Technical Specification

ISO/TS 7552-2

First edition
2024-11

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for circulating tumour cells (CTCs) in venous whole blood —

Part 2: Isolated DNA

Analyses de diagnostic moléculaire in vitro — Spécifications relatives aux processus préanalytiques pour les cellules tumorales circulantes (CTC) dans le sang total veineux —

Partie 2: ADN isolé

Reference number
ISO/TS 7552-2:2024(en)

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Published in Switzerland

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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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This document was prepared by Technical Committee ISO/TC 212, *Medical laboratories and in vitro diagnostic systems*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 140, *In vitro diagnostic medical devices*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

A list of all parts in the ISO 7552 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

Solid tumours release cells and bioanalytes into blood and other body fluids. This has opened the option of utilizing such body fluids (liquid biopsies) for a minimally-invasive procedure for tumour detection, diagnosis and characterization. Liquid biopsies can enable earlier detection and diagnosis of cancers and advance personalized patient treatment.[20,22]

These applications have become one of the fastest growing segments of the entire diagnostic market.

Circulating tumour cells (CTCs) in venous whole blood can reflect the disease complexity that evolves during tumour progression, with distinct genetic, epigenetic and gene expression biomarkers.[22]

Beside the prognostic role of CTC identification and enumeration in cancer progression, CTC molecular characterization can improve disease outcome prediction, therapeutic guidance and post-treatment monitoring of the patient.[20]

CTCs are now considered as a surrogate of tumour tissue in cancer early development, progression, and metastatic phase.[23]

Molecular characterization of CTCs can provide a strategy for monitoring cancer genotypes during systemic therapies,[24] identifying mechanisms of disease progression, identifying novel targets for biological treatment[25] and selecting targeted therapies.[20] Moreover, CTC single-cell sequencing is an important tool for tumour genomic heterogeneity analysis.[26-28] Molecular examination techniques such as qPCR, dPCR and sequencing methods including next generation sequencing (NGS) enable characterization of the CTC specific DNA features.

CTCs are fragile and tend to degrade within a few hours when collected in conventional blood collection tubes, e.g. EDTA containing tubes, without dedicated CTC stabilizers. CTCs are extremely rare, especially in early disease, e.g. less than 10 cells per 10 ml of blood, representing a ratio of approximately $1:10^7$ CTCs to white blood cells (WBCs). This low ratio represents a significant challenge to CTC enrichment required for examination. Furthermore, co-enrichment of normal blood cells causes a dilution of CTCs. The challenge is to minimize the amount of co-enriched WBCs for subsequent accurate and sensitive detection of CTC specific genetic and epigenetic alterations, especially when dealing with minor tumour cell clones.

Special measures to remove the WBCs are necessary in order to obtain good quality DNA samples characterized by high purity and thus representative of the mutational pattern within the tumour.

Standardization includes all steps of the pre-examination process, including blood collection and stabilization, transport, storage, CTC enrichment, CTC isolation (if included), and DNA isolation. This pre-examination standardization is crucial to ensure reliable examination results in current clinical use and is also critical to develop new CTC based diagnostic examinations and to establish these in clinical healthcare.[29]

An illustration of critical steps of the CTC pre-analytical workflow is provided in Annex A.

This document describes special measures to obtain appropriate quality and quantity of DNA from CTC-containing blood specimens for subsequent examination.

Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for circulating tumour cells (CTCs) in venous whole blood —

Part 2: Isolated DNA

1 Scope

This document specifies requirements and gives recommendations on the handling, storage, CTC enrichment and isolation, DNA isolation and storage, and documentation of venous whole blood specimens intended for the examination of DNA isolated from circulating tumour cells (CTCs) during the pre-examination phase before a molecular examination is performed.

This document is applicable to molecular in vitro diagnostic examinations including laboratory developed tests performed by medical laboratories. It is also intended to be used by laboratory customers, in vitro diagnostics developers and manufacturers, biobanks, institutions, and commercial organizations performing biomedical research, and regulatory authorities.

This document does not cover the isolation of genomic DNA directly from venous whole blood containing CTCs. This is covered in ISO 20186-2.

This document does not cover the isolation of specific white blood cells and subsequent isolation of genomic DNA therefrom or the pre-analytical workflow requirements for viable CTC cryopreservation and culturing.

NOTE 1 The requirements given in this document can also be applied to other circulating rare cells (e.g. foetal cells).

NOTE 2 International, national, or regional regulations or requirements can also apply to specific topics covered in this document.

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 15189, *Medical laboratories — Requirements for quality and competence*

ISO 15190, *Medical laboratories — Requirements for safety*

3 Terms and definitions

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

ISO and IEC maintain terminology 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
aliquot
portion of a larger amount of homogenous material, assumed to be taken with negligible sampling error

Note 1 to entry: The term is usually applied to fluids. Tissues are heterogeneous and therefore cannot be aliquoted.

