LightMix® Kit TREC SMA HBB Newborn

TIB Molbiol Cat. No. 40-0621-44 Roche SAP nº 09 802 533 001

Detection Kit with primer/probes for 384 (or 192) PCR reactions [dried] for use with the Roche Diagnostics LightCycler® Instruments and LightCycler® Multiplex DNA Master.

1. Content

Table 1: Kit components.

Vial	Cap Color	Description
1x PSR	**orange**	Parameter Specific Reagents (PSR) containing premixed and dried primers and probes ≤ 62 % Synthetic oligonucleotides ≥ 38 % Buffer 3 targets detected per sample

2. Intended Use

The LightMix® TREC SMA HBB Newborn kit is an *in-vitro* diagnostic test intended to be used to screen genomic nucleic acid (DNA) extracted from dried blood spots (DBS) from newborns for (1) the absence of T-cell receptor excision circles (TREC), a sign for severe combined immunodeficiency disease (SCID), (2) homozygous deletions of survival motor neuron 1 (SMN1) exon 7, the most predominant cause for severe type 1 spinal muscular atrophy (SMA) and, optional, (3) for mutations in the region of codon 6 of the beta globin gene, commonly associated with sickle cell disease (SCD) and beta-thalassemia.

In the case that the national regulations do not permit genetic thalassemia testing, the melting analysis can be skipped.

3. Storage and Stability

Shipping:

The product is shipped at ambient temperatures.

Storage upon arrival:

Store kit cooled or at room temperature (at 4 °C to 25 °C) and protected from light. **Do not freeze dry reagents.**

Dissolved stability / On-board stability:

Once dissolved, reagents can be stored refrigerated (at 2 °C to 8 °C) for daily use for up to 30 days. For long term storage, freeze at -15 °C to -25 °C (until expiry). Minimize repeat freeze-thaw cycles (< 10) and record use.
To minimize the potential for errors associated with self-labeled tubes, do not transfer to secondary vials.

4. Additional Reagents, Instruments and Consumables

4.1 Material for specimen preparation

Manual Preparation:	**Cat. No., Manufacturer/Distributor**
CX-buffer (1x PBS/0.5 % Thesit®) CE-buffer (10 mM Tris, 0.25 mM EDTA, and 2 mM NaOH)	any supplier
DNA/RNAse-free water	any supplier
Extraction: 96 well plate and seal	any supplier

Automated sample preparation:	**Cat. No., Manufacturer/Distributor**
Instruments	
MagNA Pure 96 Instrument	06 541 089 001, Roche Diagnostics
MagNA Pure 24 Instrument	07 290 519 001, Roche Diagnostics

Consumables

MagNA Pure 96 DNA and Viral NA Small Volume Kit	06 543 588 001, Roche Diagnostics
MagNA Pure 96 Processing Cartridge	06 241 603 001, Roche Diagnostics
MagNA Pure Tip 1000 µl	06 241 620 001, Roche Diagnostics
MagNA Pure 96 Output Plate	06 241 611 001, Roche Diagnostics
MagNA Pure Sealing Foil	06 241 638 001, Roche Diagnostics
MagNA Pure 96 System Fluid (Internal)	06 430 112 001, Roche Diagnostics
MagNA Pure 96 System Fluid (External)	06 640 729 001, Roche Diagnostics
MagNA Pure 96 Internal Control Tube	06 374 905 001, Roche Diagnostics
MagNA Pure 24 Total NA Isolation Kit	07 658 036 001, Roche Diagnostics
MagNA Pure 24 Processing Cartridge	07 345 577 001, Roche Diagnostics
MagNA Pure Tube 2.0 mL	07 857 551 001, Roche Diagnostics

4.2 Material for sample analysis

Reagents

LightCycler® Multiplex DNA Master	07 339 585 001, Roche Diagnostics or 07 339 577 001, Roche Diagnostics
LightMix® Universal Color Compensation Hexaplex Plus (not for LightCycler® PRO)	06 296 971 001, Roche Diagnostics or 40-0320-12, TIB Molbiol

Optional

TREC KREC Standard Row 30-621/622	09 229 230 001, Roche Diagnostics
LightMix® Modular KREC	64-0622-96, TIB Molbiol
LightMix® Modular Actin Extraction Control	07 805 993 001, Roche Diagnostics or 66-0913-96, TIB Molbiol

Instruments

LightCycler® 480 II Instrument including LightCycler® Software Version 1.5 or higher **or**	05 015 278 001, Roche Diagnostics
cobas z 480 Analyzer including LightCycler® Software Version 1.5 or higher **or**	05 200 881 001, Roche Diagnostics
LightCycler® PRO Instrument including LightCycler® Software Version 1.X.X	09 541 713 001, Roche Diagnostics

Consumables

LightCycler® 480 Multiwell Plate 96 white	04 729 692 001, Roche Diagnostics
LightCycler® 480 Multiwell Plate 384 white	04 729 749 001, Roche Diagnostics

5. Assay Principle

The LightMix® TREC SMA HBB Newborn kit is an in-vitro diagnostic test for the simultaneous detection of the absence of TREC, deletions in SMN1 gene and mutations in codon 6 region of the beta globin gene.

