TBG Biotechnology Corp.

HLAssure™ SE SBT Kit

Instructions for Use

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1. INTENDED USE

This kit provides the HLA-A, B, C, DRB1, DRB3, DRB4, DRB5, DQB1 and DPB1 typing results with high resolution by a DNA-based method.

2. SUMMARY AND EXPLANATION

The HLA molecules of the human major histocompatibility complex (MHC) antigens are key elements in restricting the specificity of T-cell mediated immune responses.[1] As HLA compatibility between donor and recipient is a major factor for determining the outcome of organ and bone marrow transplantations, accurate determination of HLA genotypes is necessary prior to transplantation. Traditionally, HLA typing has been performed by serological methods.[2] However, limitations of the traditional serological methodology become apparent as we become more knowledgeable of the HLA genes.

The HLA genes, located on the short arm of human chromosome 6, are highly polymorphic. As more DNA sequences of HLA alleles are revealed, it was clear that most HLA antigens differ from each other by only a few or one single amino acid. Due to the limitation of the traditional serological methodology differentiate these subtle antigenic differences, the use of DNA-based methods were introduced to provide a higher typing resolution. TBG Biotechnology Corp. would like to introduce the HLAssure™ SE SBT Kit as a DNA-based method for the determination of HLA alleles using **s**equence **b**ased **t**yping (SBT).

3. PRINCIPLE(S)

The HLAssure™ SE SBT Kit is for determining HLA alleles using PCR amplification with **s**equence **b**ased **t**yping (PCR-SBT) methodology. Briefly, group specific and locus specific sequence-specific primer pairs are designed to selectively amplify target sequences that are specific to a single allele or group of alleles. This amplification method is based on the principle that only primers with completely matched sequences to the target sequences result in amplified products under controlled PCR conditions. The presence of amplified DNA fragment is a positive indication of the existence of allele specific sequence within the genomic DNA. On the other hand, mismatched primers do not generate amplicons.

The PCR reaction products are examined by agarose gel electrophoresis that separates the DNA fragments by size. The amplified fragments are visualized as bands through ethidium bromide staining followed by ultraviolet light exposure.

Reactions with amplicons are purified and subjected to locus specific sequencing reactions that determine the exact DNA sequence of each sequenced allele.

The HLAssure™ SE SBT Kit supplies a panel of primer mixes that is designed to provide amplify and sequence HLA alleles. A sequence analysis software can be used to assist the determination of HLA types by analyzing the resulting sequencing reactions.

4. REAGENTS

4.1 Contents of the HLAssure™ SE SBT Kit

Each HLAssure™ SE SBT Kit provides the following contents:

4.1.1 Amplification mixes

Each amplification mix (AMP mix) contains primers to amplify specific HLA genes, consisting of locus or group specific primers. AMP mix ID with their specificity is shown in appendix 14.1.

4.1.2 Sequencing mixes

Each sequencing mix (SEQ mix) contains primers to sequence specific HLA exons, consisting of locus or group specific amplicon. SEQ mix ID with their specificity is shown in appendix 14.2.

4.1.3 EZTaq™ (5 Units/µL)

Each tube of EZTaq™ is used to amplify the HLA alleles during the amplification process.

4.1.4 Amplification buffer

Each tube of amplification buffer (AMP buffer) is used to amplify the HLA alleles during the amplification process.

Please use the designated buffer for each locus.

4.2 Warning or Caution

For Research Use Only

4.2.1 Do not use reagents past the expiration date printed on the label.

4.2.2 The test must be performed by well-trained and authorized laboratory staff.

4.2.3 Reagents should be handled in accordance with good laboratory practice using appropriate precautions.

4.2.4 Pipettes used for post-PCR manipulations should not be used for pre-PCR manipulations.

4.2.5 Biohazard Warning: the ethidium bromide used for staining of DNA is a potential carcinogen. Always wear gloves when handling stained gels.

