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Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood

Part 3: Isolated circulating cell free DNA from plasma

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National foreword

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Molekularanalytische in-vitro-diagnostische Verfahren - Spezifikationen für präanalytische Prozesse für venöse Vollblutproben - Teil 3: Aus Plasma isolierte zirkulierende zellfreie DNS

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CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

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European foreword

This document (CEN/TS 16835-3:2015) has been prepared by Technical Committee CEN/TC 140 “In vitro diagnostic medical devices”, the secretariat of which is held by DIN.

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Introduction

Molecular *in vitro* diagnostics has enabled a significant progress in medicine. Further progress is expected by new technologies analysing signatures of nucleic acids, proteins, and metabolites in human tissues and body fluids. However, the profiles of these molecules can change drastically during primary sample collection, transport, storage and processing thus making the outcome from diagnostics or research unreliable or even impossible because the subsequent analytical assay will not determine the situation in the patient but an artificial profile generated during the pre-examination process. Therefore, a standardization of the entire process from primary sample collection to circulating cell free DNA (ccfDNA) analysis is needed. Studies have been undertaken to determine the important influencing factors. This Technical Specification draws upon such work to codify and standardize the steps for circulating cell free DNA analysis from plasma prepared from human venous whole blood in what is referred to as the preanalytical phase.

1 Scope

This Technical Specification recommends the handling, documentation and processing of venous whole blood specimens intended for circulating cell free DNA (ccfDNA) analysis during the preanalytical phase before a molecular assay is performed. This Technical Specification covers specimens collected by venous whole blood collection tubes. This Technical Specification is applicable to molecular *in vitro* diagnostic examinations (e.g. *in vitro* diagnostic laboratories, laboratory customers, *in vitro* diagnostics developers and manufacturers, institutions and commercial organizations performing biomedical research, biobanks, and regulatory authorities).

Blood ccfDNA profiles can change significantly after blood collection from the donor (e.g. release of genomic DNA from white blood cells, ccfDNA fragmentation and ccfDNA quantity change). Special measures need to be taken to secure good quality blood samples for ccfDNA analysis and storage.

Different dedicated measures need to be taken for preserving blood genomic DNA. These are not described in this Technical Specification. Blood genomic DNA is covered in CEN/TS 16835-2, *Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for venous whole blood — Part 2: Isolated genomic DNA*

NOTE CcfDNA obtained from blood by the procedures suggested in this document can contain DNA present in exosomes [3] [4].

DNA from pathogens present in blood is not covered by this Technical Specification.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 15189:2012, *Medical laboratories — Requirements for quality and competence (ISO 15189:2012, Corrected version 2014-08-15)*

ISO 15190, *Medical laboratories — Requirements for safety*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN ISO 15189:2012 and the following apply.

3.1
ambient temperature
unregulated temperature of the surrounding air

3.2
analytical phase
processes that start with the isolated analyte and include all kind of parameter testing or chemical manipulation for quantitative or qualitative analysis

3.3
ccfDNA
circulating cell free DNA
extracellular human DNA present in blood, serum and plasma

Note 1 to entry: ccfDNA can include DNA present in vesicles such as exosomes [3] [4].

3.4
ccfDNA profile/s
circulating cell free DNA profile/s
amounts of different ccfDNA molecules, that are present in blood and plasma, that can be measured in the absence of any losses, inhibition and interference

3.5
cryo-precipitates
insoluble residue when frozen plasma is thawed

3.6
DNA
deoxyribonucleic acid
polymer of deoxyribonucleotides occurring in a double-stranded (dsDNA) or single-stranded (ssDNA) form

[SOURCE: EN ISO 22174:2005, 3.1.2]

3.7
pre-examination processes
preanalytical phase
preanalytical workflow
processes that start, in chronological order, from the clinicians' request and include the examination request, preparation and identification of the patient, collection of the primary sample(s), temporary storage, transportation to and within the analytical laboratory, aliquotting, retrieval, isolation of analytes, and end when the analytical examination begins

[SOURCE: EN ISO 15189:2012, 3.15, modified — An additional term was added and more details were included.]