Genomic DNA extracted from DBS on Guthrie cards, TNF cards or Whatman Genecards is analyzed by a multiplex PCR, a fragment of the genes of interest is amplified with specific primers and detected using labelled probes. The PCR combines the amplification of a 91 bp long fragment for detection of TREC in the 610 channel and an 82 bp fragment of SMN1 gene detected in the 580 channel. A 90 bp long fragment from the beta globin gene is also amplified and detected in the 530 channel using a SimpleProbe® with melting curve analysis.

Both TREC and SMN1 analysis detect target sequences present only in wild-type samples, with signal lost in samples with deleted genes. SMN1 and TREC serve as a mutual control, as it is unlikely that both SMN1 and TREC will be absent in the same sample.

Users intending to test for KREC or include Actin as the control assay can combine the respective assays within one multiplex PCR.

6. Medical Background

Newborn screening (NBS) is a well-established public health service in many countries worldwide, based on the testing of metabolites from dried blood spots (DBS). Genetic testing for primary immunodeficiency diseases (PID) has been developed in recent decades using the same DBS.

SCID

SCID refers to disorders characterized by a profound impairment in T-cell development; the immunodeficiency leads to severe and recurrent infections and is fatal if left untreated. TREC is a byproduct of T-cell receptor recombination that is absent in SCID patients. The presence of very low TREC may be an indication of less severe forms of SCID or lymphopenia.

However low TREC levels are also associated with premature birth (40 % of the cases) and maternal treatment with immunosuppressive agents (20 % of the cases). Trisomy 21 and DiGeorge syndrome may cause slightly lower TREC levels (Barbaro et al., 2017).

In case of a positive screening and consequently the absence of TREC, a diagnostic follow-up should be performed to determine whether the patient suffers from SCID or other forms of T-cell lymphopenia.

Prevalence 1:60,000

SMA

SMA is an autosomal-recessive neurodegenerative disease caused by mutations in the survival motor neuron 1 (SMN1) gene. The gene consists of nine exons and gives rise to the 38-kD SMN1 protein, which plays the major role in the assembly and regeneration of small nuclear ribonuclear proteins, mRNA splicing and axonal RNA transport. The SMN1 gene is located in an inverted, duplicated region of chromosome 5 containing two highly homologous copies of SMN, a telomeric copy (SMN1) and a centromeric copy (SMN2), which differ by five bases in total including a single coding base (c.840 C>T).

Approx. 95 % of SMA-affected Caucasian individuals have a homozygous deletion involving SMN1 exon 7 or exon 7 and 8. The SMN2 gene is unable to compensate for the homozygous loss of SMN1 because of its alternative splicing, however SMN2 copy numbers appear to have a modifying effect on the variable SMA phenotype.

This PCR test targets the c.840 C>T site for the absence of SMN1 only. In case of a positive screen, diagnostic follow-up should be performed to determine the type of SMA.

Prevalence 1:11,000 (Germany 1:4,000)

SCD and β-thalassemia

SCD and β-thalassemia testing is optional. If the national regulations do not permit genetic SCD testing, the melting analysis can be omitted so no globin gene data is acquired.

Thalassemias are among the most common recessively inherited diseases worldwide. They are characterized by reduced or absent production of hemoglobin (HB), resulting inchronic anemia of varying severity. There are two main types of thalassemia, α-thalassemia and β-thalassemia. β-thalassemia is caused by alterations in the hemoglobin β-gene (HBB) which encodes the β-chain; β-thalassemia lacks functional β-chains.

The HBB assay uses a wild type specific probe covering the gene region of codons 3 to 11. This region carries ~90 % of all globin mutations, including the more frequent rs334 (T>A) mutation encoding the HBS allele and rs33930165 (C>T) mutation encoding the HBC allele. Homozygous carriers of these alleles develop the well-known sickle cell anemia (see Table 2). In cases where the wild type specific melting peak is missing (only low Tm peaks or no peak at all), both alleles are either mutated (lower melting peak) or deleted (no peak).

Some variations are frequent and others are extremely rare. rs713040 is located outside of the probe region and is not visible. Characteristic melting profile for the most frequent variations are presented in Figure 3.