[SOURCE: ISO 20166-3:2018, 3.1]

3.2
analyte
component represented in the name of a measurable quantity

[SOURCE: ISO 17511:2020, 3.1, modified — The example has been removed.]

3.3
backflow
flow of a liquid opposite to the usual or desired direction

3.4
blood collection set
intravenous device specialized for venipuncture consisting of a stainless steel beveled needle and tube (tubing) with attached plastic wings and fitting connector

Note 1 to entry: The connector attaches to an additional blood collection device, e.g. a *blood collection tube* (3.5).

3.5
blood collection tube
tube used for blood collection, usually in a vacuum which forces blood from the vein through the needle and into the tube

3.6
circulating tumour cells
CTCs
cells present in blood, originating from a primary or metastatic site(s) of a tumour

3.7
closed system
non-modifiable system provided by the vendor including all necessary components for the analysis (i.e. hardware, software, procedures and reagents)

[SOURCE: ISO 20186-2:2019, 3.6]

3.8
CTC enrichment
method that is able to increase the ratio of *CTCs* (3.6) to other cells including white blood cells in a *sample* (3.21)

3.9
CTC isolation
method resulting in a *sample* (3.21) containing *CTCs* (3.6) without any other cell type

3.10
deoxyribonucleic acid
DNA
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: ISO 22174:2024, 3.1.6]

3.11
deoxyribonucleic acid proficiency testing program
DNA PT program
proficiency testing (3.19) for DNA based *examinations* (3.13)

3.12
diagnosis
identification of a health or disease state from its signs and symptoms, where the diagnostic process can involve *examinations* (3.13) and tests for classification of an individual's condition into separate and distinct categories or subclasses that allow medical decisions about treatment and prognosis to be made

[SOURCE: ISO 20184-1:2018, 3.6]

3.13
examination
analytical test
set of operations having the objective of determining the numerical value, text value or characteristics of a property

Note 1 to entry: Processes that start with the isolated *analyte* (3.2) and include all kinds of parameter testing or chemical manipulation for quantitative or qualitative examination.

[SOURCE: ISO 15189:2022, 3.8, modified — The original Notes to entry have been removed, and a new Note 1 to entry has been added; "analytical test" has been added as a preferred term.]

3.14
examination performance
analytical test performance
analytical performance
ability of an *examination* (3.13) procedure to measure or detect a particular *analyte* (3.2)

Note 1 to entry: Analytical performance is determined from analytical performance studies used to assess the ability of an in vitro diagnostic *examination* (3.13) procedure to measure or detect a particular *analyte* (3.2).

Note 2 to entry: Analytical performance includes such characteristics as analytical sensitivity, detection limit, analytical specificity (interference and cross-reactivity), trueness, precision and linearity.

[SOURCE: ISO 20186-3:2019, 3.11]

3.15
manufacturer
entity that is legally responsible for manufacturing a specific *workflow* (3.26) component

Note 1 to entry: For the purpose of this document, manufacturers can be *examination* (3.13) manufacturers, collection device manufacturers, *CTC enrichment* (3.8) and isolation manufacturers, nucleic acid isolation manufacturers.

3.16
needle holder
barrel used in routine venipuncture procedures to hold the *blood collection tube* (3.5) in place and to protect the phlebotomist from direct contact with blood

[SOURCE: ISO 20186-1:2019, 3.16]

3.17
pre-examination process
pre-analytical phase
pre-analytical workflow
process that starts, in chronological order, from the clinician's request and includes the *examination* (3.13) request, preparation and identification of the patient, collection of the *primary sample(s)* (3.18), transportation to and within the laboratory, cell enrichment, and isolation of *analytes* (3.2), ending when the analytical examination begins

Note 1 to entry: The pre-examination phase includes preparative processes that influence the outcome of the intended examination.

[SOURCE: ISO 15189:2022, 3.24, modified — "pre-analytical phase" and "pre-analytical workflow" have been added as preferred terms; in the definition, "user's request" has been changed to "clinician's request"; "storage, cell enrichment, isolation of analytes" has been added to the definition; Note 1 to entry has been added.]

3.18
primary sample
specimen
discrete portion of a body fluid or tissue or other *sample* (3.21) associated with the human body taken for *examination* (3.13), study or analysis of one or more quantities or characteristics to determine the character of the whole

[SOURCE: ISO 15189:2022, 3.25, modified — Note 1 to entry has been removed.]

3.19
proficiency testing
PT
evaluation of participant performance against pre-established criteria by means of interlaboratory comparisons

[SOURCE: ISO/IEC 17043:2023, 3.7, modified — Note 1 to entry has been removed.]

3.20
room temperature
temperature in the range of 18 °C to 25 °C

Note 1 to entry: Local or national regulations can have different definitions.

3.21
sample
one or more parts taken from a *primary sample* (3.18)

[SOURCE: ISO 15189:2022, 3.28]

3.22
stability
ability of a *sample* (3.21) material, when stored under specified conditions, to maintain a stated property value within specified limits for a specified period of time

[SOURCE: ISO Guide 30:2015, 2.1.15, modified — The words “reference material” were replaced by “sample material”; “specified” replaced by “stated” before “property value”. Note 1 to entry has been removed.]

