SBT RESOLVER™

Instructions for Use

PCR Amplification and Sequencing of the DRB3/DRB4/DRB5

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Conexio Genomics Pty Ltd
8/31 Pakenham St
Fremantle 6160
Western Australia
Australia

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Principle

The HLA Sequence Based Typing (SBT) procedure described here involves the locus specific PCR amplification of the HLA-DRB3/DRB4/DRB5 target amplicons which are visualised by agarose gel electrophoresis. Samples that are positive for HLA-DRB3/DRB4/DRB5 will produce two amplicons (the target amplicon and internal control) while negative samples will produce a single amplicon only (internal control). Positive samples are then further characterised by DNA sequencing following treatment with ExoSAP-IT® to remove unincorporated primers and dNTPs. The target amplicon is used as a template for direct automated fluorescent DNA sequencing using customized sequencing primers and the BigDye® 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 analysed with Assign™ sequence analysis software from Conexio Genomics Pty Ltd.

Kit Composition

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)		
Class II							
HLA-DRB3	AN-PD11.0(20)	20 tests	**DNA POL – DRB3** **HLA-DRB3 MIX**	1 x 9µL 1 x 370µL	**DRB3EX2F**	**DRB3EX2R**	1 x 44µL each
	AN-PD11.0(50)	50 tests	**DNA POL – DRB3** **HLA-DRB3 MIX**	1 x 18µL 1 x 920µL	**DRB3EX2F**	**DRB3EX2R**	1 x 110µL each
HLA-DRB4	AN-PD12.0(20)	20 tests	**DNA POL – DRB4** **HLA-DRB4 MIX**	1 x 9µL 1 x 370µL	**DRB4EX2F**	**DRB4EX2R**	1 x 44µL each
	AN-PD12.0(50)	50 tests	**DNA POL – DRB4** **HLA-DRB4 MIX**	1 x 18µL 1 x 920µL	**DRB4EX2F**	**DRB4EX2R**	1 x 110µL each

HLA-DRB5	AN-PD13.0(20)	20 tests	**DNA POL – DRB5** **HLA-DRB5 MIX**	1 x 9µL 1 x 370µL	**DRB5EX2F** **DRB5EX2R** **DRB5EX3F**	1 x 44uL each
	AN-PD13.0(50)	50 tests	**DNA POL – DRB5** **HLA-DRB5 MIX**	1 x 18µL 1 x 920µL	**DRB5EX2F** **DPB5EX2R** **DRB5EX3F**	1 x 110uL each

†The PRE-PCR component of each kit consists of a vial/s of a locus-specific PCR mix (e.g. **HLA-DRB3 MIX**) consisting of PCR buffer, dNTPs, $MgCl_2$, locus specific PCR primers, and a single vial of DNA polymerase (e.g. **DNA POL – DRB3**).

The POST-PCR kit contains sequencing primers (e.g. **DRB3EX2F**).

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, the kit components can be used until the expiry date indicated on the outer kit containers.

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 tested using the following thermal cyclers:

   MJ Research PTC 225 DNA Engine DYAD™, Applied Biosystems™ by Life Technologies™ GeneAmp® 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 biological safety cabinet or hood.
7. Table top centrifuge with plate adapters and capacity to reach 2500 x g
8. Vortex
9. Agarose gel electrophoresis apparatus
10. 1% agarose (molecular biology grade) TBE gel containing 0.1µg/mL ethidium bromide.
11. Loading buffer
12. PCR Marker suitable to cover range of 300 – 1300 bp
13. UV transilluminator

PCR Purification

14. ExoSAP-IT® (USB® Products Cat No 78200 for 100 reactions)
15. 2mM $MgCl_2$
16. Shaker

Sequencing, Purification and Denaturation.

