DEVYSER
RESULTS FOR LIFE

Devyser Thalassemia

Art. No.: 8-A106-RUO

For Research Use Only

Handbook

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1. INTRODUCTION TO DEVYSER THALASSEMIA

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1.1 Intended use

The Devyser Thalassemia kit enables analysis of sequence variants in the HBA1, HBA2 and HBB genes in human genomic DNA.

The Devyser Thalassemia kit is for research use only, not for use in diagnostic procedures.

1.2 Background

Thalassemias are a group of inherited blood disorders characterized by abnormal hemoglobin production[1,2]. The two main types, alpha- and beta thalassemia, are caused mainly by mutations in the HBA (HBA1 and HBA2) and HBB genes, respectively. In rare cases, thalassemia can be caused by mutations in regulatory elements of globin genes. In alpha thalassemia, most common mutations involve deletions in one or both HBA genes while in beta thalassemia, single nucleotide mutations is the major cause of disease[3,4].

1.3 Assay principle

The method employed by the Devyser Thalassemia kit includes multiplex PCR amplification to create a target amplicon library from each DNA sample (PCR1). The library covers, among others, the HBA1, HBA2 and HBB genes in a partly overlapping fashion, as illustrated in Figure 1.

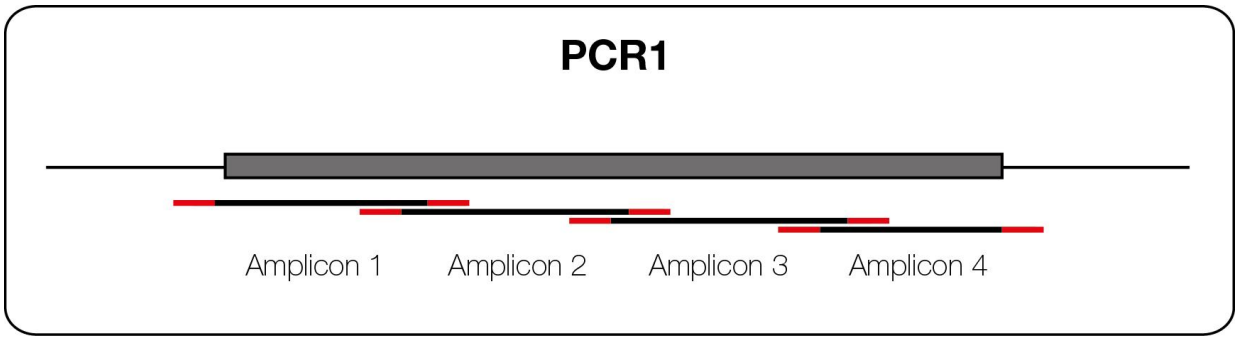

Figure 1. Multiplex target amplification

In a second PCR reaction (PCR2), sequencing adapters including unique index sequences are introduced into each amplicon (see Figure 2 below), enabling pooling of up to 96 samples. The sample pool is purified using the Devyser Library Clean (Art.No.: 8-A204). The purified sample pool is sequenced using NGS chemistry and the resulting sequences are analyzed using appropriate software for targeted sequencing.

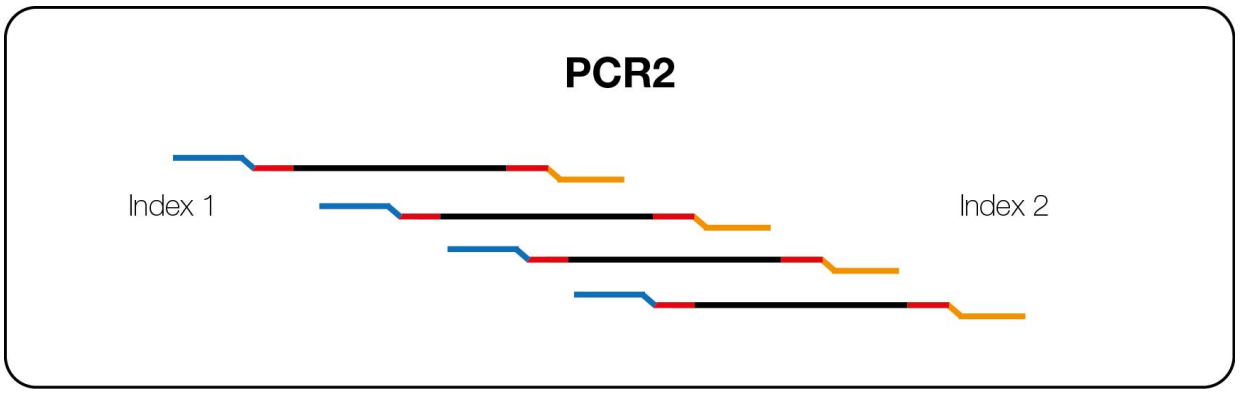

Figure 2. Introduction of adapters including unique index sequences

1.4 Assay design

Devyser Thalassemia enables target specific library generation for NGS analysis of variants (SNVs, indels and CNVs) in the HBA1, HBA2 and HBB genes. Sequencing of the HBA1, HBA2 and HBB genes includes the promoter, exon and intron regions (see table below). Regions, positioned upstream and downstream of these genes, are also targeted and used for detection of large deletions/duplications. See Figure 3 for an illustration of covered positions. Partial sequencing of the HBG1, HBG2 and HBD promotors is also performed. In addition, some common deletions in alpha- and beta thalassemia are detected with a direct detection method using primers aligned to both ends of the breakpoint (GAP-PCR). See the following tables for deletions and mode of detection.

Table 1. Genes and covered regions

Gene	No of amplicons	Covered region	Notes
HBA1	7[a]	c.-101_c.*173	c.*19_c.*74 not sequenced
HBA2	7[a]	c.-101_c.*141	
HBB	13	c.-290_c.*472	

[a]Amplicons 1-4 covering exon 1 and 2, generated from HBA1 and HBA2, have identical sequence content.

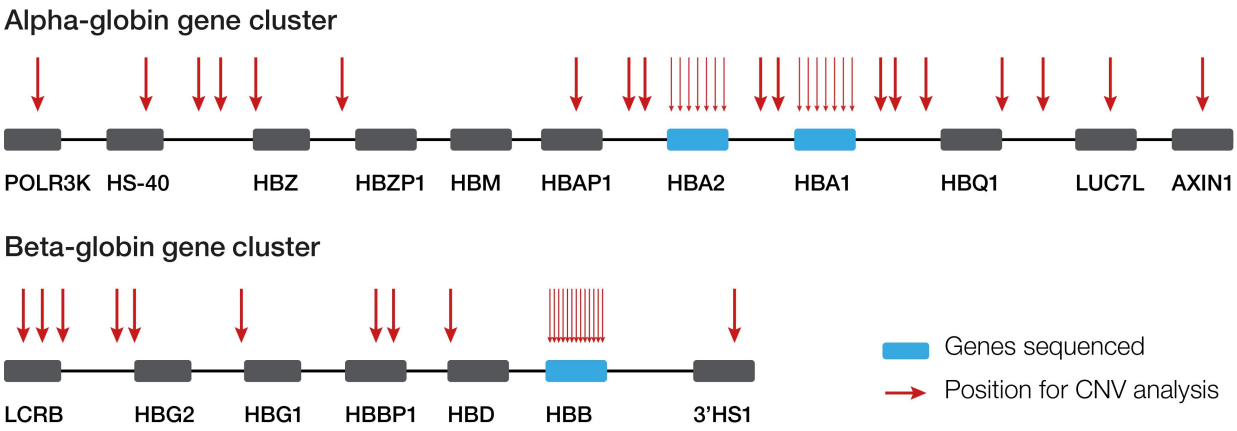

Figure 3. Illustration of covered positions in the alpha- and beta globin gene clusters

Genes in blue (HBA1, HBA2 and HBB) are fully sequenced. Vertical arrows illustrate positions where CNV analysis can be performed.

