GenVinSet

HLA CELIAC

Instructions for use

Kit for detection of alleles associated to Celiac Disease

For In Vitro Diagnostic Use

Reference GVS-DQ-48 (48 test)
GVS-DQ-24 (24 test)

Store from –18 to –30ºC

CE

Rev 06 / 28-11-2017

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Intended use

GENVINSET HLA CELIAC is a kit for the detection of alleles DQB1*02, DQB1*03:02 and DQA1*05 of HLA system and the consequent determination of DQ2 and DQ8 antigens associated with celiac disease. For the DQB1*02, it is able to determine the status of a sample as homozygous or heterozygous. Analysis based on real-time PCR technology using TaqMan® probes.

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Summary and explanation

Celiac disease is a malabsorption disorder caused by interactions between genetic and environmental factors. An environmental factor that precipitates the disease development is gluten or related proteins present in cereals like wheat, barley and rye. Immune intolerance causes a chronic inflammatory response in the mucosa of the small intestine with symptoms such as diarrhea, steatorrhea and weight loss.

It is one of the most common diseases in the Caucasian population, with a prevalence of between 1:100 and 1:500 in Europe and North America.

Susceptibility to gluten sensitivity is, in part, genetically determined. The strong predisposition is associated with HLA-DQ alleles, encoding the α and ß chains of two molecules of the Major Histocompatibility Complex (MHC) class II.

In most populations studied, 90-95% of patients carry the HLA-DQ2 heterodimer encoded by the alleles DQA1*05 and DQB1*02 in *cis* position (more common in central and northern Europe) or in trans position (more common in Mediterranean countries). The risk of an individual with the heterodimer DQA1*05:01-DQB1*02:01 to suffer the disease is 50 times greater than the average population risk.

The remaining patients (5-10%) usually carry a second heterodimer, HLADQ8 (majority among South American indigenous patients), encoded by the alleles DQA1*03:01 and DQB1*03:02. It is estimated that only 0.5% of celiac patients aren't DQ2 or DQ8. Most of them have actually a half part of the DQ2 heterodimer (DQB1*02:01).

The prevalence of celiac disease has been underestimated. Early diagnosis is important to start as soon as possible with a gluten-free diet. The absence of treatment often causes other autoimmune disorders such as type 1 diabetes or rheumatoid arthritis.

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Procedure principles

The detection method used by GENVINSET is based on three PCR amplification reactions with specific primers for DQB1*02, DQB1*03:02 and DQA1*05 HLA alleles (see section 'Alleles detected by GENVINSET HLA CELIAC', page 18) monitored with TaqMan® probes.

The detection of alleles that are a predisposition factor of celiac disease allows the determination of the haplotypes. The following table shows the HLA haplotypes which constitute the HLA-DQ2 and HLA-DQ8 antigens (the table below shows the DQAB1 and DQA1 alleles encoding the α and ß chains of the antigen, and the DRB1 allele associated):

HLA-DQ	Serological equivalent	Genotype						Frequency in celiac patients
		Haplotype 1			Haplotype 2			
		DQB1*	DQA1*	DRB1*	DQB1*	DQA1*	DRB1*	
DQ2	DR3-DQ2, -	02:01	05:01	03	-	-	-	More than 90% of celiac patients
	DR3-DQ2, DR3-DQ2	02:01	05:01	03	02:01	05:01	03:01	
	DR3-DQ2, DR7-DQ2	02:01	05:01	03:01	02:02	02:01	07	
	DR5-DQ7, DR7-DQ2	03:01	05:05	(11)	02:02	02:01	07	
	DR5-DQ7, -	03:01	05:05	(11)	-	-	-	
	DR7-DQ2, -	02:02	02:01	07	-	-	-	
DQ8	DR4-DQ8	03:02	03:01	(04)	-	-	-	2-10% of patients

Table 1. Based on references 1 – 4.

At the same time the method amplifies and detects a control gene (ß-globin) to verify the assay's result.

