OLERUP SBT™

SEQUENCE BASED TYPING

HLA Typing Kits

Instructions for Use

PCR Amplification and Sequencing of HLA Class I and II Loci

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IVD

CareDx Pty
20 Collie St.
Fremantle 6160
Western Australia
Australia

EC REP

Qarad bvba
Cipalstraat 3
B-2440 Geel
Belgium

Contents

Principle

The HLA Sequencing Based Typing (SBT) procedure described here was originally developed by D. Sayer in 2001[1] and developed into a single tube assay in 2004[2]. The procedure involves the initial amplification of the target sequence followed by enzymatic treatment to remove unincorporated primers and dNTPs. The amplicon is then used as a template for direct automated fluorescent DNA sequencing using customized sequencing primers and the Big Dye® Terminator sequencing chemistry available from Applied Biosystems™ by Life Technologies™. The extension products are purified according to the ethanol precipitation method and denatured using Hi-Di™ formamide available from Applied Biosystems™ by Life Technologies™, before separation and detection on an automated fluorescent DNA sequencer. It is recommended that the resulting data is then analysed with ASSIGN™ SBT sequence analysis software from CareDx Pty Ltd[3-5].

Intended Use

CareDx Pty Ltd's OLERUP SBT™ HLA SBT kits are used for the typing of HLA Class I (HLA-A, -B, and -C) and Class II (HLA-DRB1, -DQB1 and -DPB1) genes in a laboratory setting from genomic DNA. Each kit contains reagents that facilitate the PCR amplification and DNA sequencing of a given gene. The resultant sequencing data is then interpreted through the use of CareDx Pty Ltd's ASSIGN™ SBT software. It should be noted that these SBT kits are not used for the diagnosis of disease but can be used as part of the process in determining compatibility between donors and recipients. The test is a DNA sequencing test that produces a DNA sequence of part of a HLA gene. The intended users of the device are appropriately qualified personnel that have a knowledge of the frequency of HLA types in their population. The tests are to be performed in regulated laboratories.

Kit Composition

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)		
Class I							
HLA-A	XH-PD1.1-2(20)	20 tests	DNA POL – HLA-A	1 x 25µL	AEX1F	AEX1R	1 x 44µL each
			HLA-A MIX	1 x 352µL	AEX2F	AEX2R	
					AEX3F	AEX3R	
					AEX4F	AEX4R	
	XH-PD1.1-2(50)	50 tests	DNA POL – HLA-A	1 x 60µL	AEX1F	AEX1R	1 x 110µL each
			HLA-A MIX	1 x 880µL	AEX2F	AEX2R	
					AEX3F	AEX3R	
					AEX4F	AEX4R	
HLA-B	BS-PD2.1-2(20)	20 tests	DNA POL – HLA-B	1 x 25µL	BEX1F	BEX2F	1 x 44µL each
			HLA-B MIX	1 x 352µL	BEX2R	BEX3F	
					BEX3R	BEX4F	
					BEX4R		
	BS-PD2.1-2(50)	50 tests	DNA POL – HLA-B	1 x 60µL	BEX1F	BEX2F	1 x 110µL each
			HLA-B MIX	1 x 880µL	BEX2R	BEX3F	
					BEX3R	BEX4F	
					BEX4R		

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)	
HLA-C	HH-PD 3.2-2(20)	20 tests	DNA POL – HLA-C HLA-C MIX	1 x 25µL 1 x 352µL	CEX1F CEX1R CEX2F CEX2R CEX3F CEX3R CEX4F CEX4R CEX5F CEX5R CEX6F CEX6R CEX7F	1 x 44µL each
	HH-PD 3.2-2(50)	50 tests	DNA POL – HLA-C HLA-C MIX	1 x 60µL 1 x 880µL	CEX1F CEX1R CEX2F CEX2R CEX3F CEX3R CEX4F CEX4R CEX5F CEX5R CEX6F CEX6R CEX7F	1 x 110µL each