Table 2. Alpha globin deletions and mode of detection

Deletion	HGVS[5,6]	Mode of detection
--SEA	NG_000006.1:g.26264_45564del19301	Direct, CNV analysis
--FIL	NG_000006.1:g.11684_43534	Direct, CNV analysis
--THAI	NG_000006.1:g.10664_44164del33501	Direct, CNV analysis
-(α)20.5	NG_000006.1:g.15164_37864del22701	Direct, CNV analysis
--MED 1	NG_000006.1:g.24664_41064del16401	Direct, CNV analysis
-(α)21.9	NG_000006.1:g.[14373_36299del21927; ins29bp]	Direct, CNV analysis
-(α)27.6	NG_000006.1:g.9079_36718del27640	Direct, CNV analysis
-(α)3.7	Exact breakpoints currently not known	CNV analysis
-(α)4.2	Exact breakpoints currently not known	CNV analysis
HS-40	Several deletions	CNV analysis
Other deletions	Several deletions	CNV analysis

Table 3. Beta globin deletions and mode of detection

Deletion	HGVS[5,6]	Mode of detection
Chinese	NG_000007.3:g.48795_127698del78904	Direct, CNV analysis
Filipino	NG_000007.3:g.66258_184734del118477	Direct, CNV analysis
Yunnanese	NC_000011.10: 5182847-5249973del67127	Direct, CNV analysis
Taiwanese	NG_000007.3:g.69997_71353del1357	Direct, CNV analysis
SEA-HPFH	NC_000011.10: 5201647-5229059del27412	Direct, CNV analysis
δβ-Sicilia	NG_000007.3:g.64336_77738del13403	Direct, CNV analysis
Hb-Lepore Boston	NG_000007.3:g.63632_71046del	Direct, CNV analysis
Hb-Lepore Baltimore	NG_000007.3:g.63564_70978del	Direct, CNV analysis
Hb-Lepore Hollandia	NG_000007.3:g.63290_70702del	Direct, CNV analysis
290bp-del	HBB:c.-176_92+25del	Direct, CNV analysis
b619-del	NG_000007.3:g.71609_72227del619	Direct, CNV analysis
Other deletions	Several deletions	CNV analysis

2. MATERIALS AND EQUIPMENT

2.1 Kit configurations for Devyser Thalassemia

The Devyser Thalassemia kit is available in three configurations as follows:

Table 4. Devyser Thalassemia 24 test configuration (8-A106-24-RUO)

Component	Art.No.	Number/kit	Cap color	Storage condition
Thal mix	4-A278	1	Blue	Below −18°C
Start, 24 test	4-A280	1	Purple	Below −18°C
Dilution buffer	4-A245	3	White	-25°C to +8°C
Index mix 2, 24 test	4-A279	1	Red	Below −18°C
Index strip A2	-	1	-	Below −18°C
Index buffer	4-A258	3	Green	-25°C to +8°C
Sealer S	-	1	-	Ambient

Table 5. Devyser Thalassemia 48 test configuration (8-A106-48-RUO)

Component	Art.No.	Number/kit	Cap color	Storage condition
Thal mix	4-A278	2	Blue	Below −18°C
Start, 24 test	4-A280	2	Purple	Below −18°C
Dilution buffer, 96 test	4-A275	1	White	-25°C to +8°C
Index mix 2, 24 test	4-A279	2	Red	Below −18°C
Index plate A3	-	1	-	Below −18°C
Index buffer, 96 test	4-A277	1	White	-25°C to +8°C
Sealer L	-	1	-	Ambient

Table 6. Devyser Thalassemia 96 test configuration (8-A106-96-RUO)

Component	Art.No.	Number/kit	Cap color	Storage condition
Thal mix	4-A278	4	Blue	Below −18°C
Start, 24 test	4-A280	4	Purple	Below −18°C
Dilution buffer, 96 test	4-A275	1	White	-25°C to +8°C
Index mix 2, 24 test	4-A279	4	Red	Below −18°C
Index plate A3	-	1	-	Below −18°C
Index buffer, 96 test	4-A277	1	White	-25°C to +8°C
Sealer L	-	2	-	Ambient

2.2 Equipment and reagents required but not provided

2.2.1 Other required Devyser products

- Devyser Library Clean (8-A204), see the following table:

Table 7. Devyser Library Clean (8-A204)

Component	Art.No.	Number/kit	Cap color	Storage condition
Clean	4-A255	1	Orange	+2 to +8 °C
Wash	4-A256	1	Yellow	+2 to +8 °C
Dilution buffer	4-A245	1	White	+2 to +8 °C

2.2.2 General

- Micropipettes with aerosol barrier tips or dispenser with displacement tips dedicated for pre-PCR
- Micropipettes with aerosol barrier tips or dispenser with displacement tips dedicated for post-PCR
- Disposable powder free protective gloves
- Reaction tubes

2.2.3 DNA extraction

- DNA extraction reagents according to manufacturer's instructions for use
- QIAamp DNA Blood Mini Kit (Qiagen, cat.# 51104/51106) and QIAsymphony DSP DNA Midi Kit (Qiagen, cat.#937255) for extraction of DNA from human whole blood
- If alternative DNA extraction methods and sample materials are used, a thorough evaluation of the performance together with the Devyser Thalassemia kit should be performed

2.2.4 Determination of DNA concentration

- Determination of DNA concentration according to manufacturer's instructions for use
- Qubit™ 2 Fluorometer (cat.# Q32866) or Qubit™ 4 Fluorometer (cat.# Q33226) and required consumables (Thermo Fisher Scientific)
- Qubit™ 1X dsDNA HS Assay Kit (Thermo Fisher Scientific, cat.# Q33230/Q33231)

2.2.5 Reagent preparation and amplification

- Veriti™ Thermal Cycler with MicroAmp™ 96-Well Tray/Retainer Set (Thermo Fisher Scientific)
- If an alternative thermal cycler is used, a thorough evaluation of its performance together with the Devyser Thalassemia kit should be performed. It is of high importance that the following ramp rates are applied: heating 1,6 ˚C/s, cooling 1,6 ˚C/s
- Consumables for the thermal cycler

2.2.6 Library purification

- Devyser Library Clean (see 2.2.1)
- Magnetic rack for test tubes (DynaMag™-2 Magnet, Thermo Fisher Scientific or equivalent)
- Ethanol (96 %)

2.2.7 Sequencing

- Illumina® MiSeq™, MiniSeq™, iSeq™ 100
- User-supplied consumables needed for sequencing, according to the Illumina sequencing guide
- Illumina reagent kits, see the following tables:

Table 8. MiSeq reagent kits

Illumina reagent kit	Illumina cat #
MiSeq Reagent Nano Kit v2 (300-cycles)	MS-103-1001
MiSeq Reagent Micro Kit v2 (300-cycles)	MS-103-1002
MiSeq Reagent Kit v2 (300-cycles)	MS-102-2002
Illumina PhiX control v3	FC-110-3001

Table 9. MiniSeq reagent kits

Illumina reagent kit	Illumina cat #
MiniSeq Mid Output Kit (300-cycles)	FC-420-1004
MiniSeq High Output Reagent Kit (300-cycles)	FC-420-1003
NextSeq PhiX Control Kit	FC-110-3002

Table 10. iSeq 100 reagent kits

Illumina reagent kit	Illumina cat #
iSeq 100 i1 Reagent v2 (300-cycles)	20031371
Illumina PhiX control v3	FC-110-3001

NOTE All equipment should be tested, calibrated and maintained regularly.

2.3 Software

- SeqPilot v4.3.1 software including the SeqNext module (JSI Medical Systems GmbH). If using a different version of the software, please consult the manufacturer
- Amplicon Suite (SmartSeq) pipeline for Thalassemia

Contact Devyser support at techsupport@devyser.com for information.

2.4 Downloads

Supplementary information and files can be downloaded from www.devyser.com/ifu using the download code printed on the kit label. See the following table for details.

Table 11. Download files

Download file name	Description
Illumina double index	Index sequence information
MiSeq IEM files	Devyser setting files for sample sheet generation: • Generating a Devyser sample sheet for MiSeq.pdf • DEVYSER double Index MiSeq.txt • DevyserGenerateFASTQ.txt • DevyserGenerateFASTQ.jpg
LRM files	Devyser setting files for MiSeq, MiniSeq or iSeq 100 sample sheet generation using LRM: • Generating a Devyser sample sheet with LRM.pdf • Devyser .tsv files
SeqNext guide for Devyser Thalassemia	SeqNext guide for Devyser Thalassemia analysis
Devyser Thalassemia SeqNext .sge files	Devyser settings files (.sge files) for Thalassemia data analysis with SeqNext
Devyser Thalassemia BED files	BED files detailing amplicon positions according to Hg19/GRCh37 and Hg38/GRCh38

2.5 Other resources

2.5.1 Devyser Sequence Coverage Calculator

To plan the sequencing run with respect to coverage needs, please consult the Devyser Sequence Coverage Calculator at www.devyser.com/calculator. As displayed in the calculator, the expected minimum total number of read pairs per sample is 36 712 and minimal coverage per amplicon is 200.

After the sequencing run, the total number of read pairs per sample can be found in Illumina Sequencing Analysis Viewer (SAV), BaseSpace or Local Run Manager (LRM). The minimal coverage per amplicon can be found in the analysis software.