Table 2: Variations located next to rs334 (with frequencies).

Genomic change	AA Change	rs N°	Global	Regional	Comment
			Frequency		
NC_000011.10:g.5227002T>A	(p.Glu7Val)	rs334	1.3 %	4.7 % (Af)	HBS sickle cell anemia (HBSS)
NC_000011.10:g.5227003C>T	(p.Glu7Lys)	rs33930165	0.4 %	1.4 % (Af)	HBC sickle cell disease
NC_000011.10:g.5227003C>A	(p.Glu7Ter)	rs33930165	-	-	β-thalassemia
NC_000011.10:g.5227013G>A	(p.His3=)	rs713040	17.3 %	50.9 % (EA)	HbE

AA: Amino Acid, Af: African, EA: East Asian

7. Instructions for Use

7.1 LightCycler® 480 Instrument and cobas z 480 Analyzer

7.1.1 Dye channels and color compensation

For use with LightCycler® 480 Instruments and cobas z 480 Analyzer, use the software version 1.5 and higher. See the Instrument operator's manual for details. Program the instrument prior to reagent preparation.

Perform detection format setup based on instructions for use of LightMix® Universal Color Compensation Hexaplex Plus (40-0320-12). This color compensation is mandatory for the correct analysis. It's deactivation will generate invalid readouts of the results.

Table 3: Detection format parameter settings necessary (instrument-specific filter combinations, quantification factor and integration time) for individual channels.

Channel / Instrument	500 n.a.	530 HBB	580 SMN1	610 TREC	640 KREC*	660 Actin*
LightCycler® 480	450-500	483-533	523-568	558-610	558-640	615-670
LightCycler® 480 II	440-488	465-510	533-580	533-610	533-640	618-660
cobas z 480	-	465-510	540-580	540-610	540-645	610-670
Quant Factor *	10	10	10	10	10	10
Max Integration Time*	1 sec	1 sec	1 sec	2 sec	3 sec	3 sec

Note: *Channel 640 (KREC) and channel 660 (Actin Extraction Control) are optional.

For the HBB assay result interpretation only the melting temperature (Tm) is used. Any amplification in the 530 channel is negligible.

7.1.2 Instrument programming

Programming the cycling conditions

Detection Format: **Hexaplex**
Reaction Volume: **10 µl** (or 20 µl)

The protocol consists of 3 program steps:

1) Denaturation of sample and activation of the enzyme
2) Cycling PCR-amplification of the target DNA
3) Cooling of the Instrument

Table 4: Cycling condition programming.

Program Step:	***Denaturation***	***Cycling***			***Cooling***
Parameter					
Analysis Mode	None	Quantification mode			None
Cycles	1	45			1
Target °C	95	95	61	72	40
Hold hh:mm:ss	00:05:00	00:00:05	00:00:15	00:00:15	00:00:30
Ramp Rate °C/s **96-well**	4.4	4.4	2.2	4.4	1.5
Ramp Rate °C/s **384-well**	4.6	4.6	2.4	4.6	2.4
Acquisition Mode	None	None	**Single**	None	None

Note: Store the program and the default values as 'RUN Template', which can be loaded to start every run.

7.1.3. Optional: HBB melting analysis (second run)

Detection Format: **SimpleProbe®**
Reaction Volume: **10 µl** (or 20 µl)

The protocol consists of one program step: Melting

Table 5: Melting curve programming.

Program Step	*Melting*		
Parameter			
Analysis Mode	Melting curves mode		
Cycles	1		
Target °C	95	40	75
Hold hh:mm:ss	00:00:30	00:02:00	00:00:00
Ramp Rate °C/s **96-well**	4.4	1.5	-
Ramp Rate °C/s **384-well**	4.8	2.5	-
Acquisition Mode	-	-	Continuous
Acquisitions per °C	-	-	3

7.2 LightCycler® PRO Instrument

For use with LightCycler® PRO Instruments, LightMix® Kit TREC SMA HBB Newborn can only be used alone or in combination with LightMix® Modular Actin Extraction Control.
Use the software version 1.X.X. See the LightCycler® PRO System User Assistance for details.
For a matching LightCycler® Analysis Package (LCAP) file for LightMix® Kit TREC SMA HBB Newborn please visit navifyportal.roche.com to download.
The kit-specific run profile is part of the LCAP and equivalent to the run conditions shown above.

LightCycler® Analysis Package:	
0501_Newborn384	TREC SMA HBB Newborn, optionally with Actin extraction control Amplification and Melting, 384-well plate, 10 µl reaction volume
0502_Newborn96	TREC SMA HBB Newborn, optionally with Actin extraction control Amplification and Melting, 96-well plate, 20 µl reaction volume

Save the LCAP file in the assay folder of the SFTP or USB device.
Import and install the downloaded LCAP onto the LightCycler® PRO Instrument and Activate it.
Create or import a plate setup in the Plates tab.

