GenVinSet

DIABETES MELLITUS T1

Instructions for use

Kit for HLA-DRB1*03/DRB1*04/ DQB1*02:01/DQB1*03:02 alleles detection

Reference GVS-DMT1-48 (48 test)
GVS-DMT1-24 (24 test)

Store from –18 to –30ºC

CE 2797

Rev 02 / 28-05-2019

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Intended use

GENVINSET HLA Diabetes Mellitus Type I is a kit for HLA-DRB1*03/ DRB1*04/ DQB1*02:01/ DQB1*03:02 alleles determination by Real Time PCR using TaqMan® probes technology.

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Summary and explanation

Insulin-dependent or type 1 diabetes mellitus (IDDM) is an autoimmune disorder characterized by destruction of insulin producing beta cells in the pancreas. Progression to disease involves both genetic and environmental factors. The environmental component of IDDM susceptibility is not well understood, although viral infection has been suggested as a triggering event (1, 2).

It is the most common form of diabetes among children and young adults in populations of Caucasoid origin, where the prevalence is approximately 0.4%. The overall age-adjusted incidence of IDDM varies from 0.1/100.000 per year in China and Venezuela to 36/100.000 per year in Sardinia and Finland (3). In humans, as well as in animal models, IDDM is a multigenic disease.

In the 1970s, association and affected-sib pair linkage studies established the role of HLA genes in IDDM predisposition (4-6). The HLA region is a cluster of genes located within the major histocompatibility complex (MHC) on chromosome 6p21. The genes of the MHC region are classified into two main families, class I (-A, -B, and Cw) and class II (–DR, -DQ, -DP) (7-9). The statistically strongest genetic association with IDDM is conferred by HLA class II gene alleles.

HLA-DR/DQ are considered to be the main determinants of genetic susceptibility to IDDM. More precisely, HLA DR3/DQB1*02:01 and DR4/DQB1*03:02 haplotypes contribute to over 50% of the genetic load of this disease (10).

Initial association studies of serologically determined HLA alleles with IDDM showed increased frequencies of B8-DR3 and B15 (w62)-DR4 in Caucasians (11, 12). Both DR3 and DR4 are very strongly associated with IDDM: over 93% of IDDM patients have at least one of these alleles, compared to 43% of controls (13), with a significant increase in risk for DR3/DR4 heterozygotes (14-16).

Previous DNA-based HLA typing studies suggested that the DQ molecule may be responsible for HLA associations with IDDM, for example, the DR4 haplotype DRB1 *04:01-DQA1 *03-DQB1 *03:02 is strongly predisposing to disease, while DRB1 *04:01-DQA1 *03-DQB1 *03:01 is not (17-19).

It is clear that some combinations of HLA-DQ genes, eg those encoding DQ8 (ie. DQA1*03–DQB1*03:02) and DQ2 (ie DQA1*05:01–DQB1*02:01), and particularly those present in HLA-DQ2/DQ8 heterozygotes are associated with susceptibility to IDDM. Approximately 30% of IDDM patients are HLA-DQ2/DQ8 heterozygotes (20) From both human genetics and animal model studies there is good evidence that particular alleles of the HLA-DQB1 (*03:02 and *02:01) and DRB1 (*03 and *04) loci all are primarily involved in the genetic predispo-

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sition to IDDM (21-26). However, due to the strong linkage desequilibrium (LD) between these loci it has been very difficult to study the effect of individual HLA-DQ or –DR genes separately.

The fact that several different class II HLA alleles and combinations of alleles may be associated with IDDM can be reconciled by a hypothesis implicating a factor common to all these alleles that is central to disease susceptibility, eg presence of specific amino acid residues.

