OLERUP SBT™

SEQUENCE BASED TYPING

HARPS®

Instructions for Use

Version No: 1.1

Issue Date: 14-Sep-2017

IVD

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Contents

Principle

The OLERUP SBT™ HARPS® (Heterozygous Ambiguity Resolving Primers) are sequencing primers developed by CareDx Pty Ltd that are used to resolve heterozygous ambiguities by producing hemizygous sequence that enable the phase linking of HLA polymorphisms in locus specific sequencing based HLA typing (SBT). Following SBT with OLERUP SBT™ kits[1], the data is analysed with ASSIGN™ SBT sequence analysis software[2-4]. Once the software produces a HARPS® report, the PCR product is re-sequenced with the reported HARP(S)® and the resulting sequence data is analysed with the original data to resolve the heterozygous ambiguity.

Intended Use

CareDx Pty Ltds' OLERUP SBT™ HARPS® kits are used to assist in the resolution of heterozygous ambiguities arising from HLA sequencing based typing obtained from the use of the SBT™ typing kits. Selection of the appropriate HARP is based on the analysis of the resultant DNA sequencing data from the typing kits by CareDx Pty Ltd's ASSIGN™ SBT sequence analysis software.

Kit Composition

Each OLERUP SBT™ HARPS® product is supplied as a single vial containing a single HARP® sufficient for 20 tests (44 · L).

The OLERUP SBT™ HARPS® product names are assigned according to the following nomenclature system: Locus-terminal nucleotides, terminal nucleotide position-HARP direction. Class I HARPS® contain the "C1" prefix (e.g. C1-TT98-F), while those for Class II contain either the "RB" (HLA-DRB1), "QB" (HLA-DQB1) or "PB" (HLA-DPB1) prefixes.

For the complete list of available HARPS® please refer "Related Products" section located at the end of this document.

Storage Requirements

When stored at -20°C (temperature range of -15°C to -25°C is acceptable), the kit components can be used until the indicated expiry date and can tolerate up to 25 freeze-thaw cycles.

Accelerated stability testing for the HARPS® indicated a shelf life of five years when stored at -20°C. While confirmatory real-time testing is underway it is strongly recommended that these HARPS® are NOT to be used beyond their expiry date.

To maintain optimal kit performance, the HARP should be removed from the -20°C storage location and thawed rapidly at room temperature before use. The HARP should then be gently vortexed to ensure that the components of each tube are appropriately mixed after thawing. After use, the kits/components should be returned immediately to -20°C.

Materials, Reagents and Equipment Not Supplied

NOTE: The use of materials, reagents, equipment or procedures other than those detailed within this Instructions For Use requires validation by the user prior to use.

1. Sterile water.

2. BigDye® Terminator Cycle Sequencing Kit v3.1 or v1.1, Applied Biosystems™ by Life Technologies™.

3. 5x Sequencing Reaction Buffer (CareDx Pty Ltd, product code SEQ BUF-2.0(400) or SEQ BUF-2.0(5000)) or BigDye® Terminator v3.1 or v1.1 5X Sequencing Buffer, Applied Biosystems™ by Life Technologies™.

4. Electronic or mechanical pipettes and aerosol-resistant tips.

5. 0.2mL thin-walled thermal cycling reaction tubes (8 well strips or 96 well plates).

   Use those recommended for use with your thermal cycler.

6. Sterile 1.5mL tubes.

7. Sterile work area.

8. Table top centrifuge with plate adapters and capacity to reach 2500 x g.

9. Vortex.

10. Thermal cycler with heated lid

    These HARPS® have been validated using the following thermal cyclers:

    MJ Research PTC 225 DNA Engine DYAD™, Applied Biosystems™ by Life Technologies™ Veriti™ Thermal cycler, Gene Amp® PCR System 9700, and Eppendorf Mastercycler® Pro.