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)	
Class II						
HLA-DRB1	HH-PD5.2-5(20)	20 tests	**DNA POL – DRB1** **HLA-DRB1 MIX**	1 x 10µL 1 x 370µL	**DRB1EX2F** **DRB1EX2R-2** **DRB1EX3R-2** **RB-TG344-R**	1 x 44µL each
	HH-PD5.2-5(50)	50 tests	**DNA POL – DRB1** **HLA-DRB1 MIX**	1 x 20µL 1 x 920µL	**DRB1EX2F** **DRB1EX2R-2** **DRB1EX3R-2** **RB-TG344-R**	1 x 110µL each
	LG-PD5.2-7(20)	20 tests	**DNA POL – DRB1** **HLA-DRB1 MIX**	1 x 10µL 1 x 370µL	**DRB1EX2F** **DRB1EX2R-2** **DRB1EX3F-7** **DRB1EX3R-7** **RB-TG344-R**	1 x 44µL each
	LG-PD5.2-7(50)	50 tests	**DNA POL – DRB1** **HLA-DRB1 MIX**	1 x 20µL 1 x 920µL	**DRB1EX2F** **DRB1EX2R-2** **DRB1EX3F-7** **DRB1EX3R-7** **RB-TG344-R**	1 x 110µL each
HLA-DQB1	PQ-PD6.2-2(20)	20 tests	**DNA POL – DQB1** **HLA-DQB1 MIX**	1 x 10µL 1 x 370µL	**DQB1EX2F** **DQB1EX2R** **DQB1EX3F** **DQB1EX3R**	1 x 44µL each
	PQ-PD6.2-2(50)	50 tests	**DNA POL – DQB1** **HLA-DQB1 MIX**	1 x 20µL 1 x 920µL	**DQB1EX2F** **DQB1EX2R** **DQB1EX3F** **DQB1EX3R**	1 x 110µL each

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)	
	AN-PD6.2-3(20)	20 tests	**DNA POL – DQB1** **HLA-DQB1 MIX**	1 x 10µL 1 x 370µL	**DQB1EX2F** **DQB1EX2R-3** **DQB1EX3F** **DQB1EX3R**	1 x 44µL each
	AN-PD6.2-3(50)	50 tests	**DNA POL – DQB1** **HLA-DQB1 MIX**	1 x 20µL 1 x 920µL	**DQB1EX2F** **DQB1EX2R-3** **DQB1EX3F** **DQB1EX3R**	1 x 110µL each
HLA-DPB1	HH-PD10.1(20)	20 tests	**DNA POL – DPB1** **HLA-DPB1 MIX**	1 x 10µL 1 x 370µL	**DPB1EX2F** **DPB1EX2R**	1 x 44µL each
	HH-PD10.1(50)	50 tests	**DNA POL – DPB1** **HLA-DPB1 MIX**	1 x 20µL 1 x 920µL	**DPB1EX2F** **DPB1EX2R**	1 x 110µL each
	KD-PD10.2-1(20)	20 tests	**DNA POL – DPB1** **HLA-DPB1 MIX**	1 x 10µL 1 x 370µL	**DPB1EX1F** **DPB1EX1R** **DPB1EX2F** **DPB1EX2R** **DPB1EX3F** **DPB1EX3R** **DPB1EX4F** **DPB1EX4R** **DPB1EX5F** **DPB1EX5R** **PB-AG341-R**	1 x 44µL each

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)	
	KD-PD10.2-1(50)	50 tests	**DNA POL – DPB1** **HLA-DPB1 MIX**	1 x 20µL 1 x 920µL	**DPB1EX1F** **DPB1EX1R** **DPB1EX2F** **DPB1EX2R** **DPB1EX3F** **DPB1EX3R** **DPB1EX4F** **DPB1EX4R** **DPB1EX5F** **DPB1EX5R** **PB-AG341-R**	1 x 110µL each

†The PRE-PCR kit contains a vial of a locus-specific PCR mix (e.g. **HLA-A MIX**) consisting of PCR buffer, dNTPs, $MgCl_2$, and locus specific PCR primers, along with a single vial of DNA polymerase (e.g. **DNA POL – HLA-**).

The POST-PCR kit contains sequencing primers (e.g. **AEX1F**).

Storage Requirements

The PRE- and POST-PCR boxes may be separated and stored in designated PRE- and POST-PCR freezers. When stored at -20°C (temperature range of -15°C to -25°C is acceptable), the kit components can be used until the expiry indicated on the outer kit containers and can tolerate up to 25 freeze-thaw cycles.

Accelerated stability testing for the HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 kits indicated a shelf life of two and a half years from date of manufacture when stored at -20°C. Confirmatory real-time testing completed. Do not use beyond expiry date.

To maintain optimal kit performance, the kit components should be removed from the -20°C storage location and thawed rapidly at room temperature before use. The kit components, with the exception of the polymerase, 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

PCR

1. Sterile water
2. Electronic or mechanical pipettes and aerosol-resistant tips
3. Thermal cycler with heated lid
   These kits 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.

