GENDX

NGSgo®-MX11-3

Multiplex HLA amplification for downstream sequencing applications

Instructions For Use

Research Use Only

HLA-A, B, C, DRB1, DQB1, DPB1,

DRB3/4/5, DQA1, DPA1

Edition 3, 2021/04

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IMPORTANT NOTES AND UPDATES

Updates edition 3

- NGSengine data analysis recommendations have been added to the end of section 9.
- Assay validation information has been updated on thermal cycler compatibility.
- Troubleshooting guide has been extended.

Updates edition 2

- In protocol 1, the optimal amount of input DNA has been changed to 30 ng per reaction.
- Product use limitations have been updated.
- Troubleshooting guide has been extended.

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GenDx has made every effort to ensure that this IFU is accurate. Information in this IFU is subject to change without notice.

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

Symbol	Meaning
MAT	Material number
LOT	Batch code / Lot number
VOL	Volume
REF	Catalog number
COMP	Components
Store at -20°C	Store at -20°C
	Contains reagents for N tests
	Add liquid
	Use-by date
	Consult Instructions For Use
	Date of manufacture
	Legal manufacturer

2 KIT CONTENT

NGSgo®-MX11-3 (RUO)			Cat. no 7371864
NGSgo®-MX11-3 Mix A	96 rxn	1 tube	Mat. no 7001864.1 Red cap
NGSgo®-MX11-3 Mix B	96 rxn	1 tube	Mat. no 7001864.2 Yellow cap
NGSgo®-MX11-3 Mix C	96 rxn	1 tube	Mat. no 7001864.3 Blue cap
NGSgo®-MX11-3 Buffer A	96 rxn	1 tube	Mat. no 7001864.4 Red cap
NGSgo®-MX11-3 Buffer B	96 rxn	1 tube	Mat. no 7001864.5 Yellow cap
NGSgo®-MX11-3 Buffer C	96 rxn	1 tube	Mat. no 7001864.6 Blue cap
GenDx-LongMix PCR master mix (4x)	96 rxn	3 tubes	Mat. no. 5007652.1 Black cap
Nuclease-free water	1.25 ml	3 tubes	Mat. no. 3000000 White cap

3 SHIPPING AND STORAGE

Shipping and storage

- NGSgo-MX11-3 kits are shipped on ice or dry ice and should be stored at -20°C upon arrival.
- Changes in the physical appearance of the kit reagents may indicate product deterioration and may interfere with performance.
- In case of damaged packaging, contact us at support@gendx.com.

Shelf life

- Stability studies of the kit to support the expiration date of the kit are currently in progress. The date of manufacture is indicated on the box label.
- NGSgo-MX11-3 kit components are stable until the expiration date indicated on the labels.

In-use stability

- NGSgo-MX11-3 primer mixes (Mix A, B, C) are stable for at least 12 months after dissolving the primers in their corresponding buffers (Buffer A, B, C) when stored at -20°C.
- The NGSgo-MX11-3 kit can withstand at least 10 freeze/thaw cycles.

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 WARNINGS AND PRECAUTIONS

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.

- HLA amplification with NGSgo-MX11-3 requires ~4.5 hours, and subsequent sequencing and genotype analysis can take one to three days. Therefore, the kits are unsuitable for HLA genotyping when time is a critical factor.
- To ensure the best performance, use the NGSgo-MX11-3 kits with the materials, reagents and equipment recommended in section “Equipment and reagents to be supplied by user”. Use of

materials other than specified, must be validated by the user!

- Reconstitution or dilution of reagents in volumes other than described in this IFU can lead to incorrect results and is therefore strongly discouraged.
- GenDx cannot provide support for any problems resulting from non-adherence to this Instructions for Use document.
- Please take special note of Appendix A “Contamination control”.
- For optimal analysis, use NGSengine and select the default NGSgo-MX11-3 preference settings (NGSengine version 2.21 or higher). In case of allele ratio imbalances, the allele ratio threshold may be lowered. Successful typing of the minor allele can be achieved with Illumina NGS data up to 90:10% ratios as long as the Δsignal-to-noise is ≥10%.
- In case of a homozygous typing result, it is recommended to verify that the sample is truly homozygous or whether it contains a second allele that is underrepresented in the data. Verification of HLA-A, -B, -C, -DRB1, -DQB1 or -DPB1 can be done by using GenDx AlleleSEQR HLA, or any other validated third party HLA typing strategy.
- The HLA-DQB1*03:276N and DRB4*03:01N alleles cannot be amplified due to a deletion in these null alleles.

