



Technical Specification

ISO/TS 7552-3

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

Part 3: Preparations for analytical CTC staining

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

Partie 3: Préparations pour l'analyse par coloration des CTC

**First edition
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Contents

	Page
Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 General considerations	5
5 Activities outside the laboratory	6
5.1 Specimen collection.....	6
5.1.1 General.....	6
5.1.2 Information about the specimen donor/patient.....	6
5.1.3 Selection of the venous whole blood collection tube by the laboratory.....	6
5.1.4 Venous whole blood specimen collection from the patient/donor.....	7
5.2 Specimen storage and transport.....	7
5.2.1 General.....	7
5.2.2 Storage and transport using blood collection tubes with stabilizers.....	8
5.2.3 Storage and transport using blood collection tubes without stabilizers.....	8
6 Activities inside the laboratory	8
6.1 Specimen reception.....	8
6.2 Specimen storage after transport and reception.....	9
6.3 Enrichment of CTCs.....	9
6.3.1 General.....	9
6.3.2 Using a commercial CTC enrichment system intended for diagnostic use.....	9
6.3.3 Using the laboratory developed CTC enrichment procedure.....	10
6.4 Quality of enriched CTCs.....	10
6.5 Storage of enriched CTCs.....	10
6.6 Preparation for CTC staining.....	10
6.6.1 General.....	10
6.6.2 Pretreatment for different staining techniques (antibody, colour staining, in situ techniques).....	11
Annex A (informative) Decision guideline for critical steps of the CTC pre-analytical workflow	12
Bibliography	14

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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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.^[19,20]

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 expression features.^[21]

Besides the prognostic role of CTC identification and enumeration in cancer progression, CTC identification and analysis can improve disease outcome prediction, therapeutic guidance and post-treatment monitoring of the patient.^[19]

CTCs are now considered as a surrogate of tumour tissue in cancer early development, progression and metastatic phase.^[22]

Molecular characterization of CTCs can provide a strategy for monitoring cancer during systemic therapies,^[23] identifying mechanisms of disease progression, identifying novel targets for treatment^[24] and selecting targeted therapies^[19].

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 1:10⁷ CTCs to white blood cells (WBCs). This low ratio represents a significant challenge to CTC enrichment required for identification and examination as tumour-derived cells.

Furthermore, CTC morphology and biomolecules can change during the pre-examination process. This can lead to changes in protein quantity, integrity, modification, conformation, and localization within the cell. This can impact the validity and reliability of the examination result.

CTC examination usually requires a CTC enrichment step (e.g. based on biological properties of the CTCs, such as expression of surface molecules, or physical properties, such as size and density, or their combination) prior to cytomorphological examination or immunofluorescent staining.

CTC enrichment technologies can provide CTCs attached on a solid surface, ready for cytological examination, or CTCs in suspension, requiring extra processing steps prior to the examination. This can lead to potential cell loss.^[25]

CTC enrichment is usually followed by their identification by conventional cytochemical or protein-targeted staining procedures that allow detection of the cell traits.

Standardization includes all steps of the pre-examination process, including blood collection and stabilization, transport, storage, CTC enrichment, and CTC isolation (if included). 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.^[26]

An illustration of critical steps of the pre-analytical workflow for CTC staining is provided in [Annex A](#).

This document describes measures to standardize the pre-examination process to obtain appropriate CTC staining.

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

Part 3: Preparations for analytical CTC staining

1 Scope

This document specifies requirements and gives recommendations on the handling, storage, CTC enrichment, preparation for CTC staining, and documentation of venous whole blood specimens intended for staining of CTCs during the pre-examination phase before an 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 pre-analytical workflow requirements for viable CTC cryopreservation and culturing.

Different dedicated measures are taken for stabilizing CTCs genomic DNA and RNA that are not described in this document; they are covered in ISO 7552-1 and ISO 7552-2.

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 types

**3.10
diagnosis**

identification of a health or disease state from its signs and symptoms, where the diagnostic process can involve *examinations* (3.11) 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]

3.11

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: An examination includes the processes that start with CTC staining 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.]

3.12

examination performance analytical test performance analytical performance

ability of an *examination* (3.11) 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 procedure to measure or detect a particular analyte.

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]

3.13

immunocytochemistry

in situ detection technique that uses the principle of antibodies binding specifically to antigens in or on cells to detect the antigens (e.g. proteins) using brightfield microscopy

3.14

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* (3.11) manufacturers, collection device manufacturers, *CTC enrichment* (3.8) and isolation manufacturers, nucleic acid isolation manufacturers.

