SCID-XLA mpx RealFast™ Assay

REF 7-710 | 100 reactions

-30°C | -15°C | For research use only.

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1. Intended Use

The SCID-XLA mpx RealFast™ Assay is a non-automated real-time PCR test for the semi-quantitative detection of T-cell receptor excision circles (TREC) and kappa-deleting recombination excision circles (KREC) and beta-actin (ACTB) in a single reaction. TREC and KREC copy number determination is based on simultaneous detection and relative quantification of endogenous *ACTB* gene copies present in DNA extracted from fresh dried blood spot (DBS) samples from neonates on filter paper (Guthrie-cards).

Beta-Actin (ACTB): Ref.Seq. NG_007992.1
T-cell receptor excision circles (TRECs): Human T-cell receptor alpha delta locus: GenBank: AE000521.1
Kappa-deleting recombination excision circles (KREC): Homo sapiens immunoglobulin kappa locus, proximal V-cluster and J-C cluster (IGK-proximal) on chromosome 2: NCBI Reference Sequence: NG_000834.1

2. Introduction

Primary immunodeficiencies (PIDs) are rare inherited disorders of the immune system. Most patients present with impaired T- and/or B-cell function, resulting in defective immunity. Among PIDs, severe combined immunodeficiency (SCID) represents a heterogeneous group of life-threatening disorders defined by the absence of functional T-cells. Apart from rare non-genetic SCID-like conditions, several distinct monogenic defects - most commonly inherited in an autosomal-recessive manner - account for the majority of SCID cases. SCID can be classified according to the underlying molecular pathology, including impaired cytokine-mediated signaling, defects in V(D)J recombination, impaired T-cell receptor (TCR) signaling, increased lymphocyte apoptosis, absence or abnormal development of the thymus, and impaired calcium flux, among others. In addition to the absence of naïve T cells, most SCID genotypes exhibit a characteristic immunophenotype defined by the presence or absence of B cells and natural killer (NK) cells, leading to a stratification into T-B-NK-, T-B+NK-, T-B-NK+, and T-B+NK+ subtypes. Of these, the T-B+NK- subtype accounts for up to 50% of cases. The thymus plays a central role in generating a diverse population of peripheral T cells.
B-cell development, by contrast, occurs in the bone marrow and depends on a complex signaling cascade. Disruptions of this process may cause maturational arrest in early B-cell development, leading to humoral immunodeficiency with decreased or absent antibody production. Among primary B-cell immunodeficiencies, X-linked agammaglobulinemia (XLA) accounts for up to 85% of agammaglobulinemia cases with a defined genetic etiology. The long-term prognosis of infants with SCID and other severe immunodeficiencies can be markedly improved by early diagnosis, ideally before the onset of severe infections. In this context, T-cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs) provide valuable diagnostic markers. These circular DNA byproducts are generated during the recombination processes that create T- and B-cell receptors. Quantification of TRECs and KRECs in peripheral blood serves as a surrogate measure of thymic and bone marrow output.

3. Kit Contents

RealFast™ 4x LIT-Mix	1 vial	white cap	550 µl
SCID-XLA Assay Mix	1 vial	purple cap	275 µl
SCID Control	1 vial	green cap	75 µl
SCID Control Dilution Buffer	3 vials	transparent cap	75 µl

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

The RealFast™ 4x LIT-Mix comprises HotStart Taq DNA polymerase and dNTPs in an optimized buffer system. The SCID-XLA Assay Mix consists of gene-specific primers and dual-labeled hydrolysis probes for *ACTB*, TREC and KREC. SCID Control is a tripartite plasmid (TREC/*ACTB*/KREC) used to generate a standard curve.

4. Storage and Stability

The SCID-XLA mpx RealFast™ Assay is shipped on cooling blocks. On arrival, store the kit at -30 to -15°C. Alternatively, store at 2 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 a 82 bp fragment of the TREC sequence, a 87 bp KREC fragment and a 70 bp fragment of the *ACTB* gene. Further components are dual-labeled, sequence-specific hydrolysis probes (a **Cy5-labeled TREC probe**, a **HEX-labeled KREC probe** and a **Cy5.5-labeled *ACTB* probe**) 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.
The SCID-XLA mpx RealFast™ Assay is a semi-quantitative assay and infers the amount of all three nucleic acid targets by comparison to distinct standard curves obtained from serial dilutions of the **SCID Control** (TREC/ACTB/KREC plasmid). The *ACTB* gene serves as a reference for normalization of TREC and KREC levels.

5.2. Real-time PCR Instrument Compatibility

The SCID-XLA 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. The SCID-XLA mpx RealFast™ Assay can be combined with SMN1 RealFast™ Assay. In this case, calibration of the realtime PCR device for Cy5.5, Cy5, HEX, FAM, and ROX is required.

