times 8,8) + (\#dU \times 10,1) + (\#I \times 12,1) \\ & + (\#rA \times 14,9) + (\#rC \times 7,55) + (\#rG \times 13,6) + (\#rU \times 10) + (\#rI \times 12,1) \end{aligned} \quad (C.1)$$

where

ε is the molar extinction coefficient, in $\text{l} \times \text{mol}^{-1} \times \text{cm}^{-1}$;

$\#dA$ is the number of adenine (deoxyribonucleic acid);

$\#dC$ is the number of cytosine (deoxyribonucleic acid);

$\#dG$ is the number of guanine (deoxyribonucleic acid);

$\#dT$ is the number of thymine (deoxyribonucleic acid);

$\#dU$ is the number of uracil (deoxyribonucleic acid);

$\#I$ is the number of inosine (deoxyribonucleic acid);

$\#rA$ is the number of adenine (ribonucleic acid);

$\#rC$ is the number of cytosine (ribonucleic acid);

$\#rG$ is the number of guanine (ribonucleic acid);

$\#rU$ is the number of uracil (ribonucleic acid);

$\#rI$ is the number of inosine (ribonucleic acid).

Formula (C.1) is not applicable to modified oligonucleotides.

A different formula can be used, if this is documented and reported under 8.7.

Table C.1 — Molar mass and molar extinction coefficient of base molecules

IUPAC code	Molar mass ^a g × mol ⁻¹	Molar extinction coefficient at pH 7,0 and 260 nm l × mol ⁻¹ × cm ⁻¹
<i>dA</i>	313,20	15,2
<i>dC</i>	289,17	7,4
<i>dG</i>	329,19	11,8
<i>dT</i>	304,18	8,8
<i>dU</i>	290,16	10,1
<i>rA</i>	329,19	14,9
<i>rC</i>	305,17	7,55
<i>rG</i>	345,19	13,6
<i>rU</i>	306,15	10
<i>I</i>	314,18	12,1
<i>rI</i>	330,18	12,1
^a Molar mass is that in aqueous solution.		

C.2 Additional reference for calculating the molar mass and the molar extinction coefficient

Table C.2 is a non-exhaustive list of widely used examples of labelling technology.

Table C.2 — Labelling

Site	Modification	Molar mass g × mol ⁻¹	Molar extinction coefficient l × mol ⁻¹ × cm ⁻¹	Excitation λ _{max} nm	Emission λ nm
5'-	Cy5	534,6	250 000 (650 nm)	643	667
5'-	Cy3	507,6	150 000 (550 nm)	550	570
5'-	Cy5 (via amino modified)	817,0	250 000 (650 nm)	643	667
5'-	Cy3 (via amino modified)	791,9	150 000 (550 nm)	550	570
5'-	Amino	179,2	—	—	—
5'-	Biotin	405,5	—	—	—
5'-	Thiol	329,4	—	—	—
5'-	6-FAM	537,5	83 000 (496 nm)	496	516
3'	Cy5 (via amino modified)	848,0	250 000 (650 nm)	643	667
3'	Cy3 (via amino modified)	820,9	150 000 (550 nm)	550	570
3'	Amino	208,2	—	—	—
3'	Biotin (with TEG linker)	569,6	—	—	—
3'	Thiol (before reduction)	244,3	—	—	—
3'	TAMRA	626,7	95 000 (546 nm)	546	576
3'	7-deaza propagyl amino dA	351,26	—	—	—
3'	7-deaza propagyl amino dG	368,26	—	—	—
3'	N ₂ -aminohexyl dG	429,39	—	—	—
3'	5-aminohexyl dC	457,43	—	—	—
3'	5-aminohexyl dT	458,41	—	—	—
3'	5-fluorescein dC	874,91	—	—	—

Table C.2 (continued)

Site	Modification	Molar mass $\text{g} \times \text{mol}^{-1}$	Molar extinction coefficient $\text{l} \times \text{mol}^{-1} \times \text{cm}^{-1}$	Excitation λ_{max} nm	Emission λ nm
3'	5-TAMRA dC	815,73	—	—	—
3'	5-TAMRA dT	875,9	—	—	—
3'	5-fluorescein dT	816,72	—	—	—

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Annex D (informative)

Verification of oligonucleotide sequences using mass analysis

D.1 General

This annex provides an example of base sequence analysis of oligonucleotides by ion-source decay using matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-ToF MS) and electro spray ionization quadrupole mass spectrometer (ESI-Q-MS).