11. 125mM EDTA, pH8.0 (Available for purchase from CareDx Pty Ltd, product code EDTA-3.0(200) or EDTA-3.0(5000)).

12. Absolute and 80% Ethanol. Each run requires freshly prepared 80% ethanol consisting of absolute ethanol and sterile water. DO NOT USE DENATURED ETHANOL (also known as methylated spirits in some countries).

13. Hi-Di™ Formamide, Applied Biosystems™ by Life Technologies™, product code 4311320.

14. Automated DNA Sequencer and accessories (e.g. Applied Biosystems™ by Life Technologies™ ABI Prism® 3730), including data collection software.

These HARPS® have been tested and validated on the Applied Biosystems™ by Life Technologies™ 3100, 3730 and 3730xl capillary sequencers and software.

15. HLA Sequencing Analysis Software (e.g. ASSIGN™ SBT, version 3.6+ or higher, CareDx Pty Ltd).

Sample Requirements

Locus-specific, ExoSAP treated amplicons prepared according to the HLA OLERUP SBT™ kit Instructions for Use[1].

⚠ Warnings and Safety Precautions

- This kit must be used by trained and authorized laboratory personnel.
- All samples, equipment and reagents must be handled in accordance with good laboratory practice. In particular, all patient material should be considered as potentially infectious. The use of gloves and laboratory coats is strongly recommended. Handle and dispose of all sample material according to local and national regulatory guidelines.
- There are NO dangerous substances contained in any of the OLERUP SBT™ HARPS® products. Please refer to the MSDS that is available on the Olerup website (http://www.olerup.com).
- Do NOT use reagents beyond their expiration date.
- Use of reagents or equipment not listed under "Materials, Reagents and Equipment Not Supplied in this Kit" is NOT recommended. Such use may affect the performance of the assay.
- Care should be taken to prevent cross-contamination of specimens. Change tips between samples wherever possible. The use of aerosol-resistant tips is highly recommended.
- Pre- and Post-PCR activities must be strictly physically separated. Use specifically designated equipment, reagents and laboratory coats.

Symbols

The following non-standard symbols have been used:

Symbol	Description
C1-TT98-F	HARP® name
	Date of manufacture (required for non-EU markets).

Procedure

1. HARPS® Sequencing Reaction

1.1. Prepare a fresh solution of sequencing primer mix for each HARP® on ice each time a sequence reaction is to be performed. The composition and volumes for the mix indicated below are **per sample.**

Component	Volume
HARP®	2µL
Sterile water	11.5µL
BigDye® Terminators	1µL
5x Seq Rxn Buffer	3.5µL

1.2. Mix each sequencing reaction mixture gently by pulse vortexing.

1.3. Dispense 18µL of the sequencing reaction mix into each appropriate reaction well.

NOTE: For runs which involve few samples with many different HARPS®, it is acceptable to dispense the HARP® (2µL) directly into the individual reaction wells. A master mix may then be created composing of sterile water, BigDye® Terminators and 5x Seq Rxn Buffer, of which 16µL is to be dispensed into each reaction well. It is strongly recommended that use of this alternative procedure is validated by the user prior to implementation.

1.4. Add 2µL of purified PCR product to each appropriate well.

NOTE: Care must be taken to prevent cross-contamination of sequencing reactions.

1.5. Seal the reaction wells, mix gently and centrifuge briefly to ensure that the contents are located at the base of each reaction well.

1.6. Place the reaction wells into a thermal cycler and run according to the following profile:

Number of cycles	Temperature and time
25	96°C – 10 sec
	50°C – 5 sec
	60°C – 2 min
1	4°C - hold

1.7. Once the program is complete, remove the reaction wells from the thermal cycler and either proceed directly to purification of the reaction products or store in the dark at 4°C until required. It is recommended that samples are purified and run on the DNA sequencer within 24 hours.

2. Purification of Sequencing Reaction Products

NOTE: Purification of the reaction products may be carried out by procedures other than the ethanol precipitation method described here. It is strongly recommended that users validate these procedures before proceeding.