Note 1 to entry: The preanalytical phase may include preparative processes that may influence the outcome of the intended examination.

3.8
primary sample
specimen
discrete portion of a body fluid, breath, hair or tissue taken for examination, study or analysis of one or more quantities or properties assumed to apply for the whole

[SOURCE: EN ISO 15189:2012, 3.16, modified — The term and definition is used here without the original notes.]

3.9
room temperature
temperature which is defined as 18 °C to 25 °C for the purposes of this document

3.10
stability
ability of a sample 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".]

Note 1 to entry: The measured constituent for the purpose of this document is ccfDNA.

4 General considerations

For general statements on primary sample collection and handling (including avoidance of cross contaminations), see EN ISO 15189:2012, 5.2.6, 5.4.4. Consumables including kits shall be verified before use in examination (see EN ISO 15189:2012, 5.3.2.3); EN ISO 15189:2012, 5.5.1.2 and 5.5.1.3 can also apply.

As all steps of a diagnostic workflow can influence the final analytical performance, the entire workflow comprising the preanalytical steps, including information on sample stability and storage conditions, and analytical steps should be verified and validated (see EN ISO 15189).

Blood circulating cell free DNA profiles can change significantly after blood collection and plasma separation. The release of genomic DNA from white blood cells can change the ccfDNA profile significantly. This can impact the validity of the analytical test results. Additional post-collection effects can also occur e.g. ccfDNA fragmentation [5], [6], [7], [8]. These changes can vary individually in different donors' / patients' blood depending on pathophysiological conditions [5] [9], [10], [11].

The stability of the specific blood ccfDNA profile of interest should be investigated throughout the complete preanalytical workflow e.g. by applying the intended analytical test in time course studies reflecting the individual preanalytical workflow steps such as transport and storage.

Before or during the design of the analytical test system, it should be investigated and ensured that the blood ccfDNA profile/s intended to be analysed in the analytical test is/are not affected by the envisioned entire pre-analytical workflow.

If a commercial product is not used in accordance with the manufacturers' instructions, responsibility for its validation, verification, use and performance lies with the user.

Safety regulations on facilities, transport and handling shall be considered (EN ISO 15189:2012, 5.2.3 and 5.4.5, and ISO 15190).

5 Outside the laboratory

5.1 Primary venous whole blood collection manual

5.1.1 Information about the primary sample donor

The documentation should include, but is not limited to:

a) the primary donor / patient ID, which can be in the form of a code;

b) the health status and relevant lifestyle factors of the blood donor (e.g. healthy, gender, age, disease type, gestational age);

NOTE In particular e.g. cancer, inflammation, diabetes, hepatic disease, coronary disease, respiratory syndrome, trauma, after exhaustive exercise [5], in elderly patients suffering from acute or chronic disease, first trimester of pregnancy, placental disorders as pre-term labour, pre-eclampsia and malimplantation have been reported to affect both blood ccfDNA quantity and fragmentation [5], [9], [10], [11].

c) the information about medical treatment and special treatment prior to blood collection (e.g. anaesthetics, medications, fasting status);

d) the type and purpose of the proposed analytical test requested.

See also EN ISO 15189:2012, 5.4.4.

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

The blood ccfDNA profile can be influenced by inadequate blood collection procedures and inappropriate storage/shipping conditions, plasma separation as well as by ccfDNA isolation procedures. Specifically, the post-collection release of genomic DNA from white blood cells can change the ccfDNA profile significantly. This can impact the validity of the analytical test results.

Venous whole blood should be collected in appropriate collection devices.

Blood Collection tubes containing ccfDNA profile stabilizers are recommended when post collection release of genomic DNA from blood cells or other ccfDNA profile changes can cause impacts on the intended analytical test.

If blood collection tubes without ccfDNA profile stabilizers are used, EDTA blood collection tubes should be used in preference to other collection tubes [7], [12].