4.2.6 Biohazard Warning: all blood products should be treated as potentially infectious.

4.2.7 Caution: wear UV-blocking eye protection and do not view UV light source directly when viewing or photographing gels.

See Material Safety Data Sheets for detailed information.

4.3 Storage and Stability

The Amplification mixes and Sequencing mixes should be stored at 4°C while the Amplification buffer and EZTaq™ should be stored at -20°C. All reagents should be kept on ice during use and returned to the proper storage conditions afterwards. The Amplificatioin buffer and EZTaq™ can be used successfully after as many as 24 freezing and thawing cycles.

5. INSTRUMENT REQUIREMENTS

5.1 Programming the Thermal Cycler

The HLAssure™ SE SBT Kit is standardized for the following programs using Applied Biosystems GeneAmp® 9700 and Veriti. Thermal cyclers other than the recommended model and/or brand have to be user-validated.

HLAssure™ SBT Amplification Program

Segment	Cycle Number	Temperature	Time
1	1	95°C	5 min
2	36	93°C	30 sec
		63°C	40 sec
		72°C	2.5 min
3	1	72°C	5 min
		4°C	until removed

HLAssure™ SBT ExoSAP Program

Cycle Number	Temperature	Time
1	37°C	15~30 min
	80°C	15 min
	4°C	Until removed

HLAssure™ SBT Sequencing Program

Segment	Cycle Number	Temperature	Time
1	1	96°C	1 min
2	25	98°C	25 sec
		60°C	2 min 30 sec
3	1	4°C	Until removed

Please program the thermal cycler according to manufacturer's instruction.

5.2 Gel Electrophoresis

Refer to Section 7.6 Gel Electrophoresis.

6. SPECIMEN COLLECTION AND PREPARATION

6.1 Blood Sample Collection

The peripheral blood can be collected using blood collection tubes with sodium citrate and EDTA as anticoagulants. Contamination of the DNA by heparin can result in interference with the PCR reaction. For this reason, heparinized blood should not be used as a starting material for DNA isolation. Please refer to manufacturer's instruction for blood sample storage and stability.

6.2 DNA Isolation

Genomic DNA may be obtained from all nucleated cells. The source of cell includes, but is not limited to, whole blood, buffy coat and cultured cells. DNA isolation can be performed by any validated protocol that produces DNA with adequate quality and quantity of DNA followed by repeated user validation.

6.3 DNA Quantity

The DNA sample should be re-suspended in sterile distilled water or appropriate buffer solution at a concentration of 10~40 ng/μL. DNA should not be re-suspended in solutions containing chelating agents, such as EDTA, above 0.5 mM in concentration.

6.4 DNA Quality

The DNA sample should have an A260/A280 ratio between 1.65 and 1.8.
DNA samples may be used immediately after isolation or stored at -20^{o}C or below for extended periods of time (over 1 year) with no adverse effects on the HLA typing results.

7. DIRECTIONS FOR USE

7.1 Materials Provided

Refer to Section 4. REAGENTS

7.2 Materials Required, But Not Provided

7.2.1 Amplification, PCR clean up and sequencing reactions

7.2.1.1 Big Dye Terminator (v1.1 or v3.1)
7.2.1.2 NaOAc/EDTA (1.5M/250mM)
7.2.1.3 Ethanol absolute (＞99.5%)
7.2.1.4 ExoSAP
7.2.1.5 Distilled water or DNA diluent
7.2.1.6 Adjustable pipettes
7.2.1.7 Disposable pipette tips
7.2.1.8 Vortex mixer
7.2.1.9 Microcentrifuge
7.2.1.10 Table top centrifuge for 96 well PCR plates
7.2.1.11 Thermal Cycler with heated cover (e.g. Applied Biosystems GeneAmp 9700 and Veriti)
7.2.1.12 96 well PCR tray
7.2.1.13 Pressure Pad

7.2.2 Gel Electrophoresis

7.2.2.1 Molecular biology grade agarose
7.2.2.2 0.5x TAE or TBE buffer
7.2.2.3 Ethidium bromide solution (10 mg/mL)
7.2.2.4 Hot plate or microwave oven for heating agarose solutions
7.2.2.5 Gel tray and electrophoresis chamber Power supply
7.2.2.6 UV transilluminator
7.2.2.7 Photographic or image documentation system.

