GENDX

SBTexcellerator®

For high-resolution Sanger Sequencing-Based
HLA Typing

Instructions For Use

For Research Use Only

HLA-A, B, C, DRB1, DQB1, DPA1, DPB1, DQA1, DRB3/4/5

Edition 11 2016/12
MAT 3340000

IMPORTANT NOTES AND UPDATES

Updates of the Instructions for Use (IFU) Edition 11

- New update in section 5 “Product use limitation”. Product validation specified and equivalence explained.

- Protocol 1: Amplification
  New available GenDx-LongRange (equal to Qiagen-LongRange). Q-solution is referred to as X-solution.

- Protocol 2: PCR clean-up
  New available GenDx-ExoSAP included in SBTexcellerator workflow. Please note the change in reaction volume composition.

- Protocol 3: Sequencing
  New available GenDx-BigDye (equal to BigDye-v1.1), compatible with SBTexcellerator.

- Appendix A: Master Mix protocol
  Shelf-life of the Master Mix has been extended from 3 to 5 months. This protocol is optional and is available via the GenDx website (www.gendx.com).

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GenDx reserves the right to make improvements to this IFU and/or to the products described in this IFU, at any time without notice.

If you find information in this manual that is incorrect, misleading, or incomplete, we would appreciate your comments and suggestions. Please send them to info@gendx.com.

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1 KEY TO SYMBOLS

MAT	Material number
COMP	Components
LOT	Batch code/Lot Number
REF	Catalogue Number
	Consult Instructions For Use
	Contains reagents for N tests
	Legal Manufacturer
Store at -20°C	Store at -20°C
	Add liquid

2 KIT CONTENT

Each kit contains reagents sufficient for 50 reactions.
For full description of kit content see kit content label on the box or visit our website (www.gendx.com).

3 SHIPPING AND STORAGE

SBTexcellerator® HLA kits are:

- Shipped at ambient temperature and should be stored at -20°C upon arrival.
- Stable until the kit expiration date, indicated on the box label, when stored at -20°C.
- Stable for 5 months after dissolving primers in nuclease free H_20, when stored at -20°C.

4 TECHNICAL ASSISTANCE

For technical assistance and more information:

Email: support@gendx.com
Website: www.gendx.com
Phone: +31 30 252 3799

Or contact your local GenDx distributor (www.gendx.com).

5 PRODUCT USE LIMITATIONS

For **Research Use Only**. Not for use in diagnostic procedures. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

- To ensure the best performance please use the SBTexcellerator® kits with the materials, reagents, and equipments recommended in section 8 “Equipment and Reagents to be supplied by User”.
  Use of materials other than specified must be validated by user!
- Reconstitution or dilution of primers in volumes other than described in this IFU can lead to performance errors and is strongly discouraged!
- Please take special note of Appendix B: Contamination control.
- Before implementing SBTexcellerator® in your laboratory, please perform a validation for Sequencing-Based Typing methods using known molecular typed samples. Such samples may be obtained from the International Workshop Reference Cell Panel and the UCLA DNA Reference Panel.
- SBTexcellerator® products have been validated with
  - GenDx-LongRange PCR kit/ QIAGEN LongRange kit
  - GenDx-ExoSAP
  - GenDx-BigDye/ BDT v1.1 (Thermo Fisher Scientific)
  on Applied Biosystems GeneAmp PCR System 9700. Other thermal cyclers require different profiles and require user validation.
- Clean-ups different from the GenDx-ExoSAP must be validated by the user.
- GenDx-LongRange PCR kit is identical to the QIAGEN LR PCR kit.
- GenDx-BigDye kit is identical to the BDT v1.1 kit (Thermo Fisher Scientific).
- Protocol and procedure described in this IFU refer to the GenDx-LongRange PCR kit, GenDx-ExoSAP and GenDx-BigDye kit.