   Use of other thermal cyclers with these kits requires validation by the user.
4. 0.2mL thin-walled thermal cycling reaction tubes (8 well strips or 96 well plates).
   Use those recommended for use with your thermal cycler.
5. Sterile 1.5mL tubes
6. Sterile work area such as biological safety cabinet or hood.
7. Table top centrifuge with plate adapters and capacity to reach 2500 x g
8. Vortex

Agarose Gel Electrophoresis

9. Agarose gel electrophoresis apparatus
10. 1% agarose (molecular biology grade) TBE gel containing 0.1□g/mL ethidium bromide.
11. Loading buffer
12. PCR Marker suitable to cover range of 300 – 1300 bp
13. UV transilluminator

Purification of PCR Product

14. ExoSAP (USB® ExoSAP-IT® Cat No 78200 for 100 reactions or Illustra™ ExoProStar™ Cat No US77702 for 100 reactions)
15. 2mM $MgCl_2$ (Available for purchase from CareDx Pty Ltd, product code MgCl2-1.0(50) or MgCl2-1.0(3000))
16. Shaker

    The use of alternative PCR purification techniques requires validation by the user prior to use.

Sequencing Reaction

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

18. 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™.

Purification of Sequencing Reaction Products

19. 125mM EDTA, pH8.0 (Available for purchase from CareDx Pty Ltd, product code EDTA-3.0(200) or EDTA-3.0(5000)).

20. 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).

    The use of alternative sequencing purification techniques requires validation by the user prior to use.

Denaturation and Electrophoresis of Sequencing Reaction Products

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

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

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

    The use of other denaturation techniques and sequencing platforms requires validation by the user prior to use.

23. HLA Sequencing Analysis Software (e.g. ASSIGN™ SBT, version 4.7 or higher, CareDx Pty Ltd).

Sample Requirements

1. Sterile water (negative/ no template control)

2. High molecular weight human genomic DNA (concentration range of 20-100ng/µL in Tris/EDTA buffer and $OD_{260/280}$> 1.8) extracted from ACD or EDTA anticoagulated whole blood specimens. Do NOT use whole blood specimens containing heparin.

   The DNA Isolation method used requires validation by the user prior to use.

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 kit components.
- Do NOT use reagents beyond their expiration date.
- The use of kit components from different kit batches is NOT recommended. Such use may affect the assay's performance.
- Use of reagents not included in this kit or not listed under "Materials, Reagents and Equipment Not Supplied" (e.g. alternative *Taq* DNA polymerases) is NOT recommended. Such use may affect the performance of the assay.
- Care should be taken to prevent cross-contamination of DNA specimens. Change tips between DNA specimens.
- Pre- and Post-PCR activities must be strictly physically separated. Use specifically designated equipment, reagents and laboratory coats.
- Ethidium bromide is a potential carcinogen. Protective gloves must always be used when preparing and handling gels. Dispose of ethidium-bromide gels and buffers according to local and national guidelines.
- While viewing and photographing agarose gels under UV light, always avoid direct exposure and use appropriate UV-blocking face protection, disposable gloves and laboratory coats.

Symbols

The following non-standard symbols have been used:

Symbol	Description
HLA-X MIX	Locus specific PCR Mix
DNA POL – XXXX	DNA polymerase
AEX1F	HLA-A exon 1 forward sequencing primer. Refer to "Kit Composition" and Table 4 for other sequencing primers.
	Date of manufacture (required for non-EU markets).

Procedure

1. PCR

1.1. A separate PCR reaction will need to be set up for each locus to be amplified, and for each individual sample to be tested. Each run should include appropriate positive control/s of known genotype, and at least one negative control for each locus being amplified.

1.2. Prepare a fresh solution of PCR master mix each time a PCR is performed. Quickly thaw the locus-specific PCR mix at room temperature. Once thawed, vortex briefly.

1.3. Dispense the required volume of PCR mix and DNA polymerase into a sterile tube for the number of samples to be tested (refer to Table 1 below for the volume per reaction). Pulse vortex the solution 3-4 times.

Locus	A	B	C	DRB1	DQB1	DPB1
Locus-specific PCR Mix	16□L	16□L	16□L	16.7□L	16.7□L	16.7□L
e.g. **HLA-A MIX**						
DNA Polymerase	1□L	1□L	1□L	0.3□L	0.3□L	0.3□L
e.g. **DNA POL – HLA-A**						

Table 1: Composition of the master mix required <u>per sample</u>.

1.4. Dispense 17□L of the master mix into each reaction well.

1.5. Add 3□L of sample DNA or appropriate positive control/s to each reaction well. Add 3□L of sterile water to the negative control reaction well.

1.6. Seal the reaction wells. Mix gently by vortexing and centrifuge briefly.

1.7. Place the reaction wells into a thermal cycler and run according to the thermal cycling conditions below.

95°C – 10 mins

96°C – 20 secs
60°C – 30 secs
72°C – 3 mins
} 33 cycles

15°C - hold

1.8. Amplification takes approximately 2.5 hours to complete.

1.9. When the PCR is completed, remove the reaction wells/plate from the thermal cycler and either proceed directly to gel electrophoresis or store at 4°C until required.