3.23
storage
prolonged interruption of the pre-examination *workflow* (3.26) of a *sample* (3.21) or *analyte* (3.2) respectively, or of their derivatives e.g. stained sections or tissue blocks, under appropriate conditions in order to preserve their properties

Note 1 to entry: Long-term storage typically occurs in laboratory archives or in biobanks.

[SOURCE: ISO 20166-3:2018, 3.21]

3.24
validation
confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled

Note 1 to entry: The term “validated” is used to designate the corresponding status.

[SOURCE: ISO 9000:2015, 3.8.13, modified — The original Notes 1 to 3 to entry have been removed.]

3.25
verification
confirmation, through the provision of objective evidence, that specified requirements have been fulfilled

Note 1 to entry: The term “verified” is used to designate the corresponding status.

Note 2 to entry: Confirmation can comprise activities such as:

— performing alternative calculations;

— comparing a new design specification with a similar proven design specification;

— undertaking tests and demonstrations;

— reviewing documents prior to issue.

[SOURCE: ISO 9000:2015, 3.8.12, modified — The original Notes 1 and 2 to entry have been removed and Note 2 to entry has been added.]

3.26
workflow
series of activities necessary to complete a task

[SOURCE: ISO 20166-3:2018, 3.25]

4 General considerations

Refer to ISO 15189, ISO/IEC 17020 or ISO/IEC 17025 for general statements on medical laboratory quality management systems. In vitro diagnostics (IVD) manufacturers should follow ISO 13485. General quality management system requirements can be found in ISO 9001. For other general requirements on pre-examination processes, including pre-collection activities, collection, transport, receipt, and handling of specimen, see ISO 20658 and ISO 15189:2022,7.2.

All steps of a diagnostic workflow can influence the final analytical test result. Thus, the entire workflow including biomolecule stability and both specimen and sample storage conditions shall be specified, verified, and validated during the development of the examination including the development of in vitro diagnostic medical devices. A risk assessment of relevant workflow steps including their potential impact on the analytical test performance shall be performed and mitigation measures shall be established to enable the required analytical test performance. Guidance is provided in ISO 14971 and ISO 35001.

CTC analysis usually involves a CTC enrichment step (e.g. by size, immunomagnetic-, or microfluidic-based approaches) prior to DNA isolation (Annex A). Depending on the requirements of the examination, enriched CTCs can undergo additional steps after CTC enrichment such as further characterization and selection prior to DNA isolation (see 6.4, 6.6 and Annex A). Due to the nature of the specimen/sample and the complexity of the procedure potentially affecting the yield, purity and integrity of DNA, appropriate measures shall be taken during the pre-examination workflow to obtain suitable quantity and quality of DNA derived from CTCs for the examination.

The degree of contamination of CTCs with WBCs or other cells is critical. The presence of WBCs in a CTC enriched sample is unavoidable and can strongly affect the performance of the examination e.g. the sensitivity of detection of a somatic mutation. To overcome this problem, an isolation step can be necessary to obtain a pure CTC sample for DNA isolation.

During the whole pre-examination process, precautions shall be taken to avoid cross contamination between either different specimens or samples, e.g. by using single-use material whenever feasible or by using appropriate cleaning procedures between processing of either different specimens or samples.

Safety instructions for the whole pre-examination process shall be in place and followed. They shall be in accordance with requirements specified in ISO 15189 and ISO 15190.

The manufacturer's material safety data sheet shall be considered before first use of any potentially hazardous material (e.g. chemicals in stabilizers).

For all pre-examination steps, the examination manufacturer's instructions shall be followed.

Where, for justified reasons (e.g. unmet patient needs), a commercial product is not used in accordance with the manufacturer's instructions, responsibility for its verification, validation, use and performance lies with the laboratory.

5 Activities outside the laboratory

5.1 Specimen collection

5.1.1 General

For the collection of the blood specimen, the requirements for the intended molecular examination (e.g. type of blood collection tube, collection procedure) laid out in Clause 6 shall be followed.

5.1.2 Information about the patient/specimen donor

The documentation shall include the ID of the specimen donor/patient, which can be in the form of a code.

The documentation should include, but is not limited to:

a) the relevant health status of the specimen donor/patient (e.g. healthy, disease type, concomitant disease, demographics such as age, sex and gender);

b) the information about medical treatment and special treatment prior to blood collection;

c) the type and purpose of the examination requested;

d) the appropriate consent from the specimen donor/patient (see also ISO 15189);

e) time point of the blood draw where relevant (e.g. patients rest or active times).

NOTE A recent study demonstrated a higher CTC concentration in blood during the rest phase of breast cancer patients.[33]

5.1.3 Selection of the venous whole blood collection tube by the laboratory

Due to the low number of CTCs, a high recovery efficiency is required during enrichment. This can be hampered by the potential instability of CTCs during transport and storage, leading to a reduction of the CTC number in the specimen or reduced compatibility with the enrichment system.[34]

Therefore, venous whole blood should be collected in appropriate collection tubes with stabilizers maintaining the integrity of the CTCs for enabling sensitive DNA examination.

