inno-train DIAGNOSTIK GMBH Niederhöchstädter Str. 62 D-61476 Kronberg / Taunus Tel. +49-6173-607930	Instructions for Use	CE 0123
Version 1.1	HLA-FluoGene^{NX}	QMS 08.18

HLA-FluoGene^{NX}

Intended Use:
Analysis of HLA Class I and II Alleles
by End-Point Fluorescence Detection based on the PCR Method as well as real-time PCR

IVD

Article No.	Article	Reactions/ Test	Plate / Test	Tests/ Kit
002 082 010	HLA-FluoGene^{NX} ABCDRDQ	96	1	10
002 083 005	HLA-FluoGene^{NX} Match	192	2	5

CHANGES TO WORKFLOW OF CONVENTIONAL FLUOGENE ASSAYS

- Due to a reduced reaction volume (8 µl instead of 15 µl) the FluoGene^{NX} plate has to be shortly centrifuged after adding the FluoMix-DNA mixture (see page 5).
- Please be aware of different PCR-protocols for FluoGene^{NX} assays!
  Page 5, chapter 3.4.1 PCR Program (for use with FluoVista Analyzer)
  Page 6, chapter 3.4.2 PCR Program (for use with FluoQube Real-Time Instrument)

CHANGES TO VERSION 1.0:

- Changes cover page, header
  Version 1.1 & QMS 08.18
- Page 4, chapter 2.3, page 5, chapter 3.4, page 7, chapter 6, addition of important notes
- Page 3, chapter 1.2, page 4, chapter 3.4, Negative control is on tray position H1

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1 GENERAL PRODUCT INFORMATION

1.1 Principle of Method

inno-train's molecular FluoGene^{NX} detection system is based on PCR SSP (SSP: "*Sequence Specific Priming*"). The Polymerase chain reaction (PCR) enables amplification of defined DNA sequences [1]. After successful amplification, the DNA target sequence from genomic DNA is present in detectable concentration. SSP is a variant of PCR in which only the sequences of the primers are responsible for a specification of alleles to be detected [2, 3, 4, 5]. For complete PCR SSP analysis therefore, several amplifications are performed in parallel. PCR samples in which the primer binds to its specific target have a specific amplificate following PCR, while samples without this primer specific binding do not. Primers are also present for amplification of an internal control (gene sequence of the human growth hormone, HGH). If no specific product is present after PCR, the amplificate of this positive control must be clearly detectable. Detection of the PCR products is performed by measuring fluorescence signals and not by gel electrophoresis as in conventional PCR systems.

Each PCR test contains at least two fluorochromes coupled to oligonucleotide probes which can be differentiated in the emission spectrum of the fluorochrome. At least one probe is specific for the target sequence and one for the internal HGH control sequence.

Fluorescence detection can be performed as an endpoint method in the fluorescence reader (**inno-train** FluoVista) or in real time during the PCR run in the real-time PCR cycler (**inno-train** FluoQube).

Endpoint detection detects the emissions of the various fluorochromes in the FluoVista before and after PCR and calculates the difference using the FluoGene^{NX} analysis software.

In the FluoQube, the increase in emissions of the various fluorochromes during the PCR run is detected. The Q values and the CT values (cycle threshold, beginning of the exponential growth of an amplification curve) are determined from these fluorescence values. These are evaluated as positive or negative using the FluoGene^{NX} evaluation software. The increase in fluorescence and Q values above specific thresholds reflects a positive amplification.

1.2 HLA-FluoGene^{NX} Test Systems

inno-train's HLA-FluoGene^{NX} test systems enable low resolution typing (1st field) of HLA Class I (HLA-A*, -B*, -C*) as well as HLA Class II (HLA-DRB1*, -DRB3*, -DRB4* -DRB5*, -DQA1*/DQB1*, -DPA1*/DPB1*) markers in reference to the "common alleles" (CWD 2.0, http://igdawg.org/cwd.html).
The HLA-FluoGene^{NX} Match test system enables detection of HLA-A, -B, -C, -DRB1, 3, 4 & 5, -DQA1, -DQB1, -DPA1 and DPB1 with a resolution comparable to single antigen antibody tests.
Different oligonucleotide mixes that are pre-aliquoted and dried in PCR trays enable the amplification of genetic markers. Each of these oligonucleotide mixes contains primers and probes marked with different fluorescence dyes for:

- A HLA specific probe (fluorochrome 1)
- A HGH specific probe for amplification control (fluorochrome 2)
- Possibly an additional HLA specific probe in case of multiplex reactions (fluorochrome 3)

The negative/contamination control containing a HGH specific oligonucleotide mix is on tray position H1 and is marked by a red-stained oligonucleotide mix. The HLA-FluoGene^{NX} Match test system contains 192 reactions on two 96-well plates (plate A and plate B), both containing a negative/contamination control.