7.3 Sample Preparation

Refer to Section 6. SPECIMEN COLLECTION AND PREPARATION.

7.4 Reagent/Equipment Preparation

Refer to Section 5. INSTRUMENT REQUIREMENTS.

7.5 Amplification reaction

7.5.1 Calculate the total number of amplification reactions required based the Amplification Primer Specificity Table in appendix 14.1. If the low resolution HLA typing results are known, please select the corresponding Group Specific AMP mix (GSA) for minimal ambiguity. Otherwise, please select the Locus Specific AMP mix (LSA).

7.5.2 Remove the AMP mix and EZTaq™ from freezer and thaw on ice. Prepare a solution of Buffer/Taq mix according to the calculated total number of reactions required and table below:
Please use the designated buffer for each locus.

Total Reactions	AMP Buffer	EZTaq™
1	6 µL	0.14 µL
10	60 µL	1.4 µL
20	120 µL	2.8 µL
30	180 µL	4.2 µL

7.5.3 Add 6µL of Buffer/Taq mix to bottom of each well on a clean empty 96 well PCR tray.

7.5.4 Add 3µL of genomic DNA (10~40 ng/µL) to the lower rim of each reaction well.

7.5.5 Add 3µL of each AMP mix to the upper rim of each reaction well. Please refer to the Amplification Primer Specificity Table in section 14.1 for more information.

7.5.6 Optional Step: Add mineral oil to avoid evaporation.

7.5.7 Cover the Typing Tray with PCR Plate Cover Membrane. Make sure all tubes are properly covered by the membrane to prevent evaporative loss during PCR process. Make sure the dispensed solutions are combined.

7.5.8 Place a pressure pad on top of PCR Plate Cover Membrane and close heated thermal cycler lid. Start the SBT Amplification program shown in section 5.1.

7.5.9 After PCR process is completed, you may proceed to gel electrophoresis immediately or store the tray at –20 °C and continue to gel electrophoresis at a later time.

7.6 Gel Electrophoresis

7.6.1 Gel Preparation

7.6.1.1 Prepare sufficient 2% agarose gel to resolve total number of PCR reactions according to original gel electrophoresis system manufacture's instructions.

7.6.1.2 Make appropriate adjustments to ensure that the gel contains a final ethidium bromide concentration of 0.5 µg/mL.

7.6.1.3 Make sure that each well of gel creates a minimum volume of 15µL.

7.6.1.4 Make sure that the running buffer used for electrophoresis is consistent with the buffer used for gel preparation.

7.6.2 Performing Gel Electrophoresis

7.6.2.1 Mix 2µL of each PCR reaction with the proper quantity amount of loading dye and transfer into the agarose gel.

7.6.2.2 The electrophoresis takes place at 10V/cm until the dye front has migrated about 1.4-2.0 cm into the gel.

7.6.2.3 After electrophoresis is completed, place the agarose gel on an UV transilluminator. Photograph for documentation of PCR quantity and quality affirmation.

7.7 Purification of PCR Product (ExoSAP) :

7.7.1 Add 4µL of ExoSAP to each reaction well containing PCR product to eliminate residual primers and dNTPs

7.7.2 Briefly centrifuge, seal with PCR cover membrane

7.7.3 Close heated thermal cycler lid and start HLAssure™ SBT ExoSAP program shown in Section 5.1 of this manual.

7.8 Sequencing reactions:

7.8.1 Estimate the quantity of PCR products in each well based on the Gel documentation image. ***See table below to ensure the appropriate amount of PCR products are used for sequencing***.