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3. STORAGE REQUIREMENTS

- Store the Devyser Thalassemia kit in a freezer below −18°C (−28°C to −18°C) or the individual kit components as specified on the label (see 2.1)
- Store the components of the Devyser Library Clean kit at +2 to +8 °C
- Do not use components beyond the kit lot expiration date
- If handled and stored properly, kit components will remain stable until the expiration date of the kit or according to in use stability specified in this handbook in section 7
- Frozen kit components should be thawed in a refrigerator or at room temperature before use
- Avoid repeated freezing-thawing

4. WARNINGS AND PRECAUTIONS

- Use of this product should be limited to personnel trained in PCR, NGS techniques and NGS data analysis
- The procedure should be performed according to this handbook
- Deviations from the handbook will compromise the kit performance
- Modifications of software settings will compromise the kit performance
- Wear powder free disposable gloves, laboratory coat and eye protection when handling samples and kit reagents
- Do not pool reagents with different kit lot numbers or different vials of the same lot
- Do not use damaged reagent vials
- Frozen components should be completely thawed in a refrigerator or at room temperature before use
- Use, storage and disposal of kit components and samples, should be in accordance with the procedures defined by national biohazard safety guidelines and in accordance with country, federal, state and local regulations
- Avoid microbial contamination of reagents when removing aliquots from reagent vials
- The use of sterile disposable aerosol barrier pipette tips is recommended
- It is recommended using different sets of pipettes for the initial addition of DNA samples and for diluting and handling samples after PCR amplification
- Highly concentrated amplicons produced during PCR amplification must be handled with care to avoid contamination in the laboratory environment
- The workflow in the laboratory should proceed in a unidirectional manner, beginning in the reagent preparation area, moving to the DNA extraction area, then to the amplification area and finally to the sequencing area
- Supplies and equipment should be dedicated to each activity and not used for other activities or moved between areas
- Gloves should be changed between activities

5. PROCEDURAL LIMITATIONS

- The Devyser Thalassemia kit is for research use only, not for diagnostic procedures
- Sequence variants that may be present in other genes than what is described to be covered in this handbook will not be detected using Devyser Thalassemia
- Results obtained with the Devyser Thalassemia kit can only be directly applied to the tissue or specific sample material tested
- Rare primer site sequence variants may affect the function of individual PCR primers used in the Devyser Thalassemia kit and may result in reduced or no amplification of the affected amplicon. Copy number changes in a single amplicon that cannot be attributed to primer site mutations require confirmation by another method
- The following parameters might affect the overall performance and CNV analysis
    - Quality, sample type and concentration of the DNA
    - Deviations from the protocol
    - The number of control samples used in the analysis
    - The type of control samples used
    - Sequencing depth
    - Bioinformatic pipeline
- Coverage-based CNV analysis cannot detect changes that lie outside the target sequence of the probes and will not detect copy number neutral deletions, inversions or translocations. For example, the combination of a deletion on one chromosome and a similarly sized duplication on the other chromosome may result in false negative CNV results, i.e. in the case of an α3.7-deletion and ααα-anti-3.7-triplication
- It is recommended that only samples that have been processed and sequenced together and with the same kit lot should be analyzed together for a CNV analysis
- The bioinformatic characterization of CNVs is based on different computational strategies. None of the strategies can guarantee correct detection of all CNVs
- Running several family members or samples with the same deletions can affect CNV analysis and should be avoided
- HBA1 and HBA2 genes have high sequence homology in the region covered by amplicons 1 to 4 and therefore it is not possible to discriminate between the two genes. In addition, skewed variant dosage can be observed: 25% if heterozygous in one of the genes, 50% if homozygous in one of the genes or heterozygous in both genes
- In the case of large deletions, regions not affected by the deletion may have a preferential amplification thus generating more product compared to normal (no large deletion) which may be detected as a duplication. In the case of large duplications, the opposite applies

- Accurate detection of homopolymers ≥ 10 bp is not reliable
- Patients who have undergone a previous allogenic bone marrow transplant should not be tested with the Devyser Thalassemia kit

6. SAMPLE REQUIREMENTS

DNA concentration, integrity and purity are important parameters for successful testing using the Devyser Thalassemia kit. DNA should be free from contaminating proteins, salts and other PCR inhibitors, e.g. residual ethanol from DNA extraction procedures. Poor quality DNA may result in amplification failure and/or increased background signals.

6.1 Samples

The Devyser Thalassemia kit has been tested using human genomic DNA extracted from whole blood.

6.1.1 DNA extraction from whole blood sample

According to the manufacturer's instructions for use.

6.1.2 Determination of DNA concentration

- High quality DNA is important for accurate and reproducible determination of DNA concentration
- All DNA concentrations referred to in this handbook were determined using the Qubit Fluorometer and the Qubit 1X dsDNA HS Assay Kit
- The DNA concentration determined for a DNA sample may differ between Qubit systems and between the Qubit system and other techniques. It is important to verify that the technique used for determination of DNA concentration correlates to the actual results obtained with the Devyser Thalassemia kit

6.1.3 Dilution of DNA

- Adjust the concentration of extracted DNA to 2 ng/µL (see 7.1.2)

NOTE	The use of high-quality DNA with carefully determined concentration enables direct pooling of equal volumes from each sample library prior to purification and quantification of the library pool (see 7.3).

6.2 PhiX control

Include PhiX control v3 library DNA (see 2.2.7) in each sequencing run to ensure that the sequencing pool has the required diversity for high quality sequencing (see 8.3).

6.3 Internal system control

We recommend to perform regular internal system control of all equipment and software used in this procedure. Samples with pre-characterized HBA and/or HBB gene sequence variants (in-house developed or externally sourced) are suitable as system controls.

7. INSTRUCTIONS FOR USE

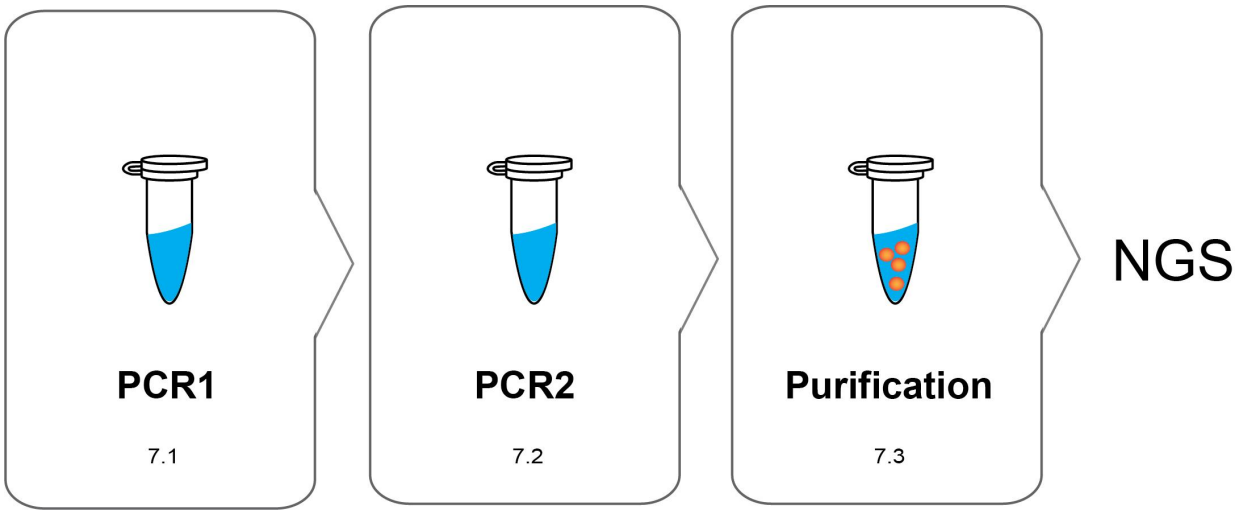


Figure 4. Schematic overview of the Devyser Thalassemia library preparation procedure (7.1 - 7.3)

The Devyser Thalassemia library preparation procedure consists of the following steps:

PCR1 (7.1)
The amplicon library is generated in one multiplex PCR reaction for each sample.

PCR2 (7.2)
Index addition to the PCR1 library is performed in PCR2 to allow pooling of multiple sample libraries for sequencing.

Purification (7.3)
Unique sample libraries generated in PCR2 are pooled and purified in a single tube. The purified library pool is analyzed by NGS.

Each step (7.1 to 7.3) is followed by a suitable stopping point where the procedure can be paused and restarted within 30 days.

NOTE	This kit is available in three test configurations: 24, 48 and 96. Each **Start** and **Thal mix** tube in the 24, 48 and 96 test configuration is sufficient for 24 reactions. Determine the required number of each component tube before starting.