Indeed, certain amino acids of the HLA-DQB1 and DRB1 chains correlate well with disease susceptibility and resistance. These residues are known to be critical for the peptide-binding function of the class II molecule (27-31). In particular, aspartic acid (Asp) at residue 57, which is in pocket 9 of the HLA-DQB1 molecule, is encoded by the HLA-DQB1*06:02 protective allele, whilst an alanine, valine or serine residue at the same position characterises predisposing alleles. In the absence of aspartic acid the charge at the 'right hand' end of the peptide-binding pocket becomes more positive (32). Nevertheless, and despite the fact that residue 57 has indeed a pronounced role in the function of class II molecules with respect to peptide binding, (33,34) it cannot completely account for all the complexity of HLA and IDDM associations, e.g.: Asp 57 is not associated with IDDM in Japanese (35,36). Interestingly, the major DQB1 allele associated with IDDM in Japan, *04:01, has a different residue 56 than other Asp57 bearing allotypes, and it was recently suggested that residue 56, which is also in pocket 9, may influence the structure and function of this pocket in peptide binding and IDDM susceptibility (27,31).

Nevertheless, class II genes are not the only responsible factor of IDDM. Several other loci, inside and outside the HLA region, are known to modify the IDDM risk, as well as some environmental factors.

Various guidelines and different authors highly recommend complementing the genetic diagnosis of IDDM with other tests, such as the serological antibody detection (anti-IAA, anti-ICA, anti-GAD65, anti-IA-2 and anti-ZnT8 immunoglobulin), the oral glucose tolerance test or the detection of the glycosylated haemoglobin levels, among others (37-39).

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Procedure principles

The detection method used by Genvinset is based in a primer specific PCRs, which anneal to specific HLA-DRB1*03, *04, DQB1*03:02, and *02:01 group of alleles, respectively, monitored with Taqman probes.

At the same time the method amplifies and detects a control gene (ß-globin) for each assay to verify the results.

This technique provides high resolution, high sensitivity, specificity and reproducibility.

(*) See Procedure Limitations (section 13)

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Kit contents

Reference: GVS-DMT1-24 (24 test)

- GVS-DMT1-PM1: 1 vial x 110 µL Primer Mix 1 (PM1) -> Determination of DRB1*03
- GVS-DMT1-PM2: 1 vial x 110 µL Primer Mix 2 (PM2) -> Determination of DQB1*02:01
- GVS-DMT1-PM3: 1 vial x 110 µL Primer Mix 3 (PM3) -> Determination of DRB1*04
- GVS-DMT1-PM4: 1 vial x 110 µL Primer Mix 4 (PM4) -> Determination of DQB1*03:02
- GVS-DMT1-MM1: 1 vial x 138 µL Master Mix 1 -> Determination of DRB1*03
- GVS-DMT1-MM2: 1 vial x 138 µL Master Mix 2 -> Determination of DQB1*02:01
- GVS-DMT1-MM3: 1 vial x 138 µL Master Mix 3 -> Determination of DRB1*04
- GVS-DMT1-MM4: 1 vial x 138 µL Master Mix 4 -> Determination of DQB1*03:02
- GVS-DMT1-C+: 1 vial x 20 µL Positive Control (C+)
- GVS-RB: 1 vial x 100 µL Reaction Blank (RB)

Reference: GVS-DMT1-48 (48 test)

- GVS-DMT1-PM1: 2 vial x 110 µL Primer Mix 1 (PM1) -> Determination of DRB1*03
- GVS-DMT1-PM2: 2 vial x 110 µL Primer Mix 2 (PM2) -> Determination of DQB1*02:01
- GVS-DMT1-PM3: 2 vial x 110 µL Primer Mix 3 (PM3) -> Determination of DRB1*04
- GVS-DMT1-PM4: 2 vial x 110 µL Primer Mix 4 (PM4) -> Determination of DQB1*03:02
- GVS-DMT1-MM1: 2 vial x 138 µL Master Mix 1 -> Determination of DRB1*03
- GVS-DMT1-MM2: 2 vial x 138 µL Master Mix 2 -> Determination of DQB1*02:01
- GVS-DMT1-MM3: 2 vial x 138 µL Master Mix 3 -> Determination of DRB1*04
- GVS-DMT1-MM4: 2 vial x 138 µL Master Mix 4 -> Determination of DQB1*03:02
- GVS-DMT1-C+: 1 vial x 20 µL Positive Control (C+)
- GVS-RB: 1 vial x 100 µL 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-DRB1*03-PM; GVS-DRB1*04-PM; GVS-DQB1*02:01-PM; and GVS-DQB1*03:02-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, P100 & P10)
- 1.5 mL Autoclaved eppendorf tubes
- RT-PCR instrument specific reagents (in the case of using RotorGene Q, only 0.1mL tubes are allowed).