8. Experimental Protocol

8.1 Specimen preparation

Manual workflow (for DNA purification protocol see also Czibere et al, 2020)

Use 3.2 mm diameter DBS from the Gutherie card, TNF card (Ahlstrom-Munksjö) or Whatman Genecard. Transfer the DBS into a 96-well PCR plate. Soak the DBS at room temperature (19 °C to 24 °C) for 10 min in 50 µl water on a shaker (200 rpm), add 150 µl CX-buffer and shake for another 10 min. Centrifuge for 5 min at 1,000 rpm, remove the supernatant and add another 150 µl CX-buffer. Centrifuge for 5 min at 1,000 rpm, remove the supernatant and add 150 µl water. Centrifuge for 5 min at 1,000 rpm, remove the supernatant and add 50 µl CE-buffer. Seal the plate and incubate for 10 min at 92 °C.

Automated workflow

Homogenize three punches from a DBS for a Guthrie, TNF card or Whatman Genecard in 600 µl of PBS, transfer 200 µl of the supernatant into a MagNA Pure processing cartridge. Perform nucleic acid extraction on the Roche Diagnostics MagNA Pure 96 instrument, using the 'DNA and Viral NA Small Volume Kit' following the MagNA Pure instrument instructions. Alternatively the nucleic acid extraction may be performed on the Roche Diagnostics MagNA Pure 24 instrument, using the ‘MagNA Pure 24 Total NA Isolation Kit’.

Sample Material: purified genomic DNA in aqueous solution

8.2 Preparation of PSR and Reaction Mix

8.2.1 Preparation of PSR

Check the tube for the colored pellet. Add 200 µl PCR-grade water, mix the solution (vortex) and spin down. For robotic pipetting the volume can be extended to 210 µl (signals will decrease by ~ 5 %).

►**Use 0.5 µl PSR per 10 µl reaction**, or 1 µl PSR per 20 µl reaction.

8.2.2 Preparation of the LightCycler® Multiplex DNA Master

Thaw the solutions and briefly spin vials in a micro centrifuge before opening. Mix carefully by pipetting up and down or vortex briefly and spin down. Keep the solution cooled.

8.2.3 Preparation of the Reaction Mix

The table below describes reaction mix preparation for the triplex reactions. Include at least one 'No Template Control' (NTC) in every run.

In a cooled tube, prepare the reaction mix by multiplying the single reaction volumes by the number of reactions including the controls plus one additional reaction; the smallest recommended pipetting volume is 1 µl.

Table 6: Preparation of the Reaction Mix.

Reaction Mix to be used with LightMix® Kit TREC SMA HBB Newborn		
LightCycler® Multiplex DNA Master	Roche Cat. No. 07 339 577 001 Roche Cat. No. 07 339 585 001	
One reaction 10 µl	**Component**	**One reaction 20 µl**
2.5 µl	Water, PCR-grade	10.0 µl
0.5 µl	PSR LightMix® Kit TREC SMA HBB Newborn (orange cap)	1.0 µl
--	PSR LightMix® Modular KREC – substitute water	--
--	PSR LightMix® Modular Actin Extraction Control – substitute water	--
2.0 µl	qPCR Reaction Mix, 5x conc. (LightCycler® Multiplex DNA Master)	4.0 µl
5.0 µl	**Volume of Reaction Mix**	**15.0 µl**

Mix gently, spin down and **transfer 5 µl** (or 15 µl) reaction mix to each well in a cooled 384 (or 96) well PCR plate.

Add 5 µl of sample or control DNA (not provided in this kit) to each well for a final reaction volume of 10 µl (or 20 µl). Use PCR grade water instead of sample to perform at least 1 NTC. Seal plate and centrifuge for 3 min at 2000 × g.

LightCycler® PRO Instrument: Assign the plate setup. The plate setup is automatically assigned when you load a plate with a barcode that matches the plate ID of an existing plate setup.

Start the run.

Do not touch the sealing foil without gloves.
Avoid prolonged waiting periods before starting the run.

9. Data Analysis

9.1 Data Analysis on LightCycler® 480 Instrument and cobas z 480 Analyzer:

The use of a color compensation file generated with the LightMix® Color Compensation Hexaplex Plus kit is mandatory for multiplex PCR on LightCycler® 480 Instruments and cobas z 480 Analyzers, but not for LightCycler® PRO Instruments.

Perform data analysis as described in the LightCycler® 480 Instrument Operator's Manual. Use the Second Derivative Maximum method (“Abs Quant / 2nd Derivative Max”).

