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IVD

For professional use only

NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit

INSTRUCTION FOR USE

"DNA-Technology Research & Production", LLC,

142281, Russia,

Moscow Region, Protvino,

Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

Customer service department

E-mail: hotline@dna-technology.ru

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1. INTENDED USE

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** is *in vitro* DNA test. The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** is designed to detect homozygous deletion of exon 7 of the SMN1 gene and assess the level of T- cell receptor excision circles (TREC) and kappa-deleting recombination excision circle (KREC) in newborn biological material (whole blood, dried blood spots) for screening for spinal muscular atrophy and primary immunodeficiencies by real-time PCR.

Indications for the assay: screening of newborns for proximal spinal muscular atrophy (SMA) and primary immunodeficiencies (PIDs) associated with the T- and B- cells disorders (forming of newborns' risk groups). There are no contradictions for use.

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** is designed for screening of newborns.

The application of the kit does not depend on population and demographic aspects. There are no contradictions for use of the **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit.**

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** can be used in clinical and diagnostic laboratories of medical institutions and in research practice.

Potential users: personnel qualified in molecular diagnostics methods and working in the clinical and diagnostic laboratory.

It is necessary to apply the kit only as directed in this instruction for use.

2. METHOD

Method: multiplex polymerase chain reaction (PCR) with real-time results detection; qualitative analysis (exon 7 of the SMN1 gene); relative quantitative analysis (TREC, KREC DNA).

The method is based on amplification of TREC, KREC, exon 7 of the SMN1 gene, and a fragment of the normalizing gene (endogenous internal control (IC)) by multiplex polymerase chain reaction (PCR).

The amplification process consists of a series of repeated cycles of temperature denaturation of DNA, annealing of primers with complementary sequences, and subsequent elongation of polynucleotide chains from these primers with Taq polymerase.

To increase the sensitivity and specificity of the reaction, a "hot" start is provided by the technique of preparing a reaction mixture consisting of two layers separated by a layer of paraffin and using Taq polymerase blocked by antibodies. The polymerase chain reaction only starts when the paraffin melts and the Taq polymerase and antibody complex dissociates in temperature, which eliminates nonspecific annealing of primers on the DNA target during the initial heating of the test tube.

DNA probes, each containing a fluorescent label and a fluorescence quencher, are introduced into the reaction mixture for amplification. When a specific product is formed, the DNA probe is destroyed and the quencher stops affecting the fluorescent label, which leads to an increase in the fluorescence level. The number of destroyed probes (and hence the fluorescence level) increases in proportion to the number of specific amplicons formed. The fluorescence level is measured at each amplification cycle in real time.

To amplify exon 7 of SMN1, a fluorescent sequence-specific primer-probe is used; its 3'-end closely matches the sequence of the initial fragment of exon 7 of SMN1, but differs from the similar fragment of SMN2.

The DNA probe used to detect the TREC DNA amplification product includes the Fam fluorescent tag.

The DNA probe used to detect the KREC DNA amplification product includes the Rox fluorescent tag.

The primer-probe used for amplification and detection of the amplification product of the target fragment of the SMN1 gene includes the SIMA fluorescent tag (amplification registration is performed on the Hex detection channel).

The DNA probe used to detect the amplification product of the endogenous internal control (IC) gene fragment includes the Cy5 fluorescent tag.

The use of several fluorescent dyes allows to reduce the number of tubes and the amount of biomaterial, since it makes it possible to simultaneously register the results of different amplification reactions taking place in the same tube. Table 1 shows the detection channels of amplification products.

Table 1. Detection channels of amplification products

Fam	Hex	Rox	Cy5
TREC	SMN1 (exon 7 of the SMN1 gene)	KREC	IC*

* IC – endogenous internal control, a single-copy genomic locus, evaluates the genome equivalents of nucleated blood cells (leukocytes) and provides quality control of DNA preparation.

The automatic analysis is available on "DNA-Technology" made instruments: DTlite or DTprime REAL-TIME Thermal Cyclers **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** (see the catalogue at https://www.dna-technology.com to see available supply options). The current version of the software is available for download at https://www.dna-technology.com/software.

The level of TREC and KREC in the test sample is estimated by the formulas:

1. ***TREC*** level **(copies per 10^5 leukocytes) = $10^{((Cp\ IC - Cp\ TREC)/3,4)}$ X 200000 (1)**

where IC Cp is the value of the IC indicator cycle (Cp);

where TREC Cp is the value of the TREC indicator cycle (Cp).

If no TREC Cp value is specified for the sample, the TREC level is 0 copies per 10^5 leukocytes.

2. ***KREC*** content **(copies per 10^5 leukocytes) = $10^{((Cp\ IC - Cp\ KREC)/3,4)}$ X 200000 (2)**

where IC Cp is the value of the IC indicator cycle (Cp);

where KREC Cp is the value of the KREC indicator cycle (Cp).

If no KREC Cp value is specified for the sample, the KREC level is 0 copies per 10^5 leukocytes.

Analysis of SMN1 exon 7 deletion is based on the estimation of the indicator cycle difference (Δ Cp) between the Cp of SMN1 (Hex channel) and IC (Cy5 channel) **(ΔCp = Cp (Hex) – Cp (Cy5))**.

Method limitations:

For correct estimation of TREC and KREC excision circles and genotyping for exon 7 of the SMN1 gene, the amount of genomic DNA per amplification tube should be at least 1.0 ng of genomic DNA (acceptable Cp value per Cy5 (IC) channel ≤ 31).

Nucleic acid extraction kits manufactured by "DNA-Technology" are recommended:

Whole blood	Dried blood spots
PREP-MB MAX REF P-103-N/4EU, P-103-A/8EU	**PREP-CITO DBS** REF P-029-N/2EU **PREP-MB-DBS DWP** REF P-128-N/9EU, P-128-P/9EU, P-129-P/9EU

3. CONTENT

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** contents is represented in Tables 2, 3.

