Instructions for use

Coeliac ImmuneKit

Ref. IMG-420

Manufactured by:
HEALTH IN CODE, S.L.
Calle de la Travesía s/n, 15E Base 5, Valencia 46024, España
+34 963 212 340 - info@healthincode.com

healthincode.com

Code: HIC-PT-KIT 03-F-03 V.01

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All Health in Code, S.L. products undergo strict quality control. The Coeliac ImmuneKit has passed all internal validation tests, thus guaranteeing the reliability and reproducibility of each assay.

If you have any questions about the use of this product or its protocols, feel free to contact our Technical Department:

+34 963 212 340

tech.support@healthincode.com

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01 General information

Coeliac disease is a complex immune disorder with a strong genetic influence. In genetically predisposed individuals, an intake of gluten (a protein found in wheat, rye, and barley) triggers an immune response that attacks the small intestine, damaging villi and leading to gut inflammation and inability to absorb nutrients, which results in a number of symptoms including diarrhea and abdominal pain and distention.

The main genes involved in the development of coeliac disease belong to the major histocompatibility complex (MHC), located in 6p21. The MHC comprises hundreds of genes belonging to the human leukocyte antigen (HLA) complex, which encode glycoproteins that can recognize exogenous and/or endogenous peptides in immune cells, thus promoting cell apoptosis when peptides are recognized as foreign. In coeliac disease, MHC genes involved in gluten recognition and presentation to CD4+ T cells are those that encode receptors HLA-DQ8, HLA-DQ2 (2.2 o 2.5), and HLA-DQ7.5. This type of receptor is composed of two subunits, α and β, which constitute heterodimeric protein DQ$\alpha\beta$. Each subunit is encoded by a different gene: *HLA-DQA1* and *HLA-DQB1* respectively.

Around 90% of coeliac patients show the heterodimer HLA-DQ2.5, responsible for the immune response to gluten (genotype HLA-DQA1*05 and HLA-DQB1*02), while most of the remaining patients show the heterodimer HLA-DQ8 (genotype HLA-DQA1*03 and HLA-DQB1*03:02). Heterodimer HLA-DQ7.5 (genotype HLA-DQA1*05 and HLA-DQB1*03:01) is the one with the lowest genetic risk and the most uncommon among the affected population. Moreover, there is a dosage effect in genes *HLA-DQB1*02* and *HLA-DQB1*03*; therefore, it is recommended to determine genetic load (one or two copies).

Genetics HLA			
Alleles *HLA-DQA1*	Alleles *HLA-DQB1*	HLA-DQ haplotype	DQ protein
*05	*02	DQ2.5	DQ2.5
*03	*03:02	DQ8	DQ8
*02	*02	DQ2.2	DQ2.2
*05	*03:01	DQ7.5	DQ7.5

Table 1. HLA-DQ alleles and haplotypes that confer risk of coeliac disease, including the proteins they encode

Coeliac disease is estimated to affect 1 in 100 people worldwide; most of these individuals are not diagnosed and are therefore at risk of long-term health complications. Coeliac disease can be developed at any age upon intake of food or drugs containing gluten. If untreated, it can result in additional severe health issues. In children, malabsorption can also affect growth and development.

As knowledge about this disease increased, the study of the HLA system has gained relevance as a diagnostic tool, resulting in its inclusion in the most recent diagnostic recommendations for children and adolescents proposed by the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN), as well as in adult guidelines.

References

- *Martínez-Ojinaga E, et al. 2018. HLA-DQ distribution and risk assessment of celiac disease in a Spanish center, Revista Española de Enfermedades Digestivas, 110(7), pp. 421-426. doi: 10.17235/reed.2018.5399/2017*
- *Núñez, C. et al. 2018. Recommendations to report and interpret HLA genetic findings in coeliac disease, Revista Española de Enfermedades Digestivas, 110(7), pp. 458–461. doi: 10.17235/reed.2018.5269/2017*

02 Intended use

The Coeliac ImmuneKit uses a combination of oligonucleotides and fluorescent hydrolysis probes for quantitative real-time PCR testing, validated for the simultaneous detection of the genotypes most commonly associated with a higher susceptibility to coeliac disease, Specifically, this assay allows detecting alleles *DQA1*02, DQA1*03, DQA1*05, DQB1*03:01*, and *DQB1*03:02*, as well as discriminating between allele *DQB1*02* and the remaining *DQB1* alleles (*DQB1*03, DQB1*04, DQB1*05,* and *DQB1*06*) by real-time PCR using TaqMan™ probes.

