Instructions for use

Imegen® Coeliac

Ref. IMG-341

CE IVD

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.03

healthincode

Health in Code, S.L. guarantees that its products are free of defects, both in materials and workmanship. This guarantee remains in force until the expiration date, if the conservation practices described in this manual are adhered to.

Our products are intended for *in vitro* diagnostic use. Health in Code, S.L. provides no guarantee, whether explicit or implicit, that extends beyond the proper functioning of the components of this kit. Health in Code, S.L. sole obligation, in relation to the aforementioned guarantees, shall be to either replace the product or reimburse the cost of it, per the client's preference, provided that materials or workmanship prove to be defective Health in Code, S.L. is not liable for any cost or expense, direct or indirect, or damage or harm incurred by the customer or user as a result of use of the product by the buyer or user.

All Health in Code, S.L. products undergo strict quality control. The Imegen® Coeliac kit 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, please contact our Technical Department:

+34 963 212 340

tech.support@healthincode.com

Instructions for Use (IFU) modifications		
Version 09	DEC 2023	Review and update of section "3. Technical characteristics".
Version 08	SEP 2023	Modifications in Tables 3 and 5 and content revision.
Version 07	DEC 2022	Modification of the storage and shipping temperature of the GENERAL MASTER MIX reagent (Section 4).
Version 06	NOV 2022	Change in manufacturer's address: Health in Code, S.L., Calle de la Travesía s/n, 15E Base 5, Valencia 46024, Spain
Version 05	NOV 2022	Change in manufacturer's identification: from Imegen to Health in Code, S.L.
Version 04	DEC 2021	Modification of master mixes number in Section 7
Version 03	SEP 2021	Ct modification in negative results Ct DQ < (Ct β-Globin +6 Ct) changes to Ct DQ > (Ct β-Globin +6 Ct). Recommendations for the interpretation of coeliac disease (page 17)
Version 02	AUG 2021	Modification of the PCR program (Table 4. Page12). Recommendation for result interpretation (page 18).

index

01 General information

Coeliac disease is a complex immune disorder with a strong genetic influence. In genetically predisposed individuals, ingestion of gluten (a protein found in wheat, rye, and barley) triggers an immune response that targets the small intestine and causes damage to villi, bowel inflammation, and inability to absorb nutrients, leading to symptoms including diarrhea, abdominal pain, and abdominal distension.

The main genes involved in the development of coeliac disease belong to the major histocompatibility complex (MHC), located in 6p21. The MHC contains hundreds of human leukocyte antigen (HLA) genes, which encode glycoproteins able to recognize endogenous and/or exogenous peptides in immune cells, triggering cell apoptosis when these peptides are recognized as foreign. In coeliac disease, the MHC genes involved in gluten recognition and its presentation to T CD4+ cells are those that encode the HLA-DQ8 and HLA-DQ2 receptors. The latter is composed of two subunits, α and β, which conform the heterodimeric protein DQαβ; these subunits are encoded by two genes, *HLA-DQA1* and *HLA-DQB1*, respectively.

Around 90% of coeliac patients carry heterodimer HLA-DQ2 (genotype *HLA-DQA1*05* and *HLA-DQB1*02*), while the remaining patients mainly carry heterodimer HLA-DQ8 (genotype *HLA-DQA1*03* and *HLA-DQB1*03:02*), responsible for the immune response to gluten. Moreover, there is a gene dosage effect for *HLA-DQB1*02* and *HLA-DQB1*03*; therefore, it is advised to report the genetic load (one or two copies).

HLA genetics			DQ protein
HLA-DQA1 alleles	HLA-DQB1 alleles	HLA-DQ haplotype	
*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 and the proteins encoded by them.

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 develop at any age after ingesting food or drugs containing gluten. If untreated, it can develop into additional severe health issues. In children, malabsorption can also affect growth and development.

As the knowledge about this disease has expanded, HLA testing has gained importance as a diagnostic tool, having been recently incorporated into the new diagnostic guidelines for children and adolescents proposed by the *European Society for Paediatric Gastroenterology, Hepatology and Nutrition* (ESPGHAN). While its inclusion into adult-oriented guidelines is variable, it generally constitutes a tool to support diagnosis in many cases.