7.1 Library generation (PCR1) for Devyser Thalassemia 24, 48 and 96-test kits

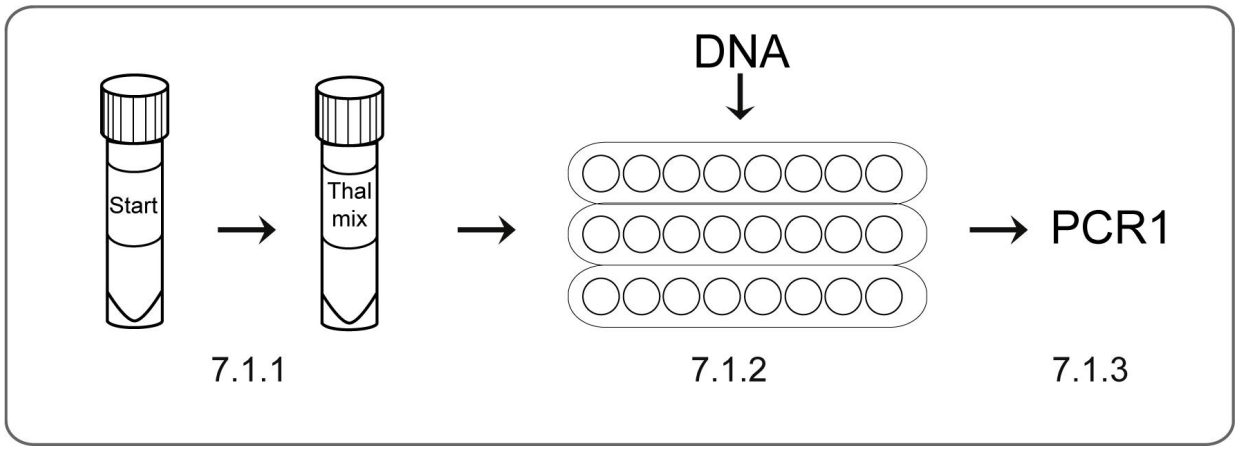


Figure 5. Schematic overview of step 7.1.1 to 7.1.3

7.1.1 Thal mix preparation for 24, 48 and 96-test

Determine the number of **Start, 24 test** and **Thal mix** tubes required. Each tube is sufficient for 24 reactions.

Required kit components: **Start, 24 test (4-A280), Thal mix (4-A278)**

A. Ensure that the **Start, 24 test** and the **Thal mix** are completely thawed before use
B. Vortex the **Start, 24 test** tube(s) briefly
C. Briefly centrifuge the **Start, 24 test** and **Thal mix** tube(s) to collect the content
D. Add 150 µL of **Start, 24 test** to the **Thal mix** tube(s) to obtain an activated **Thal mix**
E. Vortex the activated **Thal mix** tube(s) and then centrifuge it briefly to collect the content
F. Dispense 10 µL of the activated **Thal mix** into separate PCR reaction tubes or separate wells in a plate. Cap the tubes or seal the plate
G. Store the dispensed **Thal mix** at +2°C to +8°C and continue to 7.1.2
H. Any remaining activated **Thal mix** can be stored in a freezer below −18°C for 90 days. Do not aliquot the activated mix

7.1.2 Preparation and addition of DNA

Required kit component: **Dilution buffer (4-A245)** or **Dilution buffer, 96 test (4-A275)**

A. Determine the DNA concentration of each DNA sample (see 6.1)
B. Ensure that the **Dilution buffer** is completely thawed before use
C. Dilute the DNA samples to a final concentration of 2 ng/µL using the provided **Dilution buffer**
D. Add 5 µL of diluted DNA from each sample to the separate PCR reaction tubes or the separate wells in the plate containing activated **Thal mix** from 7.1.1
E. Mix by pipetting
F. Cap the tubes or seal the plate and centrifuge briefly to collect the content
G. Continue to 7.1.3

7.1.3 Thermal cycling PCR1

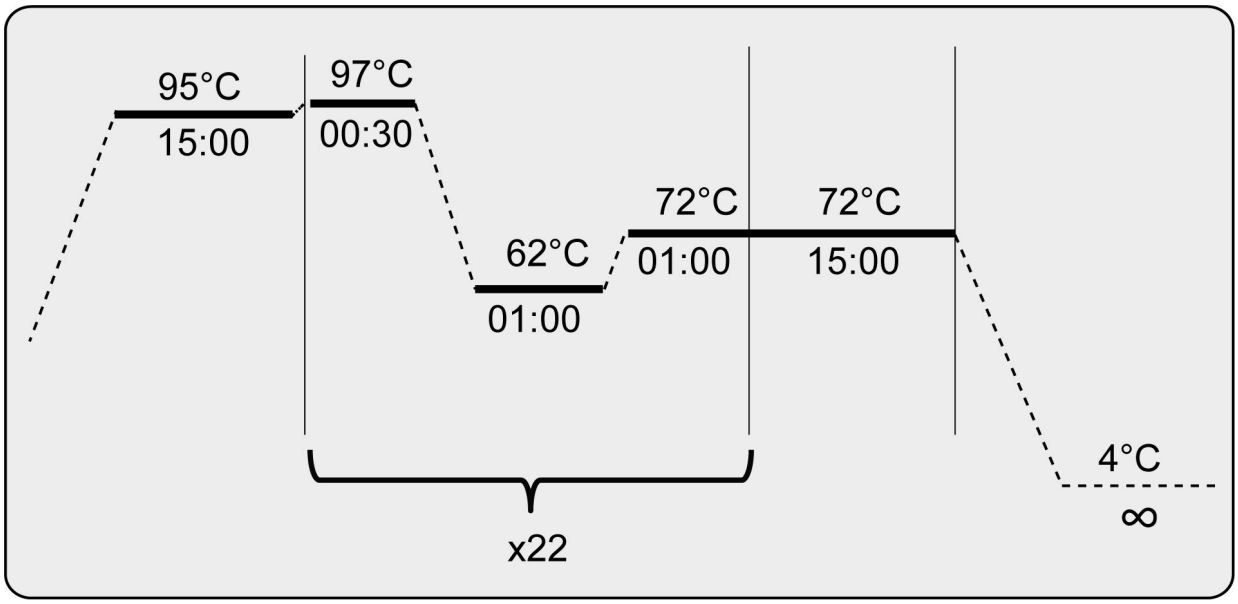


Figure 6. Thermal profile PCR1

A. Program the thermal cycler according to the PCR1 thermal profile in the figure above
B. Set the ramp rates to heating 1,6 ˚C/s and cooling 1,6 ˚C/s
C. Set the reaction volume to 15 µL
D. Place the tubes or the plate in the thermal cycler
E. Start the amplification (duration approximately 1 hr 45 min)
F. Following amplification, centrifuge briefly if necessary, to collect the content and continue to 7.2

The PCR1 library can be stored in a freezer below −18°C for 30 days.

SUITABLE STOPPING POINT

NOTE	It is of high importance that the following ramp rates are applied: heating 1,6 ˚C/s, cooling 1,6 ˚C/s.

NOTE	To program the correct ramp rate for the Veriti Thermal Cycler: In the "Tools Menu" select "Convert a Method". In the next select "9700 Max Mode" and then enter the PCR profile as outlined in section 7.1.3.

NOTE If using tubes/strips in a Veriti Thermal Cycler they should first be placed in the MicroAmp 96-Well Tray/Retainer Set for Veriti Systems.

7.2 Library indexing (PCR2)

For the Devyser Thalassemia 24 test kit proceed 7.2.1
For the Devyser Thalassemia 48 test kit proceed 7.2.2
For the Devyser Thalassemia 96 test kit proceed 7.3.3

7.2.1 Library indexing - Devyser Thalassemia 24 test kit

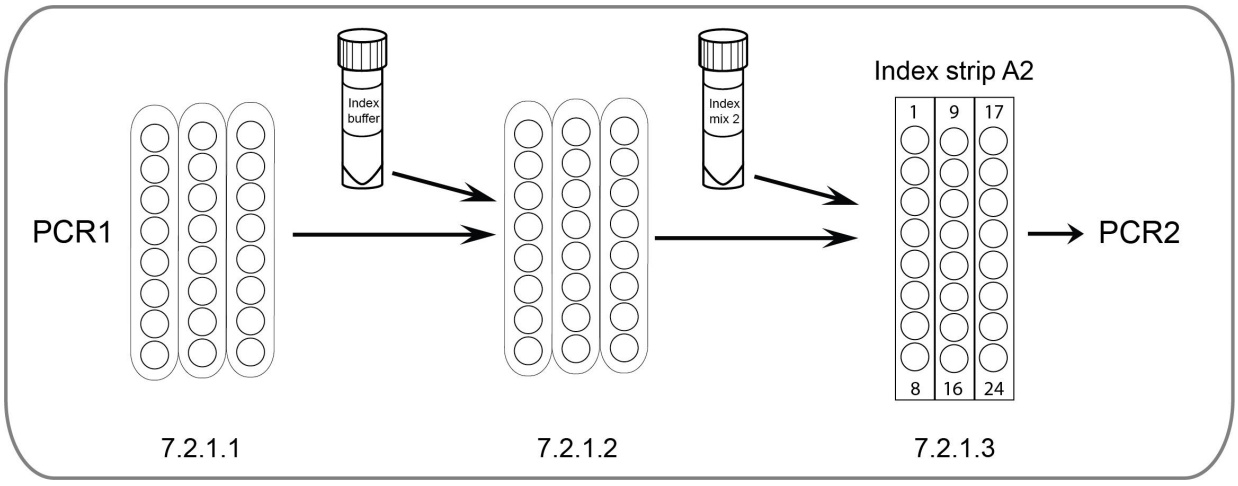


Figure 7. Schematic overview of step 7.2.1.1 to 7.2.1.3

7.2.1.1 PCR1 library dilution

Required kit component: **Index buffer (4-A258)**

A. Ensure that the **Index buffer** is completely thawed
B. For each PCR1 library to be diluted, dispense 198 µL **Index buffer** to a new tube
C. Add 2 µL of each PCR1 library to the separate dilution tubes containing 198 µL **Index buffer**. Make sure no liquid remains in the tip by pipetting repeatedly in the **Index buffer**
D. Mix the diluted PCR1 libraries thoroughly by pipetting (using a pipetting volume of at least 100 µL)