D.2 Experimental protocol of MALDI-ToF MS

a) Sample pre-treatment:

- Each analyte is dissolved in pure water to make a final concentration of 50 pmol/ μ l. 1 μ l of sample solution, and 0,5 μ l of matrix solution are spotted onto a MALDI plate. Then, the MALDI plate is air-dried, loaded into the device and finally mass spectroscopy is performed.

NOTE The matrix solution is 20 mg/ml 2,4-dihydroxyacetophenone (DHAP) and 70 mM ammonium citrate dibasic in 50 % acetonitrile solution.

b) MALDI-ToF MS testing condition:

- The testing conditions are described in [Table D.1](#).

Table D.1 — MALDI -ToF MS testing condition

Testing device used	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer
Feature	Light source: Nitrogen laser ($\lambda = 337,1$ nm)
Acceleration voltage	20 kV
Delayed extraction	Optimized for m/z 3 000
Flight mode	Linear mode (positive ion detection mode)
Measurement range	m/z 1 - 15 000

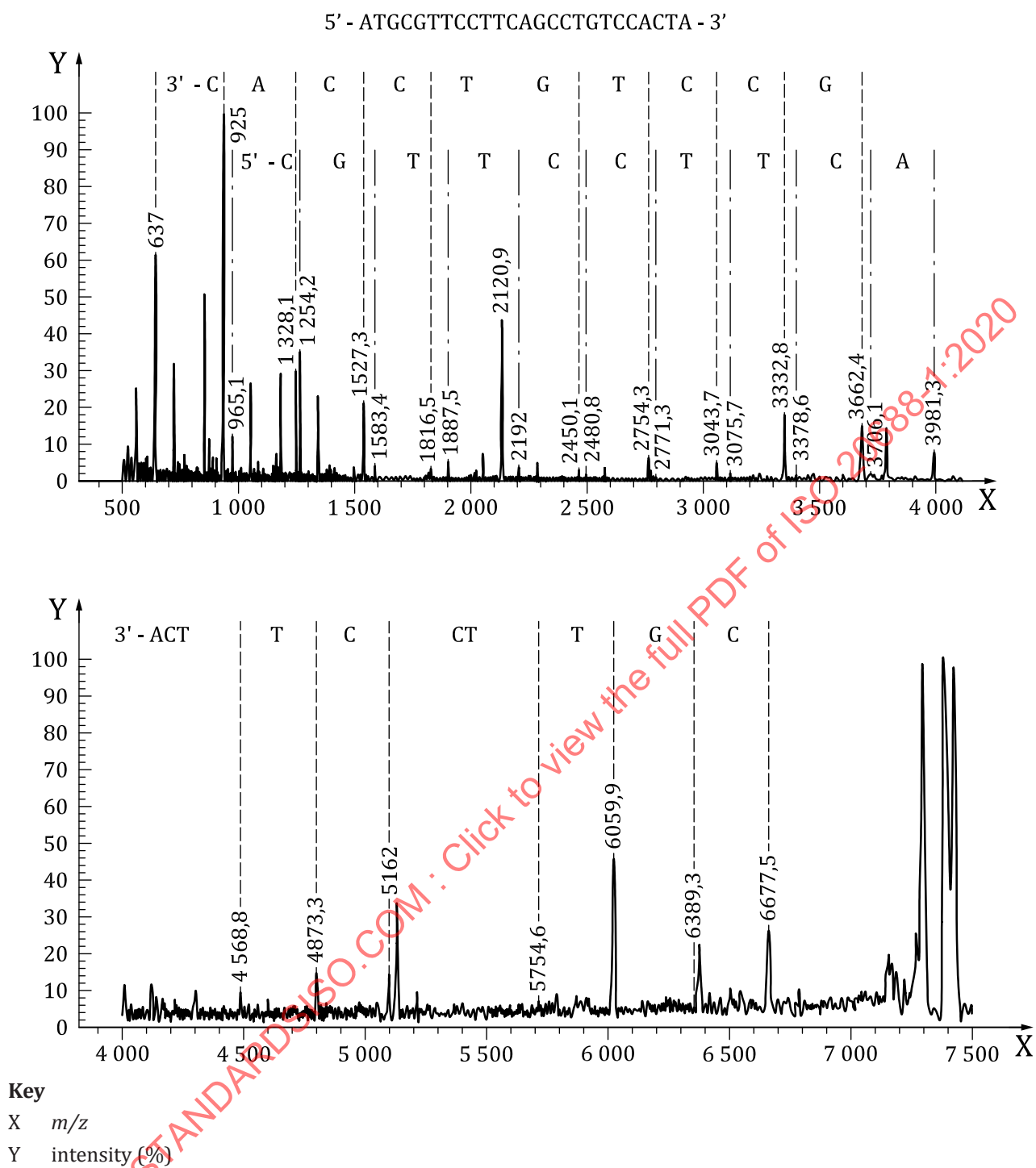


Figure D.1 — Examples of mass spectroscopy results for the synthetic oligonucleotide

