HbS-HbC mpx RealFast™ Assay

REF 7-280 | Σ 100 reactions

-30°C -15°C | For research use only.

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1. Intended Use

The HbS-HbC mpx RealFast™ Assay is a non-automated real-time PCR test for the detection of the human *hemoglobin subunit beta* (*HBB*) gene variants c.19G>A (HbC) and c.20A>T (HbS). The assay is a first-tier test for screening sickle cell disease (SCD) and hemoglobinopathies such as hemoglobin C disease or hemoglobin S-C disease. The qualitative assay determines the homozygous or heterozygous presence of *HBB* c.19G>A and c.20A>T in a human genomic DNA extract from blood. Reference sequence: NG_059281.1

2. Introduction

Sickle cell disease (SCD) is a monogenic red blood cell disorder, where normal hemoglobin (HbA) is replaced by sickle hemoglobin (HbS). SCD is inherited as an autosomal codominant trait. Common types of SCD include homozygous hemoglobin SS disease, hemoglobin SC disease, and sickle beta-thalassemia. Globally, there are approximately 300,000–400,000 newborns with SCD, with over 75% of them in Africa. The World Health Organization (WHO) has estimated that SCD may contribute up to 16% of under-five mortality in some high-burden African countries where 20–30% of the population has sickle cell trait (SCT) and 1–2% of infants have SCD. Apart from HbS, there are other Hb variants (non-S sickling Hb variants) that cause sickle cell disease.

3. Kit Contents

		100 rxn
RealFast™ 4x LIT Mix	☐ 1 vial white cap	550µl
HbS-HbC mpx Assay Mix	■ 1 vial purple cap	275µl
HBB WT-Control	■ 1 vial green cap	75µl
HbSC MUT-Control	■ 1 vial red cap	75µl

The RealFast™ 4x LIT Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The HbS-HbC Assay Mix consists of *HBB* gene-specific primers and dual-labeled hydrolysis probes for *HBB, HbC and HbS*. A wild-type and a mutant control are supplied with the kit.

The kit contains reagents for 100 reactions in a final volume of 20 µl each.

4. Storage and Stability

HbS-HbC mpx RealFast™ Assay is shipped on cooling blocks. On arrival, store the kit at -30°C to -15°C. Alternatively, store at 2°C to 8°C for short-term use within one month. The kit withstands up to 20 freeze/thaw cycles with no loss of activity. Avoid prolonged exposure to intense light. If stored correctly, the kit will retain full activity until the expiration date indicated on the label.

5. Product Description

5.1. Principle of the Test

The test is based on the fluorogenic 5' nuclease assay, also known as TaqMan® assay. Each reaction contains gene-specific primer pairs that amplify an 85 bp fragment of the *HBB* gene, which also serves as the PCR control. Further components are two dual-labeled, gene-specific hydrolysis probes that hybridize to the target sequence of the corresponding fragment. The proximity of the 5'-fluorescent reporter and 3'-quencher dye on intact probes prevents the reporter from fluorescing. During the extension phase of PCR the 5' – 3' exonuclease activity of the Taq DNA polymerase cleaves the 5'-fluorescent reporter from the hybridized probe. The physical separation of the fluorophore from the quencher dye generates a fluorescent signal in real time, which is proportional to the accumulated PCR product.
In samples positive for HBB, the **Cy5.5-labeled HBB** probe binds to the gene fragment and a strong fluorescence signal is detected in the Cy5.5 channel (711 nm). In samples positive for HbC, (HBB c.19G>A) the **HEX-labeled HbC** probe binds to the gene fragment and a strong fluorescence signal is detected in the HEX channel (556 nm). In samples positive for HbS (HBB c.20A>T), the **Cy5-labeled HbS** probe binds to the gene fragment and a strong fluorescence signal is detected in the Cy5 channel (670 nm). In samples negative for HbC and HbS only the Cy5.5-labeled HBB control probe hybridizes to the *HBB* gene fragment. Accordingly, a strong fluorescence signal is detected in the Cy5.5 channel and no or only baseline signals in the HEX and Cy5 channels. In samples compound heterozygote for HbC and HbS, strong fluorescence signals are detected in the HEX and Cy5 channels and no or only a baseline signal in the Cy5.5 channel.

5.2. Real-time PCR Instrument Compatibility

The HbS-HbC mpx RealFast™ Assay is validated for use with the Bio-Rad CFX 96, Bio-Rad CFX Opus 96, and Applied Biosystems Quantstudio 5 real-time PCR instruments calibrated for Cy5.5, Cy5 and HEX. The HbS-HbC mpx RealFast™ Assay can be combined with SMN1 RealFast™ Assay (REF 7-700). In this case, calibration of the realtime PCR device for Cy5.5, Cy5, HEX, FAM, and ROX is required.

- ✓ AB QuantStudio 5 (Applied Biosystems)
- ✓ CFX96 (Bio-Rad)
- ✓ OPUS 96 (Bio-Rad)

The kit is supplied **without** PCR-grade water.

5.3. Assay Performance Specifications

The HbS-HbC mpx RealFast™ Assay is for research use only.
Analytical performance data: currently not available.
Limit of detection: data not available.
Recommended DNA concentration: 0.2 to 10 ng/µl genomic DNA.