This genetic test allows the user to detect the presence or absence of these genotypes in four multiplexed real-time PCR reactions, which include the simultaneous amplification of the reference gene, β-globin, as qualitative and quantitative control for DNA.

The Coeliac ImmuneKit studies the germline genotype; therefore, the optimal sample type for this analysis is genomic DNA extracted from a peripheral blood sample.

Coeliac ImmuneKit is intended solely for research use and is aimed at professionals working in molecular biology.

03 Technical characteristics

The Coeliac ImmuneKit has been validated by analyzing samples previously diagnosed by genotyping using a different technique, as well as by the use of synthetic vectors (GenScript) containing the sequences of interest. These vectors are provided as positive controls to guarantee the correct setup and operation of the PCR system.

Analytical specifications:

- The necessary type of material for this study is genomic DNA from peripheral blood
- Total necessary amount of DNA: 200 ng in total
- Limit of detection: 1 ng DNA

The Coeliac ImmuneKit is compatible with real-time PCR platforms with FAM™ and VIC™ fluorescence channels.

Coeliac ImmuneKit is intended solely for research use and is aimed at professionals working in molecular biology.

04 Safety warnings and precautions

- It is recommended to strictly follow the instructions in this manual, especially regarding the handling and storage conditions of the reagents.
- Do not mouth-pipette.
- Do not smoke, eat, drink, or apply cosmetics in areas where kits and samples are handled.
- Any cuts, abrasions, and other skin injuries must be properly protected.
- Do not pour the remains of reagents down the drain. It is recommended to use waste containers established by the legal norm and manage their treatment through an authorized waste management facility.
- In the event of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous membranes and rinse with a large amount of water.
- Safety data-sheets (MSDS) of all dangerous substances contained in this kit are available on request.
- This product requires the manipulation of samples and materials of human origin. It is recommended to consider all materials of human origin as potentially infectious and manipulate them according to level 2 of the OSHA norm on biosafety and bloodborne pathogens or other practices related to biosafety of materials that contain or are suspected to contain infectious agents.
- The reagents included in this kit are not toxic, explosive, infectious, radioactive, magnetic, corrosive, or environmental biological pollutants.
- This kit has been validated with specific equipment and under specific conditions that may vary widely among laboratories. Therefore, it is recommended that each laboratory verify compliance with the technical specifications of the manufacturer when the kit is to be used for the first time.
- The manufacturer assumes no responsibility for any damage or failure of the assay caused by substituting reagents included in the kit for ones not provided by Health in Code S.L.
- The manufacturer does not guarantee the assay's reproducibility when the user uses reagents that have not been validated by Health in Code S.L. but are considered by the user equivalent to those provided in the kit.

05 Content and storage conditions of the kit

This kit contains sufficient reagents to perform 48 real-time PCR reactions with each premaster analyzed in this assay. The reagents included in this kit are as follows:

- **Coeliac Master Mix I**: PCR-specific Master Mix containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow for the simultaneous detection of allele *HLA-DQA1*05* and the endogenous gene β-globin, respectively, with the latter serving as endogenous PCR control.
- **Coeliac Master Mix II**: PCR-specific Master Mix containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow for simultaneous discrimination between allele *HLA-DQB1*02* and the remaining *HLA-DQB1* alleles.
- **Coeliac Master Mix III**: PCR-specific Master Mix containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow for simultaneous discrimination between allele *DQB1*03:02* and allele *HLA-DQA1*03*, respectively.
- **Coeliac Master Mix IV**: PCR-specific Master Mix containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow for simultaneous discrimination between allele *DQA1*02* and allele *DQB1*03:01*, respectively.
- **Master Mix General**: PCR master mix with the nucleotides, $MgCl_2$, enzyme and buffer required to perform real-time PCR.
- **Positive control**: synthetic DNA containing the sequences of the amplified regions matching all the alleles included in the kit.