Table 2. The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** content, package S, strips, for R1-H810-S3/9EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless transparent liquid under waxy white fraction	1920 µL (20 µL in each tube)	12 8-tube strips
TechnoTaq MAX polymerase	Colorless transparent viscous liquid	50 µL	1 tube
PCR-buffer	Colorless transparent liquid	1.0 mL	1 tube
Mineral oil	Colorless transparent viscous oily liquid	2.0 mL (1.0 mL in each tube)	2 tubes
Positive control C+ №1	Colorless transparent liquid	130 µL	1 tube
Positive control C+ №2	Colorless transparent liquid	130 µL	1 tube
Strip's caps	12 8-caps		

Table 3. The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** content, package S, tubes, for R1-H810-23/9EU

Reagent	Description	Total volume	Amount
Paraffin sealed PCR-mix	Colorless transparent liquid under waxy white fraction	1920 µL (20 µL in each tube)	96 tubes
TechnoTaq MAX polymerase	Colorless transparent viscous liquid	50 µL	1 tube
PCR-buffer	Colorless transparent liquid	1.0 mL	1 tube
Mineral oil	Colorless transparent viscous oily liquid	2.0 mL (1.0 mL in each tube)	2 tubes
Positive control C+ №1	Colorless transparent liquid	130 µL	1 tube
Positive control C+ №2	Colorless transparent liquid	130 µL	1 tube

All components are ready to use and do not require additional preparation for operation.

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** in package S is intended for single use and is designed for 96 tests (no more than 24 runs), including test samples, negative and positive controls.

4. REAGENTS AND EQUIPMENT REQUIRED BUT NOT PROVIDED

4.1. Specimen collection

– For blood collection: 2.0 - 4.0 mL Vacuette blood collection tubes with anticoagulant, for example, salt of ethylenediaminetetraacetate (EDTA) at a final concentration of 2.0 mg/mL or sodium citrate anticoagulant.

Please use only salt of EDTA or sodium citrate as an anticoagulant, since other substances can provide PCR

inhibition.

- Filter paper for taking and storing blood samples in the form of dried blood.

4.2. DNA extraction and PCR

Preamplification-specimen and control preparation area

4.2.1. Dried blood spots

Nucleic acid extraction kit **PREP-CITO DBS** REF P-029-N/2EU, manufactured by “DNA-Technology”

- Biological safety cabinet class II;
- Refrigerator;
- Vortex mixer;
- Automatic or hand puncher for punching 3.0-3.2 mm discs from DBS samples;
- High speed centrifuge (RCF(g) at least 16,000);
- Thermostat (temperature range 50-98 °C);
- Tube rack for 1.5 mL tubes;
- 1.5 mL tubes;
- Single channel pipettes (dispensers covering 20-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 200 µL, 1000 µL);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Nucleic acid extraction kit **PREP-MB-DBS DWP** REF P-128-N/9INT, P-128-P/9INT, P-129-P/9INT, manufactured by “DNA-Technology”

- Biological safety cabinet class II;
- Refrigerator with freezer;
- Centrifuge (RCF(g) at least 150) with bucket rotor and adapter for 96 deep-well plates (except package N REF P-128-N/9INT);
- Single channel pipettes (dispensers covering 20.0-1000 µL volume range);
- 8-channel pipettes (dispensers covering 30.0 - 300 µL volume range);
- RNase and DNase free filtered pipette tips (volume 200 µL, 300 µL, 1000 µL);
- Pipette rack;
- Automatic or hand puncher for punching 3.0-3.2 mm discs from DBS samples;
- System for automatic nucleic acid extraction in 96 Deep-Well Plate (for example, Auto-Pure 96 (Hangzhou Allsheng Instrument Co., LTD, China));
- 96-well deep-well 2.2 mL plate (for placing and pretreatment of DBS discs);
- 96 deep-well 2.2 mL plates (6 pcs) (for package N, REF P-128-N/9INT);
- thermal seal for 96-well microplates (for package N, REF P-128-N/9INT);
- 96 tip comb (for package N, REF P-128-N/9INT);

- 8-channel pipettes (dispensers covering 5.0 - 50 µL volume range) (for dosing DNA samples from 96-well deep-well plate);
- RNase and DNase free filtered pipette tips (dispensers covering 5.0 - 50 µL volume range) (for dosing DNA samples from 96-well deep-well plate);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

4.2.2. Whole peripheral blood

Nucleic acid extraction kit **PREP-MB MAX** REF P-103-N/4EU, P-103-A/8EU, manufactured by "DNA-Technology"

- Biological safety cabinet class II-III;
- Refrigerator;
- High speed centrifuge (RCF(g) at least 16,000) (for package N REF P-103-N/4EU);
- Vortex mixer;
- Solid-state thermostat (temperature range 25-98 °C) (for package N REF P-103-N/4EU);
- Magnetic homogenizer (for package N REF P-103-N/4EU);
- Single channel pipettes (dispensers covering 2.0-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- 1.5 mL tubes (SSI-1260 are recommended);
- Tube rack for 1.5 mL tubes;
- Electric laboratory aspirator with trap flask for the removal of supernatant (for package N);
- RNase and DNase free non-filtered pipette tips for aspirator with trap flask (for package N);
- Specialized forceps or plastic forceps for arranging the magnetic rods (for package N);
- DTstream *L4 automated pipetting system manufactured by DNA-Technology R&P, LLC;
- Single use filter tips for DTstream dosing unit (volume 1000 µL or recommended for similar dosing instrument);
- Container for used pipette tips, tubes and other consumables;
- Powder-free surgical gloves;
- Disinfectant solution.

Preamplification-reagent preparation area:

- UV PCR cabinet;
- Refrigerator with freezer;
- Vortex mixer;
- Vortex rotor for 0.2 mL strips;
- PCR tube rack for 0.2 mL tubes or strips;
- 1.5 mL tubes;

- Tube rack for 1.5 mL tubes;
- Single channel pipettes (dispensers covering 2.0-1000 µL volume range);
- RNase and DNase free filtered pipette tips (volume 20 µL, 200 µL, 1000 µL);
- Powder-free surgical gloves;
- Disinfectant solution;
- Container for used pipette tips.