7.2.1.2 Index preparation

Required kit components: **Index mix 2, 24 test (4-A279), Index strip A2**

A. Ensure that the **Index mix 2, 24 test** is completely thawed before use
B. Vortex and then briefly centrifuge the **Index mix 2, 24 test** tube to collect the content
C. Carefully remove the transport seal of **Index strip A2**. **Note! Do not reuse the transport seal**
D. Add 20 µL of **Index mix 2, 24 test** to each of the 24 wells of **Index strip A2**. **Note! Tips must be changed between each individual well**

7.2.1.3 Addition of diluted PCR1 libraries to Index strip A2

Required kit component: **Sealer S**

A. Add 5 µL of each diluted PCR1 library (from 7.2.1.1) to separate wells in **Index strip A2** (prepared 7.2.1)
B. Mix thoroughly by pipetting to dissolve the colored reagent pellets, using a pipetting volume of at least 10 µL. **Note! Make sure that the colored reagent pellets are completely dissolved before proceeding to the next step.** Avoid bubbles
C. Cut a piece of **Sealer S** to completely cover **Index strip A2**
D. Carefully seal **Index strip A2** and make sure that all wells are covered
E. Centrifuge briefly to collect the content
F. Continue to 7.2.4

7.2.2 Library indexing - Devyser Thalassemia 48 test kit

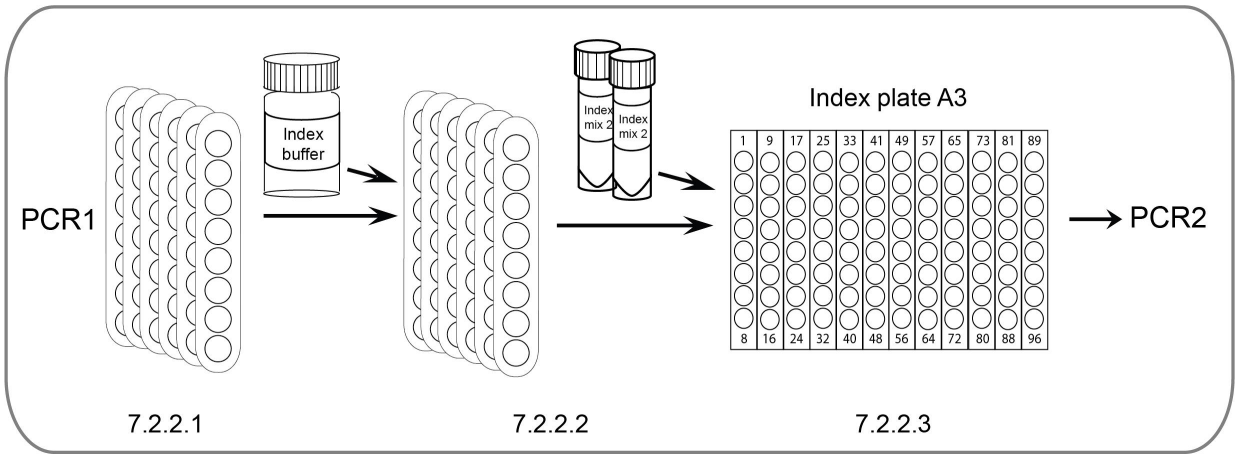


Figure 8. Schematic overview of step 7.2.2.1 to 7.2.2.3

7.2.2.1 PCR1 library dilution

Required kit component: **Index buffer, 96 test (4-A277)**

A. Ensure that the **Index buffer, 96 test** is completely thawed before use
B. For each PCR1 library to be diluted, dispense 198 µL **Index buffer, 96 test** to a new tube
C. Add 2 µL of each PCR1 library to the separate dilution tubes containing 198 µL **Index buffer, 96 test**. Make sure no liquid remains in the tip by pipetting repeatedly in the **Index buffer, 96 test**
D. Mix the diluted PCR1 libraries thoroughly by pipetting (using a pipetting volume of at least 100 µL)

7.2.2.2 Index preparation

Required kit components: **Index mix 2, 2 x 24 test tube (4-A279), Index plate A3**

A. Ensure that the **Index mix 2, 24 test** is completely thawed before use
B. Vortex and then briefly centrifuge the **Index mix 2, 24 test** tube(s) to collect the content
C. Carefully remove the transport seal of **Index plate A3**. **Note! Do not reuse the transport seal**
D. Add 20 µL of **Index mix 2, 24 test** to each of the wells to be used in **Index plate A3**. **Note! Tips must be changed between each individual well**

7.2.2.3 Addition of diluted PCR1 libraries to Index plate A3

Required kit component: **Sealer L**

A. Add 5 µL of each diluted PCR1 library pool (from 7.2.1.1) to separate wells in **Index plate A3** (prepared 7.2.1.2)
B. Mix thoroughly by pipetting to dissolve the colored reagent pellets, using a pipetting volume of at least 10 µL. **Note! Make sure that the colored reagent pellets are completely dissolved before proceeding to the next**. Avoid bubbles
C. Cut a piece of **Sealer L** to completely cover **Index plate A3**
D. Carefully seal **Index plate A3** and make sure that all wells are covered
E. Centrifuge briefly to collect the content

7.2.3 Library indexing - Devyser Thalassemia 96 test kit

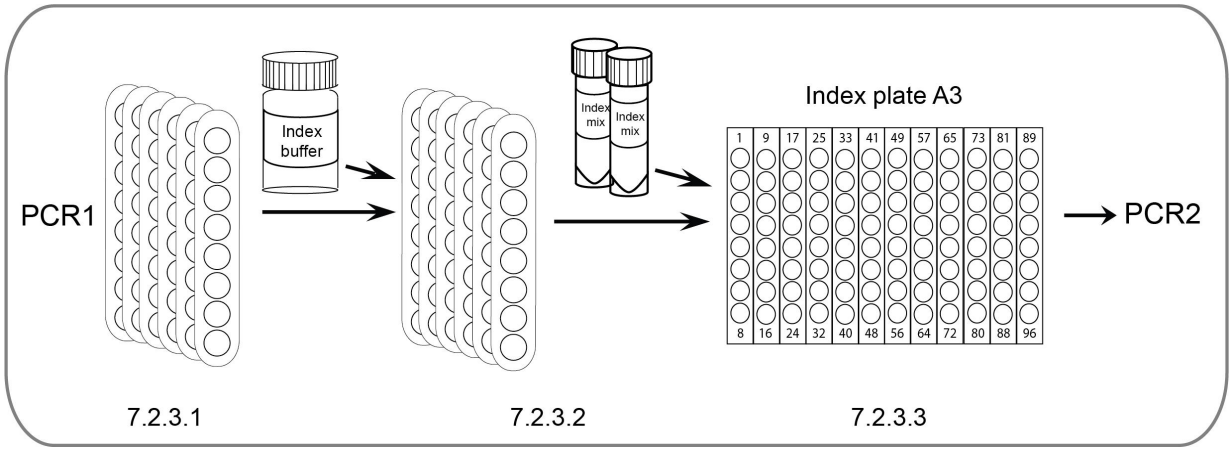


Figure 9. Schematic overview of steps 7.2.3.1 to 7.2.3.3

7.2.3.1 PCR1 library dilution

Required kit component: **Index buffer**, **96 test (4-A277)**

A. Ensure that the **Index buffer**, **96 test** is completely thawed before use
B. For each PCR1 library to be diluted, dispense 198 µL **Index buffer**, **96 test** to a new tube
C. Add 2 µL of each PCR1 library to the separate dilution tubes containing 198 µL **Index buffer**, **96 test**. Make sure no liquid remains in the tip by pipetting repeatedly in the Index buffer
D. Mix the diluted PCR1 libraries thoroughly by pipetting (using a pipetting volume of at least 100 µL