Equipment

- RT-PCR instrument.
- Vortex
- Pipettes (P200, P100 & 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.

This technique is compatible with other DNA extraction systems. Before validating results with other extraction systems, perform a technique validation test (assay).

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

- Thaw 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 use one negative and one positive samples for HLA-DRB1*03, DRB1*04, DQB1:02:01, and DQB1:03:02 in the corresponding assays.

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

2. Prepare mixtures of each one of the Primer Mixes (PM1, PM2, PM3 and PM4) with the corresponding Master Mix (MM1, MM2, MM3 and MM4) to n+1 samples:

	Vol. per sample (µL)
Master Mix 1, 2, 3 or 4	5
Primer Mix 1, 2, 3 or 4	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	40	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

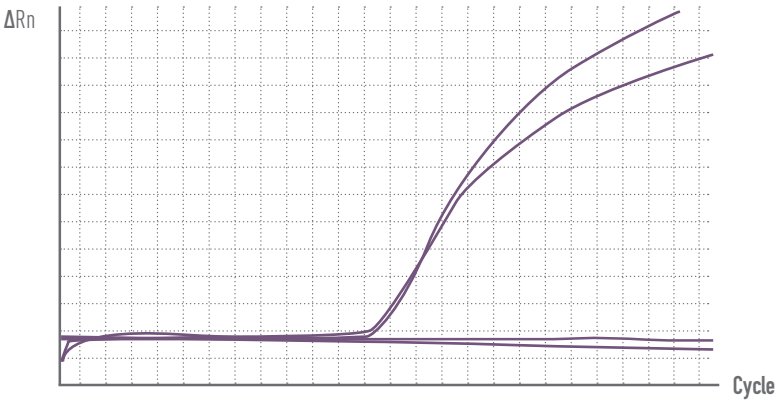
GENVINSET HLA-Diabetes Mellitus Type I kit is a qualitative technique to identify presence or absence of the HLA-DRB1*03, DRB1*04, DQB1*02:01, and DQB1*03:02 group of alleles.

It is not necessary to select any passive reference.

The results of this technique can be obtained as follows:

HLA-DRB1*03 – Reaction 1 results:

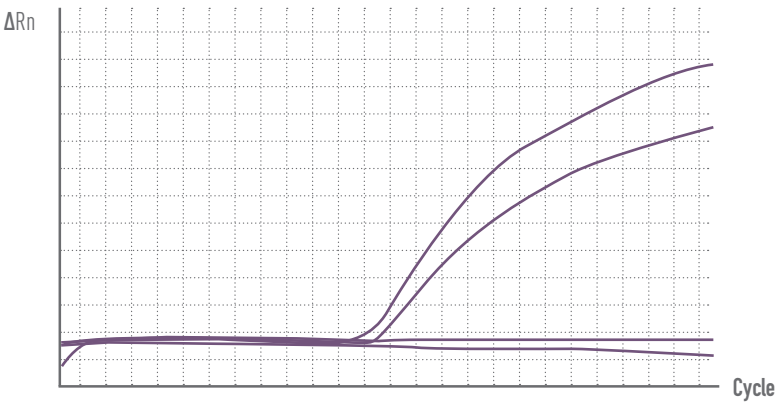
Selecting FAM channel in Amplification Plot, you will see a pattern of curves similar to the next one, in which we show the results of 2 positive samples for DRB1*03 and 2 negatives for the same target:



Samples generating an amplification curve are DRB1*03 positive and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in that fluorescence can be detected.