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Kit contents

Reference GVS-DQ-48 (48 tests)

- GVS-DQ-PM1: 2 vials x 110 µL Primer Mix 1. (PM1)
- GVS-DQ-PM2: 2 vials x 110 µL Primer Mix 2. (PM2)
- GVS-DQ-PM3: 2 vials x 110 µL Primer Mix 3. (PM3)
- GVS-DQ-MM: 6 vials x 138 uL Master Mix. (MM)
- GVS-DQ-C+: 1 vial x 15 uL Positive Control (C+)
- GVS-RB: 1 vial x 100 uL Reaction Blank. (RB)

Reference GVS-DQ-24 (24 tests)

- GVS-DQ-PM1: 1 vial x 110 µL Primer Mix 1. (PM1)
- GVS-DQ-PM2: 1 vial x 110 µL Primer Mix 2. (PM2)
- GVS-DQ-PM3: 1 vial x 110 µL Primer Mix 3. (PM3)
- GVS-DQ-MM: 3 viales x 138 uL Master Mix. (MM)
- GVS-DQ-C+: 1 vial x 15 uL Positive Control (C+)
- GVS-RB: 1 vial x 100 uL Reaction Blank. (RB)

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Kit storage

All of the kit's reagents should be stored from -18ºC to -30ºC and they are stable at this temperature until its expiration date, as indicated in the bottle. Do not perform more than 3 freezing/thawing cycles to the Primer Mix vials (GVS-DQ-PM) as this could reduce the assays sensitivity and change results.

Due to the reagent's photo sensitivity nature, avoid continuous exposure to light.

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Materials required but not supplied

General

- Gloves
- Lab coat

Consumables

- Filter tips (P200 & P10)
- 1.5 mL Autoclaved eppendorf tubes
- RT-PCR instrument specific reagents (only 0.1mL. tubes accepted in case of using Rotor Gene-Q)

Equipment

- RT-PCR instrument
- Vortex
- Pipettes (P200 & P10)

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Sample collection and preparation

This test should only be performed with complete blood samples treated with EDTA anti coagulation agents or citrate. Heparin can interfere with the PCR process and should not be used in this procedure.

The technique is compatible with any conventional DNA extraction system. Before emiting results with diagnostic purposes, perform a validation test of the samples obtained with the extraction system.

Caution

All biological and blood samples should be treated as possibly infectious. When manipulating them, observe all basic (universal) precautions. All sample manipulation should be done with gloves and appropriate protection.

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Usage procedures

A) PCR preparation

Precautions

- Unfreeze all of the kits components before starting the assay, mix and centrifuge.
- Work over ice or a cold block.
- The PCR should be assembled in the Pre-PCR area.
- Use only filtered tips and autoclaved tubes 1.5 mL.
- Use gloves and lab coat at all times.
- In each session it is recommended to test the contamination control included in the kit (Reaction Blank), and the Positive Control provided with the kit (GVS-DQ-C+) whose typing is DQB1*02/03:02 and DQA1*05.

1. Take the samples out of the freezer. Vortex (or use finger knocks).

2. Prepare mixtures of the Master Mix and each one of the Primer Mixes (PM1, PM2 and PM3) to n+1 samples:

	Vol. per sample (µL)
Master Mix	5
Primer Mix 1, 2 or 3	4

3. Pipette 9 µL of these mixtures in an optical plate/tubes and add 1 µL of DNA sample or negative control in the contamination control well.

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4. Seal the plate with convenient sealer, spin down the volume by centrifuging 1 min. 360 xg.

5. Place the plate in the thermal cycler and start the program.

B) Thermal cycler configuration

1. Set up the next amplification program:

	Cycle Number	Temperature (ºC)	Time (mm:ss)	Ramp (%)	Analysis
Denaturalization	1	95	05:00	100	X
Cycles	50	95	00:15	100	X
		64	1:00	100	Single
Cooling	1	15	∞	100	X

2. Set up the reading channels.

The emitted fluorescence must be read in FAM (495-520 nm) and HEX (535-554 nm) channels. Both fluorescences should be detected in every well (biplex reaction).

NOTE – Special settings for Rotor Gene Q:

a. Open the Rotor-Gene Q – Pure Detection software. Select the tab "Advanced" in the window New Run, and click "New".

b. Select the type of rotor used (only 0.1 mL tubes accepted, see section 'Materials required but not suplied', page 8). Select the "Locking Ring Attached" box and continue by clicking "Next".

c. Type the "Reaction Volume" as 10 uL, and identify the operator and the sample.

d. Click "Edit Profile" and set up the amplification program (see subsection 'B) Thermal Cycler Configuration'). Select the step 60 sec at 64 ºC, and clic "Acquiring to Cycling A". Select the channels for fluorescence acquisition "Green" and "Yellow". Then "OK". Click "OK" to accept and close the "Edit Profile" window.