Induced clotting process in serum tubes can lead to a leucocytes lysis and therefore the use of serum tubes should be avoided [13].

— EDTA prevents clotting and allows for extended storage compared to other anticoagulants. Consult the specifications by the analytical test provider for details.

— Venous whole blood collection tubes containing specific blood ccfDNA profile stabilizers can be able to stabilize the ccfDNA profile for up to several days at room temperature or 2 °C to 8 °C [6], [7], [12]. Consult also the specifications by the analytical test provider for details.

5.1.3 Primary venous whole blood collection from the patient and stabilization procedures

1. The identity of the person collecting the primary sample and the time of blood collection according to EN ISO 15189:2012, 5.4.4.3, f) shall be documented.

2. For the labelling (sample identification) of the blood collection tube a routine procedure (EN ISO 15189:2012, 5.4.4.3, e)) or a procedure with additional information (e.g. 2D-barcode) shall be used.

3. Standard venipuncture technique can be used. Steps for preventing possible backflow may be required. The manufacturers' instructions for using the blood collection tubes shall be followed. A blood collection set and needle holder can be required when using blood ccfDNA profile stabilizer containing tubes. In this case, the instructions of the collection set and needle holder manufacturer shall be followed.

   NOTE There is no known specific effect of venous whole blood draw procedure on the blood ccfDNA profile. Routine procedures can therefore be used.

4. Blood collection tubes shall be filled in accordance to the manufacturers' instructions and attention should be drawn to the correct positioning of the collection tube during the blood draw as well as the required volume.

5. Blood collection tube manufacturers' instructions for mixing or inverting the tube immediately after blood collection shall be followed.

   NOTE Unless additives are homogenously mixed with the blood sample, the blood ccfDNA quality can be compromised, which can impact the validity and reliability of the analytical test results.

6. Any tampering with and/or additions to the primary sample shall be documented.

Until clean plasma is generated, special care need to be taken to avoid lysis of blood cells. Therefore, it is essential that the primary sample is not frozen or shaken vigorously [6].

5.1.4 Information on the primary blood sample and storage requirements at the blood collection facility

Blood cell lysis after blood collection contaminates the sample with cellular DNA [6] and can affect the validity and reliability of the analytical test result. The documentation on the primary blood sample shall therefore include the date and the time of blood collection [6], [13].

For storing the primary blood samples collected in blood collection tubes with blood ccfDNA profile stabilizers, the blood collection tube manufacturer's instructions on storage conditions shall be followed (e.g. temperature and storage duration). Where the analytical test provider's instructions are more stringent, these shall be followed. The storage conditions (including the storage duration and temperature) shall be documented.

Where using blood collection tubes without blood ccfDNA profile stabilizers, the analytical test provider's instructions on storage conditions shall be followed. This may require documentation of storage conditions.

Where using blood collection tubes without blood ccfDNA profile stabilizers and no requirements on the storage conditions are available, the primary blood samples should be transferred immediately to 2 °C to 8 °C in order to minimize the release of DNA from leucocytes into the blood. The storage duration allowed at 2 °C to 8 °C shall be validated by analysing the potential impact on the analytical test. The storage conditions (including the storage duration and temperature) shall be documented.

NOTE Some studies could show that the storage at 2 °C to 8 °C for up to 6 h [6] had no negative impact on results obtained by the applied analytical test/s.

The temporary storage duration in the blood collection facility contributes to the total duration for storage.

5.2 Transport requirements

The required transport conditions shall be documented including any deviations therefrom.

Where using blood collection tubes with blood ccfDNA profile stabilizers, the tubes' manufacturers' instructions on transport conditions shall be followed (including the transport duration and temperature). Where the analytical test providers' instructions are more stringent, these shall be followed.

Where using blood collection tubes without blood ccfDNA profile stabilizers, the analytical test providers' instructions on transport conditions shall be followed. This can require the documentation of transport conditions (including the transport duration and temperature).