- The DQB1*03:01:01:03 allele cannot be amplified due to a SNP in the UTR.
- Long-term DNA storage should be at -20°C. Long-term storage of DNA at 4°C will decrease DNA quality and impair PCR amplification.

Assay Validation

- The assay has been validated with library preparation reagents of NGSgo compatible with Illumina, and sequencing platform MiSeq (Illumina). Other Illumina sequencing platforms using a similar chemistry as the MiSeq (e.g. iSeq, MiniSeq, HiSeq, NextSeq) are compatible with this assay, optimal conditions on these platforms have to be determined by the user.
- The assay has been validated for use on gDNA extracted from blood.
- The assay is compatible with the Applied Biosystems Proflex thermal cycler (ramp speed at 5°C/sec) and on the Applied Biosystems Veriti thermal cycler (ramp speed at 100%). Other thermal cyclers require end-user validation.
- Before implementing the NGSgo workflow for HLA typing by NGS in your laboratory, please perform a validation of sequencing-based typing methods using known molecular typed samples. Such samples

may be obtained from the International Workshop Reference Cell Panel or the HLA reference panel from the Coriell institute.

Safety Information

- When working with chemicals always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, as well as waste disposal considerations, please consult the appropriate material safety data sheets (MSDSs) available from www.gendx.com.
- If any serious incident has occurred in relation to this product, please report this to GenDx as soon as possible.

6 PRINCIPLE

NGSgo-MX11-3 consists of PCR amplification reagents to amplify Human Leukocyte Antigen (HLA) genes.
The reagents enable the multiplexed amplification of the following HLA genes:

Mix A		
HLA-A	whole gene	3.1 kb
HLA-DRB1	whole gene*	2.5 + 5 kb
HLA-DPB1	exon 1	5 kb
HLA-DRB3	whole gene*	2.5 + 5 kb
HLA-DQA1	whole gene	5.8 kb

Mix B		
HLA-B	whole gene	3.4 kb
HLA-DQB1	whole gene	6.7 kb
HLA-DRB5	whole gene*	2.6 + 4.8 kb

Mix C		
HLA-C	whole gene	3.4 kb
HLA-DPB1	exon 2-5	5.7 kb
HLA-DRB4	exon 2-3	4.3 kb
HLA-DPA1	whole gene	5.5 kb

* *except for part of intron 1*

7 PROCEDURE

HLA locus-specific amplification is performed in a thermal cycler using the NGSgo-MX11-3 primer mixes, template genomic DNA and the GenDx-LongMix PCR. The resulting HLA locus-specific amplicons can subsequently be used for identification of HLA alleles by means of Next-generation sequencing.

8 EQUIPMENT AND REAGENTS TO BE SUPPLIED BY USER

- Cold block or ice
- Pipettes and pipette tips with hydrophobic filters
- Thermal cycler
- Microcentrifuge
- Vortex
- PCR tubes or PCR plate (use thin-walled 0.2 ml PCR tubes recommended by the manufacturer of your thermal cycler)
- Agarose gel electrophoresis system

9 PROTOCOLS

PROTOCOL 1: AMPLIFICATION

Important Notes Before Starting

- Purified DNA should have 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 30 ng. However, template DNA in the range of 15 - 80 ng (in 1 - 3 µl) can be used without affecting results. When using less than 15 ng or more than 80 ng DNA input, consult troubleshooting guide at the end of this IFU.
- To streamline the process, validate your DNA purification procedure so you can use a set volume corresponding to 30 ng DNA.
- Blood samples should be collected in tubes with ACD or EDTA as an anticoagulant. <u>Do NOT use heparinized samples</u>. Heparin has an inhibitory effect on a PCR.

Primer preparation

- An orange primer pellet should be visible prior to use. Centrifuge the primer Mix A, Mix B, and Mix C tubes for at least one minute before opening for the first time to ensure that the orange primer pellet is at the bottom of the tube.
- Resuspend the primer mixes:
  Resuspend Mix A in 212 µl of Buffer A
  Resuspend Mix B in 212 µl of Buffer B
  Resuspend Mix C in 212 µl of Buffer C
- Invert the tubes a couple of times, thoroughly vortex, and briefly centrifuge. Repeat this step at least two times. Resuspended primer mixes should be stored at -20 °C

Protocol

1. Set up all reactions on a cold block or ice.

2. Thaw 4x GenDx-LongMix master mix (black cap), nuclease-free H_2O (white cap), and NGSgo-MX11-3 primer Mix A, B, and C (red, yellow, and blue cap).