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e. Clic "Gain Optimisation" in the "Run New Wizard" dialog box to open the "Auto-Gain Optimization Setup" window. In the scroll menu of "Channel Settings" select "Acquiring Channels" and then "Add". In the window "Auto-Gain Optimisation Channel Settings", set the following parameters for each channel ("Green" and "Yellow"):

- Tube position = 1
- Target Sample Range: 5 FI up to 10 FI
- Acceptable Gain Range: -10 to 10

6. Activate the option "Perform Optimisation Before 1st Acquisition", and click "Close".

7. Select "Next" and then "Start Run" in the "New Run Wizard" window.

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Results

GENVINSET HLA CELIAC is a qualitative technique to identify presence or absence of the HLA DQB1*02, DQB1*03:02 and DQA1*05 alleles.

The presence of any of the alleles associated with celiac disease will be determined by the positivity or negativity of the three test reactions (presence or absence of DQB1*02, DQB1*03:02 and DQA1*05 alleles).In the case of DQB1*02, the positivity of the reaction marked with FAM will indicate the presence of, at least, one allele of DQB1*02; the positivity of the reaction labeled with HEX will indicate the presence of, at least, one allele different to 02.

It's necessary to configure the plate so that each sample has three wells, one for each type of reaction (1, 2 and 3). Define 5 measurement channels or 'targets', as follows:

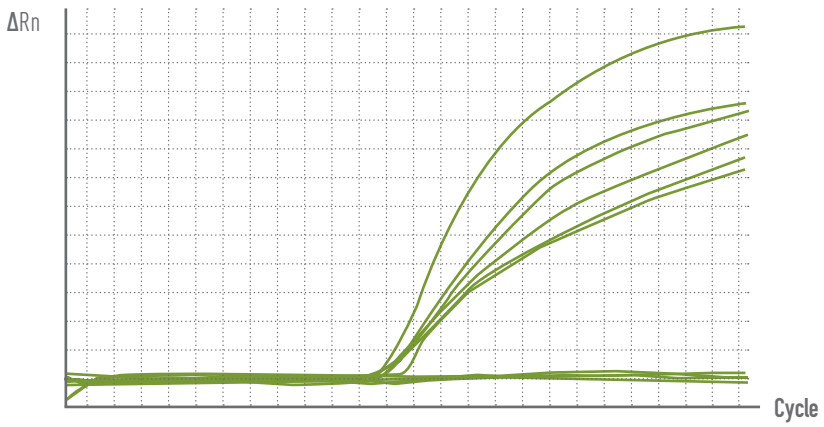
Target Name	Reporter
b-globin	HEX / VIC
DQB1*02	FAM
No DQB1*02	HEX/VIC
DQA1*05	FAM
DQB1*03:02	FAM

It is not necessary to select any passive reference.

The results of this technique are obtained as follows:

DQB1*02 (PM1) results

Selecting DQB1*02 channel (FAM) in the PM1 reaction wells, we can see an Amplification Plot similar to the next one:



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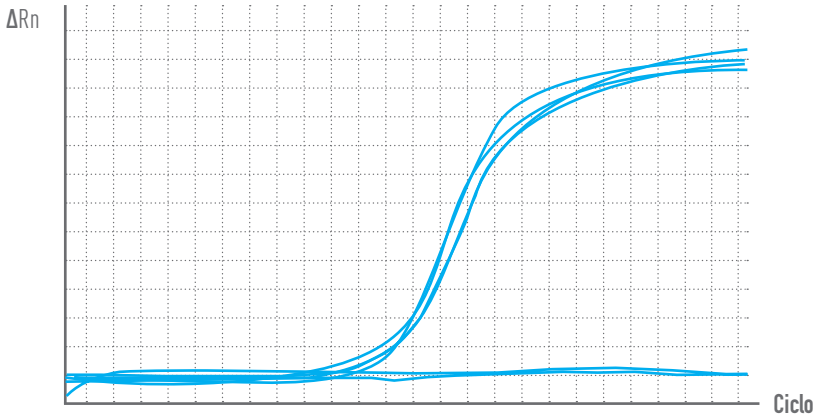
Samples generating only an amplification curve in FAM channel are DQB1*02 homozygous, and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in which fluorescence can be detected as positive. When only an amplification curve in HEX channel appears, the sample is negative for DQB1*02. In the event that both channels show curves, the sample is heterozygous for DQB1*02 (it contains a DQB1*02 allele, and other allele DQB1 different.