The examination manufacturer instructions should contain specifications on the blood collection tube(s) to be used. Where the examination manufacturer specifies usage of dedicated blood collection tube(s), these shall be used.

Where the examination manufacturer does not provide such specifications, but either the CTC enrichment or the isolation manufacturer specifies a dedicated blood collection tube, this can serve as a basis for the laboratory's tube verification for the examination. Where such specified blood collection tube does not meet the examination requirements or none of the manufacturers specifies a blood collection tube, the blood collection tube shall be specified, verified and documented by the laboratory. Due to the post-collection variability of the CTC (numbers and morphology) venous whole blood should be collected in commercially available venous whole blood collection tubes containing CTC stabilizers enabling a sufficient DNA yield (CTC/DNA stabilizer) for the intended examination.

The blood collection tube catalogue and lot number should be documented.

Blood collection tubes not containing any CTC/DNA stabilizers should be used only if specified by the examination manufacturer's instructions. In these cases, conventional blood collection tubes, e.g. EDTA containing tubes, should be used, although EDTA does not prevent changes of CTCs but it prevents blood clotting, which can minimize the potential impact of blood clots on the CTCs. Blood micro clots can impact some CTC enrichment procedures thus changing the CTC subpopulation, or even strongly hamper the entire

CTC enrichment procedure. The examination manufacturer's specifications shall be considered for further details.

NOTE 1 Studies have shown that CTC detection is possible in EDTA-collected venous whole blood within 4 h after blood draw from patients with different tumour types.[35–40]

NOTE 2 There are also alternatives to conventional blood collection-based CTC enrichments. These systems allow for in vivo and ex vivo CTC sampling from larger blood volumes.[41,42]

5.1.4 Venous whole blood specimen collection from the patient/donor

The identity of the person collecting the specimen shall be documented. This can be documented in form of the name or a code. The date and time of blood collection shall be documented.

For the labelling (both specimen and sample identification) of the blood collection tube, a routine procedure (e.g. ISO 15189 for medical laboratories or ISO 20387 for biobanks) or a similar procedure with optional additional information (e.g. 2D-barcode) shall be used.

Standard venipuncture techniques can be used, if not specified differently by the blood collection tube manufacturer. For avoiding significant changes of the CTC population during the blood collection (e.g. lysis of CTCs by shearing), a suitable needle of sufficiently large gauge diameter shall be specified and verified during the development of a blood collection tube intended for collecting CTCs contained in blood.

Steps for preventing possible backflow into the donor's/patient's body can be required.

The blood collection tube manufacturer shall provide specified and verified instructions on the blood collection procedure. These shall be followed. A blood collection set and needle holder can be required when using CTC/DNA profile stabilizer containing tubes. In this case, the instructions of the collection set and needle holder manufacturer shall be followed, as long as not specified and verified differently (e.g. by the blood collection tube manufacturer).

Blood collection tubes shall be filled in accordance with the manufacturer's instructions and attention should be drawn to the correct positioning of the collection tube during the blood draw as well as the required blood volume. Blood collection tube developers and manufacturers shall specify and verify the limits of tube underfilling. The examination manufacturers shall verify the specification for the dedicated examination.

NOTE 1 The integrity of CTCs can be influenced by inadequate venous whole blood collection procedures.

NOTE 2 Underfilling of blood collection tubes containing CTC/DNA stabilizers can compromise the function of the stabilizers due to an unfavourable blood to stabilizer ratio. This can in itself compromise the CTCs, which can impact the validity and reliability of the examination results.

The blood collection tube manufacturer's instructions for mixing or inverting the tube immediately after blood collection shall be followed. If no information about mixing or inverting is given by the manufacturer's instructions, each tube should be gently inverted 8 to 10 times.

Either incorrect or insufficient mixing can be one of the most frequent pre-examination variables. Unless additives in the blood collection tubes are homogenously mixed with the specimen, the CTCs and DNA can be compromised, which can impact the validity and reliability of the examination results. Correct mixing shall therefore be a focus during education and periodic training of all personnel involved in blood collection.

The blood collection procedure shall be documented, as requested by the medical laboratory. Both any tampering with and any addition to the specimen shall be documented.

5.2 Specimen storage and transport

5.2.1 General

When selecting and using transport packages (e.g. box for storing and transportation), transport regulations can apply. A specified and verified procedure for specimen storage and transport and written instructions

shall be in place. The specified storage and transport conditions (e.g. temperature and duration) shall be followed and documented including any deviations from them.

Temperature monitoring should be applied in a suitable manner in case the specified storage and transport conditions cannot be ensured e.g. by the specified transport packages. The duration of temporary storage in the blood collection facility and the duration of transport to the laboratory contribute to the total duration of storage and transport. Special care should be taken to avoid CTC lysis as this will change the CTC population. Therefore, the specimen shall not be frozen or shaken vigorously.