3.15

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* (3.11) 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”; “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* (3.11), 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, such as 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 diagnostic (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 CTC staining. Due to the nature of the specimen/sample and the complexity of the procedure potentially affecting the morphology and integrity of CTCs, appropriate measures shall be taken during the pre-examination workflow to maintain the CTCs features required 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 affect the performance of the examination e.g. the CTC staining due to nonspecific binding of detection components to WBC count. To overcome this problem, an isolation step can be necessary to obtain a pure CTC sample.

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.

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.

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, if provided.

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 specimen donor/patient

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.^[30]

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

The CTC staining can be influenced by inadequate venous whole blood collection procedures and inappropriate storage/transport conditions, as well as by enrichment and isolation procedures.

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.^[13]

Therefore, venous whole blood should be collected in appropriate collection tubes with stabilizers maintaining the integrity of the CTCs (CTC stabilizer).

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 preserving CTC morphology and integrity.

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

Blood collection tubes not containing any CTC 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.^[32-36]

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.^[37,38]

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 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 stabilizers can compromise the function of the stabilizers due to an unfavourable blood to stabilizer ratio. This can in itself compromise the CTC 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 homogeneously mixed with the specimen, the CTCs 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 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 the 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](#)) 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](#)) 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.^[23] 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 cells ratio. Moreover, different enrichment methods can select different CTC subpopulations (e.g. epithelial, mesenchymal.^[22] 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 recovery 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 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 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 pretreatment system(s) for CTC staining 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.

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

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 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 Preparation for CTC staining

6.6.1 General

After CTC enrichment, CTCs are stained and further characterized by immunocytochemistry or other techniques. CTCs possess morphologic features consistent with the type of tumour they derive from.^[39] CTCs exhibit a high degree of inter- and intra-patient heterogeneity in cytological appearance consistent with the morphologic heterogeneity of cells commonly found in the primary and metastatic tumour.^[40-42]

Although CTCs are morphologically distinct from normal white blood cells (WBCs) and can be identified according to standard diagnostic cytopathology criteria (i.e. cell dimension, nuclear-to-cytoplasmic ratio)^[43], immunochemical staining of tumour specific proteins should be considered for CTC identification.

6.6.2 Pretreatment for different staining techniques (antibody, colour staining, in situ techniques)

6.6.2.1 General

Where pretreatment steps for staining techniques are needed, these shall be specified and verified (e.g. length of incubation, buffer/enzyme concentrations and temperature). This shall be done for each CTC stabilization and enrichment method specified for the intended examination (e.g. specific antigen/epitope and antibody, or the nucleic acid target and probe).

CTC stabilizers in blood collection tubes and/or applied during CTC enrichment or any other pre-examination step can contain chemicals which modify the CTC intracellular and surface markers (e.g. crosslinks by fixatives such as formaldehyde or similar compounds). Depending on the examination requirements, it can be necessary to revert these modifications or make them compatible with the examination by dedicated pretreatments for CTC staining. Specifically, for staining techniques based on antibodies or other affinity binders (e.g. immunocytochemistry, immunofluorescence staining), it shall be determined if an antigen retrieval (AR) [also called epitope retrieval (ER)] is required.

Where processing CTCs without having applied a stabilizer in any pre-examination step, specific pretreatments are generally not needed. However, some examinations can require post-enrichment fixation of CTCs as the applied examination staining antibody only recognizes modified surface markers.

For classical staining (i.e. haematoxylin and eosin), pretreatment steps are usually not necessary. In contrast examinations detecting intra-cellular targets usually require pretreatments for permeabilization of CTCs. Specifically, for nucleic acid hybridization-based techniques, it shall be determined if a pre-hybridization step including permeabilization of the cells is required.

6.6.2.2 Using a commercial pretreatment system for CTC staining procedure intended for diagnostic use

The examination manufacturer shall provide specified and verified instructions on pretreatment for CTC staining 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 enrichment manufacturer has/have specified and verified one or several dedicated commercially available pretreatment system(s) for CTC staining, these can serve as a basis/framework for the laboratory's examination specific verification.

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

Where the selected pretreatment for CTC staining procedure does not sufficiently support the specified examination performance characteristics, the laboratory should modify it accordingly (e.g. by modifying the pretreatment temperature or by changing the concentration of permeabilizing reagents).