HLA- DQB1*02:01 – Reaction 2 results:

Selecting FAM channel in Amplification Plot, you will see a pattern of curves similar to the next one, in which we show the results of 2 positive samples for DQB1*02:01 and 2 negatives for the same target:



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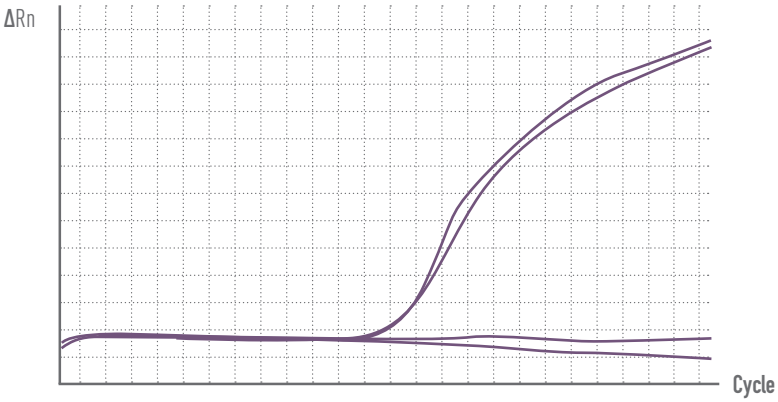
Samples generating an amplification curve are DQB1*02:01 positive and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in that fluorescence can be detected.

HLA-DRB1*04 – Reaction 3 results:

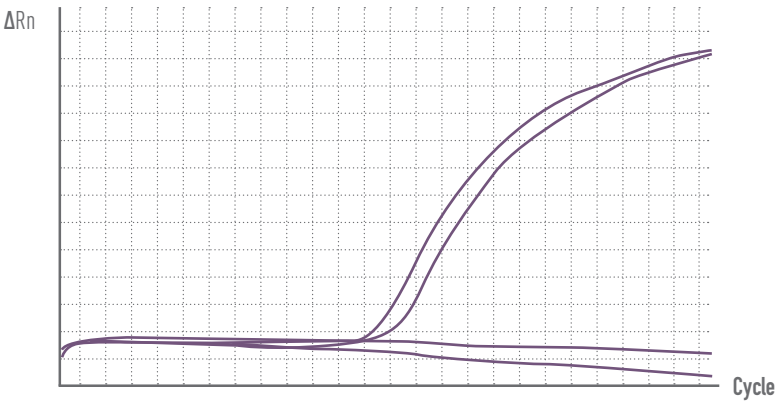
Selecting FAM channel in Amplification Plot, you will see a pattern of curves similar to the next one, in which we show the results of 2 positive samples for DRB1*04 and 2 negatives for the same target:



Samples generating an amplification curve are DRB1*04 positive and they are identified by a numeric value called Crossing Point (Cp) corresponding with the cycle in that fluorescence can be detected.

HLA-DQB1*03:02 – Reaction 4 results:

Selecting FAM channel in Amplification Plot, you will see a pattern of curves similar to the next one, in which we show the results of 2 positive samples for DQB1*03:02 and 2 negatives for the same target:



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

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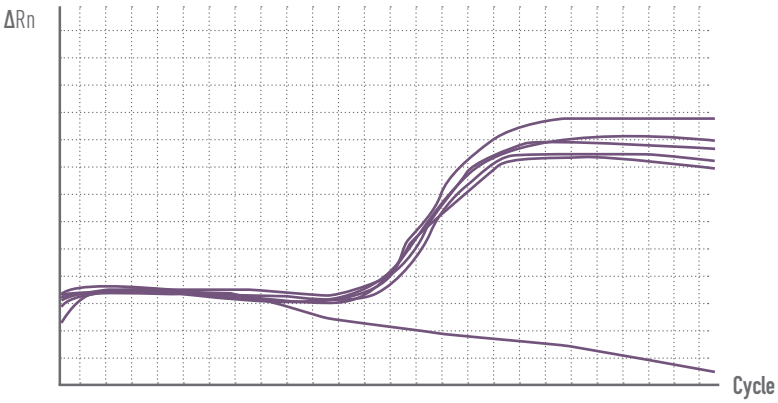
ß-globin results:

Selecting VIC/HEX channel in Amplification Plot, of the corresponding assay, you will see a pattern of curves similar to the next one, in which we show the results of 7 DNA samples (positives for ß-globin) and a negative control (water):

Samples generating an amplification curve are positive for internal control (ß-globin).