The kit is compatible with various common real-time PCR instruments capable of recording HEX, Cy5 and Cy5.5 fluorescence:

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

5.3. Assay Performance Specifications

The SCID-XLA 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 HEX (556 nm), Cy5 (670 nm) and Cy5.5 (694 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, molecular grade water, DNA extraction system, freezer, biohazard waste container.

7. Experimental Protocol

7.1. DNA Extraction

DNA extraction reagents are **not supplied** with the kit.
Analytical performance data concerning DNA isolated from dried blood spots is under evaluation and not available yet. Ensure extracted DNA is suitable for amplification in terms of concentration, purity, and integrity.
For accurate quantification, the DNA amount per reaction should be within the range of 1 to 50 ng for all samples.

7.2. No Template Control

Always include a **No Template Control** (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.

7.3. SCID Control

Always prepare a fresh dilution series of **SCID Control**. The **SCID Control** contains $2x10^5$ copies/µl of each target (ACTB, TREC and KREC). To obtain the required precision, it is recommended to pipette each dilution in duplicate. For the $2x10^4$ copies/µl dilution, mix 45 µl of **SCID Control Dilution Buffer** with 5µl of **SCID Control** ($2x10^5$ copies/µl). For the $2x10^3$ copies/µl dilution, mix 45 µl of **SCID Control Dilution Buffer** with 5µl of **SCID Control** ($2x10^4$ copies/µl). Proceed with $2x10^2$, $2x10^1$ and $2x10^0$ dilution steps using the same principle. Also include a 0-copy control with only **SCID Control Dilution Buffer** as input.

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

7.4. Preparation of SCID-XLA 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 + SCID Control dilutions + 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
2x SCID-XLA Assay Mix	2.5 µl	62.5 µl
PCR grade water *)	7.5 µl	187.5 µl
Master Mix	**15 µl**	**375 µl**

*) In case of combination of the SCID-XLA mpx RealFast™ Assay with the SMN1 RealFast™ Assay (REF 7-700) mix 5µl PCR grade water + 2.5µl SCID-XLA ASM + 2.5µl SMN1 ASM + 5µl 4x LIT Mix.

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.* «

7.5. PCR Program

Program the real-time PCR instrument according to the manufacturer's instructions for standard curve experiments with three targets / reporter dyes. **SCID Control** contains $2x10^5$ copies/µl of each target and should be assigned as standard for 10^6 copies within the instrument software (e.g. a field for defining concentrations). Continue by assigning the **SCID Control** dilution containing $2x10^4$ copies/µl as standard for 10^5 copies. Proceed with $2x10^2$, $2x10^1$ and $2x10^0$ dilutions using the same principle. 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 HEX, Cy5 and Cy5.5 channels

8. Data Analysis / Interpretation of Results

The copies of TRECs, KRECs and *ACTB* are determined by measuring the Cq value for each target in the corresponding fluorescence channel (**Cy5 = TREC**, **HEX = KREC**, **Cy5.5 = *ACTB***) and by subsequently comparing these values with a standard curve, which is based on standard curve dilution points of known concentrations (**SCID Control** dilutions). This is done automatically by most real-time PCR software and starting quantities (or mean starting quantities) are presented in a tabular view. Import these data in a spreadsheet software and calculate the number of TRECs and KRECs per 10^6 blood mononuclear cells (PBMC) for each unknown sample by using the following formula:

$$[(\text{quantity of TRECs or KRECs}) / (\text{quantity of } \textit{ACTB}/2)] \times 10^6$$

Results are of categorical nature, which means a sample is either positive or negative, or the result is inconclusive. Low or absent TRECs, or combined low/absent TREC and KREC levels, suggest SCID, whereas an isolated reduction of KRECs with normal TREC levels may indicate XLA. Cutoffs for TREC and KREC, as well as for *ACTB,* need to be established by the laboratory, as they are sensitive to certain parameters, e.g. the extraction method. It is recommended to test >100 healthy newborns, using the same dried blood spot cards (DBS) and extraction method to adjust the TREC- and KREC cutoffs to the 1st percentile of the dataset (per target; copies per 10^6 PBMC). Likewise, the acceptable minimum amount of genomic DNA extracted from a DBS sample can be established via the number of *ACTB* copies expressed by a Cq value. Once cutoffs are defined, samples can be categorized. In case of inconclusive results, it is recommended to retest samples in duplicates.

Verify the quality of the standard curve for each target. For a successful quantitation, individual parameters should meet the following criteria:

Efficiency > 91
Coefficient of determination (R^2) > 0.998
Slope: between -3.55 and -3.32

Recommendations for Threshold Settings (Cq): automatic

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

9. Warnings and Precautions

- For research use only.
- 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.