Where using blood collection tubes without blood ccfDNA profile stabilizers and no analytical test providers' instructions are available, the primary blood sample should be transported at 2 °C to 8 °C in order to minimize the release of DNA from leucocytes into the blood. The transport duration allowed at 2 °C to 8 °C shall be validated by analysing the potential impact on the analytical test.

NOTE Some studies could show that the storage at 2 °C to 8 °C for up to 6 h [6] had no negative impact on results obtained by the applied analytical test/s.

If the blood collection tube manufacturer or the analytical test provider requires a dedicated packaging, samples shall be transported accordingly. If there are no such requirements, the sample should be packed in tissues, airbags, paper or the like to protect it from shaking during transport, including accidental dropping of the package.

The sample shall not be frozen or shaken strongly [6].

See also EN ISO 15189:2012, 5.4.5.

The transport duration to the laboratory contributes to the total duration for storage.

6 Inside the laboratory

6.1 Primary sample reception

The blood sample reception time shall be documented. Nonconformities of labelling, transport conditions and blood volume differences to specifications, leaking/broken tubes etc. shall be documented.

Where there are nonconformities in labelling, transport conditions, overall storage, transport time or blood volume that could affect the validity and reliability of the analytical test result, a new sample should be obtained.

6.2 Storage requirements for venous whole blood sample

The storage temperature and time interval between primary sample receipt and sample processing for plasma generation shall be documented. Storage temperature and total storage duration shall not exceed specifications identified in 5.1.4 and 5.2.

The primary blood sample total storage duration shall include the duration for storage at the blood collection facility (5.1.4), for transportation to the laboratory (5.2) and for further storage at the laboratory or other institutions.

6.3 Plasma preparation

Where using blood collection tubes with blood ccfDNA profile stabilizers, the manufacturers' instructions to obtain plasma separation shall be followed.

Where using blood collection tubes without ccfDNA profile stabilizers, and if dedicated analytical test provider's instructions for plasma preparation are available, these shall be followed.

Where using blood collection tubes without ccfDNA profile stabilizers, and no dedicated analytical test provider's instructions are available, EDTA blood samples should be centrifuged at 1 600 g to 2 500 g at 2 °C to 8 °C for 10 min. Plasma shall be carefully transferred into a new tube without disturbing the plasma-cellular interface layer in order to avoid contamination from genomic DNA and cellular RNA derived from leucocytes. A second centrifugation should be performed at 14 000 g to 16 000 g at 2 °C to 8 °C for 10 min. If the second centrifugation step is performed, the supernatant shall be carefully transferred into a new tube without disturbing the pellet [7], [14]. If high g-force centrifugation is not possible, e.g. due to lack of appropriate centrifuges, the analytical test shall be validated when carrying out the second centrifugation at a lower g-force e.g. 3 000 g to 5 000 g for 20 min at 2 °C to 8 °C.

6.4 Storage requirements for plasma sample

The storage temperature and time interval between the plasma generation and the isolation of the ccfDNA shall be documented.

The plasma samples should be processed for analytical down-stream tests immediately. Depending on the analytical test specifications and performance, for short-term storage, plasma may be stored at 2 °C to 8 °C for a maximum of 24 h. For long-term storage plasma should be stored frozen at −20 °C or below [6], [14].

Frozen plasma samples shall not be thawed more than once [6], [14], [15]. Therefore, the plasma samples should be aliquoted into cryo-vials or other suitable vials if further testing is needed [5]. See also Table 1.

Where dedicated analytical test providers' instructions on storage of plasma are available, these shall be followed (see Table 1).