7.2.3.2 Index preparation

Required kit components: **Index mix**, **4 x 24 test tubes (4-A279)**, **Index plate A3**

A. Ensure that the **Index mix, 24 test** is completely thawed before use
B. Vortex and then briefly centrifuge the **Index mix** tube to collect the content
C. Carefully remove the transport seal of **Index plate A3**. **Note! Do not reuse the transport seal**
D. Add 20 µL of **Index mix, 24 test** to each of the wells to be used in **Index plate A3**. **Note! Tips must be changed between each individual well**

7.2.3.3 Addition of diluted PCR1 libraries to Index plate A3

Required kit component: **Sealer L**

A. Add 5 µL of each diluted PCR1 library pool from 7.2.3.1 to separate tubes in Index plate A3 prepared in 7.2.3.2
B. Mix thoroughly by pipetting to dissolve the colored reagent pellets, using a pipetting volume of at least 10 µL. **Note! Make sure that the colored reagent pellets are completely dissolved before proceeding to the next step**. Avoid bubbles
C. Use **Sealer L** to completely cover **Index plate A3** or cut a piece of **Sealer L** to cover wells in use
D. Carefully seal **Index plate A3** and make sure that wells in use are covered
E. Centrifuge briefly to collect the content

7.2.4 Thermal cycling PCR2

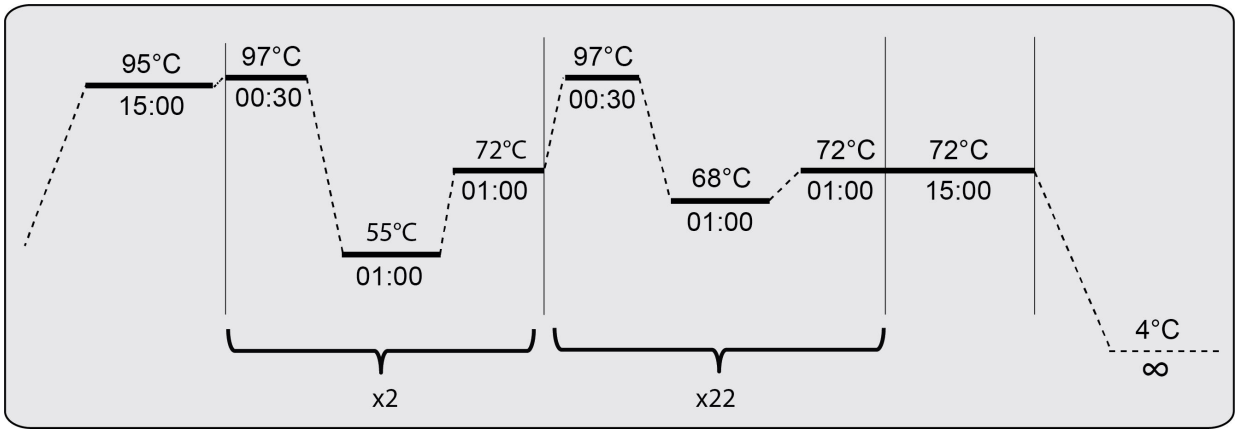


Figure 10. Thermal profile PCR2

A. Program the thermal cycler according to the PCR2 thermal profile in the figure above
B. Set the ramp rates to heating 1,6 ˚C/s and cooling 1,6 ˚C/s
C. Set the reaction volume to 25 µL
D. Place the tubes or the plate in the thermal cycler
E. Start the amplification (duration approximately 1 hr 55 min)
F. If proceeding with sequencing the same day, prepare sequencing reagents (see note in 7.3)
G. Following amplification, centrifuge briefly if necessary, to collect the content

PCR2 libraries can be stored in a freezer below −18°C for 30 days.

SUITABLE STOPPING POINT

NOTE	It is of high importance that the following ramp rates are applied: heating 1,6 ˚C/s, cooling 1,6 ˚C/s.

NOTE	To program the correct ramp rate for the Veriti Thermal Cycler: In the "Tools Menu" select "Convert a Method". In the next select "9700 Max Mode" and then enter the PCR profile as outlined in 7.2.4.

NOTE	If using tubes/strips in a Veriti Thermal Cycler they should first be placed in the MicroAmp 96-Well Tray/Retainer Set for Veriti Systems.

7.3 Pooling and purification of libraries using the Devyser Library Clean kit (8-A204)

NOTE	Defrost the MiSeq, MiniSeq or iSeq 100 reagent cartridge well in advance prior to sequencing according to the procedure described in the current MiSeq, MiniSeq or iSeq 100 System Guide[9,12,13].

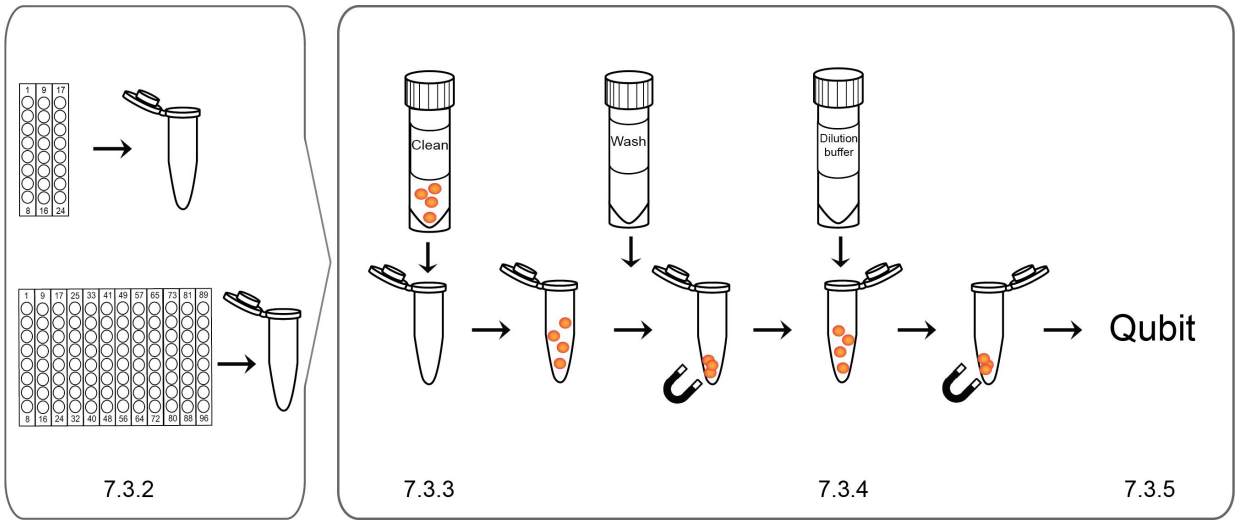


Figure 11. Schematic overview of 7.3.2 to 7.3.5

7.3.1 Preparation of Wash solution

Required kit: **Devyser Library Clean (8-A204)**
Required kit component: **Wash (4-A256)**

A. Prepare the **Wash** solution by adding 1500 µL of 96 % ethanol to the **Wash** tube
B. Mix thoroughly by vortexing
C. Tick the box on the **Wash** tube label to indicate that ethanol was added
D. **Note! The Wash solution should be stored at +2 °C to +8 °C and used within 3 months from day of activation**

7.3.2 Pooling

A. To obtain a library pool volume of at least 80 µL for subsequent purification, pool 5 µL from each of the PCR2 libraries (from 7.3.3) into a single tube
B. Mix thoroughly by vortexing and then briefly centrifuge the library pool to collect the content
C. Transfer 80 µL of the library pool into a new tube suitable for placing on a magnetic rack

NOTE	If less than 16 libraries are pooled, add equal volumes of each PCR2 library to obtain a library pool volume of 80 µL. If the total pooled volume is less than 80 µL (less than 4 samples), use equal volumes of the pooled PCR2 libraries and **Clean**. For elution, use half the library pool volume of **Dilution buffer** (see 7.3.4). However, do not use less than 25 µL of **Dilution buffer** for elution.

NOTE	The library pool should consist of libraries from samples processed together with the same Devyser library kit. If using different Devyser library kits, library pools should be prepared and purified separately.