Post-Amplification – Amplification detection area:

Real-time PCR thermal cycler.

- DTprime detecting thermal cycler (version DTprime *M*) manufactured by DNA-Technology R&P, LLC;
- DTlite detecting thermal cycler (version DTlite *S*) manufactured by DNA-Technology R&P, LLC (only for manual dosing with strips).

Software:

The most recent version of the DT thermal cyclers software can be downloaded from https://www.dna-technology.com/software.

The OS supported: all versions of Windows starting from 7.

5. STORAGE AND HANDLING REQUIREMENTS

Expiry date – 12 months from the date of production.

All components of the **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit,** except for TechnoTaq MAX polymerase, shall be stored in a refrigerator or a freezer at the temperature from 2 °C to 8 °C throughout the shelf life of the kit. The TechnoTaq MAX polymerase shall be stored in a freezer at the temperature from minus 18 °C to minus 22 °C throughout the shelf life of the kit. PCR-mix shall be stored in a refrigerator or a freezer at the temperature from 2 °C to 8 °C and out of light throughout the shelf life of the kit.

The excessive temperature and light can be detrimental to product performance.

Transportation of the reagent kit is carried out in thermocontainers with ice packs by all types of roofed transport at the temperature inside the container corresponding to the storage conditions of the components included in the kit throughout the shelf life of the kit.

It is allowed to transport the kit, except for TechnoTaq MAX polymerase, in thermocontainers with ice packs by all types of roofed transport at the temperature inside the container from 2 °C to 25 °C for no longer than 5 days and store at temperatures from 2 °C to 8 °C immediately on receipt.

It is allowed to transport the TechnoTaq MAX polymerase in thermocontainers with ice packs by all types of roofed transport at the temperature inside the container up to 25 °C for no longer than 5 days and store at temperatures from minus 18 °C to minus 22 °C immediately on receipt.

Reagent kits transported with violation of temperature conditions shall not be used.

Shelf-life of the kit following the first opening of the primary container:

- components of the kit shall be stored at the temperature from 2 °C to 8 °C throughout the shelf life of the kit;
- PCR-mix for amplification shall be stored at the temperature from 2 °C to 8 °C and out of light throughout the shelf life of the kit;
- TechnoTaq MAX polymerase shall be stored at the temperature from minus 18 °C to minus 22 °C

throughout the shelf life of the kit.

The kit stored under undue regime should not be used.

An expired **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** shall not be used.

We strongly recommend to follow the given instructions in order to obtain accurate and reliable results.

Conformity of **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** to the prescribed technical requirements is subject to compliance of storage, transportation and handling conditions recommended by manufacturer.

Contact our official representative in EU by quality issues of **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit**.

6. WARNINGS AND PRECAUTIONS

Only personnel trained in the methods of molecular diagnostics and the rules of work in the clinical and diagnostic laboratory are allowed to work with the kit.

Handle and dispose all biological samples, reagents and materials used to carry out the assay as if they were able to transmit infective agents. The samples must be exclusively employed for certain type of analysis. Samples must be handled under a laminar flow hood. Tubes containing different samples must never be opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. The reagents must be handled under a laminar flow hood. The reagents required for amplification must be prepared in such a way that they can be used in a single session. Pipettes used to handle reagents must be exclusively employed for this specific purpose. The pipettes must be of the positive dispensation type or be used with aerosol filter tips. The tips employed must be sterile, free from the DNases and RNases, free from DNA and RNA. Avoid direct contact with the biological samples reagents and materials used to carry out the assay. Wear powder-free surgical gloves. Wear protective clothing (work clothes and personal protective equipment) working with microorganisms classified as particularly pathogenic. The protective clothing and personal protective equipment must comply with the work to be performed and health and safety requirements. Avoid producing spills or aerosol. Any material being exposed to biological samples must be treated for at least 30 minutes with disinfecting solution or autoclaved for 1 hour at 121 °C before disposal.

Molecular biology procedures, such as nucleic acids extraction, PCR-amplification and detection require qualified staff to avoid the risk of erroneous results, especially due to the degradation of nucleic acids contained in the samples or sample contamination by amplification products.

All oligonucleotide components are produced by artificial synthesis technology according to internal quality control protocol and do not contain blood or products of blood processing.

Positive control is produced by artificial DNA synthesis technology. Positive control does not include parts of infectious agents.

All the liquid solutions are designed for single use and cannot be used more than once in amplification reactions. Plastic tubes do not contain phthalates. Do not breathe gas/fumes/vapor/spray produced by the components of the kit. Do not eat/drink components of the kit. Avoid contact with eyes. Only use the reagents provided in the kit and those recommended by manufacturer. Do not mix reagents from different batches. Do not use reagents from third party manufacturers' kits. All laboratory equipment, including pipettes, test tube racks, laboratory glassware, lab coats, bouffant caps, etc., as well as reagents should be strictly stationary. It is not allowed to move them from one room to another. Equip separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions. Wear lab coats, gloves and tools, which are exclusively employed for the extraction/preparation of the amplification reaction and for the amplification/detection of the amplification products. Never transfer lab coats, gloves and tools from the area designed for

amplification/detection of the amplification products to the area designed for extraction/preparation of amplification reactions. Amplification products must be handled in such a way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be exclusively employed for this specific purpose. Remove PCR waste only in a closed form. Remove waste materials (tubes, tips) only in a special closed container containing a disinfectant solution. Work surfaces, as well as rooms where NA extraction and PCR are performed, must be irradiated with bactericidal irradiators for 30 minutes before and after the work.

Do not open the tubes after amplification. Waste materials are disposed of in accordance with local and national standards. All surfaces in the laboratory (work tables, test tube racks, equipment, etc.) must be treated daily with disinfecting solution.