2.1. Briefly centrifuge the reaction wells/plates before proceeding. If reusable lids/caps have been used during thermal cycling, label the lids/caps to avoid cross-contamination.

2.2. Carefully remove the seals.

2.3. To each reaction well add 5µL of 125mM EDTA, pH8.0. Ensure that the EDTA reaches the base of the reaction well.

2.4. Add 60µL of 100% ethanol to each reaction well. Seal the wells/plate and vortex briefly but thoroughly to ensure thorough mixing.

2.5. Pellet the extension products by centrifuging at 2000g for 45 minutes. **IMMEDIATELY PROCEED TO THE NEXT STEP**. If this is not possible, re-centrifuge for an additional 10 minutes before proceeding.

2.6. Remove the seals to the reaction wells and discard the supernatant by inverting the reaction wells onto paper towel or tissues.

2.7. Place the inverted reaction wells and paper towel or tissue into the centrifuge. Centrifuge at 350g for 1 minute to remove any residual supernatant.

2.8. Remove the reaction wells from the centrifuge and replace in an upright position on the work bench. Discard the paper towel or tissues.

2.9. Prepare fresh solution of 80% ethanol with absolute ethanol and sterile water.

2.10. Add 60µL of 80% ethanol to each reaction well. Reseal the wells and vortex briefly.

2.11. Spin at 2000g for 5 minutes.

2.12. Repeat steps 2.6 and 2.7.

2.13. Remove the reaction wells from the centrifuge and discard the paper towel. Reseal the reaction wells and proceed to the denaturation step. Otherwise store at -20°C in the dark. It is recommended that the extension products are run on the DNA sequencer within 24 hours of setting up the sequencing reactions.

3. Denaturation & Electrophoresis of Sequencing Reaction Products

NOTE: The procedure for the denaturation of extension products in Hi-Di™ Formamide described here may not be necessary if purification procedures other than ethanol precipitation have been used. It is strongly recommended that users validate alternative procedures before proceeding.

3.1. Add 12µL of Hi-Di™ Formamide to each reaction well. Vortex and centrifuge the wells/plate briefly.

3.2. Incubate the reaction wells at 98°C for 5 minutes. Following incubation, ensure that the reaction wells are cooled quickly to room temperature (e.g. place on ice or use the thermal cycler to perform the denaturation and cooling steps) before being placed on the sequencer. If it is not possible to run the plates immediately, store at 4°C until required.

NOTE: Ensure that there are no air bubbles in the reaction wells. These can enter and damage the capillary.

3.3. Load the reaction wells/plate onto the automated sequencer and prepare the data collection file according to the sequencer manufacturer specifications.

3.4. The following instrument parameters have been validated by the manufacturer using Big Dye® Terminator Sequencing Kit v3.1 and POP-7™. These parameters may require user validation for other polymers, sequencing chemistries and instruments. Please refer to the appropriate instrument user's manual for detailed instructions and guidance (e.g. ensure that the dye set setting is appropriate for the chemistry used, for example v1.1 Big Dye® Terminator sequencing chemistry will require a different dye set).

Parameter	**Setting**
Dye set	Z_BigDyeV3
Mobility file	KB_3730_POP7_BDTV3
Basecaller	KB.bcp
Run Module	Regular FastSeq50_POP7
Injection time	15 sec
Run time	3000 sec

3.5. Use the instrument's data collection software to process the raw collected data and create the sequence files. Please refer to the appropriate instrument user's manual for detailed instructions and guidance.

4. Editing and analysis of electropherograms

The OLERUP SBT™ HARPS® product range were designed, developed and validated using the OLERUP ASSIGN™ SBT software developed by CareDx Pty Ltd. Users are recommended to use ASSIGN SBT v3.6+, ASSIGN SBT V4.7 or OLERUP ASSIGN SBT V471 as these versions of the software utilise setting and reference files specifically designed for the OLERUP SBT™ typing kits and HARPS®. For more details in relation to the operation of these software please refer to the applicable user manuals available for download on the Olerup website (http://www.Olerup.com).