The synthetic oligonucleotide is analysed with MALDI-ToF MS using the conditions described in [D.2](#). Each peak shown in [Figure D.1](#) is related to each nucleotide at the designated position in the oligonucleotide by using original analysis software.

D.3 Experimental protocol of ESI-Q-MS

a) Sample pre-treatment:

- 1) Each analyte is dissolved in pure water to a final concentration of 10 pmol/ μ l. Mass loads on column are kept constant at 50 pmol or 5 μ l injections.
- 2) Mobile phase A: 15 mM TAE buffer, 400 mM hexafluoro-2-propanol (HFIP) prepared in H₂O, pH 8,0.
- 3) Mobile phase B: 15 mM TAE buffer, 400 mM HFIP prepared in methanol (MeOH).

Table D.2 — HPLC gradient table

Time min	Flow rate ml/min	%A	%B
Initial	0,2	81,0	19,0
15,0	0,2	73,5	26,5
16,0	0,2	50,0	50,0
17,0	0,2	81,0	19,0
21,0	0,2	81,0	19,0

b) ESI-Q-MS testing condition (see [Table D.3](#)):

Table D.3 — ESI-Q-MS testing condition

Testing device used (LC)	Ultra high performance liquid chromatography
Testing device used (Detector)	Mass spectrometer
Column	Oligonucleotide separation technology column, 1,7 μ m, 2,1 mm, 50 mm
Column temperature	60 °C
Sample temperature	10 °C

c) Results:

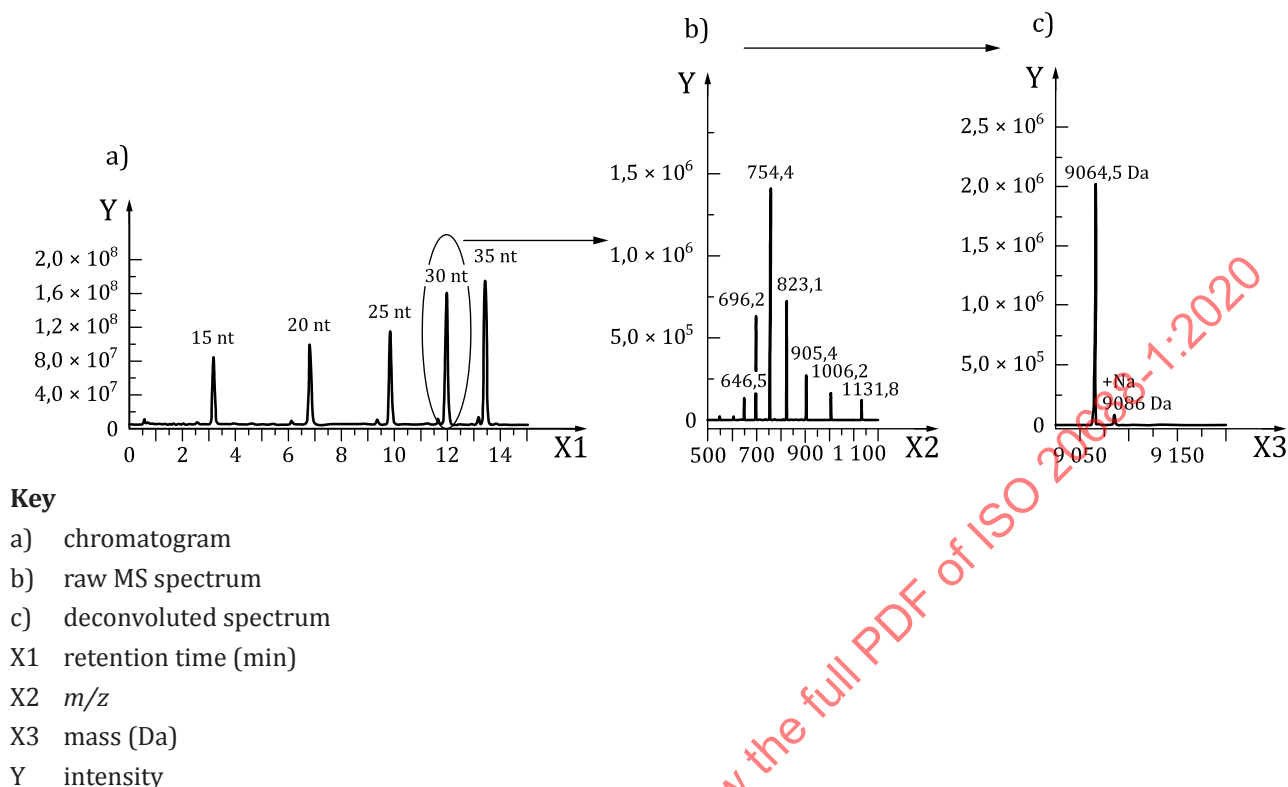


Figure D.2 — Examples of mass spectroscopy (ESI-Q-MS) results for the synthetic oligonucleotides

The synthetic oligonucleotide is analysed with ESI-Q-MS using the conditions described in [D.3](#). Deconvolution of the raw MS spectrum of the 30 nt standard resulted in a parent peak mass of 9 064,5 Da (+0,7 Da) and a minor sodium (Na^+) adduct peak with a relative intensity less than 6 % as shown in [Figure D.2](#).

D.4 Experimental protocol of ESI-Q-MS/MS

a) Sample pre-treatment:

- 1) 21 nt oligoRNA (5'-rUrCrGrUrCrArArGrCrGrArUrUrArCrArArGrGrTT-3') was used in this experiment (5 pmol/ μl in water).
- 2) Mobile phase A: 15 mM TAE buffer, 400 mM HFIP prepared in H_2O , pH 8,0.
- 3) Mobile phase B: 15 mM TAE buffer, 400 mM HFIP prepared in MeOH.

b) ESI-Q-MS/MS testing conditions:

- Testing conditions are described in [Tables D.4](#), [D.5](#) and [D.6](#).

Table D.4 — HPLC gradient table

Time min	Flow rate ml/min	%A	%B
Initial	0,2	87,0	13,0
10,0	0,2	77,0	23,0
10,1	0,2	50,0	50,0
11,1	0,2	50,0	50,0
11,2	0,2	87,0	13,0
16,0	0,2	87,0	13,0

Table D.5 — ESI-Q-MS/MS testing conditions

Testing device used (LC)	Ultra high performance liquid chromatography
Testing device used (Detector)	Qtof mass spectrometer
Column	Oligonucleotide separation technology column, 1,7 µm, 2,1 mm, 50 mm
Column temperature	60 °C
Sample temperature	6 °C