Table 1 — Summary of storage requirements for venous whole blood collection tubes with or without blood ccfDNA profile stabilizers

Blood collection tube	**Blood collection, transport and storage**		**Plasma Storage**	
	Duration	Temperature (°C)	Duration	Temperature (°C)
With blood ccfDNA profile stabilizer	- Blood collection tube manufacturer's instructions - Analytical test providers' instructions[a]		- Blood collection tube manufacturer's instructions - Analytical test providers' instructions[a]	
EDTA blood collection tubes without blood ccfDNA profile stabilizer	- Analytical test providers' instructions - within 6 h	- Analytical test providers' instructions - 2 °C to 8 °C	- Analytical test providers' instructions - Up to 24 h	- Analytical test providers' instructions - 2 °C to 8 °C
			Long term storage	- Analytical test providers' instructions - −20 °C or below

[a] If more stringent than blood tube manufacturer's instructions.

6.5 Isolation of the ccfDNA

The laboratory shall validate the entire process comprising sample collection, storage, transportation and isolation of ccfDNA to the final analytical test result according to its internal quality management system (see EN ISO 15189).

To avoid a cross contamination with amplified DNA, the isolation of ccfDNA should not be performed in the same area as the amplification and post-amplification steps, unless a closed system is used.

CcfDNA is usually of shorter length than genomic DNA. Therefore, specific dedicated ccfDNA isolation procedures should be used.

Different blood ccfDNA isolation procedures may show significant different ccfDNA yields [14], [16], [17], (Figure A.1), and size distribution patterns [14], [16], (Figure A.1) from the same sample. Therefore, this aspect should be specifically considered during the validation process.

Where using blood collection tubes containing blood ccfDNA profile stabilizers, kits specified by the manufactures of the blood collection tube should be used for the isolation of blood ccfDNA. Alternative extraction procedures can be used if verified for the same requirements and validated for the same intended use.

NOTE 1 Where using alternative extraction procedures, dedicated measures and technologies might be needed in order to avoid carrying over blood ccfDNA stabilization molecules to the final ccfDNA eluate. Stabilization molecules carry over can lead to an inhibition of the analytical test reaction.

Blood collection tube and kit manufacturers' instructions or the instructions for the validated alternative for isolating the ccfDNA shall be followed.

NOTE 2 Dedicated procedures can be included in the manufacturers' instructions for processing frozen plasma samples.

Where using blood collection tubes not containing any blood ccfDNA profile stabilizers, the analytical test manufacturers' instructions or validated alternatives for blood ccfDNA isolation shall be followed.

Where using blood collection tubes not containing any blood ccfDNA profile stabilizers, and where there are no analytical test manufacturers' instructions available, the laboratory shall validate the entire blood ccfDNA isolation process. Specific blood ccfDNA isolation kits or alternatives validated to the same requirements shall be used for reliably isolating the different fragment lengths of the blood ccfDNA profile. The kit manufacturers' instructions or the instructions for the validated alternative for isolating the blood ccfDNA shall be followed.

The reagents and consumables coming in touch with the sample shall be DNase-free.

6.6 Quality assessment and quantity measurement of isolated ccfDNA

The blood ccfDNA quantity and quality should be checked according to diagnostic kits manufacturer's instructions or according to validated procedures prior to performing the analytical test. These may include one or more of the following:

— Quantity: ccfDNA is usually at very low concentration, which makes the use of UV absorbance reading such as spectrophotometers unreliable and therefore should be avoided. Often, blood ccfDNA isolation procedures use 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. The currently most used method for ccfDNA quantification is therefore qPCR, targeting a known sequence of a single copy gene such as RNaseP [18], [19], other target genes [14], or a conserved non-coding sequence [20]. For the standard curve of the qPCR target gene assay Standard Reference Material (SRM) 2372 Human DNA Quantitation Standard [21] can be used.

— Quality: Due to the low concentration and the heterogeneity of blood ccfDNA profiles, there is no generic method for quality assessment. Depending on the analytical test requirements dedicated quality assessment test may therefore be required to be performed, e.g. percentage of fetal DNA within the total ccfDNA [22].

The blood ccfDNA isolation performance should be tested in a ccfDNA proficiency test program where available [23].

6.7 Storage of isolated ccfDNA

For storing and archiving the isolated blood ccfDNA, the ccfDNA isolation kit providers' specific instructions should be followed.