3. Always vortex all reagents to mix thoroughly, and centrifuge briefly before use.

4. Prepare 3 reaction mixes as shown in Table 1a, 1b, and 1c. The volume of each mix should be ~10% greater than required for the total number of assays to be performed. Do not add the template DNA yet at this step.

Important: Include a negative control that lacks template nucleic acid to detect possible contamination.

Table 1a. Composition of reaction mix A.

Component	Volume
Nuclease-free H_2O (white cap)	6.25 - 8.25 µl
GenDx-LongMix (4x) (black cap)	3.75 µl
NGSgo-MX11-3 Mix A (red cap)	2 µl
Template DNA (~30 ng)	1 - 3 µl
Total Volume	15 µl

Table 1b. Composition of reaction mix B.

Component	Volume
Nuclease-free H_2O (white cap)	6.25 - 8.25 µl
GenDx-LongMix (4x) (black cap)	3.75 µl
NGSgo-MX11-3 Mix B (yellow cap)	2 µl
Template DNA (~30 ng)	1 - 3 µl
Total Volume	15 µl

Table 1c. Composition of reaction mix C.

Component	Volume
Nuclease-free H_2O (white cap)	6.25 - 8.25 µl
GenDx-LongMix (4x) (black cap)	3.75 µl
NGSgo-MX11-3 Mix C (blue cap)	2 µl
Template DNA (~30 ng)	1 - 3 µl
Total Volume	15 µl

5. Vortex reaction mixes to mix thoroughly, and centrifuge briefly.

6. Dispense each of the reaction mixes into separate PCR tubes or wells. The appropriate volume is 15 µl minus the amount of DNA added in the next step.

7. Add 1 - 3 µl template DNA (~30 ng) to each tube containing reaction mix.

8. Vortex reaction mixes to mix thoroughly, and centrifuge briefly.

9. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 2.

Important: A hot start should <u>not</u> be applied.

Table 2. Cycling protocol for amplification.

Step		Temp	Time
	Initial denaturation	95°C	3 min
30 cycles 3-step cycling	Denaturation	95°C	15 sec
	Annealing	65°C	30 sec
	Elongation	67°C	6 min
	Final elongation	67°C	10 min
	Cooling	15°C	∞

10. Confirm the PCR products using an appropriate detection system such as agarose gel electrophoresis. Prepare a 1% w/v agarose gel according to your laboratory protocol, and analyze 2 µl of each PCR assay. The approximate size of the amplicons ranges from 3.1 to 6.7 kb.

Note: After the assay has been validated, gel electrophoresis is optional. For large sample panels it is preferred to continue directly with library preparation. In that case, amplification dropouts can be identified during data analysis.

PROTOCOL 2: LIBRARY PREPARATION

1. Combine equal volumes of PCR products from Mix A, Mix B, and Mix C. For example, combine 2 µl of each, to obtain a total volume of 6 µl.

2. From the combined pool, use 2 µl as input for library preparation, which has an expected concentration of around 150 ng/µl (~300 ng in total). Quantification of the amplicons is not necessary.

3. For further library preparation for Illumina sequencers, see the Instructions for Use of the NGSgo Library Full Kit. Start with protocol 3A (Fragmentation and adapter ligation).

SEQUENCING RECOMMENDATIONS

For sequencing on a MiSeq (Illumina), perform paired-end sequencing using MiSeq V2 sequencing reagents. Suitable flow cells and their expected sample capacity are listed in Table 3. For sequencing instructions, see the NGSgo Sequencing Instructions for Use. For sequencing on other NGS platforms, contact GenDx Support (support@gendx.com) for more information.