Result analysis involves a general-use software which has not specifically been adapted for analysis of these assays Crossing Point (Cp) value of each sample is calculated automatically. The instrument software tentatively calls results as Positive (red plot), Negative (green plot), or Uncertain (blue plot) in the amplification plot view.

Software Positive, Negative, and Uncertain results must be verified by the operator to avoid the reporting of false results.

9.1.1 Run validity criteria:

The amplification of the SMN1 and the TREC gene can be used as control reaction since the normal status of a sample is a positive PCR result (no disease risk).

- The NTC must show no amplification (and no melting curve if a second run with melting was performed) otherwise the entire run is invalid and must be repeated.
- If a positive control is used it must be positive in the respective channel.

Check for amplification curves of samples in channels 580 and 610:

- Results for each sample are valid if the reaction signal in one channel is absent but an amplification is present in the other channel
- If the reaction signal is absent in both the 580 and 610 channels the sample preparation and detection should be repeated to confirm the result.
- If the reaction signals are absent for all samples, the run is not valid and results cannot be used.

9.1.2 Amplification analysis:

- Visually inspect each sample in 580 and 610 channels and identify clearly visible amplification curves.
- Document all samples with amplification shown in both channels.
- Document all samples with amplification in only one channel.
- Compare Cp values of both channels for each sample. signals in 610 channel usually are delayed by 1-4 cycles compared to signals in 580 channel.
- Identify samples which show very delayed signals in 610 channel compared to 580 channel.

9.1.3 Melting curve analysis (optional for HBB analysis):

If a second run with melting was performed, open a new analysis for “Tm Calling”.
The results for the melting curve analysis will show one, two, and very rarely no melting peaks.
Identify and document the Tm values for each sample.

9.2 Data Analysis on LightCycler® PRO Instrument

Perform data analysis as described in the LightCycler® PRO System User Assistance.
Review and approve the results in the Target Results tab. It is necessary to approve the results of internal controls before approving the results of unknown samples. Visually inspect each sample in R6G and Red 610 channels and identify clearly visible amplification curves. It is possible to overwrite results, overwritten results will be flagged. In the Sample Results tab approved results can be released.

10. Result Interpretation

10.1 TREC – Channel 610 / Red 610:

- If a signal is present, the sample contains detectable TREC DNA.
- If a signal is absent, the sample is missing the T-cell receptor extinction cycles. A diagnostic follow up should be performed to determine whether the patient suffers from SCID or other forms of T-cell lymphopenia.
- Very delayed TREC signals can be associated to SCID but also to premature birth and other factors. Further analysis should be performed.

10.2 SMN1 gene – Channel 580 / R6G:

- If a signal is present, the sample does not contain a homozygous SMN1 deletion.
- If a signal for SMN1 is absent but amplification is present in channel 610, a follow up diagnostic should be performed to verify and determine the type of SMA.

10.3 HBB mutation – Channel 530 / FAM:

- One peak with a Tm of ~66 °C indicates no mutation detected within the probe binding region (wild-type could be expected).
- Two peaks; one peak with a Tm ~66 °C and one peak with a Tm ≤ 66 °C is a heterozygous genotype. The most frequent mutation rs334 melts at ~61 °C and indicates a heterozygous carrier of the HbS allele and is indicative for SCD.
- If the wild type peak with Tm ~66 °C is missing (one or two peaks with lower Tm are present or no peak detected at all) both alleles are mutated and the result indicates SCD and/or β-thalassemia.

This is a screening-test to identify the cases of potential SCD or other β-thalassemia. Forward these samples to an additional test, like MSMS, HPLC or sequencing, to verify if a SCD or β-thalassemia will be developed in this newborn.

The targeted region of the globin gene is rich in polymorphisms. Neighbored mutations, in particular rs33930165 (70613 G>A) or rs35497102 (70619-70620 delAA) as well as rs34548294 (70624-70625 insT) will cause also lower Tm peaks. If the sample generates an unexpected melting curve profile report the deviations to service@tib-molbiol.de.

Table 7: Result interpretation for individual channels. The value of the Tm has been established on the LightCycler® 480 Instrument and may vary between different instruments.