References

- *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.*
- *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.*
- *Dieli-Crimi et al., 2015. The genetics of celiac disease: A comprehensive review of clinical implications. J Autoimmune; 64:26-41. doi: 10.1016/j.jaut.2015.07.003.*
- *Haboubi et al., 2006. Coeliac disease and oats: a systematic review. Postgraduate Medical Journal BMJ; 82: 672-678. doi: 10.1136/pgmj.2006.045443.*

02 Intended use

The Imegen® Coeliac kit uses a combination of oligonucleotides and fluorescent hydrolysis probes in a qualitative real-time PCR test validated for simultaneous detection of the genotypes most frequently associated with a higher susceptibility to coeliac disease. Specifically, this test allows detecting alleles *HLA-DQA1*05*, *HLA-DQA1*03*, and *HLA-DQB1*03:02* and enables allelic discrimination between *HLA-DQB1*02* and the remaining *HLA-DQB1* alleles (*DQB1*03, DQB1*04, DQB1*05*, and *DQB1*06*).

This genetic test allows the user to determine the presence or absence of the aforementioned genotypes by two multiplexed PCR reactions, which include amplifying the reference gene, β-globin, for DNA quantity and quality control.

The Imegen® Coeliac kit studies the germline genotype; therefore, the optimal sample type for this test is genomic DNA.

The results from this assay can guide the clinician's diagnosis of the patient's genetic susceptibility to gluten. However, confirmation by serologic testing (detection of specific antibodies) is also necessary for the definitive diagnosis of the disease.

The Imegen® Coeliac kit can only be used for *in vitro* diagnosis and is aimed at professionals in molecular biology.

03 Technical characteristics

The Imegen® Coeliac kit has been developed in collaboration with the *C.H.U. Insular* (*Las Palmas de Gran Canaria*, Spain) using already diagnosed samples previously genotyped with a different technique, as well as with synthetic vectors (*GenScript*) containing the sequences of interest. These vectors are provided as a positive control to guarantee the correct setup and functioning of the PCR system. The complete validation provides a robust and specific diagnosis method. As a result of this agreement, Health in Code, S.L holds an exclusive, worldwide license on the know-how of these products for their manufacturing and commercial use.

To use Imegen® Coeliac kit, the *real-time PCR thermal cycler* must be compatible with FAM™ and VIC™.

The necessary type of material for this analysis is genomic DNA from peripheral blood. The necessary amount of DNA is 150 ng.

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 case of an accidental spill of any of the reagents, avoid contact with the skin, eyes, and mucous mebranes 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 to 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 could noticeably vary among laboratories. Therefore, it is recommended that each laboratory conduct an internal validation 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 enough reagents to perform 48 real-time PCR reactions for each of the two master mixes analyzed by this assay. The reagents included in this kit are the following:

- **Coeliac Master Mix 1**: specific *PCR Master Mix* containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow simultaneously detecting *HLA-DQA1*05* alleles and the endogenous gene β-globin, used as internal positive control for the PCR reaction.
- **Coeliac Master Mix 2**: specific *PCR Master Mix* containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow simultaneously discriminating between allele *HLA-DQB1*02* and the remaining *HLA-DQB1* alleles.
- **Coeliac Master Mix 3**: specific *PCR Master Mix* containing the oligonucleotides and fluorescent hydrolysis probes (FAM™ and VIC™) that allow simultaneously discriminating between allele *HLA-DQB1*03:02* and allele *HLA-DQA1*03*.
- **General Master Mix**: *PCR Master Mix* containing the necessary nucleotides, $MgCl_2$, enzyme, and buffer to perform real-time PCR.
- **Positive control**: positive control for the amplification of all targets of the kit.

Reagents	Color indicator	Quantity	Conservation
Master Mix 1 Coeliac	Red cap	2 x 180 µl	-20°C
Master Mix 2 Coeliac	Yellow cap	2 x 180 µl	-20°C
Master Mix 3 Coeliac	Purple cap	2 x 180 µl	-20°C
General Master Mix	White cap	3 x 600 µl	-20°C*
Positive Control	Black cap	2 x 100 µl	-20°C

Table 1. Components of the Imegen® Coeliac kit

(*) General Master Mix: Recommended to be kept frozen until first use, protected from light, and stored between 2- 8 °C after first use.

06 Equipment, reagents and material not included in the kit

Equipment:

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

Reagents:

- Nuclease-free water

Materials:

- Filter pipette tips (10 µL, 20 µL, and 200 µL)
- Sterile 1.5 mL tubes
- Fungible optical material compatible with the real-time PCR thermal cycler
- 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 To perform the test, two PCR master mixes must be prepared, adding the following reagents to 1.5 mL tubes:

Reagent	Amount per reaction		
	Coeliac Master Mix 1	Coeliac Master Mix 2	Coeliac Master Mix 3
Coeliac Master Mix 1	7.5 µL	–	–
Coeliac Master Mix 2	–	7.5 µL	–
Coeliac Master Mix 3	–	–	7.5 µL
General Master Mix	12.5 µL	12.5 µL	12.5 µL

NOTE: In order to estimate the necessary amount of reagents, the number of samples and controls to be analyzed simultaneously must be taken into account. We recommend performing the calculations either considering one extra reaction or increasing the volume of each reagent by 10%.