DQA1*05 (PM2) results

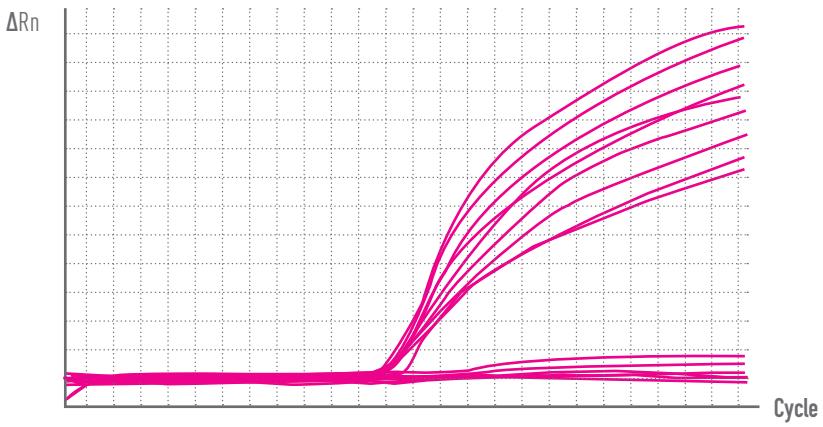
By selecting DQA1*05 channel (FAM) in the PM2 reaction wells, we can see an Amplification Plot similar to the next one:



Samples generating an amplification curve in FAM channel are DQA1*05 positive, and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in which fluorescence can be detected as positive.

DQB1*03:02 (PM3) results

By selecting DQB1*03:02 channel (FAM) in the PM3 reaction wells, we can see an Amplification Plot similar to the next one:



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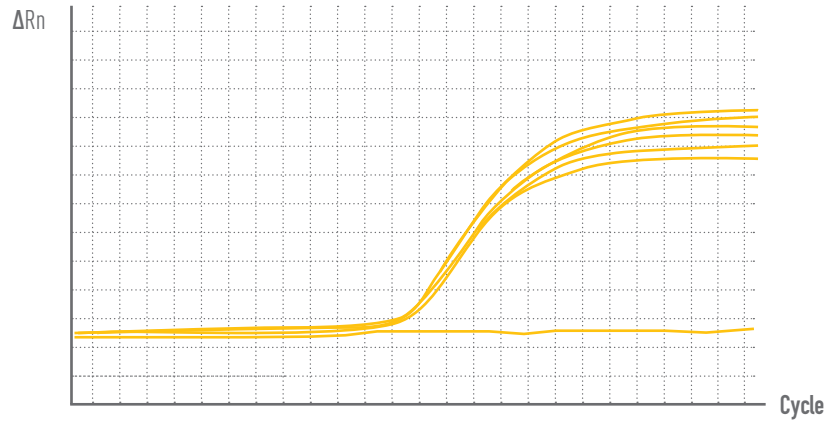
Samples generating an amplification curve in FAM channel are DQB1*03:02 positive, and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in which fluorescence can be detected as positive.

ß-globin results

By selecting the VIC/HEX channel in the reaction wells of any of the PMs (Primer Mix 2 or Primer Mix 3), we can see an Amplification Plot similar to the next one:



ß-Globin is a control gen, and it sequence must be in every DNA sample. Samples generating an amplification curve for HEX/VIC channel are positive for internal control (ß-globin) making the assay valid. Criteria established in section 'Quality Control' (page 16) must be applied.

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Quality control

Due to the qualitative nature of this test, it will not be necessary to perform a calibration.

It is recommended to perform a contamination control by replacing the DNA for the Reaction Blank supplied in the kit and the Positive Control also included (sample with DQB1*02/03:02 and DQA1*05 typing).

The following criteria should be known for the assay to be considered valid:

- The contamination control (Reaction Blank) must provide negative results both for DQB1*02 / DQB1*03:02 / DQA1*05 and for ß-globin. Cp (Crossing Point) values >35 would be consider as negative result. Cp values <35 inform us about a contamination in the session so results should be discarded.
- The Positive Control sample must provide positive results for both DQB1*02 / DQB1*03:02 / DQA1*05 and for ß-globin.
- DNA samples should always be positive for ß-globin (Cp<35).
- DNA samples generating results with Cp>35 for ß-globin and/or DQB1*02 / DQB1*03:02 / DQA1*05 loci must be considered as doubtful and must be retested performing a new extraction of DNA.

The assay must be made according to the kits recommendations, as well as other quality control procedures that comply with local, federal and/or certifying agencies specifications.