2 MATERIAL

2.1 Components of the HLA-FluoGene^{NX} Kits

- **White, barcoded 96-well PCR trays with labelled cover foil**

Contain the dyed and dried oligonucleotide mixes consisting of HLA sequence specific and internal control primers as well as fluorescence labelled oligonucleotide probes. One typing comprises a defined number of reactions (see table 1).

Table 1: Kit specific components

Kit	Typings/ Kit	PCRs/ Typing	No. PCR Trays	No. FluoMix tubes	No. optical foils
HLA-FluoGene^{NX} ABCDRDQ	10	96	10	10	10
HLA-FluoGene^{NX} Match	5	192	10 (5x plate A, 5x plate B)	10	10

- **Reaction tubes with FluoMix**
  One tube is sufficient for one typing, except for HLA-FluoGene^{NX} Match where one tube is sufficient for one FluoGene^{NX} plate.
  FluoMix contains dNTPs, PCR buffer and *Taq* Polymerase
- **Optical foils for sealing**
- **Instructions for Use**
- **Kit/Lot No. documentation**
  - Quality certificate
  - Limitations, locus cross reactivities and CWD table
  - Specificity table
  - Information sheet with changes to the previous lot
- **Versioned Lot files Update CD containing:**
  Kit and lot specific files and allele databases for import into the FluoGene Software with instructions

2.2 Storage and Shelf-Life

HLA-FluoGene^{NX} kits are despatched chilled. After receipt the kits should be stored at at least –20°C. The shelf-life of the individual components and the entire system are stated on the respective label.

2.3 Additionally Required Materials

For Amplification	For Detection and Evaluation
• Distilled water (without fluorescence) e.g. "LiChrosolv" (Merck) • Sample DNA (Ratio $A_{260/280}$: 1.8 ± 10%) • Pipettes (1 – 1000 µl) • Pipette tips with filters • Dispenser with fixed volume • Thermocycler for endpoint detection (see 3.4.2) • FluoPad, compression pad for PCR (**inno-train**, Art. No.: 002 07C P01) • FluoApp, applicator for application of the optical foils (**inno-train**, Art. No.: 002 07F A01) • Plate centrifuge	• **inno-train** FluoVista Analyzer (Art. No.: 006 010 001) or • **inno-train** FluoQube Real-Time-PCR Instrument (Art. No.: 005 010 001)* • FluoGene Software

* HLA-FluoGene^{NX} ABCDRDQ is only validated for use with FluoQube.

3 PROCEDURE

3.1 Obtaining Samples

EDTA or citrate blood should be used as starting material for the procedure since heparin residues following DNA extraction can inhibit the PCR. In DNA extraction from blood, the leukocytes serve as DNA source, but in general genomic DNA of any origin can be used for the method (hair, buccal swab, etc.).
The concentration of the sample DNA has to be determined prior to test setup. The DNA should be dissolved and diluted in distilled water (without fluorescence) in a concentration of approx. 1 ng/µl. Alternatively the DNA can be mixed undiluted with the FluoMix and be adjusted with H2O to a final concentration of 0.5 ng/µl. The purity ratio OD_{260}/OD_{280} should be 1.8 ± 10%.

3.2 Precautions

Due to the closed HLA-FluoGene^{NX} system there is no risk of contamination as no PCR product can leak out after the PCR preparation. According to existing regulations we recommend the following safety measures:

To avoid contamination within the PCR method:

- Spatial separation of the pre-PCR area (DNA isolation, storage, PCR sample) from the post-PCR area (thermocycler, fluorescence reader)
- Components of the post-PCR area must not get into the pre-PCR area.
- Use pipette tips with aerosol protection in the pre-PCR area.