NOTE: Purification of amplicons by ExoSAP treatment should occur within 24 hours of completion of PCR.

2. Agarose Gel Electrophoresis

2.1. Confirm successful amplification by agarose gel electrophoresis using 2□L of each PCR product combined with 5□L loading buffer (alternative volumes of loading

buffer should be validated prior to use). The use of 1% agarose gels is recommended.

2.2. The number and expected sizes of the resultant amplicons will vary according to the locus and sample genotype. Expected PCR amplicon sizes are indicated in Table 2.

Locus	Expected band sizes
HLA-A	≈ 2 kbp
HLA-B	≈ 2 kbp
HLA-C	≈ 1.1 kbp and 1.4 kbp
HLA-DRB1	≈ 450 bp - 850 bp (HH-PD5.2-5)
	≈ 630 bp - 980 bp (LG-PD5.2-7)
	Banding pattern will vary depending on the presence of specific allele groups
HLA-DQB1	≈ 300 bp and 500 bp (PQ-PD6.2-2)
	≈ 400 bp and 500 bp (AN-PD6.2-3)
HLA-DPB1	≈ 400 bp (HH-PD10.1)
	≈400 bp, ≈780 bp and ≈1470 bp (KD-PD10.2-1)

Table 2: Expected product sizes for each assay.

3. Purification of PCR Product

NOTE: Purification systems other than ExoSAP-IT® or ExoProStar™ (e.g. Agencourt® AMPure® XP or column-based systems) can be used to purify these PCR products. It is strongly recommended that users validate these procedures before proceeding. If ExoSAP treatment is to be used it is recommended that users follow the procedure described below.

3.1. Prepare a mastermix consisting of 4µL of ExoSAP-IT® or ExoProStar™ and 8µL of 2mM $MgCl_2$ per sample to be purified. Gently pulse vortex to mix. Dispense 12µL of the mastermix into the reaction well of each reactive sample. Seal the wells, vortex and then either place on a shaker or gently vortex for 2 minutes. Centrifuge briefly before placing into the thermal cycler. Run the thermal cycler according to the following profile:

37°C – 30 mins
80°C – 15 mins
4°C - hold

3.2. Upon completion, dilute the purified product 1:4 with sterile water. This dilution step will ensure that there is sufficient template to perform the sequencing reactions and ensure that the concentration of the template is sufficient to produce good quality sequence data.

NOTE: A higher dilution factor (e.g. 1:8) may be required if consistently high signals and associated noise and artefacts are observed. Weaker PCR products may require a lower dilution factor.

3.3. ExoSAP treated samples may be stored at 4°C until ready for use. These samples can be stored at 4°C for up to a week before use, but should be stored at -20°C for long term storage [9].

4. Sequencing Reaction

NOTE: In instances where heterozygous ambiguities are to be resolved with hemizygous sequencing primers such as HARPS®, please refer to the OLERUP SBT™ HARPS® Instructions for Use.