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Quality control

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

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

- The Reaction Blank must provide negative results both for the corresponding HLA allele tested (DRB1*03, *04, DQB1*02:01, and *03:02, respectively), 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.
- A positive control sample must provide positive results for both, the corresponding allele tested and for ß-globin.
- DNA samples should always be positive for ß-globin. (Cp<35).

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 Diabetes mellitus T1', page 19.

2. Analytical sensitivity

Once performed a dilution assay using 1:4 serialized dilutions of several DNA samples with positive and negative DRB1*03/04 and DQB1*02:01/03:02 typings, obtained by conventional extraction system, the following results were obtained as for analytical sensitivity of these alleles detection:

- Reaction 1 – DRB1*03: Detection Limit = 1,15 ng/uL
- Reaction 2 – DQB1*02:01: Detection Limit = 1,15 ng/uL
- Reaction 3 – DRB1*04: Detection Limit = 0,74 ng/uL
- Reaction 4 – DQB1*03:02: Detection Limit = 1,65 ng/uL

DNA sample obtained by conventional extraction system:

Detection Limit = 1,65 ng/µL (*)

(*) Cp < 35

3. Diagnostic sensitivity and specificity

In several studies of human genomic DNA, 154 samples for each reaction were analyzed. These samples were previously determined for HLA-DRB1 and DQB1 loci, by HLA-SSO or NGS.

All samples could be validated (positive amplification of the ß-globin control gene). A total of 36 samples were called as positive for DRB1*03 and

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DQB1*02:01. Using reaction 3, 49 samples were positive for DRB1*04, while using rection 4, 38 resulted positive for DQB1*03:02 alleles.

		DRB1*03 – Rx1		DQB1*02:01 – Rx2		DRB1*04 – Rx3		DQB1*03:02 – Rx4	
Previous result		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
	Pos.	36	0	36	0	49	0	38	0
	Neg.	0	118	0	118	0	105	0	116

There is a 100% match in the results obtained with GENVINSET HLA Diabetes mellitus T1 and previous information of samples typed by SSO (Sequence Specific Oligonucleotide probes) or NGS methodologies.

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Alleles detected by GENVINSET HLA DIABETES MELLITUS (IMGT-HLA 3.36.0)

Reacción 1	Reacción 2	Reacción 4
DRB1*03:01:01:01	***DQB1*02:01:01***	***DRB1*04:01:01:01***
DRB1*03:01:01:02	DQB1*02:02:01:02	DRB1*04:01:01:02
DRB1*03:01:01:03	DQB1*02:109	DRB1*04:01:01:03
DRB1*03:02:01	DQB1*02:53Q	***DRB1*04:03:01:01***
	DQB1*02:79	DRB1*04:03:01:02
	DQB1*02:81	***DRB1*04:04:01***
	DQB1*02:82	***DRB1*04:05:01:01***
	DQB1*02:83	DRB1*04:05:01:02
	DQB1*02:96N	DRB1*04:05:01:03
		DRB1*04:06:01
		DRB1*04:07:01:01
		DRB1*04:07:01:02
		DRB1*04:10:01
		DRB1*04:10:03

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* The detection of alleles whose intronic regions have not been sequenced cannot be evaluated.