To perform molecular biological methods in HLA typing

- Experience in HLA and DNA diagnostics is necessary.

3.3 Create a Job in the FluoGene Software

For the FluoGene^{NX} PCR trays and DNA samples to be tested a job has to be created in the evaluation software according to the FluoGene Software Manual. The FluoGene Software automatically generates a PCR protocol as a guideline for the PCR setup of the test.

3.4 PCR / Amplification

The procedure is the same for all HLA-FluoGene^{NX} kits:

⇨ Store the optical foils at room temperature to avoid a condensation on the foils.

⇨ Thaw PCR trays, FluoMix and DNA samples.

⇨ The negative/contamination control is on tray position H1 and marked by a red-stained oligonucleotide mix.

⇨ The FluoMix is ready-to-use and aliquoted for one typing per tube except HLA- FluoGene^{NX} Match test system: the FluoMix is aliquoted for one plate. Before adding DNA, vortex FluoMix thoroughly and pipette 4 µl into the negative control well.

⇨ Add 4 µl of distilled water (without fluorescence) into the negative control well.

⇨ Afterwards add the diluted DNA (conc. 1 ng/µl ± 50%) to the remaining FluoMix (according to table), again vortex thoroughly and distribute 8 µl aliquots to the remaining tubes of the typing (preferably with a hand dispenser at the top edge of the tube walls).

As an alternative you can also directly pipette the DNA into the FluoMix tube and dilute to the final volume with water. The required final DNA amounts and the final end volume are stated in the table below.

For a better visibility the already pipetted reactions show a slight coloring (the contamination control is reddish, the specific reactions blueish).

Kit	**Volume FluoMix**	**Volume DNA sample** (1 ng/µl ± 50%) / Test	**DNA amount** (± 50%)	**Final volume FluoMix, H_2O and DNA**
HLA-FluoGene^{NX} ABCDRDQ, Match	420 µl	420 µl	420 ng	840 µl

⇨ Cover the PCR tray with the adhesive optical foil and close by pressing firmly (use disposable lab gloves and an applicator, e.g. FluoApp by **inno-train**). Avoid finger prints and dirtying the foil.

⇨ **Centrifuge the tray shortly**

⇨ Avoid liquid splashes and condensate on the foil during the whole procedure. If necessary transfer the tray to thermocycler and incubate for approx. 30 sec. with heated lid.

⇨ **For using the FluoVista Analyzer: The pre-read has to be performed within 15 minutes after PCR setup**. Transfer the tray to the FluoVista Analyzer and perform the pre-read. Please note that the correct orientation of the PCR tray is provided by metal bolts on top of the FluoVista Carrier Plate. It is automatically pulled into the device when orientation is correct (jagged side to the right) and must not be pushed or pulled out by force.

⇨ Transfer the PCR tray to thermocycler or FluoQube, cover with a clean PCR pressure pad, e.g. FluoPad by **inno-train**, make sure the heated lid is clean and start PCR program.

Note: HLA-FluoGene^{NX} ABCDRDQ is only validated for use with FluoQube.

⇨ **For using the FluoVista Analyzer: The post-read has to be performed within 15 minutes after end of PCR.** The tray can be measured immediately after 3 minutes cooling to 20°C.

3.4.1 PCR Program (for use with FluoVista Analyzer)

1x Initial	**40 Cycles**	**Cooling Down**	
96°C 2 min.	96°C 15 s 60°C 30 s 68°C 5 s	20°C 3 min	20°C max. 15 min.

Validated thermocyclers and notes:

Manufacturer	**Model**	**Ramp Rate**	**Important Note**
Applied Biosystems	GeneAmp PCR System 9700	Max.	Aluminium block not suitable
Applied Biosystems	Veriti	9700 Mode	-
Analytik Jena	Biometra TProfessional 96	2.5°C	-

The use of other thermocyclers has to be validated by the user.

3.4.2 PCR Program (for use with FluoQube Real-Time Instrument)

All settings for PCR and parameters for measurement are already preset and are used automatically for FluoGene^{NX} from a secured template.

1x Initial	38x Cycles
96°C 2 min.	96°C 15 sec. 60°C 18 sec. (+ Read*) 68°C 5 sec.