Channels				Results
530 / FAM HBB	580 / R6G SMN1	610 / Red 610 TREC	530 / FAM HBB MELT	
not relevant	Amplification	Amplification	One peak Tm ~66 °C	No mutation detected
not relevant	Amplification	No Amplification	One peak Tm ~66 °C	TREC absence detected
not relevant	No Amplification	Amplification	One peak Tm ~66 °C	SMN1 deletion detected
not relevant	Amplification	Amplification	Two peaks Tm ~61 °C & ~66 °C	heterozygous HBB mutation detected
not relevant	Amplification	Amplification	One peak ~61 °C	homozygous HBB mutation detected
No Amplification	Amplification	Amplification	No peak	HBB deletion detected
not relevant	No Amplification	No Amplification	No peak	Failure/Repeat

Notes: The values of the Tm may vary ± 2.5 °C between different runs. The ΔT between the melting peaks for heterozygous genotypes may vary ± 1.5 °C. Approx. 0.1 % of all tested samples are reported to give very low TREC (or KREC) levels.

11. Typical Data for Amplification and Melting

These assays are designed to show no amplification if the target is absent or deleted. The amplification and melting curves displayed below show the presence of the target.

11.1 Data generated on LightCycler® 480 Instruments

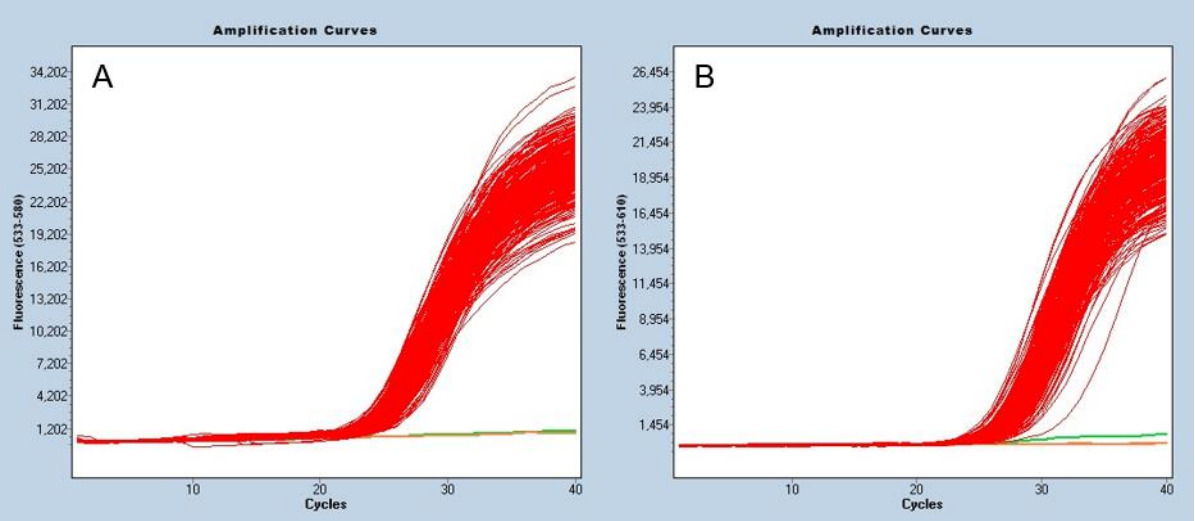

Figure 1: Amplification curves in the presence and absence of target.
A) SMN1 detection in channel 580 (red) and SMN1 deletion (green) or NTC (orange).
B) TREC detection in channel 610 (red) and TREC absence (green) or NTC (orange).