Reagents	Color indicator	Quantity	Conservation
Master Mix I Coeliac	Red cap	2 x 180 µl	-20 °C
Master Mix II Coeliac	Yellow cap	2 x 180 µl	-20 °C
Master Mix III Coeliac	Purple cap	2 x 180 µl	-20 °C
Master Mix IV Coeliac	Green cap	2 x 180 µl	-20 °C
General Master Mix	White cap	4 x 600 µl	-20 °C *
Positive control	Black cap	2 x 100 µl	-20 °C

Table 2. Components of Coeliac ImmuneKit

(*) General Master Mix: It is recommended to confirm the dilution to 10 ng / µL with a fluorimeter.

06 Equipment, reagents and material not included in the kit

Equipment:

- Real-time PCR thermal cycler (FAM and VIC channels)
- Micropipettes (10 µL, 20 µL and 200 µL)
- Vortex mixer
- Centrifuge

Reagents:

- Nuclease-free water

Materials:

- Optical 96-well plates or 0.2 ml optical tubes compatible with the real-time PCR thermal cycler used.
- Optical film for 96-well plates or optical lids for 0.2 ml tubes compatible with the real-time PCR thermal cycler used.
- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 ml tubes
- Powder-free latex gloves

Complementary kits

For sensitive and specific detection of other HLA alleles with different clinical targets, Health in Code S.L. has developed Imegen® HLA-B57:01 **(REF: IMG-306)** and Imegen® HLA-B27 **(REF: IMG-289)**.

07 Assay protocol

07.1 | Preparation of amplification reactions

01 Thaw all kit reagents and DNA samples.

02 Vortex each reagent and keep cold.

03 Four PCR mixes must be prepared to run the assay by adding the following reagents to 1.5 mL tubes:

Reagents	Amount per reaction			
	Coeliac Master Mix I	Coeliac Master Mix II	Coeliac Master Mix III	Coeliac Master Mix IV
Coeliac Master Mix I	7.5 µL	–	–	–
Coeliac Master Mix II	–	7.5 µL	–	–
Coeliac Master Mix III	–	–	7.5 µL	–
Coeliac Master Mix IV	–	–	–	7.5 µL
General Master Mix	12.5 µL	12.5 µL	12.5 µL	12.5 µL

NOTE: To estimate the amount of reagents needed, the number of samples and controls to be analyzed simultaneously must be taken into account. To perform the calculations, it is recommended either to add a sufficient amount of reagents to perform one extra reaction or to add an extra 10% of each reagent.

04 Vortex and spin both PCR mixes and dispense 20 µL into the corresponding wells of the optical consumables.

05 Once the PCR mixes have been dispensed, add the following amounts to the corresponding wells:

- 5 µL of genomic DNA samples (10 ng/µL)
- 5 µL of positive control
- 5 µL of nuclease-free water (negative control for PCR)

NOTE: It is recommended to add one negative PCR control to rule out reagent contamination, as well as one positive control to ensure the correct functioning of the PCR reaction

06 Place the tubes or plates into the real-time PCR thermal cycler and configure settings for the amplification program as indicated in the next section.

07.2 | Settings for the real-time PCR program

Fluorophores of TaqMan® probes

Master Mix	Hydrolysis probe	Receptor	Genotyping	Quencher
Coeliac I	DQA1*05	FAM™	Allele *HLA-DQA1*05*	MGB
	β-globin	VIC™	β-globin	
Coeliac II	DQB1*02	FAM™	Allele *HLA-DQB1*02*	
	DQB1	VIC™	Allele *HLA-DQB1* (except *HLA-DQB1*02*)	
Coeliac III	DQB1*03:02	FAM™	Allele *HLA-DQB1*03:02*	
	DQA1*03	VIC™	Allele *HLA-DQA1*03*	
Coeliac IV	DQA1*02	FAM™	Allele *HLA-DQA1*02*	
	DQB1*03:01	VIC™	Allele *HLA-DQB1*03:01*	

Table 3. Information about hydrolysis probes

7500 FAST and StepOne Plus Real-Time PCR system (Applied Biosystems)

- **Type of experiment**: Quantitation —Standard curve
- **Ramp rate**: standard
- **Reaction volume**: 25 µL
- **ROX™ baseline reference**: included
- Set the Cycle threshold (Ct) at 0.1 for the analysis of results

Fields	Phase 1 Enzymatic activation	Phase 2 PCR	
No. of cycles	1 initial cycle	40 cycles	
		Denaturation	Annealing/extension
Temperature	95°C	95°C	60°C
Time	10 minutes	15 seconds	1 minute*

Table 4. Optimal PCR program for 7500 FAST and StepOne real-time PCR systems (Applied Biosystems)

(*) Fluorescence detection

08 Analysis of results

The following recommendations should be followed to ensure an adequate analysis of results.