Emergency actions

Inhalation: Inhalation of the PCR-mix contained within this kit is unlikely, however care should be taken.

Eye Contact: If any component of this kit enters the eyes, wash eyes gently under potable running water for 15 minutes or longer, making sure that the eyelids are held open. If pain or irritation occurs, obtain medical attention.

Skin Contact: If any component of this kit contacts the skin and causes discomfort, remove any contaminated clothing. Wash affected area with plenty of soap and water. If pain or irritation occurs, obtain medical attention.

Ingestion: If any component of this kit is ingested, wash mouth out with water. If irritation or discomfort occurs, obtain medical attention.

Do not use the kit:

- When the transportation and storage conditions are breached;
- When the reagents' appearance does not respond to the kit passport;
- When the kit components packaging is breached;
- After the expiry date provided.

Significant health effects are **NOT** anticipated from routine use of this kit when adhering to the instructions listed in the current manual.

7. SAMPLES

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** is designed to detect DNA extracted from dried blood spots (DBS) or whole blood.

ATTENTION! Intravenous injections of heparin, infusions of parenteral nutrition are not allowed if done less than 6 hours before the test.

Sampling, sample processing procedures and storage are carried out in accordance with the instructions to the DNA extraction kit from biological material.

7.1. Interfering substances

The presence of PCR inhibitors in a sample of biological material can cause doubtful and unreliable results. Signs of PCR inhibition are the absence of internal control amplification, distortion of a curve shape (curve does not have the S-shaped, does not reach the plateau phase) or late Cp values.

Anticoagulants used for blood sampling, as well as interfering substances of endogenous nature (hemoglobin, bilirubin, cholesterol and triglycerides) are referred to PCR inhibitors. The maximum allowable concentrations of potential interfering substances in a sample of original biomaterial are given in Table 4.

Potential inhibitors in DNA preparations include: wash solution №2 of the **PREP-MB MAX** reagent kit, cresol red contained in elution solution from **PREP-MB-DBS DWP** extraction kit, residual hemoglobin (for

DNA preparations obtained using **PREP-CITO DBS** extraction kit).

No effect of inhibition of wash solution, cresol red or hemoglobin is observed if the recommended reagent kits for DNA extraction are strictly followed.

NOTE. The maximum amount of the **PREP-MB MAX** reagent kit wash solution №2 that does not inhibit PCR is 25% or less of the DNA sample volume. The maximum amount of the **PREP-MB-DBS DWP** reagent kit wash solution No. 2 that does not inhibit PCR is 12.5% or less of the DNA sample volume. The maximum amount of the **PREP-MB-DBBS DWP** cresol red that does not inhibit PCR is 0.0125 ng/ml. The maximum hemoglobin concentration that does not inhibit PCR is 0.5 mg/mL or less in the DNA sample.

Cellulose fiber and residual amounts of chemical components of filter paper impregnation and marking in samples of dried blood spots have no inhibitory effect.

Table 4. Maximum allowable concentrations of potential interfering substances in the original biomaterial sample.

Biomaterial type	**Interfering substance**	**Studied concentration in sample**
Exogenous		
Whole blood	K2, K3 EDTA	3.60 mg/mL
	Sodium citrate	0.26 mol/L
Endogenous		
Whole blood, dried blood spots	Hemoglobin	250 g/L
	Bilirubin	500 µmol/L
	Cholesterol	12 mmol/L
Dried blood spots	Triglycerides	500 mg/L

To reduce the amount of PCR inhibitors it is necessary to follow the rules of biological material collection.

If the presence of PCR-inhibitory impurities in the sample is confirmed, it is recommended to either repeat DNA extraction and PCR or to take biological material again (performed sequentially).

7.2. Sample collection

Dried blood spots (DBS)

The blood is applied to a filter paper in an amount sufficient to obtain a stain with a diameter of at least 1.0 cm. The blood should soak through the paper. After applying the sample, filter card is dried horizontally on a clean, degreased surface for at least 2 hours without additional heat treatment.

ATTENTION! Direct sunlight (ultraviolet light), heat and moisture exposure during DBS drying and storage is not allowed!

Whole peripheral blood

Whole peripheral blood sampling is carried out in vacuum plastic tubes. It may be 2.0 - 4.0 mL Vacuette blood collection tubes with anticoagulant, for example salt of ethylenediaminetetraacetate (EDTA) at a final concentration of 2.0 mg/mL, or sodium citrate anticoagulant. After taking the material, it is necessary to mix the blood with anticoagulant turning the tube 2 – 3 times.

ATTENTION! It is not allowed to use heparin as an anticoagulant.

Transportation and storage of samples

To prevent contamination and cross-contamination, filter cards with blood spots are individually packed after drying.

Transportation and storage of DBS samples on filter cards should be done according to the manufacturer's instructions.

Whole peripheral blood can be transported and stored at temperatures from 2 °C to 8 °C for no longer than 24 hours. If the material cannot be delivered to the laboratory within 24 hours, single freezing is allowed. It is allowed to store frozen material at temperatures from minus 18 °C to minus 22 °C for 6 months.

ATTENTION! The detailed description of sampling and sample processing procedures as well as sample storage and transportation requirements are cited in **PREP-CITO DBS, PREP-MB-DBS DWP** and **PREP-MB MAX** DNA extraction kits user manuals.

7.3. Sample preparation

Dried blood spots (DBS)

ATTENTION! For DNA extraction three discs from DBS sample 3.0-3.2 mm in diameter are required.

To analyze DBS sample, three discs 3.0 mm in diameter should be obtained from central part of filter card using a puncher or similar equipment (discs 3.2 mm in diameter can be used).

NOTE. It is recommended to punch discs from DBS immediately before DNA extraction procedure.