5.2.2 Storage and transport using blood collection tubes with stabilizers

The examination manufacturer shall provide specified and verified instructions for the storage and transport of the collected blood specimen (e.g. duration, temperature) and these shall be followed.

Where the examination manufacturer does not provide such specifications (e.g. due to less stringent legal frameworks), the procedure shall be specified, verified and documented by the laboratory.

Instructions shall be written accordingly for the user and followed. The blood collection tube manufacturer specifications on storage and transport conditions can serve as a basis/framework for the laboratory's own specific verification for the intended examination.

5.2.3 Storage and transport using blood collection tubes without stabilizers

Where the examination manufacturer specifies usage of blood collection tubes without stabilizers, they shall provide specified and verified instructions for the storage and transport of the collected blood specimen (e.g. duration, temperature) and these shall be followed.

Where the examination manufacturer does not provide such specifications (e.g. due to less stringent legal frameworks), the procedure shall be specified, verified and documented by the laboratory. This should be done by time course studies analysing the stability of the targeted examination analyte after blood draw.

NOTE A time course study involves repeated observations of the same variables at specific intervals over a relevant time-period (e.g. time 0, 2 h, 6 h, 12 h, 24 h, 36 h, 48 h). This reflects any knowledge on the stability of the analyte(s) of interest. Typically, this involves multiple aliquots from the same donor taken from the same blood draw repeated for several donors.

Depending on the results of the time course studies, it can be necessary to process specimens without delay or after only a short storage duration to minimize the DNA changes and to maximize the CTC recovery. Instructions shall be written accordingly for the user and followed.

The maximum duration and temperature of storage shall be specified and verified for the intended examination.

6 Activities inside the laboratory

6.1 Specimen reception

The identity of the person receiving the specimen or sample shall be documented. This can be documented in form of the name or a code. The correct identity of the specimen or sample shall be checked. This should include the clinical information (see 5.1.1 and 5.1.2), hospital admission number, name of the patient or donor, and date of birth of the patient or donor. In certain instances, e.g. in research studies, it can be necessary to only work with a code. The arrival date, time and nonconformities of labelling, storage and transport conditions (e.g. temperature, duration) and blood volume differences to specifications, leaking/broken tubes, etc. shall be documented. A procedure for handling nonconformities shall be in place.

Where there are nonconformities, e.g. usage of non-specified blood collection tubes, in transport conditions, overall storage, and transport duration or blood volume or accidental freezing that can affect the validity and reliability of the examination result, a new specimen should be obtained.

6.2 Specimen storage after transport and reception

Where further storage in the laboratory is needed, the storage temperature and the date and time when starting either specimen or sample storage shall be documented.

Storage temperature and total storage duration shall not exceed specifications identified in 5.2.

The specimen total storage duration includes the duration of storage at the blood collection facility (5.1.4), of transport to the laboratory (5.2.2) and of further storage at the laboratory or other institutions.

The maximum storage duration specified by the examination manufacturer or, if this is not provided, by the laboratory (see 5.2.2) shall not be exceeded.

6.3 Enrichment of CTCs

6.3.1 General

CTC examinations usually require an enrichment of CTCs from other cell types, typically white blood cells. CTC enrichment is achieved based on the physical (e.g. cell size, cell deformability) or the biological properties (e.g. presence of specific epitopes) of these cells.[24] More details on CTC enrichment procedures can be found in Annex A. Different methods for CTC enrichment can result in different yields (number of recovered CTCs) and different CTC to white blood cell ratio. Moreover, different enrichment methods can select different CTC subpopulations (e.g. epithelial, mesenchymal[23]). These aspects should be considered during the design, verification, and validation of an examination (e.g. by specifying the minimum number of required CTCs, the maximum percentage of contaminating white blood cells that is acceptable). This can be done by analysing the DNA of spiked-in cancer cells from established cell lines prior and after the enrichment procedure. Where unacceptable CTC yield occurs, action should be taken to minimize changes e.g. by adding a CTC stabilizer before starting the enrichment.

The CTC/DNA stabilizer in a blood collection tube can also be effective during the CTC enrichment depending on the chemical characteristics of the stabilizer. If the CTC/DNA stabilizer used in the blood collection tube is not effective anymore during CTC enrichment, an additional stabilizer should be added for the CTC enrichment procedure.

To minimize cross contamination with amplified nucleic acids, the enrichment of CTCs should not be performed in the same area as the nucleic acid amplification steps of the examination process, unless closed systems are used, which are verified to avoid cross-contamination for the intended application.

6.3.2 Using a commercial CTC enrichment system intended for diagnostic use

The examination manufacturer shall provide specified and verified instructions on CTC enrichment and these shall be followed.