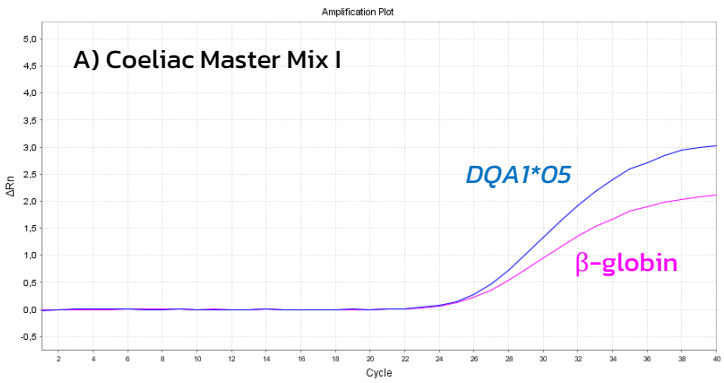
NEGATIVE CONTROLS

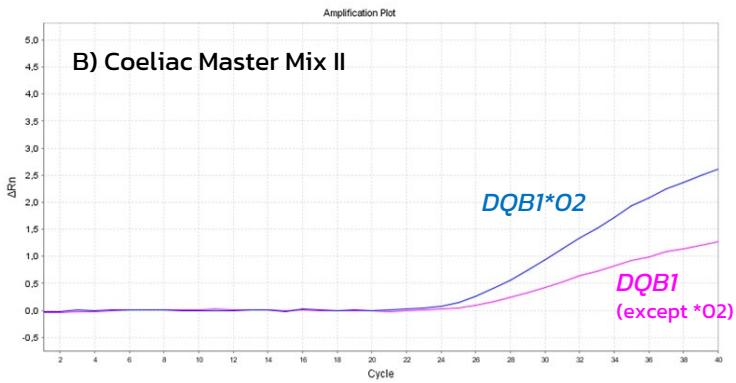
- Verify the lack of amplification in negative controls (NTC). If amplification is detected, it is recommended to repeat the test to rule out accidental contamination.

POSITIVE CONTROL

- Verify that the positive control amplifies all the expected alleles with the four reagent mixes. If no amplification is detected in the positive control, see Section 9 of this document.

  The expected results of the positive control for all kit mixes are shown below:





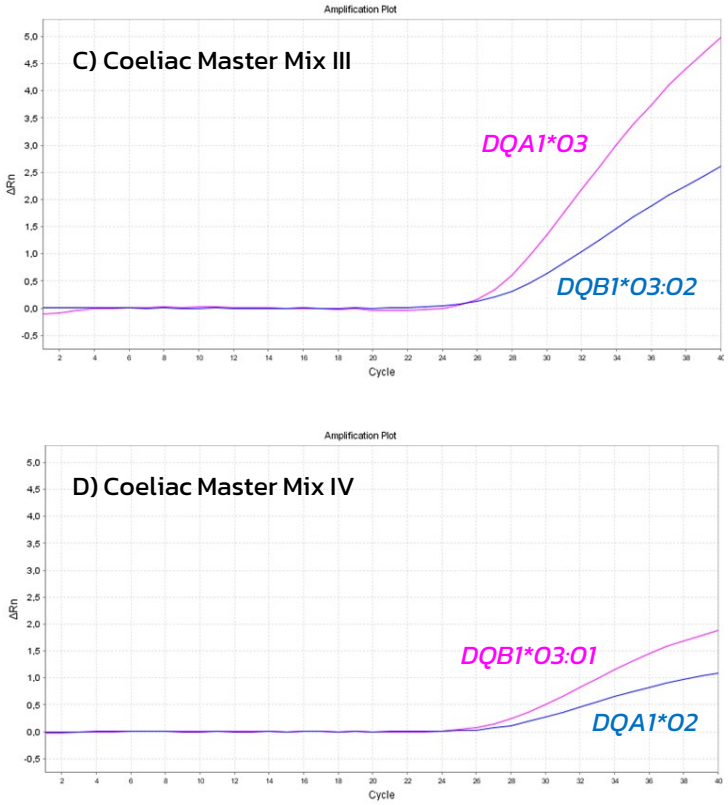


Figure 1. Expected results of the positive control for the 4 mixes included in the kit: ***A) Coeliac Master Mix I:*** *DQA1*05 (FAM), β-globin (VIC);* ***B) Coeliac Master Mix II:*** *DQB1*02 (FAM), remaining alleles DQB1 (VIC);* ***C) Coeliac Master Mix III:*** *DQB1*03:02 (FAM) and DQA1*03 (VIC);* ***D) Coeliac Master Mix IV:*** *DQA1*02 (FAM), DQB1*03:01 (VIC).*