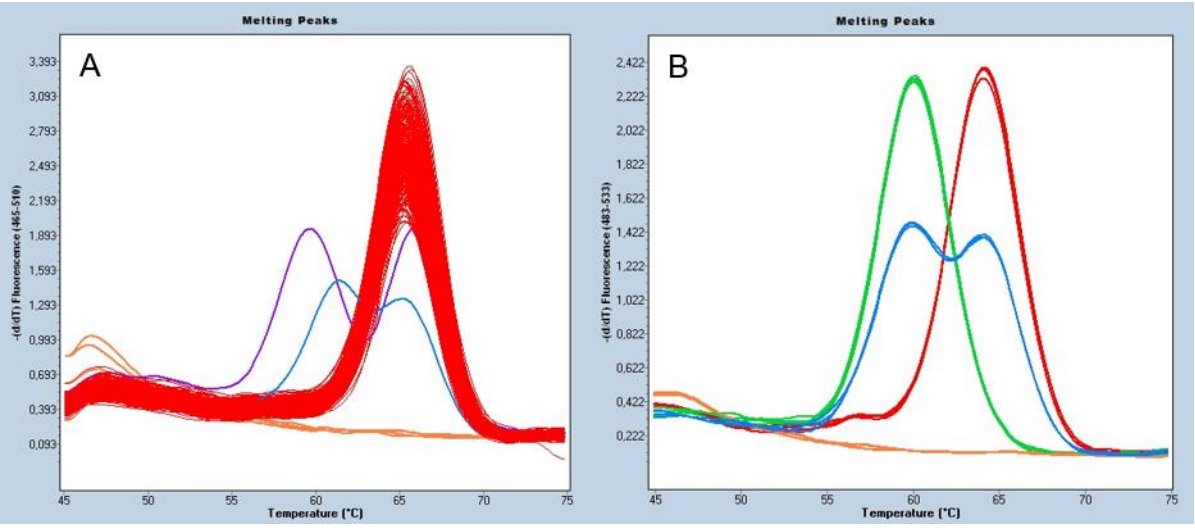

Figure 2: Melting curve analysis results for HBB in channel 530.
A) One peak for wild type (red), two peaks for heterozygous HBB/HBS (blue) and HBB/HBC (violet), NTC are shown in orange.
B) One peak with Tm of ~66 °C (red) for wild type. One peak with Tm ~61 °C (green) double mutated rs334 (SCD). Two peaks (blue) show heterozygous (HBB/HBS). No peaks (orange) for NTC.

11.2 Data generated on LightCycler® PRO Instruments

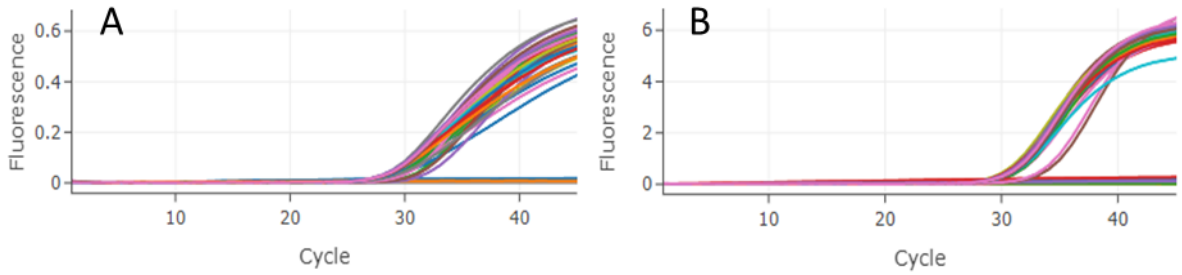

Figure 3: Amplification curves in the presence and absence of target.
A) SMN1 detection in channel R6G (Reactive) and SMN1 deletion (Non-Reactive) or NTC (Valid).
B) TREC detection in channel Red 610 (Reactive) and TREC absence (Non-Reactive) or NTC (Valid).

Cq values and Fluorescence levels generated on the LightCycler® PRO Instruments may differ compared to Cp values and Fluorescence levels generated by LightCycler® 480 Instruments or cobas z 480 Analyzers due to different calculation methods.

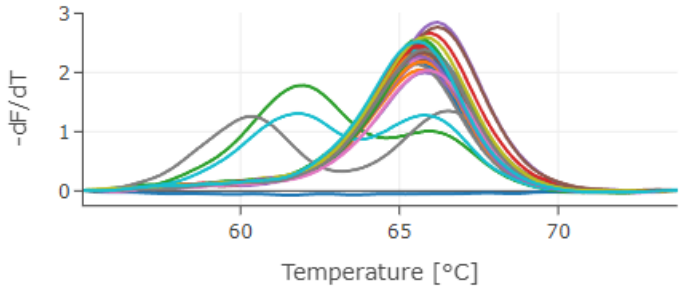

Figure 4: Melting curve analysis results for HBB in channel FAM.
One peak for wild type, two peaks for heterozygous HBB/HBS (green and teal) and HBB/HBC (grey), NTC are shown in blue.

12. Performance Characteristics

The shown data were generated on LightCycler® 480 Instruments.

Analytical sensitivity:

Every lot is verified to detect at least ten (10) copies per reaction.

The analytical sensitivity has been determined in multiplex PCR to be in the range of 7 copies for TREC and 9 copies for SMN1.

Analytical specificity:

The specificity to the targeted genes and the suitability of the PCR amplification was demonstrated by in-silico analysis and sequencing of the amplicons.

Evaluation studies:

A study performed in 2017 of 1,000 samples tested by PCR reported a sensitivity and specificity of 100 % for the detection of TREC absence.

A study performed in 2018, that included 200,000 DBS for the detection of SMN1 gene deletion, reported a sensitivity and specificity of 100 % (Czibere et al., 2020).

A study performed in 2019, that included 5,000 samples that were tested for TREC / KREC / SMA reported a Sensitivity and specificity of >99.99 % for both TREC and SMA.

Table 8: Summary of the performance data.