Where the examination manufacturer does not provide such specifications (e.g. due to less stringent legal frameworks), but the blood collection tube manufacturer and/or the CTC isolation manufacturer and/or the DNA isolation kit manufacturer has/have specified and verified one or several dedicated commercially available CTC enrichment system(s), these can serve as a basis/framework for the laboratory's examination specific verification.

NOTE Commercial CTC enrichment systems can sometimes be integrated into parts of commercially available pre-examination workflows together with CTC DNA, CTC RNA, and CTC protein isolation kits, while other commercial solutions are stand-alone procedures that end with CTC enrichment.

Where none of these manufacturers has specified and verified a specific CTC enrichment system, the laboratory shall select, specify, verify, and document an appropriate CTC enrichment system approved for diagnostic use, where available. Instructions for use shall be written accordingly and followed.

Where the selected CTC enrichment procedure does not sufficiently support the specified examination performance characteristics, the laboratory should modify it accordingly (e.g. by increasing the volume of either the blood specimen or sample, by modifying the pressure applied for filtration, by adjusting the quantity of the capture antibody or using an additional antibody for CTC enrichment).

6.3.3 Using the laboratory developed CTC enrichment procedure

Where no commercially available CTC enrichment procedure intended for diagnostic use can be successfully verified with the intended examination (see 6.3.2), the laboratory shall develop its own procedure by either:

— modifying an existing CTC enrichment procedure for diagnostic use;

— using a commercially available system for research use only that can be modified as required; or

— developing its own CTC enrichment system.

The procedure chosen from the list above shall be specified, verified, and eventually validated based on the outcome of the risk assessment (see Clause 4) for the intended use of the examination. Instructions for use shall be written accordingly and followed.

6.4 Quality of enriched CTCs

Where the intended examination requires a CTC quality assessment, the examination manufacturer shall provide specified and verified instructions and these shall be followed.

If these are not provided (e.g. due to less stringent legal frameworks), but the CTC isolation manufacturer and/or the CTC DNA isolation kit manufacturer provides such instructions, these can serve as a basis/framework for the laboratory's examination specific verification. Where this verification is not successful, the laboratory should modify the CTC enrichment quality assessment procedure or develop its own.

Where the laboratory develops its own CTC enrichment quality assessment procedure, generally accepted image analysis of the enriched CTCs, e.g. by haematoxylin and eosin staining to determine typical tumour cell specific morphological features such as cell dimension and nucleus to cytoplasm ratio (see ISO/TS 7552-3 and Reference [43]) or more specific surface protein staining by fluorescently labelled antibodies can be used.

Staining can impact the CTC DNA profile examination by causing DNA degradation or by interfering with the examination. This risk can be mitigated by applying DNA-stabilizers and/or using DNAse free staining reagents. Compatibility of the CTC staining procedure with the DNA profile examination shall therefore be verified during the development of the examination.

6.5 Storage of enriched CTCs

The examination manufacturer shall provide specified and verified instructions for storage of enriched CTCs and these shall be followed.

Where there are no examination manufacturer's instructions provided (e.g. due to less stringent legal frameworks), but the CTC enrichment kit manufacturer and/or the blood collection tube manufacturer and/or the isolation kit manufacturer and/ or the CTC DNA isolation kit manufacturer has/have specified storage conditions, these can serve as a basis/framework for the laboratory's examination specific verification.

Where these instructions cannot be successfully verified with the examination, or where no such instructions are provided, the storage conditions shall be specified, verified, and documented by the laboratory. Instructions for use shall be written accordingly and followed.

The maximum storage duration, at defined temperature (e.g. room temperature or 2 °C to 8 °C), of the enriched CTCs should be determined for ensuring that there are no negative impacts on the examination performance characteristics. This should be done by running a time-course experiment analysing potential changes in the number of enriched CTCs over time e.g. by analysing this after 30 min, 60 min, 90 min and 120 min of storage.

6.6 Isolation of CTCs

6.6.1 General

CTC isolation implies the separation of CTCs from all other blood components, usually after CTC enrichment. CTC isolation is mainly based on physical principles, e.g. achieved by dielectrophoresis or micromanipulation.[44]

The choice of the CTC isolation system is dependent on the compatibility with the used CTC enrichment system and with the examination requirements.

Changes in the number of recovered CTCs can occur during CTC isolation. Therefore, suitable control procedure to verify the CTC isolation efficiency should be applied e.g. by visualizing the presence of the isolated CTCs.

6.6.2 Using a commercial CTC isolation system intended for diagnostic use

The examination manufacturer shall provide specified and verified instructions on CTC isolation and these shall be followed.

Where there are no examination manufacturer's instructions provided (e.g. due to less stringent legal frameworks), but the blood collection tube manufacturer and/or the CTC enrichment manufacturer and/or the DNA isolation kit manufacturer has/have verified one or several dedicated commercially available CTC isolation system(s), these can serve as a basis/framework for the laboratory's examination specific verification.

NOTE Commercial CTC isolation systems can sometimes be integrated into parts of commercially available pre-analytical workflows together with CTC DNA, RNA, and protein isolation kits, while other commercial solutions are standalone procedures that end with isolated CTC.