6 PRINCIPLE

SBTexcellerator® HLA Kits are primer/oligonucleotide sets dedicated to high-resolution HLA Sequencing-Based Typing (SBT) for identification of alleles of the Human Leukocyte Antigens (HLA).

SBTexcellerator® products are available for HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB1, -DQA1, -DPB1, -DPA1 and -G loci.

Core kits consist of amplification primers and sequencing primers for exons 2, 3 and 4 (exon 4 sequencing not available for all loci).
Extended kits contain sequencing primers for additional regions and several group-specific sequencing primers (GSSPs). Use of these is only required when ambiguities need to be resolved. See www.gendx.com for exon coverage of the amplification and sequencing primers.

In case of DQA1 and DRB1, the core kit consists of exon 2 sequencing primer sets that separate specific allele groups to allow for enhanced (sequencing) resolution.
DQA1 SEQ Ex2Fa1/ Ra1: for DQA1*01 and –DQA1*03 alleles. DQA1 SEQ Ex2Fa2/ Ra2: others.
DRB1 SEQ Ex2Fa2: for DRB1*04/07/09. DRB1 SEQ Ex2Fa1: others.

7 PROCEDURE

1. HLA locus-specific amplification is performed in a thermal cycler using the amplification primer mix (red caps), template DNA, and the GenDx-LongRange PCR Kit.

2. Before sequencing the PCR products are cleaned up using Exonuclease I and Shrimp Alkaline Phosphatase (or alternative methods) to remove unincorporated primers and nucleotides.

3. Sequencing is performed using sequencing primers (yellow and green caps), Life Technologies BigDye® Terminator sequencing chemistry, with subsequent analysis on ABI PRISM 3100/3700 or Applied Biosystems 3130/3730 Genetic Analyzers.

4. The sequence products are purified using Sephadex G-50 Superfine (or alternative methods) to remove unincorporated sequencing primers and residual nucleotides.

5. Denatured samples are loaded on an automated genetic analyzer.

8 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

The following list includes the essential equipment and reagents.

General
- Thermal cycler
- Sequencer
- Micro centrifuge
- Vortex
- Deionised water
- Centrifuge (rotor and adapters for 96-well microtiter plates)
- Pipettes and Pipette tips (use of tips with hydrophobic filters is strongly recommended)
- Multichannel pipette (optional to facilitate handling)
- Ice

HLA Locus Amplification
- GenDx-LongRange PCR kit
- PCR tubes (use thin-walled 0.2 ml. PCR tubes recommended by the manufacturer of your thermal cycler)
- Agarose gel electrophoresis system

Sequencing of HLA Loci
- 1.5 or 2 ml. micro centrifuge tubes
- GenDx-BigDye (catalog # 5345555) compatible with SBTexcellerator

Clean-up and Analysis of HLA Sequencing Products
- GenDx-ExoSAP (catalog # 5341151, 5341551)
- Sephadex G-50 Superfine
- Multiscreen 45 µl Column Loader
- Multiscreen Column Loader Scraper clear
- Multiscreen-HV
- Multiscreen Centrifuge Align Frame Blue
- EU Frosted Sub Skirted Thin-wall 96x 0.2 ml. plates
- Capillary sequencer (e.g., ABI PRISM® 3100/3700 or Applied Biosystems 3130/3730)
- SBTengine® software to analyze sequence files and to create HLA typing reports

9 IMPORTANT NOTES BEFORE STARTING

Sample Preparation

- It is recommended to use purified DNA that has an A260/A280 ratio of ~1.8.
- If necessary, DNA should be diluted in nuclease free H_2O before use.
- The optimal amount of template DNA to use in the reaction is 100 ng. However, template DNA in the range of 50–200 ng (in 1–4 µl) can be used without affecting results.
- To streamline the process, validate your DNA purification procedure so you can use a set volume corresponding to 50–200 ng DNA.
- Blood samples should be collected in ACD or EDTA anti-coagulated tubes. Do NOT use heparinized samples. Heparin has an inhibitory effect on a PCR.