*The read is performed during the 60°C step. The following color channels must be selected: "Blue", "Green" and "Orange". The ramp rate of the FluoQube must be adjusted to 2.5 °C/s. These settings are defined by using the "FluoGene.rts" template.

The use of the qTOWER³ (Analytik Jena) is possible under certain conditions. To do this, prior to using the FluoGene^{NX} method a service visit from **inno-train** must take place in order to make certain settings on the device and to install the corresponding software to be acquired. For further information please ask your local inno-train representative.

3.5 Fluorescence Detection

⇨ The endpoint fluorescence detection takes place in the **inno-train** FluoVista Analyzer (post-read). Please make sure that steaming up of the optical foil by condensation is avoided (see above) and reading is performed max. 15 min. after thermocycling.

⇨ The fluorescence detection of the **inno-train** FluoQube Real-Time Instrument is carried out directly during the PCR run.

3.6 Calculation of the Data

⇨ Is carried out automatically by the FluoGene Software.

4 EVALUATION

The evaluation can be carried out after endpoint fluorescence detection (pre- and post-read) or after real-time PCR.
For the endpoint detection with the FluoVista, the background fluorescence of the different fluorochromes measured during the pre-read before PCR is automatically subtracted from the fluorescence values of the post-read by the FluoGene Software.
In the FluoQube, the increase in emissions of the various fluorochromes during the PCR run is detected. The Q values and the CT value (cycle threshold, beginning of the exponential growth of an amplification curve) are determined from these fluorescence values.
These calculated values are evaluated as positive or negative using the FluoGene evaluation software. The increase in the fluorescence or the Q values above specific thresholds reflects thereby a positive amplification. If the respective value is below the threshold value, the reaction is evaluated as negative.
In positive HLA specific reactions the internal control reaction may be suppressed because of competitive primer effects. In the event that the specific reaction is negative and the internal control drops out, the reaction would not be considered for the result and marked with a red question mark.
Further information is provided in the FluoGene Software manual.

4.1 Reference to international standards

According to the established EFI guidelines homozygous results should be verified with other methods and/or family studies. The report generated by the FluoGene Software displays only one proven allele group, i.e. HLA-C*03.

5 PERFORMANCE DATA/PERFORMANCE ASSESSMENT

As part of performance assessment studies, all HLA-FluoGene^{NX} kits were tested with pre typed (molecular biological) samples. In all kits the results agreed 100%.
In quality controls each oligonucleotide mix is tested for correct positivity and negativity.
A safe typing result is obtained if 7.5 ng of DNA are used per well. The composition of the oligonucleotide mixes allows reliable identification of the HLA alleles listed in the specificity tables. HLA-FluoGene^{NX} enables at least a low-resolution typing (1st field) of HLA class I (HLA-A *, -B *, -C *) as well as HLA class II (HLA-DRB1 *, -DRB3 *, -DRB4 * - DRB5 *, -DQA1 * / DQB1 *, -DPA1 * / DPB1 *) regarding the "Common Alleles" (CWD 2.0 http://igdawg.org/cwd.html). The accuracy and reproducibility of the reactivity of the individual oligonucleotide mixes were checked using control samples with known alleles.
The quality of the results that are achieved with the HLA-FluoGene^{NX} kits depends largely on the reagents and materials used. For this reason it is recommended that the tests are performed using the additionally required materials described in chapter 2.3. Any deviations from the procedure (e.g. use of alternative thermocycler) must be validated by the user.

6 GENERAL WARNINGS AND PRECAUTIONS

- ⇨ In samples of human origin there is still a potential risk of infection even after DNA extraction. Therefore gloves and lab coats should be worn when performing the FluoGene^{NX} method and all recommendations on handling infectious material should be followed.
- ⇨ Inadequate patient material may affect the results of analysis.
- ⇨ The fluorophores are photosensitive. Therefore the FluoGene^{NX} plates should be processed quickly.
- ⇨ Reagents should not be used after their expiry date.
- ⇨ Only use optical foils delivered with the kits for sealing the FluoGene^{NX} plates.
- ⇨ Reagents should not be used after their expiry date.
- ⇨ Only use the **inno-train** FluoVista Analyzer or the **inno-train** FluoQube Real-Time PCR Instrument for measuring fluorescence.
- ⇨ The kit HLA-FluoGene^{NX} ABCDRDQ is only validated for use with FluoQube.
- ⇨ Reagents must be used lot specific.
- ⇨ HLA-FluoGene^{NX} tests have to be exclusively evaluated by the FluoGene Software.
- ⇨ The specifications given in these Instructions for Use for the FluoGene^{NX} workflow have to be strictly followed. Deviations may lead to false positive or false negative reactions of the primer-/probe mixes and therefore cause incorrect typing results.