Where none of these manufacturers has specified and/or verified a specific CTC isolation system, the laboratory shall select, specify, verify, and document an appropriate CTC isolation system approved for diagnostic use, where available. Instructions for use shall be written accordingly and followed.

Where the selected CTC isolation procedure does not sufficiently support the specified examination performance characteristics, the laboratory should modify it accordingly (e.g. by imaging of the isolated cell(s) as a quality control of the presence of the CTCs, by reducing the reagent dead volume of the isolation devices).

6.6.3 Using the laboratory-developed CTC isolation procedure

Where no commercially available CTC isolation procedure intended for diagnostic use can be successfully verified with the intended examination, the laboratory shall develop its own procedure by either:

— modifying an existing CTC isolation procedure for diagnostic use;

— using a commercially available system for research use only that can be modified as required; or

— developing its own CTC isolation system.

The procedure chosen from the list above shall be verified and eventually validated based on the outcome of the risk assessment (see Clause 4) with the intended examination. Instructions for use shall be written accordingly and followed.

6.7 Isolation of DNA from an enriched CTC sample

6.7.1 General

Isolated single CTCs and pooled isolated CTCs are usually not processed for DNA isolation due to the very low quantity of DNA. Instead, the isolated CTCs are introduced to the examination directly or only a lysis step is

performed prior to the examination, depending on the examination design. In this case, the pre-examination process ends in 6.6.

DNA isolation can be performed for isolated CTC pools containing high cell numbers.

To avoid a cross contamination with amplified material, the isolation of DNA should not be performed in the same area as the amplification steps of the examination process, unless a closed system is used, which is designed to avoid cross-contamination. During the development of the examination it shall be verified that cross-contamination does not impact the examination. Appropriate no template controls should be used to monitor the cross-contamination risk.

6.7.2 Using a commercial DNA isolation kit intended for diagnostic use

The examination manufacturer shall provide specified and verified instructions on CTC DNA isolation and these shall be followed.

Where there are no examination manufacturer's instructions provided (e.g. due to less stringent legal frameworks), but the CTC enrichment kit manufacturer and/or the CTC isolation kit manufacturer and/or the blood collection tube manufacturer has/have specified kits for DNA isolation, these can serve as a basis/framework for the laboratory's examination specific verification.

Where none of these manufacturers has specified and/or verified dedicated kits for CTC DNA isolation, the laboratory shall select, specify, verify, and document an appropriate kit for CTC DNA isolation approved for diagnostic use, where available. Instructions for use shall be written accordingly and followed.

Where the selected DNA isolation procedure does not sufficiently support the specified examination performance characteristics, the laboratory should modify it accordingly (see 6.8.3).

For information on storage of isolated DNA, see 6.9.2.

6.7.3 Using a laboratory-developed CTC DNA isolation procedure

Where no commercially available CTC DNA isolation kit intended for diagnostic use can be successfully verified with the intended examination, the laboratory shall develop its own procedure by either:

— modifying an existing CTC DNA isolation kit for diagnostic use;

— using a commercially available kit for research use only; or

— developing its own procedure.

The procedure chosen from the list above shall be specified, verified, and eventually validated based on the outcome of the risk assessment (see Clause 4) for the intended use of the examination.

Instructions for use shall be written accordingly and followed.

An RNA removal step, such as RNase treatment should be incorporated into the DNA isolation procedure if the examination is sensitive to RNA contamination in the CTC DNA eluate. The RNase, other reagents, and consumables coming in contact with the CTC DNA should be DNase-free.

NOTE Where using modified DNA isolation kits or a laboratory's own developed procedure, dedicated measures and technologies can be needed in order to avoid carrying over CTC/DNA stabilization molecules to the final DNA eluate in case of using tubes with blood CTC/DNA profile stabilizer. Stabilization molecules carry-over can impact the performance of the examination.

The isolated CTC DNA should be kept at 2 °C to 8 °C (e.g. cooling block) or on wet ice and should be examined without delay, unless specified and verified differently.

For more information on storage of isolated CTC DNA, see 6.9.3.

6.8 Quantity and quality assessment of isolated DNA from enriched or isolated CTCs

6.8.1 General

During the design and development of the pre-examination and/or examination workflows, appropriate methods for assessing the quantity and quality of DNA isolated from CTCs shall be specified, developed and verified, where required for ensuring examination performance. Due to the low DNA yield usually obtained from CTC samples, contrived samples can support the assessment where appropriate. Where appropriate, generally accepted methods/technologies can be used (see 6.8.2 and 6.8.3). The CTC DNA isolation kit manufacturer's instructions on determining the CTC DNA quantity and quality can also serve as a basis/frame, where available.

NOTE Some stabilizers such as cross-linking reagents can have negative impacts on reliability of DNA quantity and quality measurements, especially if these are amplification based.