17. BigDye® Terminator Cycle Sequencing Kit v3.1 or v1.1, Applied Biosystems™ by Life Technologies™.
18. BigDye® Terminator v1.1 & v3.1 5X Sequencing Buffer, Applied Biosystems™ by Life Technologies™.
19. 125mM EDTA, pH8.0

20. Absolute Ethanol. Each run needs freshly prepared 80% ethanol solution consisting of absolute ethanol and sterile water. DO NOT USE DENATURED ETHANOL.

Other PCR and Sequencing purification procedures require validation by the user prior to use.

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

22. Automated DNA Sequencer and accessories (eg 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 sequencing platforms requires validation by the user prior to use.

23. HLA Sequencing Analysis Software (eg Assign SBT™, version 3.5 or higher, or Assign ATF™ (Conexio Genomics 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.

⚠ 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 biological 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" (eg alternative 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 wherever possible.
- 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.

Procedure

1. PCR

1.1. Set up one reaction for each sample, for each loci being amplified. Include appropriate positive and negative amplification controls of known genotype and at least one no template control for each group of samples being amplified.

1.2. Prepare a fresh solution of PCR master mix each time a PCR is performed. Thaw the required number of vials of the appropriate PCR Mix. Once thawed vortex briefly.

1.3. Dispense the required amount of PCR mix and DNA polymerase into a sterile tube for the number of samples to be tested. Refer to Table 1 below. Pulse vortex the solution 3-4 times.

Locus	**DRB3**
Locus-specific PCR Mix e.g. **HLA-DRB3 MIX**	16.7µL
DNA Polymerase e.g. **DNA POL-DRB3**	0.3µL

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

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

1.5. Add 3µL of sample DNA or appropriate control sample to each reaction well. Add 3µL of sterile water to the no template 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 amplify the target sequence 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 plate from the thermal cycler and either proceed directly to gel electrophoresis or store at 4°C until required.

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

2. *Agarose Gel Electrophoresis*

2.1. Confirm successful amplification of the internal control amplicon in for all DNA samples tested, and the DRB3/DRB4/DRB5 target amplicon in positive control and positive DNA samples by agarose gel electrophoresis using 5μL of each PCR product combined with 5μL of loading buffer (alternative volumes of loading buffer should be validated prior to use). The use of 1% agarose gels is recommended.

2.2. All samples tested using the DRB3/DRB4/DRB5 PCR mixes should amplify the internal control amplicon regardless of HLA-DRB3/DRB4/DRB5 genotype. HLA-DRB3/DRB4/DRB5 positive samples should amplify both the internal control amplicon plus the target amplicon. The expected sizes of each amplicon are listed in Table 2.

Locus	Expected band sizes
DRB3 target amplicon	≈ 640 bp
DRB4 target amplicon	≈ 460 bp
DRB5 target amplicon	≈ 470 bp
Internal control band	≈ 400 bp

Table 2: Expected product sizes for the DRB3/DRB4/DRB5.

3. *Purification of Positive DRB3/DRB4/DRB5 PCR Product*

NOTE: Purification systems other than EXOSAP-IT® (eg 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-IT® 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® and 8μL of 2mM $MgCl_2$ per sample. Dispense 12μL of the mastermix into the reaction well of each positive sample. Seal the tubes, vortex, and place on a shaker or gently vortex for 2 mins. Centrifuge briefly before placing into the thermal cycler. Run the thermal cycler according to the following profile:

37°C - 30mins
80°C - 15mins
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 (eg 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-IT® treated samples may be stored at 4°C until ready for use.

4. Sequencing Reaction

NOTE: Only DRB3/DRB4/DRB5 positive samples identified by gel electrophoresis should be sequenced using the following procedure.

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

Locus	Sequencing Primers
DRB3	DRB3EX2F DRB3EX2R
DRB4	DRB4EX2F DRB4EX2R
DRB5	DRB5EX2F DRB5EX2R DRB5EX3F

Table 3: Sequencing primers provided to sequence the positive samples for each locus.