For further information regarding the ASSIGN™ reference files to be used for analysis, please refer to the OLERUP SBT™ kit Instructions For Use[1].

Performance Characteristics

Well characterised samples that contained unresolved heterozygous ambiguities were sequenced using the recommended HARP® reported from ASSIGN™ SBT v3.6+ and higher. Each HARP produced sufficient hemizygous sequence for the resolution of heterozygous ambiguities.

Limitations and Cautions

- These products are for professional use only.
- It is strongly recommended that these products are validated by the user prior to implementation in the laboratory using samples whose HLA type has been determined by other molecular based procedures. In particular, any deviations from this procedure (e.g. the use of alternative DNA sequencing purification procedures) must be validated by the user prior to implementation.
- ASSIGN™ SBT, v3.6+ and higher (ASSIGN™ SBT V4.7 and OLERUP ASSIGN™ SBT V471), calculates the HARP(S)® required to resolve an ambiguity and includes a score based on the sequence differences at the HARPS® annealing site. The higher the score, the greater the sequence differences and the highest probability of producing hemizygous sequence.

- For further information, including exceptions and cautions regarding specific HARPS®, please refer to *OLERUP SBT™ HARPS® Technical Notes*[5] downloadable from the Olerup website (http://www.olerup.com).

Bibliography

1. *OLERUP SBT™ Typing Kits IFU*, CareDx Pty Ltd.
2. *ASSIGN™ SBT v3.6+ Operator Manual*, CareDx Pty Ltd.
3. *ASSIGN™ SBT v4.7 Operator Manual*, CareDx Pty Ltd.
4. *OLERUP ASSIGN™ SBT v471 Operator Manual*, CareDx Pty Ltd.
5. *OLERUP SBT™ HARPS® Technical Notes,* CareDx Pty Ltd.
6. Current HLA alleles can be found at http://www.ebi.ac.uk/imgt/hla.

Troubleshooting

Problem	Possible cause(s)	Solution
Weak signal intensity of electropherograms	Weak PCR product	Check sequence quality obtained from the sequencing primers supplied with the SBT Resolver™ typing kits.
	Poor sequencing set up.	Ensure sequencing reactions are set up according to the manufacturer's instructions. Ensure correct addition and mixture of samples and sequencing mixture.
	Insufficient reaction products applied to sequencer	Check sequencer parameters. Injection time and voltage may need to be increased before

Problem	Possible cause(s)	Solution
		reapplying samples on the sequencer.
	Problems during purification of sequencing products	Ensure there is sufficient mixing of materials during the purification procedure. Use extreme care when discarding the supernatant as it may dislodge the pellet.
Signal intensity is too high (Presence of high fluorescent peaks – artefacts)	Too much PCR product	Check sequence quality obtained from the sequencing primers supplied with the SBT Resolver™ typing kits. Consider using a higher dilution factor following PCR purification.
	Too much reaction products applied to sequencer.	Check instrument parameters. Consider reducing the injection time and voltage before reapplying samples on the sequencer.
Noisy baseline (high background)	Poor PCR purification	Check sequence quality obtained from the sequencing primers supplied with the SBT Resolver™ typing kits. Ensure ExoSAP treatment is undertaken according to the SBT Resolver™ typing kit instructions. Consider using ExoSAP following the manufacturers procedure

Problem	Possible cause(s)	Solution
		(increasing the amount of enzyme), or consider an alternative purification technique. If an alternative PCR purification procedure was used, ensure that the procedure was undertaken according to standard operating procedures.
	Poor sequencing set up.	Refer to "weak signal intensity of electropherograms".
	Contaminated sequencing reactions	Ensure that all steps are taken to prevent cross contamination. Change pipette tips wherever possible. Add liquids at the top of the reaction wells. Prevent aerosols.
	Contaminated sequencing reagent(s)	Check sequence quality obtained from the sequencing primers supplied with the SBT Resolver™ typing kits. Check sequence quality of other samples using the same reagent batches/aliquots. Repeat sequencing using fresh reagents.
	Poor purification of sequencing products.	Refer to "weak signal intensity of electropherograms".