Table 3. MiSeq flow cell capacity

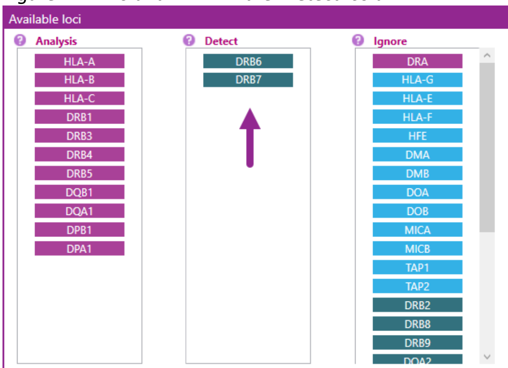
MiSeq reagent kit (300 cycles, V2)	Maximum number of NGSgo-MX11-3 samples*
Nano flow cell (0.3 Gb)	4
Micro flow cell (1.2 Gb)	16
Standard flow cell (4.5 Gb)	60

* The recommended number of samples per flow cell is based on a conservative cluster density of 800 K/mm^2, aiming for an average read depth of ~500 per locus to intercept the amplicon input variation and cluster density variation. Although a read depth of >200 reads is optimal for phasing, successful sequencing and HLA typing has been achieved with read depths as low as 50 reads using NGSengine HLA typing software.

DATA ANALYSIS RECOMMENDATIONS

NGSgo-MX11-3 can be used in combination with NGSengine software. NGSengine version 2.21 (and higher) has been optimized for analysis of NGSgo-MX11-3 data. Note, DRB6 and DRB7 need to be added to the 'Detect' column in the library settings before analysis (Fig 1). The NGSgo-MX11-3 button should be used in the locus analysis settings.

Figure 1. DRB6 and DRB7 in the 'Detect' column.



10 APPENDIX A. CONTAMINATION CONTROL

IMPORTANT:

General Physical Precautions

- Please note that opening tubes after PCR can release amplification products by means of aerosols that can contaminate your working area. For this reason the working areas for pre-amplification and post-amplification procedures must be separated, as described in EFI and ASHI Standards.
- Ideally, pre-amplification and post-amplification procedures should be performed in separate rooms.
- Resuspension of primers and preparation of the locus-specific amplification reaction mix should be performed in the pre-amplification area. Thermal cycling and all protocols thereafter should be performed in the post-amplification area.
- Use a separate set of pipettes for the pre-amplification procedures. Use of pipette tips with hydrophobic filters is strongly recommended.

- In case of contamination, laboratory benches, apparatus, and pipettes need to be decontaminated, for example with a 1% Trigene disinfectant according to the manufacturer's instructions. Preferably, contaminated samples and reagents are discarded.
- Prepare and freeze small aliquots of primer solutions. Use of fresh nuclease-free H_2O as provided in the kit is strongly recommended.

General Chemical Precautions

- PCR stock solutions can also be decontaminated using UV light. However this method is laborious 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.

11 TROUBLESHOOTING GUIDE

GenDx-LongMix was not added to the PCR mix or not mixed properly when added	Repeat amplification paying attention to the addition and mixing of the amplification reaction mix.
Poor-quality or degraded genomic DNA	Run genomic DNA on a 1% agarose gel to evaluate quality. Purified DNA should have an A260/A280 ratio of ~1.8.
Different samples have different read amounts	Make sure the template DNA input in each reaction is similar (30 ng).
High noise in class I HLA genes	Try using a lower amount of DNA input in the PCR reaction.
DNA input was too high (> 80 ng)	Successful amplification can be achieved with up to 100 ng of DNA, however, high input may increase noise in class I HLA genes and can affect allele balance. Additionally, contamination in DNA samples may interfere with the PCR reaction when adding large volumes of DNA.

DNA input was too low (< 15 ng)	Successful amplification can be achieved with as low as 7.5 ng of DNA when the DNA is of high quality, however, low input may lead to reduced yield and lower sample mappability.
HLA-C or HLA-DQB1 allele imbalance	Thoroughly vortex all tubes and reaction mixes at all the steps indicated in the amplification protocol.
HLA-DQB1 imbalanced in lower quality DNA sample	Repeat the amplification with 2.5 µl mix B instead of 2 µl. In some cases, increased concentration of mix B will improve the final allele balance for DQB1. This does not apply to mix A or mix C.
Imbalanced read depth for DRB1 exon 1 versus exon 2	The DRB1 exon 2-6 amplicon is longer compared to the exon 1 amplicon. Decreased sample gDNA quality (fragmented gDNA) may result in a relatively lower read depth in exon 2-6.

Low LRD for HLA-DRB3	Due to the similarity in sequence between HLA-DRB3 and some HLA-DRB1 alleles, reads cannot be distinguished and are removed from analysis, thereby causing a low LRD.

12 LIMITED LICENSE AGREEMENT

Use of this product signifies the agreement of any purchaser or user of the GenDx NGSgo-MX11-3kits to the following terms:

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