6. Materials Required but not Supplied

Real-time PCR instrument with Cy5.5 (711 nm), Cy5 (670 nm) and HEX (556 nm) filters*), instrument-compatible reaction vessels, disposable powder-free gloves, vortexer, mini-centrifuge for 2.0 ml tubes, tube racks, set of calibrated micropipettes (0.5 – 1000 µl), sterile tips with aerosol-barrier filter, nuclease free water, DNA extraction system, freezer, biohazard waste container.

*) *The combination of HbS-HbC mpx RealFast™ Assay and SMN1 RealFast™ Assay requires Cy5.5 (711 nm), Cy5 (670 nm), HEX (556 nm), FAM (520 nm) and ROX (605 nm) filters.*

7. Experimental Protocol

7.1. DNA Extraction

DNA extraction reagents are **not supplied** with the kit.
Analytical performance data concerning DNA isolated from various specimens (e.g., whole peripheral blood, dried blood spots) is under evaluation. Ensure extracted DNA is suitable for amplification in terms of concentration, purity, and integrity.
For accurate genotype calling, the DNA amount per reaction should be within the range of 1 to 50 ng for all samples.

7.2. PCR Controls

Always include the **HBB WT-Control** as a positive reference signal for HBB (Cy5.5 channel) and negative reference for HbC (HEX channel) and HbS (Cy5 channel).
Always include the **HbSC MUT-Control** as positive reference signals for HbC (HEX channel) and HbS (Cy5 channel).
Always include an **NTC** in each experiment to confirm the absence of potential contamination. It is advisable to run the NTC (use PCR-grade water instead of DNA) in duplicates.

*» **Note:** The Controls are potential sources of contamination. Make sure to handle them carefully. «*

7.3. Preparation of HbS-HbC mpx RealFast™ **Master Mix**
Gently vortex and briefly centrifuge all solutions after thawing. Set up PCR at room temperature. Prepare sufficient **Master Mix** for all your reactions (N samples + positive control + negative controls) plus at least one additional reaction to compensate for pipetting inaccuracies:

Component	per reaction	e.g. 24+1 reactions
RealFast™ 4x LIT Mix	5 µl	125 µl
HbS-HbC mpx Assay Mix	2.5 µl	62.5 µl
PCR grade water	7.5 µl	187.5 µl
Master Mix	**15 µl**	**375 µl**

Dispense **15 µl Master Mix** into each well. Add **5 µl** purified **DNA** or **Control** template to reach a final reaction volume of 20 µl.
To minimize the risk of contamination, always pipette templates in the following order: first NTC, then samples, and last controls. Immediately close reaction vessels.

*» **Note:** Avoid creating bubbles in the final reaction mix and avoid touching the optical surface of the cap or sealing film without gloves. Both may interfere with fluorescence measurements. Centrifuge briefly if needed. «*

7.4. PCR Program
Program the real-time PCR instrument according to the manufacturer's instructions for quantitation experiments with two targets/reporter dyes. Place the samples into the thermal cycler and run the following program:

QuantStudio 5, CFX96, CFX Opus 96, and other Peltier heating block-based instruments

Cycles	Temp	Time	Steps
1	95°C	3 min	Initial denaturation
40	95°C	15 sec	Denaturation
	60°C	1 min	Annealing/Extension – **Data acquisition** in Cy5.5, Cy5, and HEX channels

8. Data Analysis / Interpretation of Results

The presence or absence of the HbC (*HBB* c.19G>A) allele is defined by whether there is a signal in the **HEX channel** or not. The presence or absence of the HbS (*HBB* c.20A>T) allele is defined by whether there is a signal in the **Cy5 channel** or not. Successful PCR can be verified by the successful amplification of the *HBB* gene detected in the **Cy5.5 channel** (HBB) in *HBB* heterogenous or *HBB* homozygous samples. Genomic DNA samples compound positive for HbC and HbS show amplification in both the HEX and the Cy5 channel, but no or only basal Cy5.5 fluorescence signals. The signals for both the WT and MUT Control must be correct in every run. Should either Control show discrepant results, the run is invalid and needs to be repeated. Fluorescent levels and amplification curves are automatically displayed in amplification plots in the real-time PCR software.

Sample Type	Amplification in **Cy5.5** channel (711 nm)	Amplification in **Cy5** channel (670 nm)	Amplification in **HEX** channel (556 nm)	Genotype
HBB WT-Control	**YES**	NO	NO	HBB
HbSC MUT-Control	NO	**YES**	**YES**	HbC / HbS
Sample 1	**YES**	**YES**	NO	HBB / HbS
Sample 2	**YES**	NO	**YES**	HBB / HbC
Sample 3	NO	**YES**	**YES**	HbC / HbS
Sample 4	NO	**YES**	NO	HbS homozygous
Sample 5	NO	NO	**YES**	HbC homozygous
NTC	NO	NO	NO	

Some instrument software may need manual threshold settings for accurate analysis.

Recommendations for Threshold Settings (Cq):
Set threshold value for the Cy5.5, Cy5 and HEX channels just above the background fluorescent signal generated by the NTC. Samples crossing the threshold line beyond Cq 37 give invalid results and must be repeated.

To analyze acquired data, please follow your instrument software instructions.

9. Warnings and Precautions

- Always use disposable powder-free gloves and wear a lab coat when handling specimens and reagents.
- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- Use pipettes dedicated to PCR setup only, use aerosol-guarded pipette tips.
- Use instrument-compatible reaction vessels with optically clear caps or sealers.
- Do not mix reagents from different lots.
- Do not use expired kits or kit components.