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Specific operation data

1. Analytical specificity

The alignment of primers and probes in the most common HLA database (IMGT-HLA) has revealed the absence of non specific bindings. No cross-reaction phenomena with genomic DNA have been reported.

Specificity of the analysis reactions are detailed in section 'Alleles detected by GENVINSET HLA CELIAC', page 18.

2. Analytical sensitivity

Once performed a dilution assay using 1:4 serialized dilutions of several DNA samples with positive and negative DQB1*02/03:02 and DQA1*05 typings, obtained by conventional extraction system, the following results were obtained as for analytical sensitivity of the DQB1*02, DQB1*03:02 and DQA1*05 allele detection:

- Reaction PM1 – Detección DQB1*02: Detection Limit = 0,2 ng/uL
- Reaction PM2 – Detección DQA1*05: Detection Limit = 0,2 ng/uL
- Reaction PM3 – Detección DQB1*03:02: Detection Limit = 1 ng/uL
- DNA sample obtained by conventional extraction system: Detection Limit = 1 ng/µL (*)

  (*) Cp < 35

3. Diagnostic sensitivity and specificity

In a study of human genomic DNA, 36 samples obtained from a laboratory were analyzed. They were previously typed by HLA-SSO in Luminex platform.

Of the 36 samples tested, all of them were validated (positive amplification of the ß-globin control gene). Among them, 3 were homozygous for DQB1*02 and 15 were heterozygous for this allele. A total of 14 samples were called as positive for DQA1*05 allele and 7 were positive for DQB1*03:02. The rest of them, 18, 22 and 29 respectively, were called as negative due to no signal in FAM channel.

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	PM1– DQB1*02			
SSO	Samples	Homocygous	Heterocygous	Negative
	Homozygous.	3	0	0
	Heterocygous.	0	15	0
	Negative.	0	0	18

		PM2 – DQA1*05		PM3 – DQB1*03:02	
SSO	Samples	Pos.	Neg.	Pos.	Neg.
	Pos.	14	0	7	0
	Neg.	0	22	0	29

There is a 100% match in the results obtained with GENVINSET HLA CELIAC and previous information of samples typed by SSO (Sequence Specific Oligonucleotide probes) methodology.

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Alleles detected by GENVINSET HLA CELIAC (IMGT-HLA 3.30.0)

Primer Mix 1	Primer Mix 2
DQB1*02:01:01	***DQA1*05:01:01:01***
DQB1*02:02:01	DQA1*05:01:01:02
DQB1*02:02:04	DQA1*05:01:01:03
DQB1*02:53Q	DQA1*05:01:02
DQB1*02:62	DQA1*05:02
DQB1*02:79	***DQA1*05:03:01:01***
DQB1*02:80	DQA1*05:03:01:02
DQB1*02:81	DQA1*05:04
DQB1*02:82	***DQA1*05:05:01:01***
DQB1*02:83	DQA1*05:05:01:02
DQB1*02:84	DQA1*05:05:01:03
DQB1*02:96N	DQA1*05:05:01:04
	DQA1*05:05:01:05
	DQA1*05:05:01:06
	DQA1*05:05:01:07
	DQA1*05:05:01:08
	DQA1*05:05:01:09
	DQA1*05:05:01:10
	DQA1*05:06:01:01
	DQA1*05:06:01:02
	DQA1*05:07
	DQA1*05:08
	DQA1*05:09
	DQA1*05:10
	DQA1*05:11

- Detected Allele
- Non detected Allele
- Non tested allele. Possible weak amplification.
- *CWD alleles marked in bold and italics*

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Alelos detectados por GENVINSET HLA CELIAC (IMGT-HLA 3.30.0)