Size of amplicons (bp)	Quantity (ng)
200~500	5-10
500~1,000	10~20
1,000~3,000	15~50

7.8.2 Calculate total number sequencing reactions required based on the Sequencing Primer Specificity Table in section 14.2.

7.8.3 Using the table below to prepare the BDT sequencing mix.

No. of Reaction	1	10	30
*BDT Ready Reaction Premix	0.5 µL	5 µL	15 µL
5X BDT buffer	0.75 µL	7.5 µL	22.5 µL
ddH20	0.25 µL	2.5 µL	7.5 µL
Total	**1.5 µL**	**15 µL**	**45 µL**

*BDT: Big Dye Terminator (v1.1 or v3.1)

7.8.4 Add 1.5µL of BDT sequencing mix to each reaction well.

7.8.5 Add 2.5µL of each SEQ mix to each reaction well. Please refer to the Sequencing Primer Specificity Table in section 14.2 for more information.

7.8.6 Add 1 µL of purified PCR product to each reaction well.

7.8.7 Briefly centrifuge, seal with PCR sealing membrane.

7.8.8 Start the HLAssure™ SBT Sequencing program shown in section 5.1.

7.9 Ethanol Precipitation of Sequencing Reaction Product:

7.9.1 Perform ethanol precipitation to remove excess BDT.
7.9.2 Add 5μL of dH_2O and then briefly centrifuge.
7.9.3 Add 2μL of NaOAc/EDTA(1.5M/250mM) to each reaction well containing sequencing reaction product.
7.9.4 Briefly centrifuge to combine the contents.
7.9.5 Add 25μL of 100% ethanol to each sequencing mixture, apply PCR cover membrane.
7.9.6 Vortex the mixture briefly but vigorously.
7.9.7 Centrifuge at 2,000 xg for 30minutes.
7.9.8 Remove PCR Cover Membrane, centrifuge the PCR tray onto a paper towel at 100 xg for 10 seconds.
7.9.9 Add 50μL of 70~80% ethanol to each sequencing mixture.
7.9.10 Centrifuge at 2,000 xg for 5 minutes.
7.9.11 Remove supernatant by inverted spin as in step 7.9.8.

7.10 Prepare the Samples for Sequencing Electrophoresis :

7.10.1 Prepare sequencing reactions for loading onto the capillary DNA sequencer by adding 15μL of either HiDi formamide or 0.3mM EDTA to each sequencing reaction tube:
7.10.2 Centrifuge briefly to collect the solution at bottom of the tubes.
7.10.3 If dissolving in HiDi formamide, denature the sequencing reactions in a thermal cycler for 2 minutes at 95 °C.
7.10.4 If dissolving in 0.3mM EDTA, DO NOT HEAT DENATURE, simply load the reactions directly on the instruments.

7.11 Data Collection :

Perform data collection according to the instrument-specific parameters.

8. RESULTS

8.1 Data Interpretation

8.1.1 The sequence data are processed with an allele typing software program to identify the HLA alleles. A software program that is compatible with these reagents is AccuType™ (Texas BioGene Inc., Texas, USA).

8.2 Data Analysis and AccuType™ SBT Setting

8.2.1 Note that the group specific sequences are intended to be analyzed simultaneously with the locus specific sequences to provide unambiguous typing results. To obtain accurate typing result, the sequence library must be compatible with AccuType™ settings.

8.2.2 In order for AccuType™ to accurately identify specific sequences, it is necessary to include the following naming method as a part of the sample filename.

SampleName_SEQ Mix_AMP Mix.ab1

8.2.3 For example, if a sample is sequenced with SEQ Mix, A2F and amplified with AMP Mix, A02, an appropriate naming convention would be SampleName_A2F_A02.ab1.

8.2.4 For operations of the AccuType™ software, please refer to the manufacturer's instruction manual.

8.3 Limitation of Procedure

8.3.1 Quality typing results can only be guaranteed if the enclosed instructions are strictly followed.

8.3.2 All instruments, including thermal cycler and pipetting devices, must be calibrated according the manufacturer's instructions.