4.1. Table 3 lists the sequencing primers that are to be used for each locus.

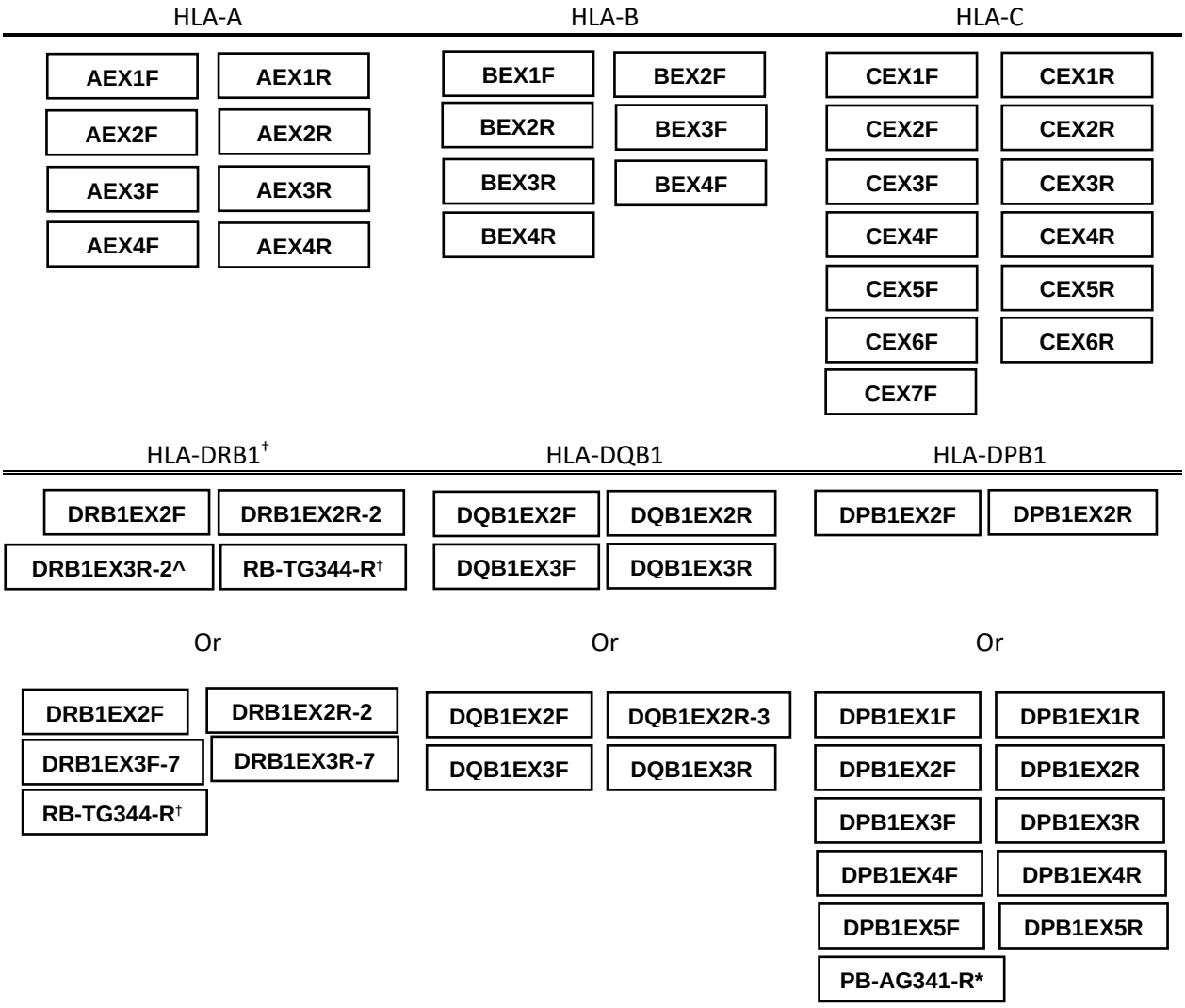

Table 3: Sequencing primers provided for use for each locus.

†RB-TG344-R is a HARP® directed to the codon 86 dimorphism. Its use is optional.

*PB-AG341-R is a HARP® directed to the codon 85 dimorphism in DPB1. Its use is also optional.

^DRB1EX3R-2 is a DRB1 sequencing primer in the HH-PD5.2-5 kits which behaves similar to a HARP and is designed to sequence the following allele groups: *03, *08, *11, *12, *13, *14, *15 and *16. This primer will produce either heterozygous, hemizygous, or no sequencing data depending on the genotype of the sample being typed. When analysing DRB1EX3R-2 data in ASSIGN™ against the DRB1-FullX2 reference, the resulting exon 3 data will be analysed in a separate layer and will allow resolution of a number of allele ambiguities in exon 3, such as the DRB1*14:01 vs *14:54 ambiguity. Its use is optional depending on the typing strategy used by the

laboratory. This is not applicable to the LG-PD5.2-7 kits as bi-directional sequencing for exon 3 is available.

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

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

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

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

NOTE: For runs which involve few samples with many sequencing primers, it is acceptable to dispense the sequencing primer (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 16uL 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.

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

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

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

4.7. 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

4.8. Once the program is complete, remove the reaction wells/plate 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.

5. 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.

5.1. 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.

5.2. Carefully remove the seals.

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

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

5.5. 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.

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

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

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

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

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

5.11. Spin at 2000g for 5 minutes.

5.12. Repeat steps 5.6 and 5.7.

5.13. 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[10]. It is recommended that the extension products are run on the DNA sequencer within 24 hours of setting up the sequencing reactions.

6. 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 the ethanol precipitation have been used. It is strongly recommended that users validate alternative procedures before proceeding.

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

6.2. 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. Once the sample has been resuspended in Hi-Di Formamide, it is recommended that it be loaded immediately on the instrument. The sample will be stable for 24 hours on the instrument[8].

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

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

6.4. 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

6.5. 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.

7. Editing and analysis of electropherograms

The OLERUP SBT™ kits are designed, developed and validated using the OLERUP ASSIGN™ SBT software developed by CareDx Pty Ltd. Users are recommended to use ASSIGN™ SBT versions 3.6+ and higher (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 CareDx website (http://www.CareDx.com).