Primer Mix 3

DQB1*03:02:01	DQB1*03:08	DQB1*03:221
DQB1*03:02:02	DQB1*03:106	DQB1*03:223
DQB1*03:02:03	DQB1*03:107	DQB1*03:224
DQB1*03:02:04	DQB1*03:11	DQB1*03:225
DQB1*03:02:05	DQB1*03:110	DQB1*03:228
DQB1*03:02:06	DQB1*03:125	DQB1*03:229
DQB1*03:02:07	DQB1*03:146	DQB1*03:233
DQB1*03:02:08	DQB1*03:161	DQB1*03:237N
DQB1*03:02:09	DQB1*03:174	DQB1*03:240
DQB1*03:02:10	DQB1*03:175	DQB1*03:245
DQB1*03:02:11	DQB1*03:178	DQB1*03:247
DQB1*03:02:12	DQB1*03:179	DQB1*03:251
DQB1*03:02:13	DQB1*03:18	DQB1*03:261
DQB1*03:02:14	DQB1*03:184	DQB1*03:263
DQB1*03:02:15	DQB1*03:185	DQB1*03:269N
DQB1*03:02:16	DQB1*03:189	DQB1*03:32
DQB1*03:02:17	DQB1*03:190	DQB1*03:37
DQB1*03:02:18	DQB1*03:199	DQB1*03:45
DQB1*03:02:19	DQB1*03:203	DQB1*03:62
DQB1*03:02:20	DQB1*03:204	DQB1*03:63
DQB1*03:02:21	DQB1*03:205	DQB1*03:64
DQB1*03:02:22	DQB1*03:210	DQB1*03:66N
DQB1*03:02:23	DQB1*03:211	DQB1*03:67
DQB1*03:02:24	DQB1*03:213N	DQB1*03:68
DQB1*03:02:25	DQB1*03:214	DQB1*03:70
DQB1*03:02:26	DQB1*03:215	DQB1*03:81
DQB1*03:07	DQB1*03:220	DQB1*03:85

- Alelo detectado
- Alelo no testado. Posible amplificación de baja señal
- Alelo no detectado
- ***Alelos CWD marcados en negrita y cursiva***

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Limitations of the procedure

- The described conditions for the PCR should be precisely controlled. Deviations from these parameters can lead to poor results.
- All GENVINSET work must be made according to general lab best practices and be adjusted to local regulations, like the EFI standard (European Federation of Immunogenetics).
- The rt-PCR thermal cycler must be calibrated according to the manufacturers' recommendations y should be used in accordance to manufacturers' instructions.
- Do not mix components from other kits or lot numbers.
- Do not use the kit after its expiration date.
- Do not use the kit if there are suspicions of possible loss of reactivity, contamination, container deterioration or any other incidence that might affect the kits performance.
- Due to the complexity of HLA typing, data and result interpretation should be revised by qualified personnel.
- Eliminate expired reagents according to applicable regulations.

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Troubleshooting guide

Problem

- **Probable cause(s)**
  - Suggested corrective measure(s).

Negative control (H_2O) is positive.

- **Primer Mix/Negative control contamination**
  - Repeat the experiment with new Primer Mix/Negative control aliquots.
  - Perform the kit components manipulation always according to usually accepted practices to avoid contamination.
  - Verify manipulation and storage conditions.
  - Discard contaminated reagents.
- **Pre-PCR area is contaminated**
  - Confirm that all necessary precautions in the PCR area have been followed.
  - Check for possible contamination problems in other PCR techniques.
  - Confirm suitability of the used reagents (1.5 mL tubes, pipette tips).
  - Confirm there is no Taq contamination.
- **Pipetting error**
  - Always check that the added sample matches the sample sheet.

Low or no signal in all samples. Control samples are OK.

- **Bad quality of DNA samples**
  - Repeat the DNA extraction.
- **Samples with very low DNA concentration**
  - Check the DNA concentration.

Fluorescence intensity too low

- **Kit degradation (Primer Mix vial)**
  - Confirm the kits correct storage (Primer Mix vial stored in darkness).
  - Avoid more than 3 freeze/unfreeze cycles of the primer mix vial.

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- Aliquote the reagents if necessary.
- Repeat the series with new reagents.

- **Taq has lost activity**
  - Confirm Taq's activity.
  - Repeat with new Taq.

Negative control sample is positive

- **Cross contamination**
  - Handle the kit's components always with all currently contamination avoidance practices.
- **Pipetting error**
  - Always check that the added sample matches the sample sheet.

Positive control sample is negative

- **Pipetting error**
  - Always check that the added sample matches the sample sheet.

Fluorescence intensity varies

- **There is dirt on the outside which interferes with the signal**
  - Manipulate the plates always wearing gloves.
- **The volume is not at the bottom of the well or there is an air bubble**
  - Centrifuge to make sure the sample is at the bottom of the well and there are no air bubbles, according to the technics protocol.
- **Pipetting error**
  - Verify the volume added in each well is correct.

There is no fluorescence signal

- **Incorrect reading channels selected**
  - Configure the correct reading channels.
- **Pipetting error or reagent absence**
  - Control the pipetting and the reactions configuration.
  - Repeat the PCR.
- **No reading channel was selected in the thermal cycler's program.**
  - Revise and modify the thermal cycler program.

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