OLERUP SBT™
SEQUENCE BASED TYPING

HLA Typing Kits

Instructions for Use

PCR Amplification and Sequencing of HLA Class I and II Loci
High Throughput

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Principle

The HLA Sequence Based Typing (SBT) procedure described here, involves the initial amplification of the target sequence followed by treatment with ExoSAP-IT® 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™ sequence analysis software from CareDx Pty Ltd.

Kit Composition

Kit	Catalogue No	Σ	PRE-PCR Contents† (No of vials)		POST-PCR Contents (No of vials)		
Class I							
HLA-A	A(1536) HT	1536 tests	DNA POL – HLA-A A MIX	4 x 230µL 4 x 3.4mL	AEX2F AEX3F	AEX2R AEX3R	1 x 1.7mL each
HLA-B	B(1536) HT	1536 tests	DNA POL – HLA-B B MIX	4 x 230µL 4 x 3.4mL	BEX2F BEX3F	BEX2R BEX3R	1 x 1.7mL each
HLA-C	C(1536) HT	1536 tests	DNA POL – HLA-C C MIX	4 x 230µL 4 x 3.4mL	CEX2F CEX3F	CEX2R CEX3R	1 x 1.7mL each

Class II						
HLA-DRB1	DRB1(1536)HT	1536 tests	**DNA POL – DRB1** **DRB1 MIX**	1 x 300µL 4 x 3.5mL	**DRB1EX2F** **DRB1EX2R-2** **RB-TG344-R**	1 x 1.7mL each

The PRE-PCR kit contains a vial of a locus-specific PCR mix (e.g. **A MIX**) consisting of PCR buffer, dNTPs, $MgCl_2$, and locus specific PCR primers, along with four vials of DNA polymerase for Class I kits (e.g. **DNA POL – HLA-A**) or one vial of DNA polymerase for Class II kits.

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

Storage Requirements

The PRE- and POST-PCR reagents 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.

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™ 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 0.1μg/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.

    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 (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 denaturation techniques and sequencing platforms requires validation by the user prior to use.

23. HLA Sequencing Analysis Software (e.g. Assign™ SBT, version 3.6+ or higher or, 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.

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 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. 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
Locus-specific PCR Mix e.g. **A MIX**	8µL	8µL	8µL	8.35µL
DNA Polymerase e.g. **DNA POL – HLA-A**	0.5µL	0.5µL	0.5µL	0.15µL

Table 1: Composition of the master mix required per sample.

1.4. Dispense 8µL of the master mix into each reaction well.

1.5. Add 2µL of sample DNA or appropriate positive control/s to each reaction well. Add 2µL 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 2μL of each PCR product combined with 5μL 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 kbp and 1.4 kbp
HLA-DRB1	≈ 450 bp - 650bp (Banding pattern will vary depending on the presence of specific allele groups)

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-IT® or ExoProStar™ is to be used it is recommended that users follow the procedure described below.

3.1. Prepare a mastermix consisting of 2μL of ExoSAP-IT® or ExoProStar™ and 4μL of 2mM $MgCl_2$ per sample to be purified. Dispense 6μL 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 - 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 (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. ExoSAPtreated samples may be stored at 4°C until ready for use. ExoSAPtreated 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.

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.**

HLA-A		HLA-B		HLA-C		HLA-DRB1†	
AEX2F	AEX2R	BEX2F	BEX2R	CEX2F	CEX2R	DRB1EX2F	DRB1EX2R-2
AEX3F	AEX3R	BEX3F	BEX3R	CEX3F	CEX3R	RB-TG344-R†	

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.

4.1. 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	1 µL
Sterile water	5.75 µL
BigDye® Terminators	0.5 µL
5x Seq Rxn Buffer	1.75 µL

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

4.2. Dispense 8µL of the sequencing reaction mix into each appropriate reaction well.

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

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

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

4.5. 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

4.6. 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 60 µL 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. 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. 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.

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

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

Assay	Product Code	Assign Reference File
HLA-A HIGH THROUGHPUT	A(1536)HT	A.xml
HLA-B HIGH THROUGHPUT	B(1536)HT	B.xml
HLA-C HIGH THROUGHPUT	C(1536)HT	C.xml or Cw.xml
HLA-DRB1 HIGH THROUGHPUT	DRB1(1536)HT	527_DRB1.xml

Performance Characteristics

CareDx Pty Ltd's OLERUP SBT™ kits are locus specific assays. Sequence analysis of PCR and sequencing primer sites and performance evaluation has not identified alleles that are not amplified through the recommended use of these kits.

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.

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.

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. The use of default mode (Standard mode) and default ramp rate (100%) are recommended where possible. 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.
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

ASSIGN™ SBT V3.6+	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

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)

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.

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