1. Mark the necessary amount of single use plastic 1.5 mL tubes together with one tube for negative control (C-) (**PREP-CITO DBS** extraction kit) or empty high profile 96-well deep-well plate (**PREP-MB-DBS DWP** extraction kit).
2. Punch three discs from each DBS sample and place them in the marked tubes or wells of plate. No paper discs are placed in the negative control tube or well (C-).

Samples obtained are ready for DNA extraction.
ATTENTION!

1. It is recommended to fill the deep-well plate with DBS discs considering the number of controls in the NeoScreen SMA/TREC/KREC assay (NeoScreen SMA/TREC/KREC assay includes 2 positive controls (“C+ №1”, “C+ №2”) and one negative control (“C-“), thus 93 of 96 wells can be filled with DBS discs).
2. The punched discs must be completely saturated with blood. It is recommended to punch out the discs without taking over the area of the markings limiting the bloodstain.
3. When using hand puncher, prior to obtaining a new DBS sample it is recommended to make 2-3 cuts on a clean piece of filter paper, which will reduce the risk of cross-contamination of samples.

8. PROCEDURE

8.1. DNA extraction from biological material

DNA extraction is carried out according to the extraction kit instructions. **PREP-CITO DBS, PREP-MB MAX** and **PREP-MB-DBS DWP** extraction kits are recommended.

ATTENTION! Simultaneously with DNA extraction from biological material a negative control must be prepared and carried through all the stages of sample preparation.

8.1.1. Dried blood spots (DBS)

DNA extraction from dried blood spots is performed using the **PREP-CITO DBS** or **PREP-MB-DBS DWP** reagent kit according to the manufacturer's instructions.

ATTENTION! Prior to DNA extraction it is required to prepare biomaterial samples (see 7.3).

The obtained DNA samples can be stored at 2 °C to 8 °C for no more than seven days or at minus 18 °C to minus 22 °C for no more than one month.

8.1.2. Whole peripheral blood

Extraction of DNA from whole blood is performed using the **PREP-MB MAX** reagent kit according to the manufacturer's instructions. It is recommended to use negative control from the **PREP-MB MAX** reagent kit as a negative control.

ATTENTION! DNA elution should be performed in 100 µL of dilution buffer.

The obtained DNA sample can be stored at 2 °C to 8 °C for no longer than seven days or at minus 18 °C to minus 22 °C for no longer than one month.

NOTE. If DNA samples will be stored for more than seven days, supernatant must be transferred to a new tube.

8.2. Assay procedure

ATTENTION! The reagents and tubes should be kept away from direct sunlight.

ATTENTION! When using package S, strips, strictly observe the completeness of the strips and caps. Do not use the caps for the strips of the other kits!

8.1 Mark one tube/striped tube with the paraffin sealed PCR-mix for each test sample, negative control (C-), positive control C+ №1, and positive control C+ № 2.

ATTENTION! The amount of reagents is calculated for no more than 24 runs, assuming variable quantity of test samples, 1 negative control and 2 positive controls per run.

Example: To test 5 samples, mark 5 test tubes, one negative control tube "C-" and two positive control tubes "C+ №1", "C+ №2". Total number of tubes is 8.

8.2 Vortex the tubes with PCR-buffer and TechnoTaq MAX polymerase for 3-5 seconds and spin in a vortex mixer for 1-3 seconds.

ATTENTION! TechnoTaq MAX polymerase should be taken out of the freezer immediately before use.

8.3 Prepare a mixture of PCR-buffer with TechnoTaq MAX polymerase. To do this, mix in a separate tube:

- 10 x (N+1) µL of PCR-buffer,
- 0.5 x (N+1) µL of TechnoTaq MAX polymerase,

where N is the number of marked tubes including "C-", "C+ №1" and "C+ №2".

Example: There are 5 test samples, "C-", "C+ №1" and "C+ №2". There are 8 marked tubes.

Prepare a mixture of PCR-buffer and TechnoTaq MAX polymerase for 9 (8+1) tubes, i.e. 90 µL of PCR-buffer + 4.5 µL of TechnoTaq MAX polymerase.

8.4 Vortex the tube with the mixture of PCR-buffer and TechnoTaq MAX polymerase and spin in a vortex mixer for 3-5 seconds.

ATTENTION! Prepare a mixture of PCR-buffer and TechnoTaq MAX polymerase immediately before use.

8.5 Add 10 µL of PCR-buffer and TechnoTaq MAX polymerase mixture to each marked tube (including "C-", "C+ №1" and "C+ №2"). Avoid paraffin layer break.

ATTENTION! Strips with PCR-mix cannot be stored after adding a mixture of PCR-buffer and TechnoTaq MAX polymerase, proceed to pp. 8.6—8.12 immediately after dosing.

8.6 Add one drop of mineral oil (~20 µL) to each tube. Close the tubes/strips.

8.7 Vortex the tubes with the DNA sample, positive controls "C+ №1" and "C+ №2", and negative control "C-" for 3-5 seconds and spin in a vortex mixer for 1-3 seconds.

ATTENTION!

1. If DNA samples were stored at minus 18 °C — minus 22 °C, thaw them at room temperature (18 °C — 25 °C) or at 2 °C — 8 °C. Only one thawing is allowed.
2. If using PREP-MB-DBS DWP extraction kit, spin deep-well plate with DNA at RCF(g) 150 for 30 seconds, remove protective seal and mix them by pipetting 3—5 times.
3. If using PREP-CITO DBS extraction kit, vortex tubes with DNA samples and negative control "C-" for 3—5 seconds and spin on vortex for 1—3 seconds.
4. If using PREP-MB MAX extraction kit, spin tubes with DNA sample and negative control "C-" for

1—3 seconds, then place tubes with DNA into magnetic homogenizer. If supernatant containing extracted DNA was transferred into new tubes, vortex tubes with DNA sample and negative control "C-" for 3—5 seconds and spin on vortex for 1—3 seconds.

5. To prevent contamination, only open the caps of the tubes where DNA sample is going to be introduced, and close them before proceeding to the next sample. If using strips, close the strip cap after introducing samples before proceeding to the next strip. Close the tubes/strips tightly. Use filter tips.