Assay Set-up

- **Set up all reactions on ice**.
- Prepare a volume of reaction mix at least 10% greater than required for the total number of assays to be performed.

SBTexcellerator® Primer Preparation

1. Briefly centrifuge all tubes before opening for the first time to ensure that the pellet is at the bottom of the tube.

2. Re-suspend each primer in nuclease free H_2O (provided), using the re-suspension volumes listed in Table 1.

Table 1: Re-suspension volumes for amplification and sequencing primers (re-suspend each primers in the provided nuclease free H_20)

HLA locus	AMP primers (red caps)	SEQ primers (yellow & green caps)
A	55 µl	55 µl
B	55 µl	55 µl
C	55 µl	55 µl
DRB1	55 µl	55 µl
DRB3/4/5	55 µl	110 µl
DQA1	55 µl	55 µl
DQB1	55 µl	55 µl
DPA1	55 µl	55 µl
DPB1	55 µl	55 µl
G	55 µl	55 µl

10 PROTOCOLS

PROTOCOL 1: AMPLIFICATION PROCEDURE

1. **Set up all reactions on ice.**

2. Prepare a separate reaction mix for each amplification primer.

3. Thaw 10x LongRange PCR Buffer (which includes 25 mM Mg^{2+}), dNTP mix (10 mM each), nuclease free H_20 and primer solutions (red caps). Mix the solution thoroughly and centrifuge briefly before use.

4. Prepare an amplification reaction mix as shown in Table 2. For HLA-DQB_1 the addition of **X-solution** (5x) and double the amount of LongRange PCR enzyme mix per reaction is required.

5. Mix the reaction mix thoroughly, and centrifuge briefly.

6. Dispense appropriate volumes into each PCR tube.

7. Add 1-4 µl template DNA (50-200 ng) to each tube containing reaction mix. Final volume is 25 µl. *Do not exceed 4 µl of template DNA to ensure optimal PCR performance.*

Table 2: Composition of reaction mix for locus-specific amplification.

Component	All loci (except DQB1)	DQB1
Nuclease free H_2O	15.85-18.85 µl	10.45-13.45 µl
X-Solution (5x)	-	5 µl
LongRange PCR enzyme mix (5 U/µl)	0.4 µl	0.8 µl
LongRange PCR Buffer (10x)	2.5 µl	
dNTP mix (10 mM each)	1.25 µl	
AMP primer (red cap)	1 µl	
Template DNA	1-4 µl	
Total Volume	25 µl	

Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 3.

Important: For a simplified hot-start place the tubes in a thermo cycler that is preheated to 95°C and start the cycling program. A hot-start will ensure PCR specificity.

After amplification samples can be stored overnight at 2-8°C. Clean-up of the PCR product should preferentially be carried out within 24 hours.

8. PCR products must be confirmed by agarose gel electrophoresis. Prepare a 1% (w/v) agarose gel according to your laboratory protocol. Analyse 3 µl of each PCR assay. Approximate sizes of PCR products are listed for individual loci in Table 4.

Table 3. Cycling protocol for amplification.

Step		Temp	Locus	Time
	Initial denaturation	95 °C		3 min
3-step cycling	Denaturation	95 °C		15 sec
	Annealing	65 °C		30 sec
	Elongation	68 °C	A,B,C and G	3 min
			DQB1	4 min
			DRB,DPA1	5 min
			DPB1,DQA1	6 min
35 cycles				
	Final elongation	68 °C		10 min
	Cooling	15 °C		∞

Table 4: Approximate size of PCR products

HLA locus	Expected size
A, B and C	3.1 to 3.4 kb
DRB1	3.7 to 4.8 kb
DRB3	3.8 kb
DRB4	0.4 kb (exon 2), 1.3 kb (exon 3)
DRB5	4.0 kb
DQA1	5.4 to 5.8 kb
DQB1	3.7 kb to 4.1 kb
DPA1	4.9 kb
DPB1	5.0 kb (exon 1), 5.7 kb (exon 2-5)
G	2.7 kb

PROTOCOL 2. CLEAN-UP OF PCR PRODUCTS

9. Set up all reactions on ice.

10. Add 1 µl of Exonuclease I and 1 µl of Shrimp Alkaline Phosphatase to each of the PCR products (Table 5).

11. Gently shake the mixture to produce a homogeneous mixture and centrifuge briefly.

12. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 6.