8.3.3 The HLAssure™ SE SBT Kit is standardized for the EZTaq™ polymerase from TBG. The use of other brands of Taq polymerase may result in non-specific false positive PCR reactions.

8.3.4 The quality and quantity of DNA samples have to be within the range as specified within instruction manual.

8.3.5 The HLAssure™ SE SBT Kit can not resolve all allele combinations. When an ambiguous result obtained from the HLAssure™ SE SBT Kit, please refer to section 10, TROUBLESHOOTING, to identify possible causes and solutions. If the ambiguous results are still not resolved, please use a typing kit with the same level of resolution from other suppliers to resolve the ambiguity. You can also contact TBG for assistance.

8.4 Quality Control

Each manufactured lot is checked against a panel of standard DNA samples. The alleles carried by these DNA samples react with the corresponding primer mixes in the kit. The report is available upon request.

9. PERFORMANCE CHARACTERISTICS

9.1 Specific Performance

The HLAssure™ SE SBT Kit was compared with other SBT typing system by testing 152 random whole blood specimens at three geographically distinct locations. There was 100% concordance (152/152) for each locus A, B, C, DRB, DQB1 and DPB1 as shown in the following table.

Allele	Number of Concordant Tests	Number of Tests	Concordance	95 % Confidence Limits	
				Lower	Upper
HLA-A	152	152	100%	100%	100%
HLA-B	152	152	100%	100%	100%
HLA-C	152	152	100%	100%	100%
HLA-DRB1	152	152	100%	100%	100%
HLA-DRB345	152	152	100%	100%	100%
HLA-DQB1	152	152	100%	100%	100%
HLA-DPB1	152	152	100%	100%	100%

9.2 Quality Control of Kit Manufacturing

Each manufactured lot is checked against a panel of standard DNA samples. The alleles carried by these DNA samples react with most of the primer mixes in the kit. The report is available upon request.

9.2 Reproducibility

Ten to 24 standard DNA samples from International Histocompatibility Working Group (IHWG) were typed using the HLAssure™ SE SBT Kit for 5 times for the within-lot and inter-lot reproducibility studies. The data show 100% reproducibility for HLA alleles.

10. TROUBLESHOOTING

Problem	Potential Cause	Solution
Weak bands or absence of band(s) after PCR amplification	Poor DNA quality	Repeat DNA purification and make sure the A260/A280 ratio is between 1.65-1.80.
	Insufficient amount of DNA	Make sure the DNA concentration is within the suggested range of 10~40ng/µL.
	Insufficient Taq polymerase activity	Make sure there is sufficient amount of Taq polymerase added and the Taq polymerase has not been degraded.
	Improperly calibrated thermal cycler	Calibrate the thermal cycler.
	Insufficient ethidium bromide staining	Re-stain the agarose gel in fresh 0.5xTBE buffer with 0.5µg/mL ethidium bromide.
Presence of false positive band(s) after PCR amplification	Overly high DNA concentration	Make sure the DNA concentration is within the suggested range of 10~40ng/µL.
	Excessive Taq polymerase activity	Make sure the amount of EZTaq™ polymerase added is as instructed on page 9.
	Improperly calibrated thermal cycler	Calibrate the thermal cycler.

11. BIBLIOGRAPHY

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3. Newton, C.R., Graham, A., Heptinstall, E., Powell, S.J., Summers, C., Kalsheker, N., Smith, J.C., and Markham, A.F. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Research 17:2503-2516, 1989.

12. TRADEMARKS USED IN THIS DOCUMENT

HLAssure™ Texas BioGene, Inc.
AccuType™
Applied Biosystems
GeneAmp®

13. PATENTS USED IN THIS DOCUMENT

This product is optimized for use in the Polymerase Chain Reaction ("PCR") Process which is covered by patents owned by Roche Molecular Systems, Inc. and F. Hoffmann-La Roche Ltd ("Roche"). No license under these patents to use the PCR Process is conveyed expressly or by implication to the purchaser by the purchase of this product. Further information on purchasing licenses to practice PCR may be obtained by contacting, in the United States, the Director of Licensing at Roche Molecular Systems, Inc. 1145 Atlantic Avenue, Alameda, California 94501, and outside the United States, the PCR Licensing Manager, F. Hoffmann-La Roche Ltd, Grenzacherstr. 124, CH-4070 Basel, Switzerland.