8.8 Add 5.0 µL of DNA sample into corresponding tubes. Do not add DNA into the tubes "C-", "C+ №1" and "C+ №2". Avoid paraffin layer break.

8.9 Add 5.0 µL of negative control (C-) which passed whole DNA extraction procedure into "C-" tube. Avoid paraffin layer break.

8.10 Add 5.0 µL of the corresponding positive control to the tubes marked "C+ №1" and "C+ №2". Avoid paraffin layer break.

8.11 Spin the tubes/strips for 1-3 seconds in a vortex mixer.

8.12 Set the tubes/strips into the real-time thermal cycler.

8.13 Launch the operating software for DT instrument[1]. Add corresponding test[2], specify the number and IDs of the samples, positive and negative controls. Specify the position of the tubes/strips in the thermal unit (see 8.12) and run PCR. See Table 5.

Table 5. The PCR program for DTlite and DTprime thermal cyclers.

Step	Temperature, °C	Min	Sec	Number of cycles	Optical measurements	Type of the step
1	80	2	00	1		Cycle
	94	5	00			
2	94	0	30	5		Cycle
	64	0	15		√	
3	94	0	10	45		Cycle
	64	0	15		√	
4	94	0	5	1		Cycle
5	10[1]	…	…	Holding		Holding
√- optical measurements [1] – holding at 25°C is allowed						

9. CONTROLS

The **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** contains positive control C+ №1 and positive control C+ №2.

The kit contains IC – endogenous internal control, a single-copy genomic locus, which evaluates the genome equivalents of nucleated blood cells (leucocytes) and provides quality control of the DNA preparation.

To reveal possible contamination, a negative control is required.

ATTENTION! A negative control sample should go through all stages of DNA extraction (see section 8.1).

[1] Please, apply to operation manual for DTprime and DTlite real-time PCR instruments PART II.
[2] Instructions for uploading "files with test parameters" can be found on "DNA-Technology's" website https://www.dna-technology.com/assaylibrary.

Validity criteria for controls are shown in Tables 6, 7, 8.

Table 6. Validity criteria for positive control №1.

Cp TREC **(Fam)**	Cp SMN1 **(Hex)**	Cp KREC **(Rox)**	Cp BK **(Cy5)**
Specified	Specified	Specified	Specified
ΔCp = |Cp Fam - Cp Cy5| ≤ 1	-	Δ Cp = |Cp Rox - Cp Cy5| ≤ 1	-

Table 7. Validity criteria for positive control №2.

Cp TREC **(Fam)**	Cp SMN1 **(Hex)**	Cp KREC **(Rox)**	Cp BK **(Cy5)**
Not specified or ≥ 39	Not specified or ≥ 35	Not specified or ≥ 39	Specified

Table 8. Validity criteria for negative control.

Cp TREC **(Fam)**	Cp SMN1 **(Hex)**	Cp KREC **(Rox)**	Cp BK **(Cy5)**
Not specified or ≥ 39	Not specified or ≥ 38	Not specified or ≥ 39	Not specified or ≥ 37

ATTENTION! If any of the specified validity conditions for the amplification of controls are not met, the results of the whole run are considered unreliable. In this case a repeated PCR run of the whole batch of samples is required.

The registration of Cp in any of the detection channels for a negative control can be the result of contamination (cross-contamination of samples during PCR preparation, contamination of the PCR box with genomic DNA samples or positive controls, or contamination of the laboratory with amplification products).

When Cp value for the negative control is beyond the acceptable limit (see Table 8), as well as Cp value for positive control C+ №2 on Fam/Rox detection channels (see Table 7), special procedures are required to identify possible cause and eliminate contamination. It is recommended to consider the results of the run unreliable, after elimination of contamination a repeated assay is required staring from sample preparation.

10. DATA ANALYSIS

Registration of the results is carried out automatically during amplification by the software provided with detecting thermocycler. It is possible to create and print a report based on the analysis results. Please refer to DTlite or DTprime thermal cycler's user manual for details on working with software.

1 Interpretation of the results for each sample is performed considering the Cp values of the TREC, SMN1 and KREC (Fam/Hex/Rox detection channels) and the endogenous internal control (IC) (Cy5 detection channel).

2 For samples that underwent PCR with an IC (Cy5 detection channel) Cp ≤ 31, the result is determined as reliable,

- TREC and KREC levels are evaluated according to formulas 1 and 2 respectively (see c.2) and interpreted according to Table 9, considering the lower threshold of the biological reference interval for TREC and KREC or threshold recommended for newborn screening (see Annex A).
- Interpret the results for the homozygous deletion of exon 7 of SMN1 according to Table 10.

3 For samples with IC Cp value (Cy5 detection channel) > 31, the result is considered unreliable.

An unreliable or doubtful result may be caused by insufficient volume or quality of primary biological material; violation of sample preparation protocol; the presence of inhibitors in the DNA preparation

obtained from biological material; incorrect implementation of the assay protocol; non-compliance with the amplification temperature regime, etc. In this case, either a repeated amplification of the DNA preparation, or a repeated extraction of the DNA preparation, or a repeated collection of biological material (performed sequentially) is required.

ATTENTION! The result of the test may be questionable in the following cases:

- blood transfusions, therapies that affect the neonate's leukopoiesis (e.g., administration of drug inhibitors of leukopoiesis);
- administration of drugs or other therapies during pregnancy that affect the formation of the fetal immune system.

ATTENTION! The results can be interpreted only in conjunction with the results of other laboratory and instrumental studies, the clinical picture, as well as the gestational age of the newborn.