Table 5. GenDx-ExoSAP for amplicon clean-up.

Component	Vol./ Reaction
Exo I (10U/ µl)	1 µl
SAP (2 U/ µl)	1 µl

Table 6. GenDx-ExoSAP cycling protocol for amplicon clean-up.

Step	Temp	Time
Enzyme activation	37 °C	30 min
Enzyme deactivation	80 °C	20 min
Cooling	15 °C	∞

PROTOCOL 3. SEQUENCING REACTION

13. Set up all reactions on ice.

14. Briefly mix and centrifuge all tubes containing the sequencing primers (yellow and green caps) before opening.

15. Prepare a separate sequencing reaction mix for each sequencing primer according to Table 7.

16. Gently mix the sequencing reaction mix to produce a homogeneous reaction and centrifuge briefly.

17. Dispense 9 µl of the sequencing reaction mix into each PCR tube.

18. Add 1 µl purified PCR product to each tube containing the sequencing reaction mix. The final volume in each PCR tube is 10 µl.

19. Program the thermal cycler according to the manufacturer's instructions. Use the conditions outlined in Table 8.

20. Place the samples immediately in the thermal cycler.

Table 7. Composition of sequencing master mix for sequencing of HLA loci.

Component	Vol./ RXN
Nuclease free H_2O	5.5 µl
GenDx-BigDye Buffer (5x)	1.5 µl
GenDx-BigDye Ready Reaction mix v.1.1	1 µl
SEQ primer (yellow or green cap)	1 µl
Total volume	9 µl

Table 8. Cycling protocol for sequencing.

Step		Temp	Time
	Initial denaturation	96 °C	10 sec
3-step cycling	Denaturation	96 °C	10 sec
	Annealing	50 °C	10 sec
	Elongation	60 °C	2 min
25 cycles			
	Cooling	15 °C	∞

PROTOCOL 4. SEQUENCING PRODUCT CLEAN-UP by Sephadex

21. Prepare Sephadex at least 3 hours before use.

22. Fill the wells of the Multiscreen (MS) 45 µl Column Loader with Sephadex G-50 and remove the remaining Sephadex G-50 by scraping with the MS Column Loader Scraper Clear.

23. Lay a MS-HV plate upside-down on top of the filled MS 45 µl Column Loader and turn it upside-down to fill the MS-HV plate with Sephadex G-50.

24. Add 300 µl deionised water to each well (avoid air bubbles) and let the Sephadex steep. The Sephadex should steep at least 3 hours before use but not longer than overnight. Store the Sephadex at 4°C when steeping overnight.
    Make sure the columns do not dry out!

25. Put the MS-HV plate containing the steeped Sephadex G-50 on a 96-wells microtiter plate placing the MS Centrifuge Align Frame Blue in between, and spin down at 750g for 5 min.

26. Replace the 96-wells microtiter plate and the MS Centrifuge Align Frame Blue by the Genetic Analyzer run plate (e.g. EU Frosted Subskirted Thin-wall 96 x 0.2 ml. plate).

Discard the flow-through and store the 96-wells microtiter plate for re-use.

27. Pipet 10 µl deionised water, followed by 10 µl of sequence sample on the MS-HV plate, in the middle of the well.

28. Spin down at 750g for 5 min. to collect the flow-through in the Genetic Analyzer run plate.

Continue to the next protocol for immediate processing of the sequencing products on the Genetic Analyzer, or cover the genetic analyzer run plate containing the sequencing products with an adhesive seal and store at -20°C for long term storage.