The sequencing based typing data generated using the OLERUP SBT™ typing kits should be analysed against the following ASSIGN™ SBT reference files which are provided by CareDx Pty Ltd:

Assay	Product Code	ASSIGN Reference File
OLERUP SBT™ HLA-A	XH-PD1.1-2	A.xml
OLERUP SBT™ HLA-B	BS-PD2.1-2	B.xml
OLERUP SBT™ HLA-C	HH-PD3.2-2	C.xml or Cw.xml
OLERUP SBT™ HLA-DRB1	HH-PD5.2-5	DRB1-FullX2.xml
	LG-PD5.2-7	527_DRB1.xml
OLERUP SBT™ HLA-DQB1	PQ-PD6.2-2	DQB1.xml
	AN-PD6.2-3	623_DQB1.xml
OLERUP SBT™ HLA-DPB1	HH-PD10.1	DPB1.xml
	KD-PD10.2-1	DPB1.xml

Performance Characteristics

Accuracy

Panels of up to 93 samples from the UCLA International DNA Exchange proficiency testing program (2008 – 2010) used for internal testing for the OLERUP SBT™ kits yielded the following results:

Locus	Number of samples tested	Diagnostic sensitivity (% of successful PCRs)	Diagnostic specificity (% of genotypes obtained)	Number of discordant samples	Number of heterozygous samples	Number of unique alleles
HLA-A	81	100%	100%	0	74	20
HLA-B	82	100%	98.8%	0	79	81
HLA-C	39	97.5%	97.5%	0	35	21
HLA-DRB1	93	96.7%	96.7%	0	84	39
HLA-DQB1 (PQ-PD6.2-2)	42	100%	100%	0	36	14
HLA-DQB1 (AN-PD6.2-3)	38	100%	100%	0	34	15
HLA-DPB1 (HH-PD10.1)	77	100%	100%	0	60	18
HLA-DPB1 (KD-PD10.2-1)	16	100%	100%	2*	14	13

* The two discordant samples contained additional sequence information outside exon 2 that was not reported by the UCLA International DNA Exchange proficiency testing program. One sample contained 131:01, but was reported as 13:01 by the UCLA International DNA Exchange proficiency testing program. The alleles differ in exons 3 and 4. The other sample contained 107:01, but was reported as 13:01 by the UCLA International DNA Exchange proficiency testing program. These alleles differ in exon 1.

For the OLERUP SBT™ HLA-DRB1 kits (product code LG-PD5.2-7), a panel of 23 well characterised samples, covering a broad range of alleles was used for internal testing. In addition, a panel of 293 externally sourced samples were also typed without *a priori* knowledge of other HLA typing data. These samples were also tested with the OLERUP SBT™ HLA-DQB1 assay (PQ-PD6.2-2). In those cases where a homozygous result was obtained, the DQB1/ DRB1 associations for those samples were examined to confirm the result as well as to detect instances where allele-drop out may have occurred.

The testing yielded the following results:

Locus	Number of samples tested	Diagnostic sensitivity	Diagnostic specificity	Number of discordant samples	Number of heterozygous samples	Number of unique alleles
HLA-DRB1	23	100%	100%	0	23	12
	286*	97.9%	99.6%	0	253	33

* Six samples failed to amplify due to poor quality DNA samples. One sample was found to contain contaminating DNA, the source of which occurred at the laboratory from which the samples were obtained. As a result of the contamination, a genotype could not be obtained for that sample.

Sequence analysis of PCR and sequencing primer sites and performance evaluation studies have not identified any common and well documented alleles that have not been amplified through the recommended use of these kits. For further information refer to the *OLERUP SBT™ Primer Analysis* document available with each OLERUP ASSIGN™ SBT reference release, downloadable from the CareDx website (http://www.CareDx.com).

Detection Limit

The recommended concentration of high molecular weight human genomic DNA is 20-100ng/µL. Internal testing has shown that samples with concentrations as low as 5ng/µL can also be used. Correct genotypes were also obtained from poor quality or sheared DNA.

Specificity

CareDx Pty Ltd's OLERUP SBT™ kits are locus specific assays. Use of the kits according to these instructions should only amplify a single locus. In most instances the use of the sequencing primers incorporated in each kit will produce a HLA typing for most samples without the need for further resolution. In those instances where heterozygous ambiguities remain, the use of resolving sequencing primers (such as OLERUP SBT™ HARPS®) is recommended.

It should be noted that mutations at amplification or sequencing primer sites are possible and may result in allele drop-out. Samples that suggest a homozygous typing result must be confirmed by alternative procedures.