6.6.2.3 Using the laboratory developed pretreatment for CTC staining procedure

Where no commercially available pretreatment for CTC staining procedure intended for diagnostic use can be successfully verified with the intended examination (see [6.6.2.2](#)), the laboratory shall develop its own procedure by either:

- modifying an existing pretreatment for CTC staining procedure for diagnostic use;
- using a commercially available system for research use only that can be modified as required; or
- developing its own pretreatment for CTC staining.

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.

Annex A
(informative)

Decision guideline for critical steps of the CTC pre-analytical workflow

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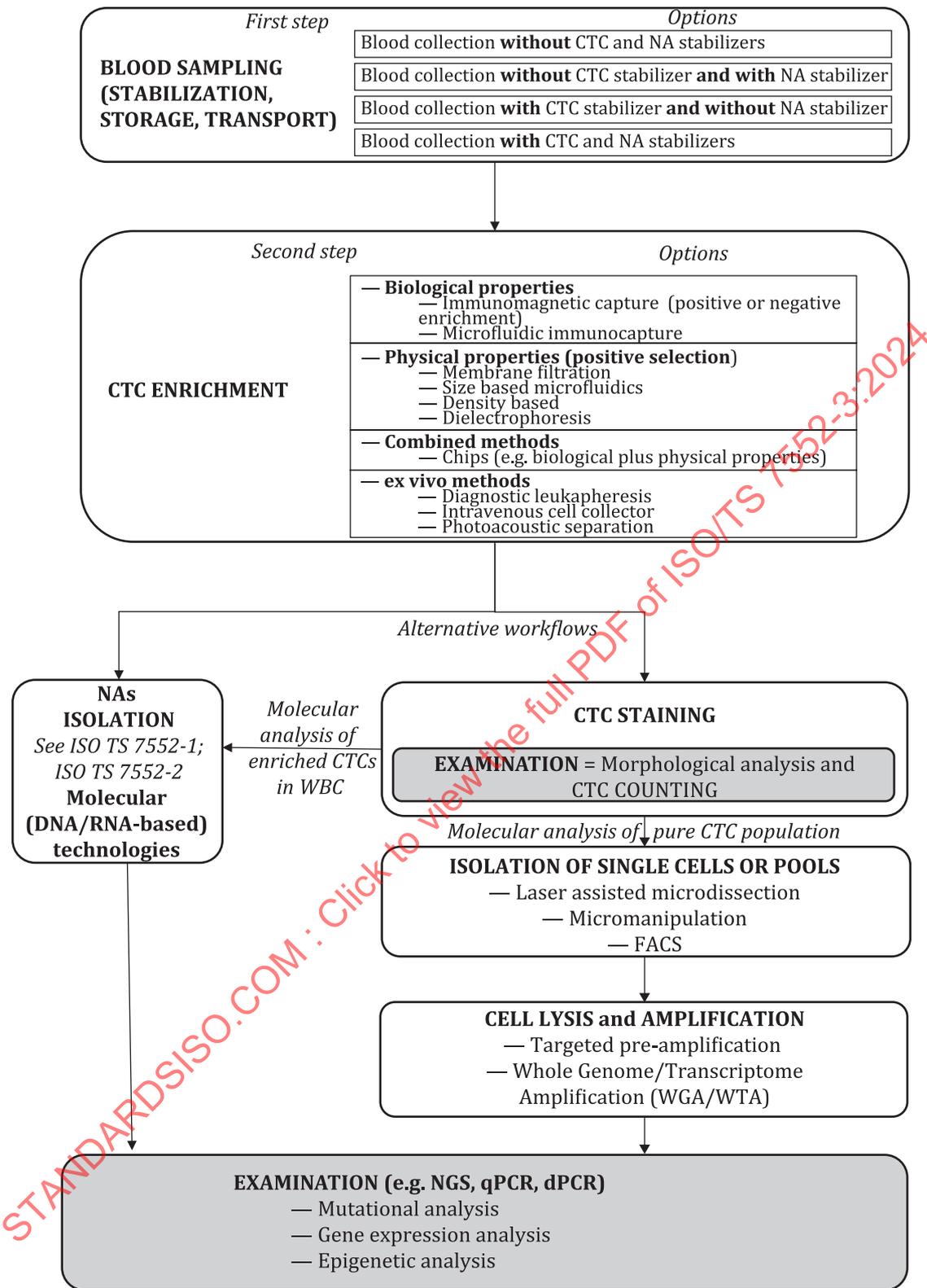


Figure A.1 — Decision guideline for critical steps of the CTC pre-analytical workflow