Problem	Possible cause(s)	Solution
	Poor or incorrect matrix	Check spectral calibration and matrix. Repeat application of sequencing products.
Presence of Dye blobs	Poor purification of sequencing products	Refer to "weak signal intensity of electropherograms". Ensure products are washed sufficiently with 80% ethanol. Ensure all traces of ethanol are removed.
Heterozygous sequence obtained.	Incorrect HARP® selected from HARPS® report.	Check HARPS® score is above recommended threshold. Check the *SBT Resolver™ HARPS® Technical Notes*[5] for any comments or exceptions that may apply to the HARP®.
	Incorrect HARP® used.	Ensure that the correct HARP® is used.
No sequence obtained	Random sequencing failure	Check sequence data obtained from SBT Resolver™ typing kit. Check sequence data obtained for other samples that were sequenced with the same HARP®. Repeat sequencing reaction and ensure that all reagents and template have been applied.
	Poor purification of	Ensure procedure is

Problem	Possible cause(s)	Solution
	sequencing products	undertaken according to the manufacturer's instructions. If an alternative purification procedure was used, ensure that it was undertaken according to standard operating procedures.
	Incorrect HARP® selected or used.	Refer to "Heterozygous sequence obtained".

Related Products

CE marked IVDs:

OLERUP SBT™ HLA Typing Kits

XH-PD1.1-2(20) XH-PD1.1-2(50)	OLERUP SBT™ HLA-A kit (20 and 50 tests)
BS-PD2.1-2(20) BS-PD2.1-2(50)	OLERUP SBT™ HLA-B kit (20 and 50 tests)
HH-PD5.2-5(20) HH-PD5.2-5(50) LG-PD5.2-7(20) LG-PD5.2-7(50)	OLERUP SBT™ HLA-DRB1 kit (20 and 50 tests)

OLERUP SBT™ HARPS®

Product codes:

C1-TT98-F(20)	C1-AC98-F(20)	C1-TC98-F(20)	C1-TA98-F(20)	C1-CA102-F(20)
C1-CT102-F(20)	C1-CC102-F(20)	C1-AG203-F(20)	C1-GT240-F(20)	C1-TT368-F(20)
C1-GG307-R(20)	C1-GT355-R(20)	C1-AT362-F(20)	C1-GG362-AR(20)	C1-GG362-R(20)

C1-GG363-AF(20)	C1-TA363-F(20)	C1-TA368-F(20)	C1-CT423-F(20)	C1-AG413-R(20)
C1-AG453-R(20)	C1-AC497-F(20)	C1-CG570-R(20)		
C1-BTA-F(20)	C1-BCG-F(20)	C1-CC144-F(20)	C1-AC206-F(20)	C1-GA206-F(20)
C1-GC209-F(20)	C1-CG285-F(20)	C1-CA309-R(20)	C1-GAA309-R(20)	C1-GAT309-R(20)
C1-CG319-F(20)	C1-AG360-F(20)	C1-AC362-F(20)	C1-GC363-F(20)	C1-GG363-BF(20)
C1-CC387-F(20)	C1-TA420-F(20)	C1-CC486-F(20)	C1-AC559-R(20)	C1-CT559-R(20)
C1-GA559-R(20)	C1-CG572-R(20)	C1-GG572-R(20)	C1-GAG601-R(20)	
C1-CT97-F(20)	C1-CT112-F(20)	C1-CG134-F(20)	C1-CA176-F(20)	C1-AG270-F(20)
C1-AC302-R(20)	C1-GC302-R(20)	C1-CC341-R(20)	C1-CA343-F(20)	C1-CG343-F(20)
C1-CC355-F(20)	C1-GA361-F(20)	C1-CT379-R(20)	C1-GG539-R(20)	C1-TG539-R(20)
C1-AG595-R(20)	C1-AA601-R(20)			
RB-01-F(20)	RB-04-F(20)	RB-09-F(20)	RB-15-F(20)	RB-52-F(20)
RB-GG125-F(20)	RB-AA197-F(20)	RB-TT197-F(20)	RB-GT196-F(20)	RB-GA196-F(20)
RB-TA164-F(20)	RB-TT227-F(20)	RB-AT258-F(20)	RB-GC258-F(20)	RB-CT257-R(20)
RB-AT257-R(20)	RB-TT321-R(20)	RB-GT344-R(20)	RB-TG344-R(20)	