Interfering Substances

CareDx Pty Ltd has identified all known potential interfering substances that could impact the test. See table below.

Inhibitor	Potential source	Risk	Comments
EDTA	TE buffer, blood collection tubes	Very low	Resuspend DNA in Tris-HCl pH8 or TE with <1mM EDTA. Use commercial Blood DNA preparation kits and/or avoid EDTA blood collection tubes
Alcohols	Ethanol, isopropanol, isoamyl alcohol	Low	Ensure DNA pellets or beads are air dried and visually inspected for ethanol droplets (1% ethanol = 1.25ul 80% ethanol in a 100ul PCR reaction).
Excess salts	KCl, NaCl, CsCl, NaAc	Very low	Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 230/260 starting genomic DNA ~2
Chaotropic salts	Guanidinium Cl; MgCL2; urea	Very low	Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 230/260 starting genomic DNA ~2
Phenol:chloroform	Organic carryover	Very low	A component of the widely used commercial Trizol DNA extraction procedure. Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 230/260 starting genomic DNA ~2
Proteins	BSA, PEG, blood	Very	Use commercial Blood DNA preparation kits. Ensure OD

Inhibitor	Potential source	Risk	Comments
	albumin	low	260/280 starting genomic DNA >1.8
Heme, hemoglobin, immunoglobulins	Blood	Very low	Avoid using blood samples exhibiting gross hemolysis. Use commercial Blood DNA preparation kits. Ensure OD 260/280 starting genomic DNA >1.8
Detergents/DDT	Na deoxycholate, sarkosyl, SDS, NP40, Tween 20, Triton X-100, N-octyl glucoside	Very low	Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 230/260 starting genomic DNA ~2
Proteases	Proteinase K, sample handling	Very low	Use commercial Blood or saliva DNA preparation kits. Wear gloves at all times
Nucleases	Sample handling, restriction enzymes, micrococcal nuclease	Very low	Use commercial Blood DNA preparation kits. Wear gloves at all times
Exogenous DNA/RNA	Carryover, contamination	Very low	Prepare genomic DNA in dedicated pre-PCR area
Carriers	RNA, heparin, glycogen	Very low	Use commercial Blood DNA preparation kits and/or avoid heparin blood collection tubes
Excess metal ions	Mg2+ from PCR buffer, Fe ions	Very low	Ensure thorough washing of DNA pellets or beads with 80% ethanol. Ensure OD 230/260 starting genomic DNA ~2
Antiviral drugs (e.g. acyclovir)	Blood	Very low	Use commercial Blood DNA preparation kits. Ensure OD 260/280 starting genomic DNA >1.8
Glove powder	Powdered gloves	Very low	Use powder free gloves
UV irradiated PCR tubes	UV treatment of PCR tubes	Very low	Avoid UV treatment of plasticware

Limitations and Cautions

- It is strongly recommended that these kits 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 PCR or DNA sequencing purification procedures) must be validated by the user prior to implementation.
- These kits have been validated using panels of samples whose genotypes cover a broad range of alleles. However, it should be noted that rare alleles and alleles with polymorphisms in amplification and sequencing primer sites may be encountered and these may not be amplified or sequenced.
- The nature of HLA sequence based typing is such that factors other than the PCR mix may result in preferential amplification or allele drop out. As a consequence, apparent homozygous typing results should be confirmed using alternative methods and/or family genotyping.
- A positive control (human DNA) and negative control (sterile water) must be included on every PCR run. The positive control must produce a PCR product of the appropriate size depending on the locus amplified and the resultant sequence must be in concordance with the sample's genotype. There must be no PCR products in the negative template

control for each experiment. If a band is evident contamination may have occurred at some level and the run must be repeated.

- Occasionally there may be additional, fainter PCR products evident. These additional bands do not interfere with sequence results or quality.

License

The OLERUP SBT™ kits contain GoTaq® Hot Start Polymerase (DNA POL) which is manufactured by Promega Corporation for distribution by CareDx Pty Ltd. Licensed to Promega under U.S. Patent Nos. 5,338,671 and 5,587,287 and their corresponding foreign patents.