PROTOCOL 5. GENETIC ANALYZER

29. Seal the Genetic Analyzer run plate with the grey flexible cover.

30. Load the Genetic Analyzer run plate containing the samples into the thermal cycler, heat the samples for 2 min. at 95°C and subsequently put them on ice until loading in the genetic analyzer.

31. Run the Genetic Analyzer according to the manufacturer's instructions.

32. Use the standard parameters to run your sequencer for your POP version and capillary length.

33. Process the collected raw sequence data with the SBTengine® software. Other HLA typing software may require user validation.

11 APPENDIX A. MASTER MIX

To decrease your hands-on time, GenDx has validated the use of Master Mixes for the preparation of reaction mixes for HLA locus amplification and sequencing.

For the complete protocol please go to our website: www.gendx.com

12 APPENDIX B. CONTAMINATION CONTROL

IMPORTANT:

It is extremely important to include at least one negative control in every PCR setup that lacks template nucleic acid to detect possible contamination.

General Physical Precautions

- Separate the working areas for setting up the PCR amplification mix and DNA handling, including the addition of starting template, PCR product analysis, or plasmid preparation. Ideally, use separate rooms.
- Use a separate set of pipettes for the PCR amplification mix. Use of pipette tips with hydrophobic filters is strongly recommended.
- Prepare and freeze small aliquots of primer solutions and dNTP mix. Use of fresh nuclease free H_2O is strongly recommended.
- In case of contamination, laboratory benches, apparatus, and pipettes can be decontaminated with a 1% Trigene disinfectant.
- Afterward decontamination benches and pipettes must be rinsed thoroughly with nuclease free H_2O.

General Chemical Precautions

- PCR stock solutions can also be decontaminated using UV light. This method is laborious however, and its efficiency is difficult to control and cannot be guaranteed. We recommend storing solutions in small aliquots and using fresh aliquots for each PCR.
- Another approach to prevent amplification of contaminating DNA is to treat individual reaction mixtures with DNAse or restriction enzymes that cut between the binding sites of the amplification primers used, before adding the template DNA sample.

Safety Information

- When working with chemicals always wear a suitable lab coat, disposable gloves, and protective goggles. For more information please consult the appropriate material safety data sheets (MSDSs) available from the product supplier.