Further information and telephone assistance at: +49 6173-607930, or by email to support@inno-train.de

7 LICENSING AGREEMENT DYES AND QUENCHER

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8 TROUBLE SHOOTING

Problem	Possible cause	Solution
Failure of reactions / false negative reactions	Aliquoted volume is too high	Check pipette setting
	Impure DNA quality	Measure OD_{260} / OD_{280}. A quotient of 1.8 is optimal.
	Magnetic beads within PCR setup	Separate beads by magnet or centrifugation
	PCR inhibitors in distilled water	Only use fresh distilled water
	Low DNA concentration	Measure DNA concentration and adjust to 1 ng/µl. For DNA dilution only use fluorescence-free water.
	Mastermix and DNA were not mixed well	Thoroughly vortex mastermix-DNA solution
	Mastermix was not pipetted in well	Optical control. After adding the mastermix the solution is colored blue.
	Mastermix was pipetted with air bubbles into well	Optical control. When air bubbles are observed centrifuge the plate shortly or knock it carefully on the table.
	Incorrect amplification conditions	Check the thermocycler. See points 3.4.2 and 3.4.3 to see which thermocyclers have been validated for performing the method.
	Wells are not correctly sealed by optical foil	Make sure that the optical foil seals each well tightly. Use the FluoApp for this purpose. The usage of the FluoPad during PCR is recommended.
	Splashes or dirt on optical foil	Change optical foil
	Fogging or dirty optical foil	Incubate the PCR tray in thermocycler with running lid heating for app. 30 sec. or replace the optical foil. Touch the foil only with clean disposable gloves. Make sure that the FluoVista is not placed directly under the flow of the air condition.
	Plate is not fixed correctly on FluoVista Carrier Plate during post-read	Check the correct position of the plate prior to each read
	Period between PCR setup and pre-read is longer than 15 minutes and/or storage between PCR setup and pre-read or respectively between thermocycler and post-read in the light	Measure the plate within 15 minutes after PCR setup and always keep in the dark.
False positive reactions	Aliquoted volume is too low	Check pipette setting
	Aliquoted DNA concentration is too high	Measure DNA concentration and set to 1 ng/µl. Only dilute DNA with fluorescence free water.
	Incorrect amplification conditions	Check the thermocycler. See points 3.4.2 and 3.4.3 to see which thermocyclers have been validated for performing the method.
	Red or yellow pens used on plate	Only use black or blue inks for writing on the plate.
	Air bubbles in well during pre-read	Destroy potential air bubbles by knocking off or centrifuging the plate.
	Evaporation due to untight foil sealing	Use FluoApp for tightly pressing the foil on the plate. Take care of tight sealing at the edges.
	Period between end of PCR and fluorescence detection is too long	Perform the post-read within 15 min after the end of thermocycling.
	Splashes or dirt on optical foil	Change optical foil
	Fogging or dirty optical foil	Incubate the PCR tray in thermocycler with

Problem	Possible cause	Solution
False positive reactions		running lid heating for app. 30 sec. or replace the optical foil. Touch the foil only with clean disposable gloves. Make sure that the FluoVista is not placed directly under the flow of the air condition.
	Period between PCR setup and pre-read is longer than 15 minutes	Measure plate within 15 minutes after the PCR setup

9 EXPLANATION OF SYMBOLS

⚠	Note accompanying documents		Expiry date
LOT	Lot number	REF	Article number
	Follow Instructions for Use		Manufacturer
	Observe upper temperature limit	IVD	In vitro diagnosticum
	Contents sufficient for <n> tests	BLOCK	PCR-tray
BUFFER	FluoMix	FOIL	optical foil
CONTROL+	positive control DNA	CONTROL-	negative control DNA

10 LITERATURE

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5. Bunce M, Young NT, Welsh KI: Molecular HLA Typing – The brave new world. Transplantation 1997;64:1505-1513.