7.3.3 Library Purification

Required kit: **Devyser Library Clean (8-A204)**
Required components: **Clean (4-A255)**, **Wash (4-A256)**

A. Briefly centrifuge the **Clean** tube to collect the content
B. Firmly tap the **Clean** tube. Make sure that the bead pellet is re-suspended and that the content is homogenous. If necessary, briefly vortex the tube but avoid extensive vortexing
C. Add 80 µL re-suspended **Clean** to the library pool from 7.3.2 and mix by pipetting. See note for use of alternative volumes of library pool and **Clean**
D. Incubate the tube at room temperature for 3 minutes
E. Place the tube onto a magnetic rack until all beads are pelleted and the solution has become clear
F. While keeping the tube on the magnetic rack, carefully remove and discard the solution. **Note! It is important to avoid touching the bead pellet during this step (see Figure 12)**
G. Add 150 µL of prepared **Wash** solution (from 7.3.1) to the tube without removing it from the magnetic rack
H. Slightly lift and rotate the tube two half circles to wash the beads
I. Place the tube onto the magnetic rack to pellet the beads
J. Carefully remove as much **Wash** solution as possible by pipetting from the bottom of the tube. **Note! It is important to avoid touching the bead pellet and the walls of the tube during this step (see Figure 12)**
K. Leave the lid open until all remaining Wash solution has evaporated and the bead pellet has changed from being luster to lusterless, approximately 5-9 minutes, while remaining on the magnetic rack. **Important! See note below**
L. Remove the tube from the magnetic rack

NOTE	It is important that all **Wash** solution has evaporated and that the pellet is dry before continuing. The pellet appearance should change from being luster to lusterless and the color should change slightly to a lighter nuance when dry. If **Wash** solution remains, briefly centrifuge the tube to collect all remaining **Wash** solution, pellet the beads using the magnetic rack, remove the residual **Wash** solution and air dry the pellet again.

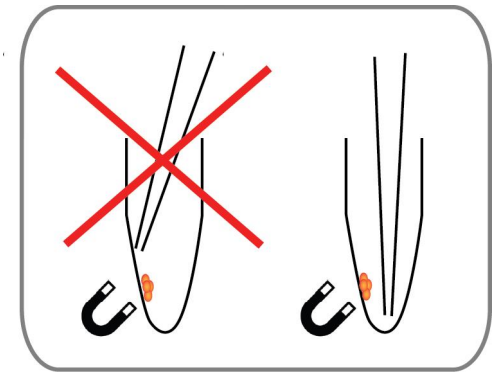


Figure 12. Bead pellet

7.3.4 Library elution

Required kit: **Devyser Library Clean (8-A204)**
Required kit component: **Dilution buffer (4-A245)**

A. Briefly centrifuge the **Dilution buffer** to collect the content
B. Add 40 µL **Dilution buffer** to the tube from 7.3.3 and re-suspend the pellet by pipetting and/or tapping the tube. If necessary, collect the liquid by a very brief centrifugation
C. Place the tube onto the magnetic rack until all beads are pelleted
D. While keeping the tube on the magnetic rack, transfer the cleared supernatant, containing the purified library pool, to a new tube

7.3.5 Library quantification

Required kit: **Qubit 1X dsDNA HS Assay Kit** (see 2.2.4)

Quantify the library as described in the current user manual for Qubit 1X dsDNA HS Assay Kits for details

A. Ensure that all Qubit 1X dsDNA HS solutions are at room temperature
B. Mix 190 µL Qubit 1X dsDNA HS working solution with 10 µL of Qubit standard 1
C. Mix 190 µL Qubit 1X dsDNA HS working solution with 10 µL of Qubit standard 2
D. Mix 190 µL Qubit 1X dsDNA HS working solution with 10 µL of the purified library pool from 7.3.4
E. Briefly vortex, centrifuge and incubate each tube for 2 minutes at room temperature
F. Measure the concentration (ng/µL) of the purified library pool on a Qubit Fluorometer

7.3.6 Library dilution

Required kit: **Devyser Library Clean (8-A204)**
Required kit component: **Dilution buffer (4-A245)**

A. Use the **Dilution Buffer** to dilute the purified library pool from 7.3.4 to a final concentration of:
   - Illumina MiSeq: 0.25 - 0.30 ng/µL
   - Illumina MiniSeq: 0.23 ng/µL
   - Illumina iSeq 100: 0.23 - 0.26 ng/µL
B. Measure the concentration (ng/µL) of the diluted library pool to confirm the concentration by repeating E to F in 7.3.5
C. Proceed to sequencing according to section 8

SUITABLE STOPPING POINT

NOTE	The concentration of the purified library pool can be adjusted to ensure that the Illumina specifications for the reagent kit are met.

NOTE	The purified and diluted library pool is not recommended to be stored for long term or to be re-used. For potential re-runs it is instead recommended to repeat the pooling and purification steps of this protocol from point 7.3.2, to ensure that the library holds the final concentration range defined in 7.3.6.

8. ILLUMINA SEQUENCING

8.1 Number of samples per flow cell

Calculate the number of samples to be sequenced per flow cell by using the Devyser Sequence Coverage Calculator (see 2.5.1).

8.2 Index description

The Illumina double indexes introduced during PCR2 are listed in the following tables. Detailed information about the index combinations and index sequences can be found online (see 2.4 for details).

Table 12. Illumina double index used in Index strip A2

	Index 1-8	**Index 9-16**	**Index 17-24**
	Index1: N701	Index1: N702	Index1: N703
Index2: N501	1	9	17
Index2: N502	2	10	18
Index2: N503	3	11	19
Index2: N504	4	12	20
Index2: N505	5	13	21
Index2: N506	6	14	22
Index2: N507	7	15	23
Index2: N508	8	16	24

Table 13. Illumina double index used in Index plate A3

	Index 1-8	**Index 9-16**	**Index 17-24**	**Index 25-32**	**Index 33-40**	**Index 41-48**	**Index 49-56**	**Index 57-64**	**Index 65-72**	**Index 73-80**	**Index 81-88**	**Index 89-96**
	Index1: N701	Index1: N702	Index1: N703	Index1: N704	Index1: N705	Index1: N706	Index1: N707	Index1: N708	Index1: N709	Index1: N710	Index1: N711	Index1: N712
Index2: N501	1	9	17	25	33	41	49	57	65	73	81	89
Index2: N502	2	10	18	26	34	42	50	58	66	74	82	90
Index2: N503	3	11	19	27	35	43	51	59	67	75	83	91
Index2: N504	4	12	20	28	36	44	52	60	68	76	84	92
Index2: N505	5	13	21	29	37	45	53	61	69	77	85	93
Index2: N506	6	14	22	30	38	46	54	62	70	78	86	94
Index2: N507	7	15	23	31	39	47	55	63	71	79	87	95
Index2: N508	8	16	24	32	40	48	56	64	72	80	88	96

8.3 Sequencing using MiSeq™

8.3.1 Sample sheet generation

Generate a sample sheet for each run in the Illumina Experiment Manager (IEM) software by using the Devyser Guide "Generating a Devyser Sample Sheet for MiSeq" (see 2.4 for details) and the Illumina document 15031335: " Illumina Experiment Manager User Guide"[7].

8.3.2 Denaturation of the purified library pool

A. Prepare 20 pM PhiX, HT1 and a fresh dilution of 0.2 N NaOH according to the current version of the Illumina document # 15039740: "Denature and Dilute Libraries Guide"[8]
B. Combine 5 µL purified library pool from 7.3.6 with 5 µL 0.2 N NaOH
C. Briefly vortex, centrifuge and incubate for 5 minutes at room temperature
D. Add 1410 µL prechilled HT1 to dilute the denatured library pool
E. To obtain a sequencing mix, add 9 µL 20 pM denatured PhiX control DNA. The added PhiX will represent approximately 1 % of the total number of reads from the sequencing run
F. Repeatedly invert and then vortex the tube to mix and briefly centrifuge to collect the content

8.3.3 Sequencing

A. Prepare the sequencing run according to the current version of the Illumina document # 15027617: "MiSeq System Guide"[9]
B. For loading the reagent cartridge, transfer 600 µL of the sequencing mix to the sample well in the reagent cartridge
C. Load the desired flow cell and execute the sequencing run
D. After completion of the sequencing run, locate the generated sequencing data files (FASTQ) and move them to the correct location for analysis (see 2.3 and section 9)

8.4 Sequencing using MiniSeq™

8.4.1 Setting up a run using the Local Run Manager

Set up a run in the Local Run Manager (LRM) software by using the Devyser Guide “Generating a Devyser sample sheet with LRM” (see 2.4 for details) and the Illumina document #1000000002702: "Local Run Manager Software Guide"[10].

8.4.2 Denaturation of the purified library pool

A. Prepare RSB Buffer, Hybridization Buffer, a fresh dilution of 0.1 N NaOH and 1.8 pM denatured PhiX (control DNA) according to the current version of the Illumina document #1000000002697: "MiniSeq System Denature and Dilute Libraries Guide"[11]

B. Combine 5 µL purified library pool from 7.3.6 with 5 µL 0.1 N NaOH

C. Briefly vortex, centrifuge and incubate for 5 minutes at room temperature

D. Add 5 µL 200 mM Tris-HCl, pH 7.0 to the denatured library pool. Briefly vortex and centrifuge

E. Add 985 µL prechilled Hybridization Buffer to the denatured library pool. Briefly vortex and centrifuge

F. Add 570 µL prechilled Hybridization Buffer to a new tube

G. Add 180 µL diluted library (from step E) to the Hybridization Buffer (from step F). The total volume is 750 µL

H. To obtain a sequencing mix, add 5 µL 1.8 pM denatured PhiX to the library. The added PhiX will represent approximately 1 % of the total number of reads from the sequencing run.