Below are the possible results obtained using the Coeliac ImmuneKit:

GENOMIC DNA SAMPLES

Coeliac Master Mix I

This PCR system detects the presence of allele *HLA-DQA1*05* and of the endogenous gene β-globin. For the correct analysis of all the results obtained with the kit, the detection of endogenous gene β-globin must first be verified in all DNA samples. β-globin is a ubiquitous gene; therefore, its presence informs the user about good DNA sample quality and integrity.

The possible results are shown below:

- Presence of allele HLA-DQA1*05 and of endogenous gene β-globin (VIC):

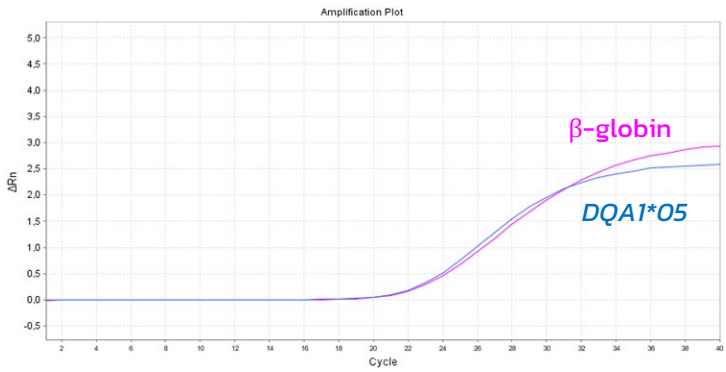


*Figure 2. Expected result for a sample containing allele DQA1*05 (FAM) and β-globin (VIC). Amplification signal is observed in all channels*

- Absence of allele HLA-DQA1*05 and presence of β-globin (VIC):

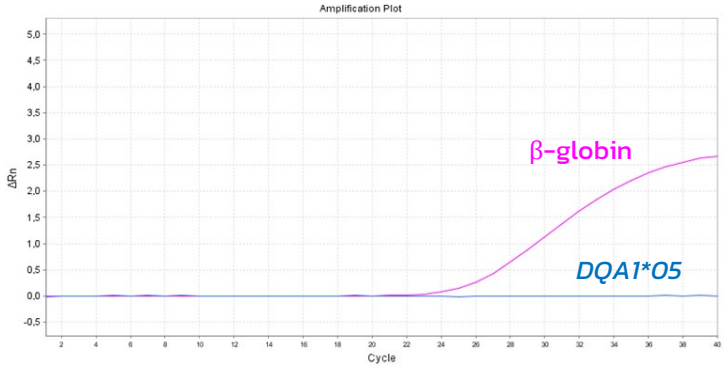


*Figure 3. Expected result for a sample that does not contain allele HLA-A1*05. Only the endogenous gene β-globin (VIC) is detected.*

Coeliac Master Mix II

This PCR system detects the genotype of the *HLA-DQB1* gene and discriminates between *HLA-DQB1*02* and the remaining *HLA-DQB1* alleles.

The possible results are shown below:

- Homozygous *HLA-DQB1*02 / HLA-DQB1*02* genotype (2 copies of the *HLA-DQB1*02* allele):

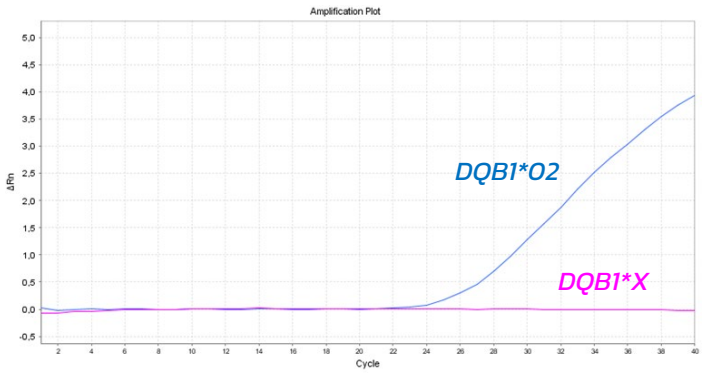


*Figure 4. Expected result for a homozygous sample for allele HLA-DQB1*02 (FAM). Amplification is only detected in the FAM channel.*

