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Biotechnology — Massively parallel sequencing —

Part 1: Nucleic acid and library preparation

Biotechnologie — Séquençage parallèle massif —

Partie 1: Acides nucléiques et préparation des collections

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Foreword

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

A list of all parts in the ISO 20397 series can be found on the ISO website.

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Introduction

Massively parallel sequencing (MPS) is a high throughput analytical technology for nucleic acid sequencing. MPS methods can process thousands to billions of nucleotide sequence reads simultaneously in a single run, allowing whole genomes, transcriptomes and specific nucleic acid targets from different organisms to be analysed in a relatively short time.

MPS is used in many life science disciplines permitting determination and high throughput analysis of millions of nucleotide bases. The biological variability of deoxyribonucleic and ribonucleic acid polymers from living organisms provides challenges in accurately determining their sequences. The quality of sequence determination by MPS depends on many factors including, but not limited to, sample quality, library preparation, and sequencing data quality.

The quality of nucleic acids and libraries prepared for MPS is critical to obtaining high quality sequence data. Controlling the upstream processing steps of MPS and evaluating nucleic acid samples and libraries for their suitability for sequencing significantly improves MPS results, downstream analyses and ultimately conclusions dependent upon the MPS data.

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Biotechnology — Massively parallel sequencing —

Part 1:
Nucleic acid and library preparation

1 Scope

This document specifies the general requirements for and gives guidance on quality assessments of nucleic acid samples. It specifies general guidelines for library preparations and library quality assessments prior to sequencing and data generation.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 20395:2019, *Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR*

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3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO 20395:2019 and the following apply.

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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 https://www.electropedia.org/

3.1
adapter
oligonucleotides of known sequence that are enzymatically added (e.g. ligase or polymerase chain reaction) to the end(s) of a DNA/cDNA fragment

3.2
barcode
index
short sequence of typically six or more nucleotides that serve as a way to identify or label individual samples when they are sequenced in parallel on a single sequencing lane, chip or both

Note 1 to entry: Barcodes are typically located within the sequencing *adapters* (3.1).

3.3
barcoding
indexing
unique DNA sequence identification
method that enables multiple samples to be pooled for sequencing

Note 1 to entry: Each sample is identified by a unique *barcode* (3.2), which enables identification of results during the parallel analysis.

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3.4
GC content
GC
percentage of guanine and cytosine in one or more nucleic acid sequence(s)

Note 1 to entry: The amount of guanine and cytosine in a polynucleotide is usually expressed as fraction (or percentage) of total nitrogenous bases. Total nitrogenous bases comprise the total number of nucleotide bases of reads from one or more MPS run.

[SOURCE: ISO 20397-2:2021, 3.15]

3.5
library
sequencing library
DNA, cDNA or RNA that has been prepared for massively parallel sequencing within a specific size range and typically containing *adapters* (3.1) and/or identifiers recognised for sequence specific priming, sequence capture, and/or identification of specific extracts

Note 1 to entry: Libraries can be DNA or cDNA. cDNA libraries are prepared for RNA sequencing on most sequencers. Some instruments can directly sequence RNA.

3.6
library preparation
sequencing library preparation
set of procedures used to prepare DNA or RNA fragments containing tags, and sequencing primer binding regions for massively parallel sequencing (MPS)

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3.7
spike-in control
spike-in process control
target sequence often of defined sequence identity and concentration that are spiked in the sample at various steps of the massively parallel sequencing protocol

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Note 1 to entry: Process controls can be used to evaluate any protocol step but are typically applied as nucleic acids controls prior to *library preparation* (3.6).

3.8
Q score
measure of the sequencing quality of a given nucleotide base

[SOURCE: ISO 20397-2:2021, 3.32, modified — The notes to entry have been deleted.]

4 Nucleic acid sample quality evaluation

4.1 General

The laboratory shall establish, implement and document a workflow for nucleic acid quantification that ensures accurate and reproducible results. Requirements for nucleic acid sample quantity and quality can vary between MPS methods. Nucleic acid purification methods can also affect the quality of nucleic acids used for library preparation.

A quality control procedure shall be developed to clearly define nucleic acid quality and library composition. This procedure shall be verified, implemented and documented, and permit accurate quantification of nucleic acid at the minimum amount of nucleic acid required for the MPS performed. The measurement uncertainty and sensitivity of the procedure used for this determination shall be determined. The quantification allows appropriate adjustment of the nucleic acid concentration for input into the MPS sequencer.

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Annex A provides a quality control checklist. Quantity, purity and integrity are major quality indicators for the prepared samples. Additional general considerations of sample quality regarding multiplex molecular testing including NGS are available in ISO 21474-1:2020.

4.2 Sample quantification

A range of methods for nucleic acid quantification are provided in ISO 20395:2019, 5.2. Other methods (e.g. electrophoresis) can also be used for the quantification.

Optimal sample amounts and concentrations appropriate for different MPS applications are listed in Table B.2.

4.3 Sample purity

Nucleic acid sample purity analysis shall be conducted in accordance with methods specified in ISO 20395:2019, 5.4.

4.4 Sample integrity

4.4.1 General

A range of methods used for assessing sample integrity is described in ISO 20395:2019, Annex B.

Gel electrophoresis and microfluidic analysis system can be used to evaluate nucleic acid sample integrity.