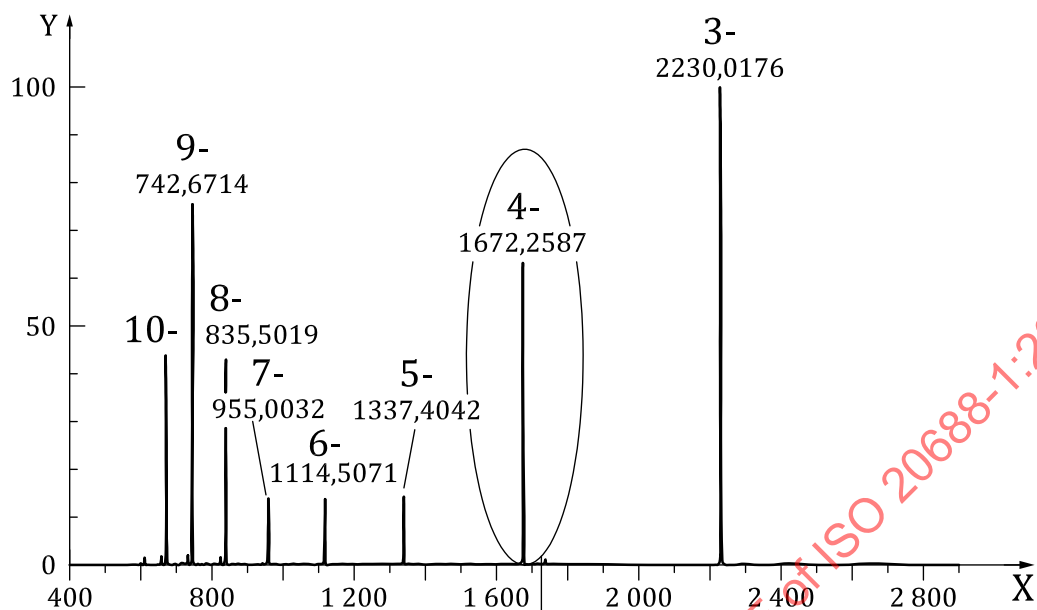
Table D.6 — ESI-Q-MS/MS condition

MS system	Qtof mass spectrometer
Data mass range	400 Da to 3 000 Da
Mode	ESI negative resolution
Cone voltage	2,0 kV
Source offset	80 V
Source temperature	125 °C
Desolvation temperature	500 °C
Desolvation gas flow	800 l/h
Lockmass	Glu Fibrinopeptide B at 100 fmol/µl in 50–50 H ₂ O–CAN, 0,1 % FA

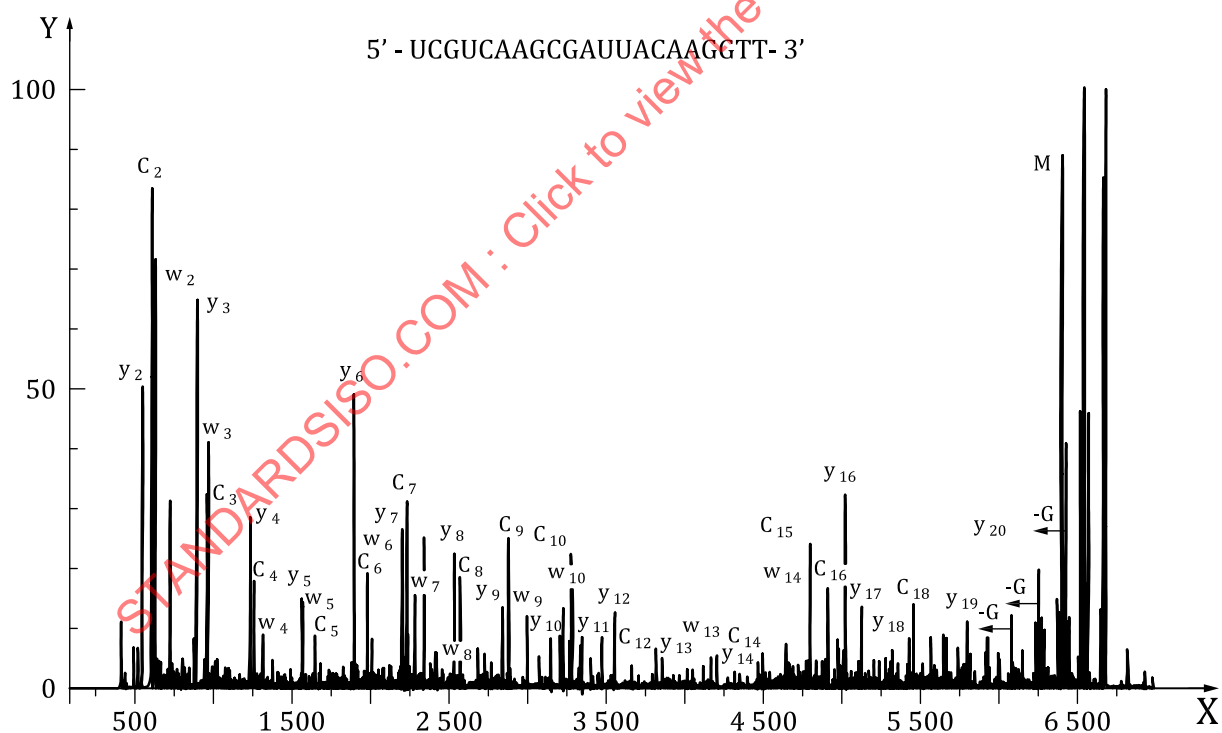
The synthetic oligonucleotide is analysed with ESI-Q-MS/MS using the conditions described in [D.4](#). The experiment was conducted using the M⁴⁻ peak [circled in [Figure D.3, a](#)] as the precursor ion. The fragmentation peak assignments were done by matching against the theoretical masses using simple calculations.

c) Results:

a)



b)

**Key**

X mass

Y intensity (%)

Figure D.3 — Examples of mass spectroscopy (ESI-Q-MS/MS) results for the synthetic oligonucleotides