- Heterozygous *HLA-DQB1*02 / HLA-DQB1*X* genotype (1 copy of the *HLA-DQB1*02* allele):

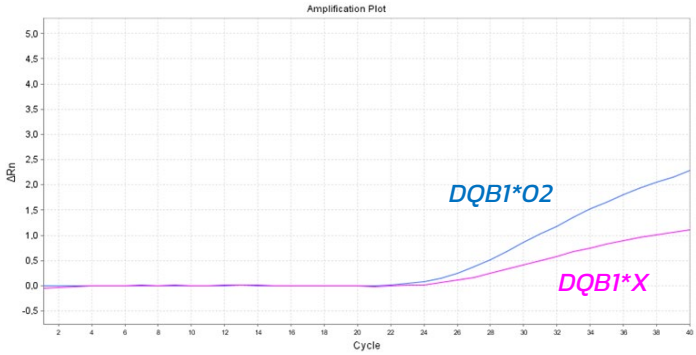


*Figure 5. Expected result for a heterozygous sample for HLA-DQB1, in which one of the alleles is HLA-DQB1*02 (FAM). Amplification is detected in both channels (FAM and VIC). Note: "X" refers to any allele other than HLA-DQB1*02 (VIC).*

Coeliac Master Mix III

This PCR system detects the genotype *HLA-DQB1*03:02* and *HLA-DQA1*03* for this gene.

The possible results are shown below:

- Presence of alleles *HLA-DQB1*03:02* and *HLA-DQA1*03*:

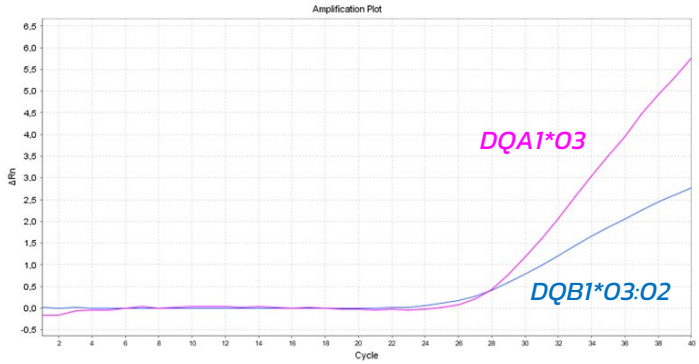


*Figure 6. Expected result for a sample that is positive for DQ8. Presence of alleles HLA-DQB1*03:02 (FAM) and HLA-DQA1:03 (VIC). Amplification is detected in both channels (FAM and VIC).*

Coeliac Master Mix IV

This PCR system detects the genotype *HLA-DQA1*02* and *HLA-DQB1*03:01* for this gene.

The possible results are shown below:

- Presence of alleles *HLA-DQA1*02* and *HLA-DQB1*03:01*:

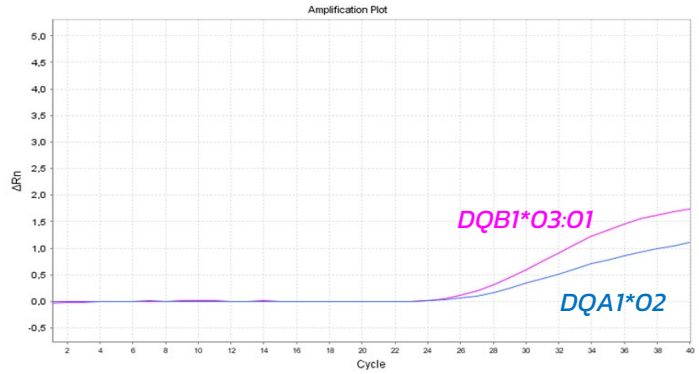


*Figure 7. Expected result for a sample containing alleles HLA-DQA1*02 (FAM) and HLA-DQB1*03:01 (VIC). Amplification is detected in both channels (FAM and VIC).*