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4.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis can be used as a method for separating and isolating different sized nucleic acid molecules. It can also be used to determine nucleotide acid integrity. For example, optimally, genomic DNA (gDNA) samples have a strong main band of high molecular mass (greater than 20 kbp[1] in size) with minimal band dispersion.

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4.4.3 Capillary gel electrophoresis

Capillary gel electrophoresis can also be used to assess nucleic acid integrity.

4.4.4 Microfluidic analysis system

Microfluidic analysis system can be used to assess the integrity of genomic DNA or RNA extracted from various materials.

NOTE 1 DNA or RNA integrity number is commonly used as a numerical quality assessment criterion. The higher the value, the better the quality.

NOTE 2 Specifies the appropriate threshold depending on the type of devices.

4.4.5 PCR method

A PCR method can be used for integrity evaluation. High quality samples can generate data that are more useful than data generated from degraded samples.

NOTE Formalin-fixed and paraffin-embedded (FFPE) samples can cause challenges for some DNA applications. Further guidance is given in the ISO 20166 series.

1) kbp = kilo base pairs.

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5 Nucleic acid library preparation

5.1 General

The laboratory shall establish, implement and document each procedure for nucleic acid library preparation that ensures accurate and reproducible results.

The quality of the MPS library is determined by the following procedures including, but not limited to:

a) fragmentation;

b) addition of universal sequences;

c) size selection;

d) amplification;

e) purification and clean up;

f) library quantification;

g) library qualification.

5.2 Fragmentation

5.2.1 General

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Some sequencing methods (e.g. short read sequencing) require the template DNA, cDNA or RNA to be fragmented as a first step prior to library preparation.

Fragmentation can be performed either mechanically or enzymatically to produce the DNA or RNA size range that is required for the particular method and sequencing platform. Chemical fragmentation is typically reserved for long RNA fragments.

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The selection of a fragmentation method should take into account the impact of the specific approach on evenness of coverage in the final libraries, e.g. to avoid the introduction of a GC bias.

The amount of starting material available and potential sample loss resulting from each approach should also be considered.

5.2.2 Mechanical fragmentation

Mechanical shearing can be performed using focused acoustic shearing devices. The resulting fragment sizes (150 bp[2] to 5 000 bp) can be controlled by varying the intensity and duration of ultrasonic acoustic waves.

Hydrodynamic shearing can be used to produce larger fragments (typically 1 kbp to 75 kbp), but requires large DNA input amounts (>1 µg) and the throughput is low.

Nebulization is another alternative, which uses compressed air to force DNA or RNA through a small hole. Although fragment size can be controlled to an extent, large amounts of input DNA (microgram quantities) are required and the method is only suitable for small sample sizes.

5.2.3 Enzymatic fragmentation

Enzymatic fragmentation methods (e.g. using fragmentases, transposases or endonucleases) have the advantage of higher throughput compared to mechanical methods and typically result in a lower

2) bp = base pairs.

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sample loss. The disadvantage of enzymatic approaches is that they can typically result in sequence bias since many enzymes have specific recognition sequences or sequence preferences.

5.2.4 Chemical fragmentation

Chemical fragmentation is typically reserved for breaking up long RNA fragments. Chemical fragmentation is performed by heating RNA with a divalent metal cation (magnesium or zinc). The length of the resulting products ranges from 115 base nucleotides to 350 base nucleotides and can be adjusted by increasing or decreasing the time of incubation.

5.2.5 Fragmented nucleic acid sample quantity

Certain library preparation protocols require the fragmented DNA or RNA to be quantified (as some material can be lost during the process). This can be performed using any of the methods described in 4.2, but most typically this is done with spectrophotometry or intercalating fluorescent dyes.

5.2.6 Fragmented nucleic acid sample purity

If there is a risk that impurities from the fragmentation process (e.g. components of enzymatic fragmentation reactions) can be carried over into the purified products, the sample purity can be assessed using the methods described in 4.3.

5.2.7 Fragmented nucleic acid size distribution

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The fragmented nucleic acid should be checked to determine whether the appropriate size range has been achieved. This can be done using the methods described for monitoring sample integrity in 4.4.

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5.2.8 Fragmented nucleic acid purification using gel electrophoresis

Purification of fragmented nucleic acids can be done by separating nucleic acids with a specific size from those with other sizes before downstream library preparation and sequencing steps. The purification can be achieved by using capillary electrophoresis, bead-based methods or other electrophoresis methods. The purified nucleic acids can be quantified as described in 4.2.

5.3 Addition of universal sequences

5.3.1 Repair

Because damage can rise from fragmentation, the nucleic acid sample shall be repaired after this process to improve efficiency in subsequent preparation steps.

NOTE The following conditions can warrant a repair procedure: abasic sites, nicks, thymine dimers, blocked 3′-ends, oxidized guanines or pyrimidines, deaminated cytosine.

The end of the fragment shall be polished (i.e. the addition of 5′-PO_4 or 3′-OH) to make it suitable for ligation.

5.3.2 Ligation of adapter

Evaluation of the adapter sequence can include, but is not limited to:

a) length;

b) the design of the adapter;

c) the ratio of adapter to DNA.

The ratio of adapter is critical and requires optimization.

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