Table 9. Interpretation of TREC and KREC results

Value	TREC	KREC
Within the biological reference interval/above the normal threshold (Annex A)	Normal	Normal
Below the biological reference interval/ newborn screening threshold (Annex A)	High risk of primary immunodeficiencies with T-cell disorders, and development of severe infectious diseases (possible PID).	High risk of primary immunodeficiencies with B-cell disorders, and development of severe infectious diseases (possible PID).
	It is recommended to repeat the assay to confirm the result, starting with sample preparation.	
Not determined (0)	Maximum risk of immunodeficiency and development of severe infectious diseases (PID, possible severe combined immunodeficiency (SCID)).	Maximum risk of immunodeficiency and development of severe infectious diseases (PID, possible severe combined immunodeficiency (SCID)).
	It is recommended to repeat the assay to confirm the result, starting with sample preparation.	
N o t e - If recorded TREC/KREC Cp ≥ 37, accuracy of TREC/KREC level determination may be reduced, in case the primary result is “Normal”, repeated assay of DBS sample is optional.		

Table 10. Interpretation of SMN1 exon 7 results.

Result on Hex detection channel	Result on Cy5 detection channel	ΔCp = Cp (Hex) – Cp (Cy5)	Interpretation
Cp specified	Cp ≤ 31	< 6.0	No deletion in exon 7 of the SMN1 gene in the homozygous state detected (N)
Cp specified	Cp ≤ 31	6.0 - 8.0	Unreliable result (?)*
Cp specified	Cp ≤ 31	≥ 8.0	Deletion of exon 7 of the SMN1 gene in the homozygous state **detected** (del)**
Cp not specified	Cp ≤ 31	Is not considered	Deletion of exon 7 of the SMN1 gene in the homozygous state **detected** (del)**
Cp specified / not specified	Cp not specified	Is not considered	Unreliable
	Cp> 31	Is not considered	
* – If a doubtful result is obtained, another assay is required, starting with the sample preparation step. If a doubtful result is reproduced, there is a high risk of a diagnosis of SMA and a quantitative MLPA or sequencing assay is recommended ** – it is recommended to confirm the result by repeating the assay starting with sample preparation N – No deletion in exon 7 of the SMN1 gene in the homozygous state detected (1 or more copies of exon 7 of the SMN1 gene determined) del – Deletion of exon 7 of the SMN1 gene in the homozygous state detected (0 copies of exon 7 of the SMN1 gene determined)			

11. SPECIFICATIONS

For biomaterial samples containing DNA of TREC, KREC, exon 7 of the SMN1 gene, and IC normalizing gene, thermal cycler software registers exponential growth of fluorescence level on the corresponding detection channels during the amplification.
For human biomaterial samples not containing DNA of TREC, KREC, or exon 7 of the SMN1 gene, there is no exponential growth of fluorescence level on the corresponding detection channels during the amplification or it is below the background value; exponential growth of fluorescence level on Hex detection channel is possible in case of homozygous deletion of SMN1 exon 7 provided the difference between Cp value on Hex channel and Cp value on Cy5 channel (Δ Cp) ≥ 8.

a. The analytical **specificity** of the **NeoScreen SMA/TREC/KREC REAL-TIME PCR Detection Kit** was confirmed using genetically engineered constructs (recombinant plasmids) corresponding to the DNA sequences of the detected indicators (TREC, KREC, SMN1, IC).

The specificity of the reagent kit was tested using bioinformatic methods. The absence of cross-reactivity for each of the oligonucleotide systems (TREC, KREC, SMN1, IC) included in the reagent kit was shown.
Specificity of TREC, KREC DNA detection in the presence of genomic (chromosomal) DNA was confirmed by testing DNA preparations of the Hela cell line, which is not characterized by rearrangement of T- and B- cell receptor genes and the presence of TREC and KREC circular molecules.
Specificity of detection of SMN1 exon 7 deletion in the homozygous state was established using two variants of genetically engineered constructs corresponding to SMN1 exon 7 and SMN2 genes, and also confirmed on clinical material.
The **NeoScreen SMA/TREC/KREC Kit** allows to detect loss of the exon 7 of the SMN1 gene in homozygous state by deletion (exon 7 or exons 7-8 of SMN1) or by conversion c.850C>T (SMN1 to SMN2 conversion).

b. Analytical **sensitivity** is 5 copies per amplification tube for each analyte (TREC, KREC, SMN1 and IC).

TREC and KREC analytical sensitivity:

- 5 copies per 10^5 leukocytes (at Cy5 Cp < 28)
- 10^3 copies per 10^5 leukocytes (at Cy5 Cp 28 ≤ Cp ≤ 31)

c. TREC and KREC measurement range:

- 15 – 10^5 copies per 10^5 leukocytes (at Cy5 Cp < 28)
- 10^3 – 10^5 copies per 10^5 leukocytes (at Cy5 Cp 28 ≤ Cp ≤ 31)

d. Accuracy characteristics:

1. Repeatability and reproducibility of TREC and KREC determinations (Lg copies/10^5 leukocytes): coefficient of variation (%CV) ≤10.

2. Correctness of TREC and KREC level determination (Lg copies/10^5 leukocytes): relative error (δx) ≤5%.

Limit (analytical) error of TREC and KREC level determination is ±0.1 Lg copies/10^5 leukocytes.
The accuracy limit of TREC and KREC level determination is ±0.3 Lg copies/10^5 leukocytes.

e. Diagnostic characteristics by TREC and KREC rate

	TREC	**KREC**
Number of samples	61	61
Diagnostic sensitivity (95% CI)	100.00% (88.78%-100.00%)	93.33% (68.05%-99.83%)
Diagnostic specificity (95% CI)	100.00% (88.43%-100.00%)	97.83% (88.47%-99.95%)

NOTE. When using the **NeoScreen SMA/TREC/KREC Kit**
- when evaluating the T cell receptor excision circles (TREC) level, the **absence or value less than the reference interval lower limit** is considered a positive result.