13 TROUBLESHOOTING GUIDE

Little or no PCR product	
a) LongRange PCR Enzyme Mix was not added to the amplification mix or not mixed properly when added	Repeat amplification paying attention to the addition and mixing of LongRange PCR Enzyme Mix with the amplification mix.
b) Cycling conditions not optimal	When using a fast thermal cycler, reduce the ramp rate to 1°C/s.
c) DNA concentration not optimal	Re-quantify the DNA and adjust to 50 ng/µl. If the sample concentration is below the recommended range and little or no amplification product is visible, sequence the sample. Acceptable sequence and typing may be achievable.
d) Poor-quality or degraded genomic DNA	Run genomic DNA on a 1% agarose gel to evaluate quality. It is recommended to use purified DNA that has an A_{260}/A_{280} ratio of ~1.8.
e) Weak amplification of HLA-DRB4 exon 3	Use the recommended amount of purified PCR product for sequencing, even if amplification of exon 3 is weak. Use 63°C annealing instead of 65°C as described in Table 3, page 19. Acceptable sequencing data should still be obtained.
f) Weak or no DQB1 amplification product	Increase amount of LongRange polymerase to 0.8ul/PCR
g) DRB1 Ex2Fa1 primer is not compatible with BDT v3.1.	We recommend using BDT v1.1 for this primer
Unusual PCR products	
a) Two PCR products visible after amplification of HLA-DRB1, HLA-DRB4, or HLA-DQB1 locus	In a heterozygous sample, 2 bands may appear for the HLA-DRB1 or HLA-DQB1 PCR products due to length polymorphism in intron regions of the HLA-DRB1 or HLA-DQB1 gene. Amplification of HLA-DRB4 normally results in 2 PCR products, except for DRB4*0301N, which does not possess exon 2.
Excessive background noise	
a) PCR products not cleaned up prior to sequencing	Clean-up the PCR products using the ExoI/SAP clean-up method before using them in the sequencing reaction.
b) No or poor clean-up of sequencing reactions	Be sure to perform clean-up of sequencing reactions using Protocol 4: Sequencing product clean-up by Sephadex. Pipette the sample directly onto the center of the gel-bed surface. Do not allow the reaction mixture or the pipette tip to contact the sides of the gel-bed or the sides of the wells of the plates.
c) Signal strength too high	See “Excessive signal strength” below.
d) Poor or incorrect matrix	Repeat the spectral calibration and re-inject samples.
e) Poor injection	Re-inject samples.
f) Injection time set too high	Reduce injection time and re-inject. Signal strengths of 100–1500 are optimal. Samples of poor quality may have lower signal strengths but may still be analyzed and typed. Some samples may have signals that are over 1500 and will not have excess background.
g) Peaks shifted or on top of each other	Incorrect mobility file chosen. Choose correct mobility file.
h) Poor sequence quality in one of the HLA-DRB1 exon 2 forward sequences	There are 2 forward sequencing primers for exon 2. Both should be used routinely. Primer R3 is specific for sequencing alleles in group DR1/2/3/5/6/8/10. Primer R4 is specific for sequencing alleles present in group DR4/7/9. With homozygous samples, sequences will be obtained from only one of the primers. In heterozygous samples possessing 2 alleles from the same set of groups (e.g. group DR1 and group DR6), heterozygous sequence data is to be expected from the primer for the relevant groups, and the other primer will generate bad or no sequence. The SBTengine® software will reject this sequence data in most cases, so it does not interfere with typing analysis.
Weak signal	
a) Injection time needs to be increased	Repeat sequencing reactions and increase injection time.
b) PCR product concentration too low	Increase the amount of PCR product in the sequencing reaction, and reduce the amount of nuclease free H_2O proportionately.

Excessive dye blobs	
a) No or poor clean-up of sequencing reactions	Be sure to perform clean-up of sequencing reactions using Protocol 4: Sequencing product clean-up by Sephadex. Pipette the sample directly onto the center of the gel-bed surface. Do not allow the reaction mixture or the pipette tip to contact the sides of the gel-bed or the sides of the wells of the plates.
b) Poor sequencing reaction due to error in pipetting or weak amplification product	Be sure that both the cleaned-up amplicon and the correct sequencing mix are added and combined. In the case of weak amplification, confirm the intensity of the amplicon by running an agarose gel.
Excessive signal strength	
a) PCR product too concentrated	Dilute the PCR product with nuclease free H_2O before sequencing.
b) Too much BDT Ready Reaction Premix in the sequencing reaction	Reduce the amount of BDT Reaction Premix and adjust the amount of BDT Buffer according to the manufacturer's instructions.
c) Injection time set too high	Reduce injection time and re-inject. Signal strengths of 100–1500 are optimal. Samples of poor quality may have lower signal strengths but may still be analyzed and typed. Some samples may have signals that are over 1500 and will not have excess background.

14 LIMITED LICENSE AGREEMENT

Use of this product signifies the agreement of any purchaser or user of the GenDx SBTexcellerator® kits with the following terms:

- The SBTexcellerator® kits may be used solely in accordance with the SBTexcellerator® IFU and for use with components contained in the kit only. GenDx grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the GenDx SBTexcellerator® IFU and additional protocols available at www.gendx.com.
- Other than expressly stated licenses, GenDx makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
- This kit and its components are licensed for one-time use and may not be re-used, re-furbished, or re-sold.
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- For updated license terms, see www.GenDx.com.

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