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Troubleshooting

Problem	Possible cause(s)	Solution
No or weak PCR product	Poor quality DNA	Assess DNA quality by gel electrophoresis. Intact DNA should be approx. 3kb with little or no evidence of smearing on gel. Re-extract DNA and repeat PCR where possible.
	Insufficient quantity of DNA added to PCR.	Check concentration of DNA is between 20-100ng/µL. Re-extract DNA and repeat PCR where possible.
	Presence of PCR inhibitors in genomic DNA	PCR inhibitors are present in all bodily fluids, including blood, serum, and plasma. PCR inhibitors represent a diverse group of substances with different properties and mechanism of action. The most common inhibitors in blood are Haemoglobin, IgG, and Lactoferrin. In addition, hormones or antiviral substances like acyclovir, as well as some antibiotics can also affect gene amplification[12,13]. Avoid the use of whole blood specimens containing heparin. For specifics, please see the referenced manuscripts[12,13]. EDTA: can alter Mg2+ concentrations and may inhibit DNA polymerase at certain concentrations[13]. Generally, Na-citrate tubes lead to high-quality DNA[11]. DNA extraction/purification methods may efficiently remove PCR inhibitors. Customer should evaluate the DNA extraction /purification methods to ensure the purity of the sample. Re-extract DNA and repeat PCR where possible.
	DNA polymerase not added to the mastermix or insufficient mixing of mastermix prior to addition to samples.	Repeat PCR. Ensure mastermix components are added and mixed sufficiently by vortexing.
	Thermal cycling problems	Check the thermal cycling run parameters. Check the run history to ensure that the run was not terminated prematurely. Ensure that the thermal cycler is operating according to manufacturer's specifications and is regularly maintained.
	No ethidium bromide added to the gel.	Submerge the gel in a staining bath containing 1X TBE with 0.5mg/mL ethidium bromide. Destain in 1X TBE before taking gel image. Ensure ethidium bromide is added to gel prior to pouring.
	DNA samples are eluted or diluted in water that can have a slightly acidic pH.	Wherever possible use sterile water with a neutral pH.
No or weak PCR product for the exon 3-5 band for	Poor quality DNA	Amplification of samples of very poor quality may result in weak amplification of the exon 3-5 amplicon. Typing can still be achieved using exon 1

the KD-PD10.2-1 assay		and 2 sequence data. Alternatively re-extract DNA and repeat PCR where possible.
Incorrect band sizes	Incorrect kit used	Check that the appropriate kit is used.
	Incorrect thermal cycling program used.	Check the thermal cycle parameters.
	PCR contamination	Check the negative control for evidence of contamination. Decontaminate work area and repeat PCR. Repeat PCR to identify source of contamination. Consider using a fresh kit. If the genomic DNA of a sample appears to be contaminated, re-extract or obtain an alternative source of DNA.
Weak signal intensity of electropherograms	Weak PCR product	Check gel image. Sequencing weak PCR bands is NOT recommended as the sequence quality may be insufficient for SBT. Consider using a lower dilution factor (e.g. 1:2, 1:3) after PCR purification.
	Insufficient reaction products applied to sequencer	Check sequencer parameters. Injection time and voltage may need to be increased.
	Problems during purification of sequencer products	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 the gel image. Consider using a higher dilution factor following PCR purification. Check the amount of DNA polymerase used in the PCR.
	Too much reaction products applied to sequencer.	Check instrument parameters. Consider reducing the injection time and voltage.
Noisy baseline (high background)	Contaminated PCR product	Refer to corrective actions listed above.
	Amplification of closely related HLA genes	Check thermal cycling parameters.
	Poor PCR purification	Ensure ExoSAP treatment is undertaken according to kit's user instructions. Ensure that the PCR mixture is mixed thoroughly with ExoSAP Consider using ExoSAP following the manufacturers procedure (increasing the amount of enzyme), or consider an alternative purification technique.
	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 primer	Check sequence quality of the other sequencing primers and other samples using the same primer. Consider using a fresh aliquot of sequencing

		primer.
	Contaminated dye terminator mix or sequencing buffer	Repeat sequencing with fresh aliquot of reagents.
	Poor purification of sequencing products.	Repeat sequencing and ensure that purification is undertaken according to manufacturer's instructions.
Presence of Dye blobs	Poor purification of sequencing products	Purify products according to kit instructions. Ensure products are washed sufficiently with 80% ethanol.

Related Products

CE marked IVDs:

ASSIGN™ SBT 3.6+ Product code: CGX0036+

ASSIGN™ SBT v4.7 Product code: CGX00470

OLERUP ASSIGN™ SBT v471 Product code: CGX00471

OLERUP SBT™ HARPS®

For full product list, please refer to the OLERUP SBT™ HARPS® Instructions for Use

For Research Use Only:

OLERUP SBT™ HLA Typing Kits

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)

Note: the products listed above are licensed as IVDs in Australia

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.

CE 0197

CE

Self-certified kits:

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

Contact Information

Manufacturer

CareDx Pty Ltd
PO Box 1294
Fremantle 6959
Western Australia
Australia
Tel: +61-08-9336-4212
Email: orders-aus@caredx.com
Website: www.CareDx.com

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