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 are indicated **per sample.**

Component	Volume
Sequencing primer	2 µL
Sterile water	11.5 µL
BigDye® Terminators	1 µL
5X Sequencing buffer	3.5 µL

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

4.4. Dispense 18µL of the sequencing reaction mix to each appropriate reaction tube/well.

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 tubes, mix gently and centrifuge briefly to ensure that the contents are located at the base of each reaction tube.

4.7. Place the reaction tubes into a thermal cycler and run according to the following profile:

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

4.8. Once the program is complete, remove the reaction tubes from the thermal cycler and either proceed directly to purification of the reaction products or store 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 tubes/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 seal.

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

5.4. Add 60 µL of 100% ethanol to each reaction well. Seal the 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 tubes and discard the supernatant by inverting the reaction tubes onto paper towel or tissues.

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

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

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

5.10. Add 60µL of 80% ethanol to each reaction tube/well. Reseal the tubes and mix by vortexing briefly.

5.11. Spin at 2000g for 5 mins.

5.12. Repeat steps 5.6 to 5.7.

5.13. Remove the reaction tubes from the centrifuge and discard the paper towel. Reseal the reaction tubes and proceed to the denaturation step. Otherwise store at -20°C for no longer than 24 hours.

6. Denaturation & Electrophoresis of Sequencing Reaction Products

Denaturation of extension products

6.1. Add 12µL of Hi-Di™ Formamide to each reaction tube. Vortex and centrifuge the tubes briefly.

6.2. Incubate the reaction tubes at 98°C for 5 minutes. Place the reaction tubes on ice for at least 3 minutes before being placed on the sequencer. If this is not possible, store at 4°C until the reactions reach room temperature or until required.

NOTE: ENSURE THAT THERE ARE NO AIR BUBBLES IN THE REACTION WELLS. THESE CAN ENTER AND DAMAGE THE CAPILLARY.

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

Electrophoresis Conditions for ABI 3730 & 3730xl automated sequencers

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. Dye set setting for v1.1 Big Dye® Terminator sequencing chemistry).

Parameter	Setting
Dye set	Z_BigDyeV3
Mobility file	KB_3730_POP7_BDTV3
Basecaller	KB.bcp
Run Module	Regular FastSeq50_POP7
Injection time	15 sec
Collection 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 SBT Resolver™ kits were developed and validated using the Assign ATF™ software developed by Conexio Genomics Pty Ltd. Sequence analysis may also be performed using Assign SBT™ software. For more details please refer to the Conexio Genomics website (http://www.conexio-genomics.com).

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.
- These Sequence Based Typing 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.
- A positive control (human DNA sample known to have HLA-DRB3/DRB4/DRB5), a negative control (human DNA sample known to be negative for HLA-DRB3/DRB4/DRB5) and no template control (sterile water) must be included on every PCR run. The positive control must produce two amplicons of the appropriate size and the resultant sequence must be in concordance with the sample's genotype. The negative control must produce a single internal control amplicon of the appropriate size. There must be no PCR products in the no 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 larger, fainter PCR products evident. These additional bands do not interfere with sequence results or quality.

License

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

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	Avoid the use of whole blood specimens containing heparin. 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.
Incorrect band sizes	Incorrect kit used	Check that the appropriate kit has been 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

Problem	Possible cause(s)	Solution
		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 (eg 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. Consider adjusting the parameters if an alternative thermal cycler is used.
	Poor PCR purification	Ensure ExoSAP-IT® treatment is undertaken according to kit's user instructions. Ensure that the PCR mixture is mixed thoroughly with ExoSAP-IT®.
	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.

Problem	Possible cause(s)	Solution
	Contaminated sequencing primer	Check sequence quality of the other sequencing primers and other samples using the same 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.

Support and Contact Details

Conexio Genomics Pty Ltd
8/31 Pakenham St
Fremantle 6160
Western Australia
Tel: +61-422-863-227
email: support@conexio-genomics.com
Skype: conexiocgx
Website: http://www.conexio-genomics.com

Or your local distributor.