Self-certified OLERUP SBT™ HLA Typing Kits:

HH-PD3.2-2(20) HH-PD3.2-2(50)	OLERUP SBT™ HLA-C kit (20 and 50 tests)
PQ-PD6.2-2(20) PQ-PD6.2-2(50) AN-PD6.2-3(20) AN-PD6.2-3(50)	OLERUP SBT™ HLA-DQB1 kit (20 and 50 tests)
HH-PD10.1(20) HH-PD10.1(50) KD-PD10.2-1(20) KD-PD10.2-1(50)	OLERUP SBT™ HLA-DPB1 kit (20 and 50 tests)

Self-certified HARPS®:

QB-TA122-F(20)	QB-GC134-F(20)	QB-CT173-F(20)	QB-TA173-F(20)	QB-TA185-F(20)
QB-GA316-R(20)	QB-CG353-R(20)	QB-GG353-R(20)	QB-GG361-R(20)	
PB-GC112-F(20)	PB-TT113-F(20)	PB-TAC121-F(20)	PB-GC194-F(20)	PB-AT251-R(20)
PB-AT251-BR(20)	PB-GT313-R(20)	PB-AG341-R(20)	PB-GG341-R(20)	

ASSIGN™ SBT software (Self-certified):

ASSIGN™ SBT v3.6+ Product code: CGX0036+

ASSIGN™ SBT v4.7 Product code: CGX00470

OLERUP ASSIGN™ SBT v471 Product code: CGX00471

CE 0197 CE

For Research Use Only (except Australia):

OLERUP SBT™

AN-PD11.0-0(20) AN-PD11.0-0(50)	OLERUP SBT™ HLA-DRB3 kit (20 and 50 tests)
AN-PD12.0-0(20) AN-PD12.0-0(50)	OLERUP SBT™ HLA-DRB4 kit (20 and 50 tests)

AN-PD13.0-0(20) AN-PD13.0-0(50)	OLERUP SBT™ HLA-DRB5 kit (20 and 50 tests)
LC-PD2.9(20) LC-PD2.9(50)	OLERUP SBT™ HLA-B57 kit (20 and 50 tests)

General Purpose Laboratory Reagents

MgCl2 – 1.0(50) MgCl2 - 1.0(3000)	2mM $MgCl_2$
SEQ BUF – 2.0(400) SEQ BUF – 2.0(5000)	5x Seq Rxn Buffer
EDTA – 3.0(200) EDTA – 3.0(5000)	125mM EDTA, pH8.0

Please contact your local distributor for further details.

Contact Information

Manufacturer

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Website: www.olerup.com

For support and ordering details, please refer to the Olerup website (http://www.olerup.com).

Method history

Version	Date	Modification	Authorised by
1.0	08/09/2017	Version 1 drafted from IFU008 to form new OLERUP version	L. Westwood

1.1	14/09/2017	Minor updates. Added OLERUP ASSIGN V471. Re-Issued	L. Westwood