6.8.2 Quantity assessment of CTC DNA

Nucleic acids isolated from CTCs are usually at low concentrations, which can make the use of UV absorbance readers such as spectrophotometers unreliable and can therefore be inappropriate. DNA isolation procedures from enriched or isolated CTCs can involve use of carrier nucleic acids [e.g. carrier RNA of a neutral sequence such as Poly(A) or Poly(C)]; this carrier will additionally interfere with the UV absorbance reading.

Therefore, other methods appropriate for the quantification or semi-quantification of DNA deriving from CTCs targeting known DNA sequences can be required, such as quantitative polymerase chain reaction (qPCR), digital PCR (dPCR) or fluorometric or chip-based methods.

The specified and implemented methods shall be verified for the intended examination.

6.8.3 Quality assessment of CTC DNA

Due to the low concentration of the DNA, there is no generic method for quality assessment. Depending on the examination requirements, it can be necessary to develop, specify and verify a dedicated quality test.

During the development and verification of the examination, it shall be ensured that the isolated CTC DNA does not contain substances interfering with the specified examination performance. A test for presence of interfering substances in the isolated CTC DNA should be specified, developed, and verified. This can be done e.g. by amplifying an endogenous DNA and/or inspecting qPCR response curves for anomalies.[45]

Developers/manufactures and medical laboratories should regularly test the CTC enrichment/isolation and DNA isolation performance in a CTC DNA proficiency test (PT) program, where available. Additional information on PT can be found in ISO 15189.

6.9 Storage of isolated DNA from enriched CTCs

6.9.1 General

The CTC DNA examination manufacturer shall provide specified and verified instructions for the storage of isolated CTC DNA, as long as the isolated DNA does not need to be processed for examination without delay. These instructions shall be followed.

Where such instructions are not provided (e.g. due to less stringent legal frameworks), the DNA isolation kit manufacturer instructions can serve as a basis/frame for the laboratory to specify and verify DNA storage instructions for the intended examination. Instructions shall be written accordingly and followed.

Where none of these instructions are provided, the DNA examination laboratory shall specify, verify and document conditions for the storage of isolated DNA e.g. temperature, duration and other conditions required. Instructions shall be written accordingly and followed.

For long-term storage, the laboratory shall have verified protocols in place on how to store the isolated DNA. Usually, the DNA is frozen. However, for DNA preservation, other validated methods for archiving can also be used (see References [46] and [47] for examples).

For long-term storage, aliquots of the isolated analyte should be generated to avoid repeated freezing and thawing or repeated recovery from other archiving systems. As amounts of isolated DNA are typically small, storage vessels with reduced DNA adsorption to the tube wall should be used.

Unintended freeze-drying of the isolated DNA during long-term storage due to water evaporation should be avoided as the DNA can degrade, which can be critical for some examinations. In addition, recovery from the storage vessel can be difficult or even impossible due to water evaporation. Therefore, appropriate storage vessels, such as screw-capped cryogenic vials, avoiding water evaporation during long-term storage, should be used and documented.

For long-term storage, a verified system and process should be in place to organize and uniquely mark the storage vessel containing the isolated DNA, making them easily retrievable and identifiable.

Traceability without loss or confusion of sample identity shall be ensured, e.g. by use of readable RFID, 1D-barcodes or 2D-barcodes on labels or pre-printed storage vessels with unique codes provided by manufacturers, suitable for low storage temperatures.

The freezer temperature shall be continuously monitored by adequate alarm systems. The freezer temperature should be monitored and recorded by verified instruments, such as a circular temperature chart or an electronic thermometer.

Samples should not be stored in a “frost-free” freezer, as the temperature can be cycled several times a day, which can cause DNA degradation.

6.9.2 Storage of DNA isolated with a commercially available kit intended for diagnostic use

The examination manufacturer shall provide specified and verified instructions on DNA storage and these shall be followed.

Where neither the examination manufacturer's nor the CTC DNA isolation kit manufacturer's instructions are provided, the laboratory shall specify and verify procedures on how to store the isolated CTC DNA until the examination.

As long as not specified and verified differently, for short-term storage, the extracted CTC DNA should be kept at 2 °C to 8 °C (e.g. cooling block) or on wet ice and should be examined the same day. For long-term storage, isolated CTC DNA should be stored at ≤ −20 °C.

Other verified methods and systems for archiving can also be used (see References [46] and [47] for examples).

6.9.3 Storage of DNA isolated with the laboratory developed procedure

Where a laboratory-developed procedure (see 6.8.3) is used, the laboratory shall have verified procedures in place on how to store the isolated CTC DNA until the examination.

Where the laboratory intends to store the isolated CTC DNA, the isolated CTC DNA shall be eluted in an appropriate buffer enabling short-term storage or long-term storage.

As long as not specified and verified differently, for short-term storage, the isolated CTC DNA should be kept at 2 °C to 8 °C (e.g. cooling block) or on wet ice and should be examined the same day.

For long-term storage, isolated CTC DNA should be stored at ≤ −20 °C. Other verified methods and systems for archiving can also be used (see References [46] and [47] for examples).