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

Alleles detected by GENVINSET HLA DIABETES MELLITUS (IMGT-HLA 3.36.0)

Reacción 3

DQB1*03:02:01:01
DQB1*03:02:01:02
DQB1*03:02:01:03
DQB1*03:02:01:04
DQB1*03:02:01:05
DQB1*03:02:01:06
DQB1*03:02:01:07
DQB1*03:02:01:08
DQB1*03:02:02
DQB1*03:02:03
DQB1*03:02:04
DQB1*03:02:05
DQB1*03:02:06
DQB1*03:02:07
DQB1*03:02:08
DQB1*03:02:09
DQB1*03:02:10
DQB1*03:02:11
DQB1*03:02:12
DQB1*03:02:13
DQB1*03:02:14
DQB1*03:02:15
DQB1*03:02:16
DQB1*03:02:17
DQB1*03:02:18
DQB1*03:02:19
DQB1*03:02:20
DQB1*03:02:21
DQB1*03:02:22
DQB1*03:02:23
DQB1*03:02:24
DQB1*03:02:25
DQB1*03:02:26
DQB1*03:02:27
DQB1*03:02:28
DQB1*03:02:29
DQB1*03:02:30
DQB1*03:07
DQB1*03:08
DQB1*03:106
DQB1*03:107
DQB1*03:11
DQB1*03:125
DQB1*03:136
DQB1*03:141
DQB1*03:146
DQB1*03:153
DQB1*03:161
DQB1*03:174
DQB1*03:175
DQB1*03:178
DQB1*03:179
DQB1*03:18
DQB1*03:189
DQB1*03:190
DQB1*03:199
DQB1*03:203
DQB1*03:204
DQB1*03:205
DQB1*03:210
DQB1*03:211
DQB1*03:213N
DQB1*03:214
DQB1*03:215
DQB1*03:223
DQB1*03:224
DQB1*03:225
DQB1*03:228
DQB1*03:229
DQB1*03:233
DQB1*03:237N
DQB1*03:240
DQB1*03:245
DQB1*03:247
DQB1*03:251
DQB1*03:261
DQB1*03:263
DQB1*03:265
DQB1*03:273
DQB1*03:274
DQB1*03:277

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

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Alleles detected by GENVINSET HLA DIABETES MELLITUS (IMGT-HLA 3.36.0)

DQB1*03:278
DQB1*03:279
DQB1*03:287
DQB1*03:295
DQB1*03:296
DQB1*03:298
DQB1*03:299
DQB1*03:300
DQB1*03:301
DQB1*03:308
DQB1*03:310N
DQB1*03:315
DQB1*03:32
DQB1*03:320
DQB1*03:321
DQB1*03:322
DQB1*03:323
DQB1*03:324
DQB1*03:333
DQB1*03:334N
DQB1*03:339N
DQB1*03:343
DQB1*03:344
DQB1*03:345
DQB1*03:348
DQB1*03:349
DQB1*03:352
DQB1*03:362
DQB1*03:364
DQB1*03:367
DQB1*03:368
DQB1*03:369
DQB1*03:37
DQB1*03:371
DQB1*03:379
DQB1*03:383
DQB1*03:386
DQB1*03:388
DQB1*03:392
DQB1*03:45:01
DQB1*03:45:02
DQB1*03:62
DQB1*03:63
DQB1*03:64
DQB1*03:66N
DQB1*03:67
DQB1*03:68
DQB1*03:70
DQB1*03:81
DQB1*03:85

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

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Procedure limitations

- 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).

Contamination control (RB) is positive is positive.

- Primer Mix / Master Mix / Reaction Blank
  - Repeat the experiment with new Primer Mix / Master Mix / Reaction Blank 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.
- Blood processed without previous frozen step
  - Repeat extraction with a new blood aliquot previously frozen.
- Samples with very low DNA concentration
  - Check the cellular lysate DNA concentration
- DNA samples with high concentration
  - Perform a DNA extraction method validation assay testing some dilution of the DNA samples.

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Fluorescence intensity too low

- **Kit degradation (Primer Mix vial)**
  - Confirm the kits correct storage (Primer Mix vial stored in darkness and between -18 and -30ºC).
  - Avoid more than 3 freeze/unfreeze cycles of the Primer Mix vial.
  - Aliquote the reagents if necessary.
  - Repeat the series with new reagents.
- **Kit degradation (Master Mix vial)**
  - Confirm the kits correct storage, between -18 and -30ºC.
  - Avoid more than 3 freeze/unfreeze cycles of the Master Mix vial

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.

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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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