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14. APPENDIX

14.1 Amplification Primer Specificity Table

HLA A AMP Mix Primers:

Group Specific AMP mix (GSA)

AMP Mix ID	HLA A Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
A-01	01, 11, 36	-	~2.4 kbp
A-02	11	-	~2.3 kbp
A-03	02	-	~2.0 kbp
A-04	03	-	~2.5 kbp
A-05	23	23:17	~2.3 kbp
A-06	23, 24	24:33	~2.0 kbp
A-07	29, 32, 74	?32:04*	~2.3 kbp
A-08	30	-	~2.8 kbp
A-09	31, 33	-	~2.6 kbp
A-10	25, 26, 34, 43, 66	-	~2.4 kbp
A-11	68, 69, 34:01, 66:02/03	?34:05*	~2.4 kbp
A-12	80:01	-	~ 2.8 kbp

***Lack of intron sequence data**

Locus Specific AMP mix (LSA)

AMP Mix ID	HLA A Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
A-L	All A alleles	-	~ 3.0 kbp

HLA B AMP Mix Primers:

Group Specific AMP mix (GSA)

AMP Mix ID	HLA B Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
B-01	07, 48, 81	48:02	~2.4 kbp
B-02	08, 42 ?35:87*	-	~2.3 kbp
B-03	13, 57	-	~2.4 kbp
B-04	14, 38, 39, 67	?67:02*	~2.3 kbp
B-05	14	-	~2.2 kbp
B-06	15, 46	15:42	~2.3 kbp
B-07	18, 37	-	~2.7 kbp
B-08	27, 40, 47 ? 35:42*	40:01/48/55 ?27:05:04*	~2.7 kbp
B-09	15:42, 35, 51, 52, 53, 58, 78 ?56:06*	51:42, ?35:87*	~2.2 kbp
B-10	35, 53, 57	-	~3.3 kbp
B-11	40	40:02/03/04/06 /08/40/55	~2.4 kbp
B-12	41	-	~2.4 kbp
B-13	44 ?51:42, 83:01*	44:27 ? 44:15/18*	~2.6 kbp
B-14	45, 49, 50	-	~2.7 kbp
B-15	54, 55, 56, 59, 82	?56:06, 59:02/03/04*	~2.3 kbp

***Lack of intron sequence data**

Locus Specific AMP mix (LSA)

AMP Mix ID	HLA B Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
B-L	All B alleles	-	~3.0 kbp

HLA C AMP Mix Primers:

Group Specific AMP mix (GSA)

AMP Mix ID	HLA C Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
C-01	01	-	~2.3 kbp
C-02	02, 15	-	~3.2 kbp
C-03	15	-	~2.3 kbp
C-04	03	-	~2.9 kbp
C-05	04	-	~2.9 kbp
C-06	05, 08	-	~3.2 kbp
C-07	05, 06, 08, 12, 15	15:43	~2.1 kbp
C-08	07	-	~2.9 kbp
C-09	14	-	~2.6 kbp
C-10	16	-	~3.1 kbp
C-11	17	-	~3.1 kbp
C-12	18	-	~2.5 kbp

Locus Specific AMP mix (LSA)

AMP Mix ID	HLA C Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
C-L	All C alleles	-	~3.1 kbp

HLA DRB1,3,4,5 AMP Mix Primers:

Group Specific AMP mix (GSA)