04 Vortex and spin both PCR mixes and dispense 20 µL into each well of the optical plate.

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

- **Type of experiment**: Quantitation —Standard curve
- **Ramp speed**: Standard

- **Reaction volume**: 25 µL
- **ROX™ baseline reference**: included
- **TaqMan® probe fluorophores**:

Probe	Hydrolysis probe	Receptor	Genotyping	Quencher
Coeliac 1	DQA1*05	FAM™	Allele *HLA-DQA1*05*	MGB
	β-Globin	VIC™	β-Globin	
Coeliac 2	DQB1*02	FAM™	Allele HLA-*DQB1*02*	
	DQB1	VIC™	Allele *HLA-DQB1* (except for *HLA-DQB1*02*)	
Coeliac 3	DQB1*03:02	FAM™	Allele *HLA-DQB1*03:02*	
	DQA1*03	VIC™	Allele *HLA-DQA1*03*	

Table 3. Information about probes

- **Optimal program**:

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 (Thermo Fisher Scientific)

(*) Fluorescence detection

08 Analysis of results

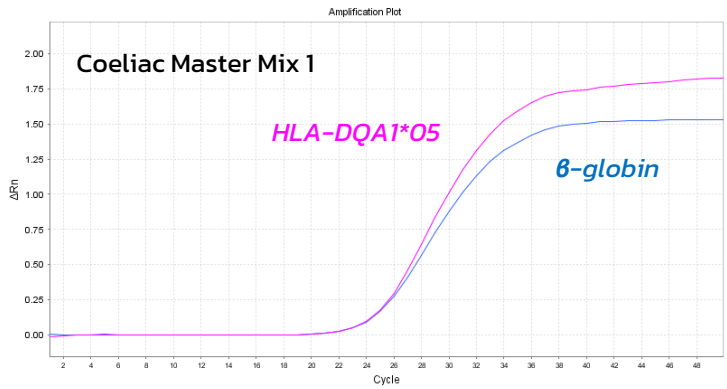
The following recommendations should be followed to ensure an adequate analysis of results:

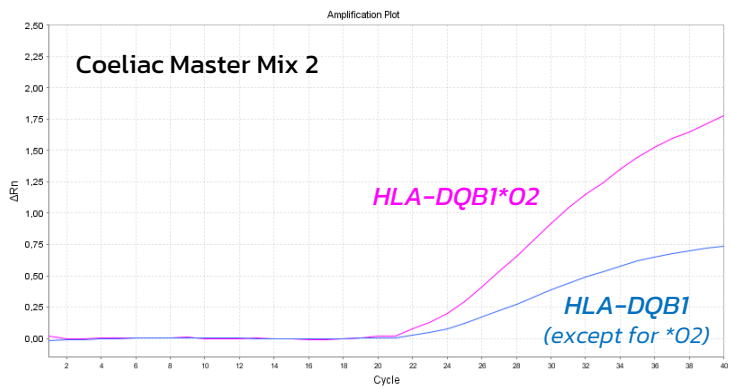
NEGATIVE CONTROLS

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

POSITIVE CONTROL

- Confirm that the positive control amplifies all the expected alleles with both master mixes. If no amplification is detected in the positive control, review section 9 of this document.





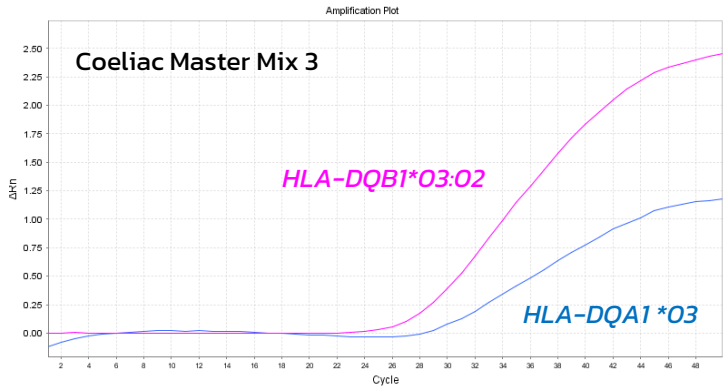


Figure 1. Expected results for the positive control. ***Coeliac Master Mix 1****: HLA-DQA1*05 (FAM),* ***β****-globin (VIC).* ***Coeliac Master Mix 2****: HLA-DQB1*02 (FAM), remaining HLA-DQB1 alleles (VIC).* ***Coeliac Master Mix 3****: HLA-DQB1*03:02 (FAM) and HLA-DQA1*03 (VIC).*

GENOMIC DNA SAMPLES

Coeliac Master Mix 1

- Confirm the detection of endogenous β-globin gene in all DNA samples. The β-globin gene is ubiquitous; therefore, its presence informs the user about the good quality and integrity of the DNA sample.
  - **Negative sample for all target alleles of the mix**: Confirm that β-globin is detected in the VIC channel.