- Presence of allele *HLA-DQA1*02* and absence of *HLA-DQB1*03:01*:

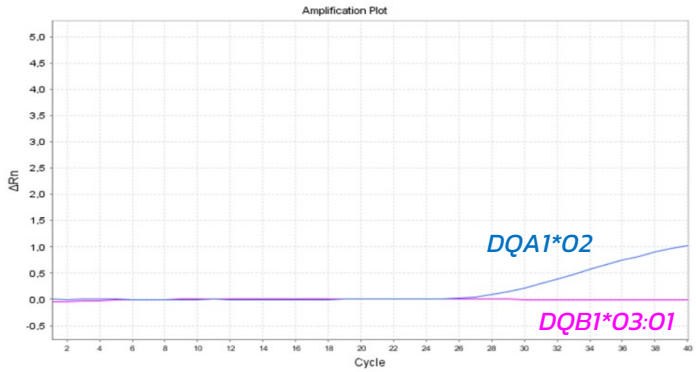


*Figure 8. Expected result for a homozygous sample for allele HLA-DQA1*02 (FAM). Amplification is only detected in the FAM channel.*

◇ Presence of alleles *HLA-DQB1*03:01* and absence of *HLA-DQA1*02*:

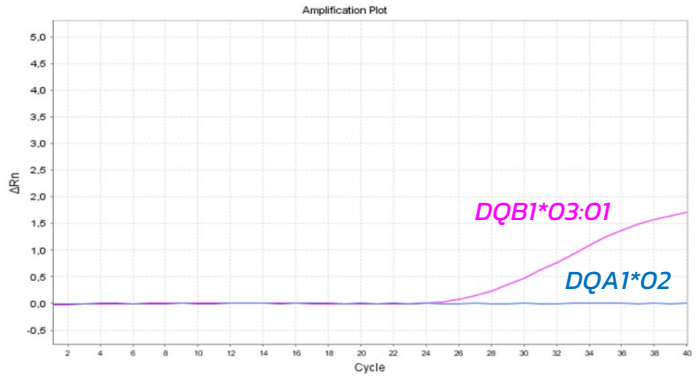
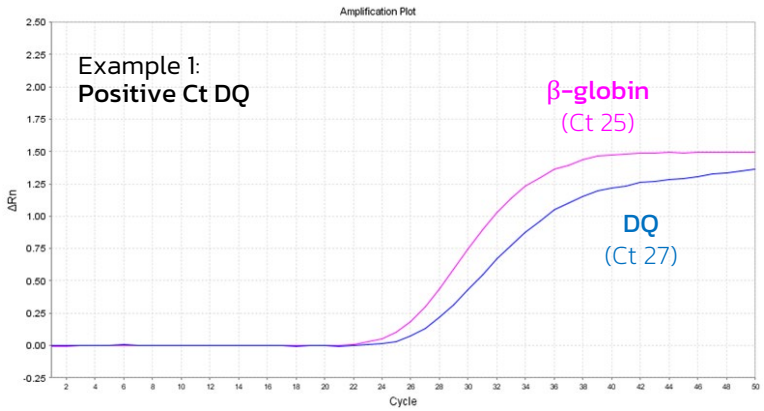


*Figure 9. Expected result for a homozygous sample for allele HLA-DQB1*03:01 (VIC). Amplification is only detected in the VIC channel.*

+ Recommendations for the clinical interpretation of results in coeliac disease

It is recommended to establish a cut-off value based on the result for the endogenous β-globin. The results obtained for each allele will be considered positive or negative depending on the result obtained for β-globin:

↘ POSITIVE Ct DQ < (Ct β-Globin +6 Ct)
↘ NEGATIVE Ct DQ > (Ct β-Globin +6 Ct)