AMP Mix ID	HLA DRB1 Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
DRB-01	01	-	~430 bp
DRB-02	15, 16	-	~730 bp
DRB-03	03, 14:02/03/06 ?13:15, 14:13/19/20*	03:07, ?03:17, 14:17*	~760 bp
DRB-04	04	-	~550 bp
DRB-05	07	-	~400 bp
DRB-06	08 ?13:17*	?08:06*	~860 bp
DRB-07	09	-	~440 bp
DRB-08	10	-	~460 bp
DRB-09	12	12:01**	~590 bp
DRB-10	03, 11, 13, 14 08:06, 12:01**, 12:17	?13:17*	~470 bp
DRB-11	03:07, 13:01/02/04/36 ?13:34, 14:17/21/33*	13:03/05/12/13	~460 bp
DRB-12	1401/04/05/10/18/54/84, 11:01*, 11:01:08, 12:17, ?11:13/17, 13:08/83*	?14:13/17/19/33*	~470 bp
DRB-L3	03, 08, 11~14	12:17	~300 bp

***Lack of intron sequence data**

****Unpublished new allele identified in specific populations**

Locus Specific AMP mix (LSA)

AMP Mix ID	HLA DRB Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
DRB-L1	All DRB1 alleles	-	~260 bp
DRB-L2	All DRB1 alleles	03:42, 04:49, 11:30, 13:67, 11:01:06, 14:39/46 ?01:07*	460 ~ 740 bp
DRB-L3	All DRB1 alleles	01, 04, 07, 09, 10, 15, 16, 12:17	~1.0 kbp
DRB3-L2	All DRB3 alleles	-	~400 bp
DRB4-L2	All DRB4 alleles	-	~400 bp
DRB4-L3	All DRB4 alleles		~400 bp
DRB5-L2	All DRB5 alleles	-	~400 bp

HLA DQB1 AMP Mix Primers:

Group Specific AMP mix (GSA)

AMP Mix ID	HLA DQB1 Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
DQB-01	02	-	~480 bp
DQB-02	03	03:02/03 ?03:05-08/11	~530 bp
DQB-03	03	03:01/04/09/19 ?03:10/12/13	~525 bp
DQB-04	04	-	~525 bp
DQB-05	05	-	~480 bp
DQB-06	06	-	~540 bp

***Lack of intron sequence data**

Locus Specific AMP mix (LSA)

AMP Mix ID	HLA DQB1 Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
DQB-L2	All DQB1 alleles (Exon2)	-	~510 bp
DQB-L3	All DQB1 alleles (Exon3)	-	~400 bp

HLA DPB1 AMP Mix Primers:

Locus Specific AMP mix (LSA)

AMP Mix ID	HLA DPB1 Allele Group	Excluded Allele(s)	Amplicon Size (kbp)
DPB-L2	All DPB1 alleles (Exon2)	-	~300 bp
DPB-L3/4	All DPB1 alleles (Exon3)	-	~1,300 bp

14.2 Sequencing Primer Specificity Table

HLA A Sequencing Primers:

SEQ Mix ID	HLA A Exon Coverage	Covered AMP Mix	Direction
A1F	1	A-01, 04, 05, 07~12, L	Forward
A2F	2	A-01 ~ 12, L	Forward
A2R		A-01 ~ 12, L	Reverse
A3F	3	A-01 ~ 12, L	Forward
A3R		A-01 ~ 12, L	Reverse
A4F	4	A-01 ~ 12, L	Forward
A4R		A-01 ~ 12, L	Reverse
A5F	5	A-01, 02, 04, 07~10, 12, L	Forward

Note:
- A minimum of SEQ Mix A2F, A3F, and A4F should be sequenced for each AMP Mix A-01~12.
- If the sample contains a low resolution typing of A*68 or A*74, SEQ mix A1F should be sequenced to reduce possible ambiguity.
- If the sample contains a low resolution typing of A*23, SEQ Mix A5F should be sequenced to reduce possible ambiguity.