Channel	530	580	610
Target	**HBB**	**SMN1**	**TREC**
LoD in multiplex (copies/rxn)	< 10	8.7	7.1
Specificity	passed	passed	passed
Diag. Sensitivity	n.d	> 99.99 %	> 99.99 %
Diag. Specificity	n.d	> 99.99 %	> 99.99 %
Prevalence (Lit.)	n.d	1:11,000	1:60,000
PPV	n.d	100 %	100 %
NPV	n.d	100 %	100 %

n.d. = not determined Limit of Detection (LoD) values for 20 µl reactions with 5 µl sample in 96 well plates.

13. Assay Limitations

All results should be interpreted by a trained professional in conjunction with the patient information (e.g. premature birth). Interpretation of results generated by this test should take into consideration the possibility of false results. Negative results do not exclude the presence of the respective target. Molecular test results should not be the sole basis of a patient treatment.

False positive results may occur from cross-contamination of nucleic acid or amplified product; a false positive result leads to the disease risk being overseen. False negative PCR results (no amplification) should be confirmed in a second test.

The test is not validated for quantitative testing.

14. Precautions and Warnings

Handling requirements

This product is an in-vitro diagnostic device that must be used by qualified personnel only. Before using this product, read the operator / safety instructions in the instruments operator's manual.

General precautions for the handling of generic laboratory materials are required.

The laboratory workflow should follow good laboratory practices. Due to the risk of contamination, PCR preparation and PCR amplification should be performed in physically separated areas.

- Do not mix reagents from different lots.
- Do not use the reagents after the expiration date.
- Use the version of the manual delivered with the kit (see kit label).

Laboratory procedures

All materials of human origin and related waste must be considered as potentially infectious.

- Thoroughly clean all work surfaces and treat with disinfectants approved by local authorities.
- Do not eat, drink or smoke in the laboratory working area.
- Do not pipet by mouth.
- Wear disposable protective gloves, laboratory coats and adequate eye protection during the handling of samples and set components.
- Always use nuclease-free techniques.
- Use of filter tips is essential
- Thoroughly wash your hands after handling the samples and the sets components.

Amplification and detection

Before using this product, please read the LightCycler® 480 Instrument Operator's Manual. In the plate set up enter sample identifiers in each position. Check LightCycler® Instrument settings and make sure that they match those reported in this manual. Do not touch the plate cover without gloves.
Please refer to all the operator and safety instructions of the LightCycler® Instrument.
LightCycler® 480 plates can be extremely hot when removed from the instrument; use appropriate protection.

Result interpretation and reporting

Molecular test results should not be the only basis for a treatment decision.

Handling of waste materials

Dispose all reagents and inactivate waste materials according to the current local guidelines.

15. References

DBS

- Optimized DNA extraction from neonatal dried blood spots: application in methylome profiling. Ghantous et al., 2014

TREC

- Assessment of thymic output in adults after haematopoietic stem-cell transplantation and prediction of T-cell reconstitution. Douek et al., 2000
- Quantification of T-cell receptor excision circle DNA using fluorescence resonance energy transfer and the LightCycler system. Loeffler et al., 2002
- Development of a routine newborn screening protocol for severe combined immunodeficiency. Baker et al., 2009
- Simultaneous quantification of recent thymic T-cell and bone marrow B-cell emigrants in patients with primary immunodeficiency undergone to stem cell transplantation. Sottini et al., 2010
- Neonatal screening for severe primary immunodeficiency diseases using high-throughput triplex real-time PCR. Borte et al., 2012
- Newborn Screening for Severe Primary Immunodeficiency Diseases in Sweden—a 2-Year Pilot TREC and KREC Screening Study. Barbaro et al., 2017

SMA

- 1000 sample comparison of MLPA and RT-PCR for carrier detection and diagnostic testing for Spinal Muscular Atrophy Type 1. Strom et al., 2013
- High-throughput genetic newborn screening for spinal muscular atrophy by rapid nucleic acid extraction from dried blood spots and 384-well qPCR. Czibere et al., 2020

16. Certificate of Origin

Product is not from human, animal or plant origin. Country of Origin: Germany.

17. Version History

Version	Event	Date
V230316	Editorial changes and minor corrections	2023-03-16
V230511	Complete revision and figure update	2023-05-11
V231115	Addition of the LightCycler® PRO and editorial changes	2023-11-15

Manufacturer and Contact Details

Report IVD device observations, deviations and problems to service@tib-molbiol.de and your local Roche representative. Please report lot number(s) and a brief description.

TIB MOLBIOL Syntheselabor GmbH | Eresburgstr. 22-23 | D-12103 Berlin | Germany
Tel. +49 30 78 79 94 55 | FAX +49 78 79 94 99 | dna@tib-molbiol.de | WWW.TIB-MOLBIOL.COM
EORI DE 4806433 | Registry Court Berlin Charlottenburg HRB 93163 B

CE IVD