If there is no information available from the ccfDNA isolation kit provider or if laboratories' own validated blood ccfDNA isolation procedures are used, the isolated blood ccfDNA should be assayed immediately. In the case that the isolated blood ccfDNA cannot be assayed immediately, the laboratory shall have verified protocols in place how to store the isolated blood ccfDNA.

Storage for archiving purposes should be at −20 °C or below [6].

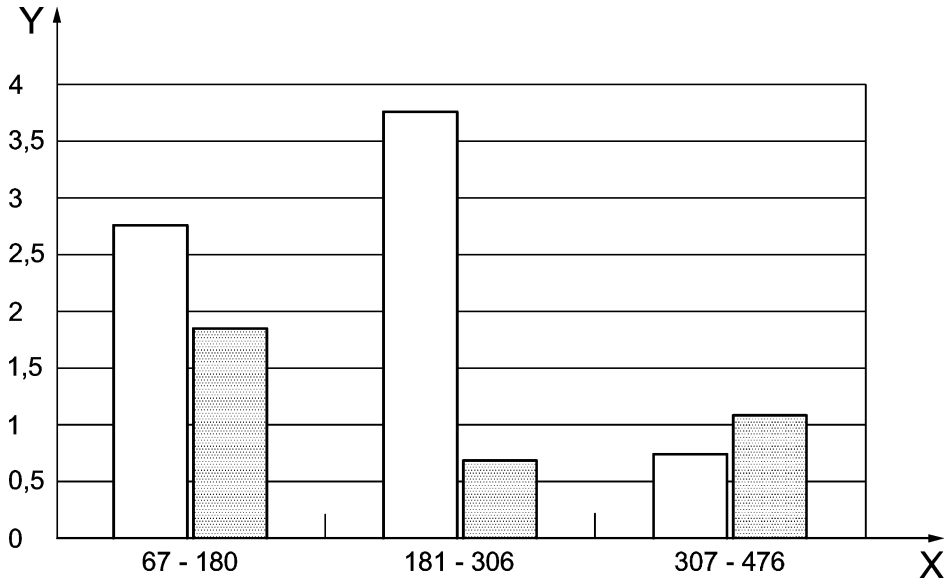
For archiving aliquots of the isolated blood ccfDNA should be generated to avoid repeated freezing and thawing [6].

Annex A
(informative)

Influence of isolation procedures on ccfDNA fragments' lengths distribution pattern in plasma samples

Figure A.1 shows ccfDNA fragments' lengths distribution pattern depending on ccfDNA isolation procedure.

The venous whole blood samples were collected from healthy volunteers (n = 15) in K_2EDTA tubes and plasma was immediately isolated by centrifugation (first centrifugation was performed at 1 600 g at 4 °C for 10 min, then plasma was recovered and submitted to a second centrifugation at 16 000 g at 4 °C for 10 min). The ccfDNA was immediately isolated from plasma by two different kits. The evaluation of ccfDNA fragments' lengths was performed by qPCR evaluating the APP DNA (Amyloid beta (A4) Precursor Protein) concentration (ng/ml plasma). For this test, four different assays with comparable amplification efficiency for 67 bp, 180 bp, 306 bp, and 476 bp amplicons were assessed [14].



Key

X fragments' lengths (bp); three classes were defined: 67 bp to 180 bp; 181 bp to 306 bp; 307 bp to 476 bp

Y ccfDNA concentration (ng/ml plasma)

▨ kit A

□ kit B

Figure A.1 — ccfDNA fragmentation pattern depending on the ccfDNA isolation procedure [14]

The ccfDNA recovery yield obtained by the two kits was different, mainly when the class between 180 bp to 306 bp was considered. Also, it demonstrated that the measured ccfDNA concentration changes on the basis on the amplicon length.

These data demonstrate that ccfDNA isolation procedures used can influence the ccfDNA fragments' lengths distribution.

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