I. Repeatedly invert and then vortex the sequencing mix and briefly centrifuge to collect the content

8.4.3 Sequencing

A. Prepare the sequencing run according to the current version of the Illumina document # 1000000002695: "MiniSeq System Guide"[12]

B. For loading the reagent cartridge, transfer 500 µL of the sequencing mix to the sample well in the reagent cartridge

C. Load the desired flow cell and execute the sequencing run

D. After completion of the sequencing run, locate the generated sequencing data files (FASTQ) and move them to the correct location for analysis (see 2.3 and section 9)

8.5 Sequencing using iSeq 100

8.5.1 Setting up a run using the Local Run Manager

Set up a run in the Local Run Manager (LRM) software by using the Devyser Guide “Generating a Devyser sample sheet with LRM” (see 2.4 for details) and the Illumina document #1000000002702: "Local Run Manager Software Guide".

8.5.2 Preparation of the purified library pool

A. Prepare RSB Buffer or 10 mM Tris-HCl, and 50 pM PhiX according to the current version of the "iSeq 100 Sequencing System Guide"[13]

B. Combine 5 µL purified library from 7.3.6 with 95 µL RSB

C. Briefly vortex and centrifuge

D. To obtain a sequencing mix, add 1 µL 50 pM denatured PhiX. The added PhiX will represent approximately 1% of the total number of reads from the sequencing run

E. Briefly vortex the tube to mix and centrifuge to collect the content

8.5.3 Sequencing

A. Prepare the sequencing run according to the current version of the "iSeq 100 Sequencing System Guide"[15]

B. For loading the reagent cartridge, transfer 20 µL of the sequencing mix to the library reservoir

C. Load the flow cell and execute the sequencing run

D. After completion of the sequencing run, locate the generated sequencing data files (FASTQ) and move them to the correct location for analysis

9. SEQUENCE DATA ANALYSIS

9.1 Supported software and sequencing mode

The Devyser Thalassemia libraries can be sequenced in paired-end mode (2 x 151 cycles) using Illumina MiSeq, MiniSeq or iSeq 100, see the following table.

Table 14. Software and sequencing mode

Software	Sequencing mode
SeqNext	Paired end 2x151 cycles*
Amplicon Suite	Paired end 2x151 cycles

*For some heterozygous genetic variants, the presented VAF values may be imprecise. See Devyser SeqNext guide for additional information (see 2.4)

9.2 Sequence data analysis using the SeqNext module of the SeqPilot software

Transfer the sequencing data files (FASTQ) to an appropriate location and start the analysis in SeqNext according to manufacturer's instructions for use. Consult the SeqNext guide for Devyser Thalassemia (see 2.4) for details on downloading settings and performing analysis and data interpretation.

9.3 Sequence data analysis using Amplicon Suite

Upload the sequencing data files (FASTQ) and start the analysis in Amplicon Suite according to manufacturer's instructions for use.

10. SYMBOLS USED ON LABELS

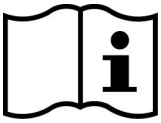
Lot or batch number

Expiry date

Number of tests

Store below temperature shown

Temperature limit



Consult instructions for use

Catalogue number

Manufacturer

Research Use Only

11. NOTICE TO PURCHASER

Purchase of this product does not provide a license to perform PCR under patents owned by any third party.

MiSeq™, MiniSeq™ and iSeq™ 100 are trademarks of Illumina® Corporation.

Qubit™, DynaMag™ and Veriti™ are trademarks of Thermo Fisher Scientific Corporation.

12. CONTACT INFORMATION

12.1 Legal manufacturer

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Instrumentvägen 19
SE-126 53 Hägersten
SWEDEN

Phone: +46-8-562 15 850
Homepage: www.devyser.com

12.2 Technical support

Phone: +46-8-562 15 850
E-mail: techsupport@devyser.com

13. REFERENCES

[1] Harteveld CL, Higgs DR. “Alpha-thalassaemia”, Orphanet J Rare Dis. 2010 May 28;5:13.

[2] Galanello R1, Origa R. “Beta-thalassemia”, Orphanet J Rare Dis. 2010 May 21;5:11.

[3] Higgs DR. “The molecular basis of α-thalassemia”. Cold Spring Harb Perspect Med. 2013 Jan 1;3 (1):a011718.

[4] Thein SL. “The molecular basis of β-thalassemia”, Cold Spring Harb Perspect Med. 2013 May 1;3 (5):a011700.

[5] http://globin.cse.psu.edu/globin/hbvar/. Nucl. Acids Res . 32 Database issue: D537-541. Cited 2020-01-09.

[6] https://www.ithanet.eu/db/ithagenes. Updated on 2020-01-07 11:55:09. Cited 2020-01-09.

[7] Illumina Experiment Manager User Guide (Document # 15031335)

[8] MiSeq System Denature and Dilute Libraries Guide (Document # 15039740)

[9] MiSeq System Guide (Document # 15027617)

[10] Local Run Manager Software Guide (Document # 1000000002702)

[11] MiniSeq System Denature and Dilute Libraries Guide (Document # 1000000002697)

[12] MiniSeq System Guide (Document # 1000000002695)

[13] iSeq 100 Sequencing System Guide (Document # 1000000036024)

14. ABBREVIATIONS

Abbreviation	Explanation
bp	base pairs
CNV	copy number variation
HBA1: hemoglobin subunit alpha 1	hemoglobin subunit alpha 1
HBA2: hemoglobin subunit alpha 2	hemoglobin subunit alpha 2
IEM	Illumina Experiment Manage
Indel	insertion and / or deletion
RUO	Research use only
NGS	next generation sequencing
PCR	polymerase chain reaction
ROI	region of interest
SNV	single nucleotide variation
VAF	variant allele frequency

15. REVISION HISTORY

Version 2021-02-11
Editorial changes

Changes to include iSeq 100 as an option for sequencing

5 Added that the kit should not be used to test patients who have undergone a previous allogenic bone marrow transplant

7.1.1 Changed duration activated Thal mix can be stored at −18°C from 6 weeks to 90 days

7.3.6 Removed instructions for storage of the purified library pool and added a note to inform that it is not recommended to store the purified and diluted library pool for long term or for re-use

Version 2020-07-01

General: Editorial changes and changes to include 96 test configuration

Chapter 1:
Added b619-del to Table 3.

Chapter 2:
Updated Table 5 Devyser Thalassemia 48 test configuration (8-A106-48-RUO)

Added Table 6 Devyser Thalassemia 96 test configuration (8-A106-96-RUO)

Chapter 7: Add note about available test configurations 8, 24 and 96 in the introduction. Updated for 96 test configuration

Added 7.2.3 Library indexing - Devyser Thalassemia 96 test kit

Version 2020-01-09

Editorial changes

1.4. Added "Partial sequencing of the HBG1, HBG2 and HBD promotors is also performed"

2.2.1 Other required Devyser products added (moved from 2.2.6)

2.2.3 DNA extraction added

Table 8. Sge file description revised

2.5. New

Chapter 3. Storage requirements. Specific storage recommendations stated for below -18 °C: -28°C to -18°C.

Chapter 5. Procedural limitations. Point 5: sample type added. Point 9 new.

7.1.1, H. Added: Do not aliquot the activated mix.

7.3.6 . Note added. Storage of purified library pool added.

Chapter 8. Illumina MiSeq Sequencing. Moved calculator text to 2.5.1. Info regarding sequencing modes moved from introduction to chapter 9. 2 x 151 bp changed to 2 x 151 cycles.

8.1. Number of samples per flow cell. New

9.1 Supported software and sequencing mode. New

Chapter 13. References 7 to 9 corrected, 10 to 12 added.

Chapter 14. List updated (VAF added)

Version 2018-12-13

Editorial changes

Updated Article number and Product name

Section 1.5 regarding Assay procedure was moved to chapter 2

Information about MiniSeq added in chapter 2, 7 and 8

Version 2018-06-20

Editorial changes.

2.2.3, 6.1.2, 7.3.5. Qubit dsDNA HS changed to Qubit dsDNA 1X HS

7.1.3. Cycles changed from 20 to 22 in the thermal profile for PCR1.

7.2.1.1. Volume **Index buffer** changed from 98 to 198 µL.

7.3.6. Concentration changed from 0.4 - 0.45 ng/µL (corresponding to approximately 2 nM) to 0.25 - 0.30 (corresponding to 1.1 - 1.3 nM).

Version 2018-04-12
New