- when evaluating the level of the kappa-deleting recombination excision circles (KREC), the **absence or value less than the reference interval lower limit** is considered a positive result.

f. Diagnostic characteristics on homozygous deletion of exon 7 of the SMN1 gene

Diagnostic characteristics of the kit on homozygous deletion of exon 7 of the SMN1 gene determined during the research in the following groups:

Group characteristics based on MLPA and sequencing data	**Number of samples in each group**	**NeoScreen SMA/TREC/KREC exon 7 of the SMN1 gene result**	
		≥ 1 copies (N)	**0 copies (del)**
Conventional norm: SMN1 amount – 2 copies, SMN2 amount – 2 copies	54	54	0
Gene duplication (SMN1): SMN1 amount – 3 copies, SMN2 amount – 0-3 copies	23	23	0
Gene deletion carriage (SMN1): SMN1 amount – 1 copy, SMN2 amount – 0-4 copies	51	51	0
Intrinsic homozygous deletion in the gene (SMN1)[1]: SMN1 amount – 0 copies, SMN2 amount – 2 copies	9	0	9
Gene conversion (SMN1)[1]: SMN1 amount – 0 copies, SMN2 amount – 3 - 4 copies	47	0	47
Hybrid genes SMN1/SMN2 with partial deletion in the SMN1[1] gene: exon 7 of SMN1 amount - 0 copies	9	0	9
Deletion in the exon 7 of SMN1 carriage and heterozygous point mutation in the SMN1 gene (c.815A> G, rs104893922)[2]	1	1	0
Other variants[1] of gene copies' / exon 7 and 8 of SMN1 and SMN2 combination	13	11	2
Total	**207**	**140**	**67**

[1] - groups containing samples with pathogenic variant of SMN1 changes (deletion and/or conversion in exon 7 of SMN1) in homozygous state (genetic diagnosis SMA 5q)

[2] – pathogenic point mutation detected by SMN1 sequencing (combination of deletion carriage and pathogenic mutation in the SMN1 second copy – genetic diagnosis SMA 5q)

N – No deletion in exon 7 of the SMN1 gene in the homozygous state detected (1 or more copies of exon 7 of the SMN1 gene determined)

del – Deletion of exon 7 of the SMN1 gene in the homozygous state detected (0 copies of exon 7 of the SMN1 gene determined).

	Deletion in exon 7 of SMN1 in homozygous state
Number of samples	207
Diagnostic sensitivity (95% CI)	100.00% (94.64%- 100.00%)
Diagnostic specificity (95% CI)	100.00% (97.40%-100.00%)

NOTE. When using the **NeoScreen SMA/TREC/KREC Kit**

- when evaluating exon 7 of the SMN1 gene, **the presence** of deletion **in the homozygous state** is considered positive result.

g. Within-batch and between-batch precision

Within-batch precision amounts (95% CI) - 100% (97.84–100%).

Between-batch precision amounts (95% CI) - 100% (97.84–100%).

Within-batch and between-batch precision of results was confirmed in clinical laboratory tests performed on 20 samples using two reagent kits from one series, one reagent kit from the second series, and three models of detection thermal cyclers.

12. TROUBLESHOOTING

Table 11. Troubleshooting

	Result	Possible cause	Solution
C+ №1. C+ №2.	Does not meet the acceptable Cp values (see Table 6, 7)	Operation error Violation of storage and handling requirements	Repeat the assay Dispose of the current batch
C+ №2. C-	Does not meet the acceptable Cp values (see Table 7, 8)	Contamination	Dispose of the current batch Perform decontamination procedures
IC	Invalid	Low amount of DNA\ PCR inhibition	Repeat the assay Resample

If you face to any undescribed issues contact our customer service department regarding quality issues with the kit:

Phone: +7(495) 640.16.93

E-mail: hotline@dna-technology.ru

https://dna-technology.com/support

13. QUALITY CONTROL

“DNA-Technology Research & Production”, LLC declares that the abovementioned products meet the provision of the Council Directive 98/79/EC for *in vitro* Diagnostic Medical Devices. The quality control procedures performed in accordance with ISO 9001:2015 and ISO 13485:2016:

- observation of quality management in manufacturing of IVDD products;
- creation of values for customers;
- maintenance of the best service quality and customer management.

Technical support:

E-mail: hotline@dna-technology.ru

https://www.dna-technology.com

Manufacturer: "DNA-Technology Research & Production", LLC,

142281, Russia, Moscow Region,

Protvino, Zheleznodorozhnaya Street, 20

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

Seller: "DNA-Technology" LLC,

117587, Russia, Moscow,

int. ter. Municipal District Chertanovo Severnoye,

Varshavskoye shosse, 125 Zh, building 5, floor 1, office 12;

Phone/fax: +7(495) 640.17.71

E-mail: info@dna-technology.com

https://www.dna-technology.com

14. KEY TO SYMBOLS

IVD	*In vitro* diagnostic medical device		Date of manufacture
	Temperature limit		Consult instructions for use
Σ	Contains sufficient for <n> tests	REF	Catalogue number
	Use-by date		Manufacturer
LOT	Batch code		Keep away from sunlight
VER	Version	NON STERILE	Non-sterile

Annex A

Biological reference interval (newborns)

Reference interval limits	TREC, copies per 10^5 leukocytes	KREC, copies per 10^5 leukocytes
Whole blood		
Upper limit (95% reference interval)	4400	4100
Lower limit (95% reference interval)	650	600
Lower limit (99% “left-sided” reference interval)	490	480
Lower limit (99.9% “left-sided” reference interval)	320	270
Dried blood spots		
Upper limit (95% reference interval)	2900	3200
Lower limit (95% reference interval)	460	270
Lower limit (99% “left-sided” reference interval)	360	210
Lower limit (99.9% “left-sided” reference interval)	210	140
Newborn screening (NBS):	Threshold value is set according to NBS recommendations	
NOTE. Reference interval for TREC and KREC concentrations was established using a sample of 3576 dry blood spots and 733 whole blood samples from newborns using the **NeoScreen SMA/TREC/KREC Kit**; data from normal TREC and KREC ranges and **threshold values** given in medical refereed publications and Newborn screening guidelines may be used to interpret the assay results.		