HLA B Sequencing Primers:

SEQ Mix ID	HLA B Exon Coverage	Covered AMP Mix	Direction
B1F	1	B-01, 03, 04, 07~12, 14, L	Forward
B2F	2	B-01 ~ 15, L	Forward
B2R		B-01 ~ 15, L	Reverse
B3F	3	B-01 ~ 15, L	Forward
B3R		B-01 ~ 15, L	Reverse
B4F	4	B-01 ~ 15, L	Forward
B4R		B-01 ~ 15, L	Reverse
B5R	5	B-01~08, 11~15, L	Reverse

Note:

- A minimum of SEQ Mix B2F, B2R, B3R, and B4F should be sequenced for each AMP Mix B-01~15.
- If the sample contains a low resolution typing of B*15/18/27/35/44/81, SEQ Mix B1F should be sequenced to reduce possible ambiguity.
- If the sample contains a low resolution typing of B*07, SEQ Mix B5R should be sequenced to reduce possible ambiguity.

HLA C Sequencing Primers:

SEQ Mix ID	HLA C Exon Coverage	Covered AMP Mix	Direction
C1R	1	C-02~04, 06~12, L	Reverse
C2F	2	C-01~ 12, L	Forward
C2R		C-01~ 12, L	Reverse
C3F	3	C-01~ 12, L	Forward
C3R		C-01~ 12, L	Reverse
C4F	4	C-01~ 12, L	Forward
C4R		C-01~ 12, L	Reverse
C5R	5	C-01, 02, 04~12, L	Reverse
C6F	6	C-02, 04, 05, 06, 08, 10, 11, L	Forward
C7R	7	C-02, 04, 05, 06, 08, 10, 11, L	Reverse

Note:

- A minimum of SEQ Mix C2F, C3R, and C4F should be sequenced for each AMP Mix C-01~12.
- If the sample contains a low resolution typing of C*03/17, SEQ Mix C1R should be sequenced to reduce possible ambiguity.
- If the sample contains a low resolution typing of C*04/07/18, SEQ Mix C5R should be sequenced to reduce possible ambiguity.
- If the sample contains a low resolution typing of C*07/08, SEQ Mix C6F should be sequenced to reduce possible ambiguity.
- If the sample contains a low resolution typing of C*04/07, SEQ Mix C7R should be sequenced to reduce possible ambiguity.

HLA DRB1,3,4,5 Sequencing Primers:

SEQ Mix ID	HLA DRB Exon Coverage	Covered AMP Mix	Direction
DRB1F	1	DRB-L1	Forward
DRB1R			Reverse
DRB2F	2	DRB-01~12, DRB-L2	Forward
DRB2R			Reverse
DRB2R-86(GTG)		DRB-01~12, DRB-L2	Reverse
DRB345-2F	2	DRB3-L2, DRB4-L2, DRB5-L2	Forward
DRB345-2R			Reverse
DRB3F	3	DRB-L3	Forward
DRB3R		DRB-L3	Reverse
DRB4-3F		DRB4-L3	Forward
DRB4-3R		DRB4-L3	Reverse

Notes:

- When the sample is singly positive for AMP Mix 04, 06 or 10 (in addition to AMP mix DRB-L), it is recommended to sequence DRB2R-86 with the AMP Mix 04, 06, or 10 PCR product to reduce possible ambiguity.

HLA DQB1 Sequencing Primers:

SEQ Mix ID	HLA DQB1 Exon Coverage	Covered AMP Mix	Direction
DQB2F	2	DQB-01~06, L2	Forward
DQB2R		DQB-01~06, L2	Reverse
DQB3F	3	DQB-L3	Forward
DQB3R		DQB-L3	Reverse

HLA DPB1 Sequencing Primers:

SEQ Mix ID	HLA DPB1 Exon Coverage	Covered AMP Mix	Direction
DPB2F	2	DPB-L2	Forward
DPB2R			Reverse
Codon 8			Reverse
Codon 85			Reverse
DPB3F	3	DPB-L3/4	Forward
DPB3R			Reverse
DPB4F	4		Reverse
DPB4R			Reverse