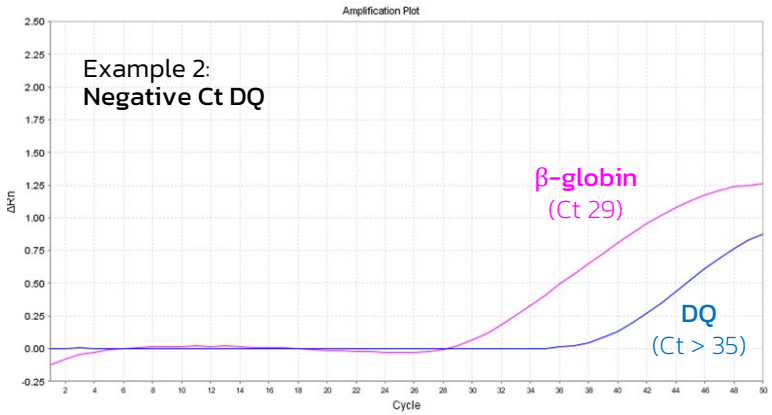


Figure 10. Example of interpretation of a coeliac disease assay to consider a sample positive (example 1) or negative (example 2). Cycler threshold at 0,1..

The risk of having CD varies depending on the HLA-DQ genotype present. Based on this, a degree of risk can be estimated for each individual.

Haplotype	DQ2		DQ7.5	DQ8	Others
	DQ2.5	DQ2.2			
	DQB1*02 DQA1*05	DQB1*02 DQA1*02	DQB1*03:01 DQA1*05	DQB1*03:02 DQA1*03	
DQ2.5	Very high	Very high	High	High	High
DQ2.2		Moderate	High	Moderate	Moderate
DQ7.5			Low	Moderate	Low
DQ8				Moderate	Moderate
Others					No risk

Table 5. Risk levels attributed to the different haplotype combinations.

Recommendations for the interpretation of genetic reports in coeliac disease

A genetic report must include the information summarized in the following points:

- Indicating whether the individual carries heterodimer HLA-DQ2, referring to DQ2.5 (presence of alleles *HLA-DQA1*05* and *HLA-DQB1*02*) and/or HLA-DQ8 (presence of alleles *HLA-DQA1*03* and *HLADQB1*03:02*) and/or HLA-DQ7.5 (presence of alleles *HLA-DQA1*05* and *HLA-DQB1*03:01*)
- In the event that the individual does not show HLA-DQ2 (DQ2.5) or DQ8 or DQ7.5, it must indicate whether any of the alleles encoding DQ2.5: *DQA1*05* or *DQB1*02* are present.

09 Troubleshooting

The table below specifies the possible test results that can be obtained for the different controls and from one sample in one run, along with their interpretation and the most likely reasons for each possible result:

Sample	HLA alleles	β-globin	Cause
Positive control	+	+	Expected result
	–	–	Failure of PCR amplification [1]
	+	–	
	–	+	
Sample	+	+	Expected result
	–	+	
	+	–	Failure of PCR amplification [1]
	–	–	Failure of sample amplification [2]
Negative PCR	–	–	Expected result
	+	+	PCR contamination with human DNA [3]

Table 6. Interpretation the possible results obtained using the Coeliac ImmuneKit

(1) Failure of PCR amplification: make sure the amplification program and fluorescence detection settings are correct. An amplification error may be due to a technical issue during PCR program setup.

(2) Failure of sample amplification: verify that sample quantification meets the recommendations; if so, the specified result may be due to a highly degraded sample.

(3) PCR contamination with human DNA: PCR contamination may be due to mishandling of the sample, the use of contaminated reagents or environmental contamination. Thoroughly clean the laboratory where the PCR was prepared, as well as the equipment and material used. If necessary, use fresh aliquots of the PCR reagents. Prepare the PCR reaction containing the positive control last, in order to avoid cross-contamination. It is recommended that the assay be repeated in this case.

10 Limitations

10.1 | Equipment

Coeliac ImmuneKit has been validated for use with the following real-time PCR platforms:

- 7500 FAST Real-Time PCR System (Applied Biosystems)
- One Real-Time PCR System (Applied Biosystems)

This kit is compatible with any real-time PCR equipment able to detect fluorescence emitted by fluorophores FAM™ and VIC™.

If a different brand or model of thermal cycler is used, the amplification program may need to be adjusted. Should you need further information or advice, please contact our technical support service.

10.2 | Reagents

Coeliac ImmuneKit has been validated using the reagents included in the kit and those recommended in section 6 of this manual (Equipment and materials not included in the kit).

10.3 | Product stability

Optimal performance of this product is achieved provided that the specified recommended storage conditions are applied, within the optimal product expiration date associated with each production batch.

For any questions about the applications of this product or its protocols, please contact our Technical Department:

tech.support@healthincode.com

+34 963 212 340

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