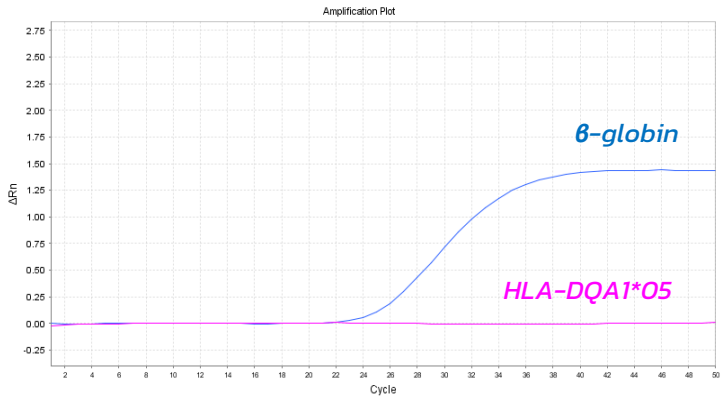


*Figure 2. Expected results for a DNA sample without the HLA-DQA1*05 allele. Only the endogenous* ***β****-globin gene is detected (VIC)*

  - **Positive sample for all target alleles of the mix**: Amplification signal is observed in all channels.

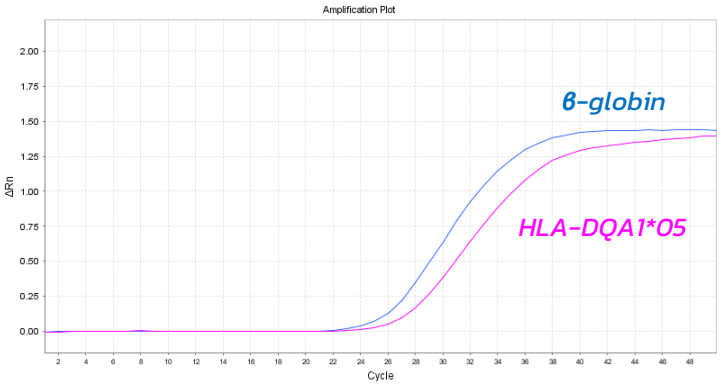


*Figure 3. Expected results for a sample with alleles HLA-DQA1*05 (FAM) and* ***β****-globin (VIC).*

Coeliac Master Mix 2

This PCR system detects the genotype for the *HLA-DQB1* gene and discriminates between *HLA-DQB1*02* and all other *HLA-DQB1* alleles. The potential results are shown below:

- Homozygous *HLA-DQB1*02 / HLA-DQB1*02* genotype:

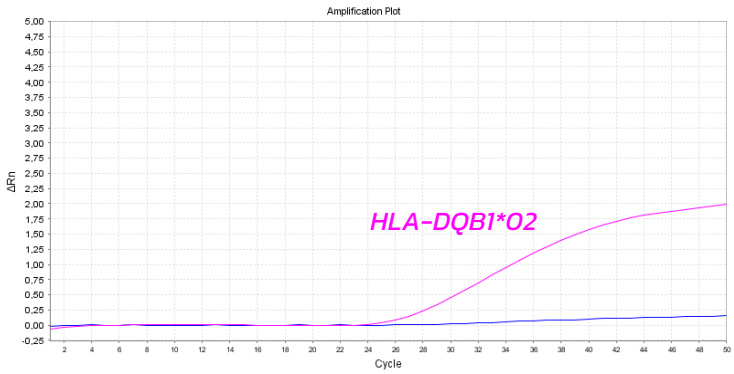


*Figure 4. Expected result for a homozygous sample for the HLA-DQB1*02 allele (FAM). Amplification is only detected in the FAM channel.*

- Heterozygous *HLA-DQB1*02 / HLA-DQB1*X* genotype:

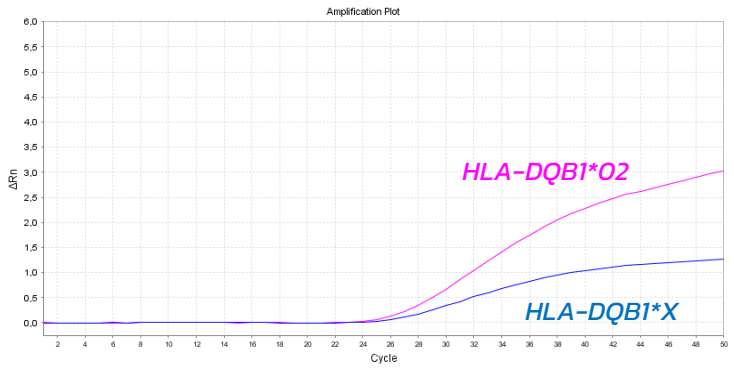


*Figure 5. Expected result for a heterozygous sample for HLA-DQB1, in which one of the alleles is HLA-DQB1*02 (FAM). Amplification is detected in both the FAM and VIC channels. Note: "X" stands for any allele other than HLA-DQB1*02 (VIC).*

- Homozygous HLA-DQB1*X genotype:

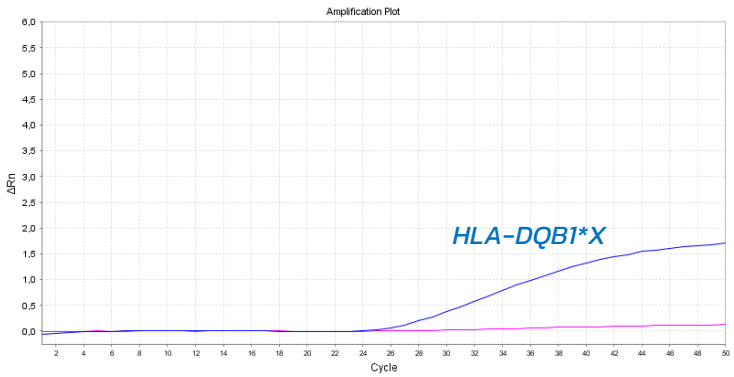


*Figure 6. Expected result for a sample with presence of any HLA-DQB1 allele except for HLA-DQB1*02. In this case, the specific genotype is unknown. Amplification is only detected in the VIC channel. Note: "X" stands for any allele other than HLA-DQB1*02 (VIC).*

Coeliac Master Mix 3

This PCR system detects the genotype for the *HLA-DQB1*03:02* allele and the *HLA-DQA1*03* allele. The possible results are shown below:

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

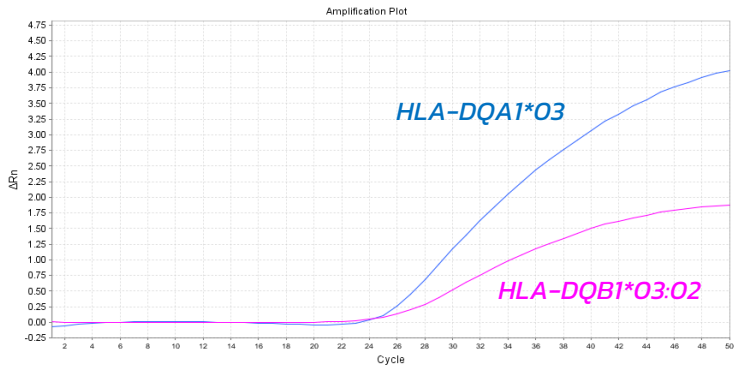


*Figure 7. Expected result for a DQ8-positive sample. Presence of alleles HLA-DQB1*03:02 (FAM) and HLA-DQA1:03 (VIC). Amplification is only detected in the FAM channel.*

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

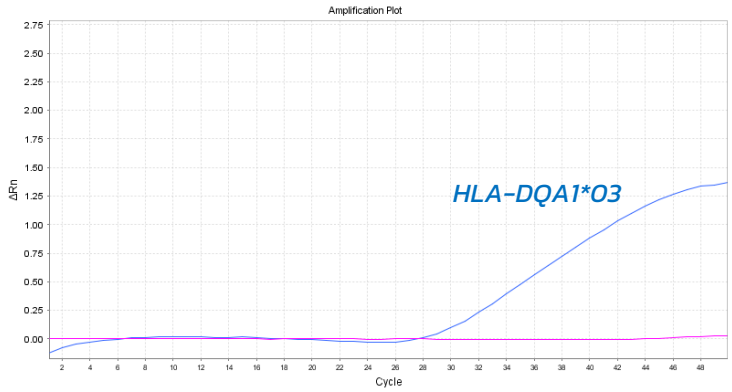


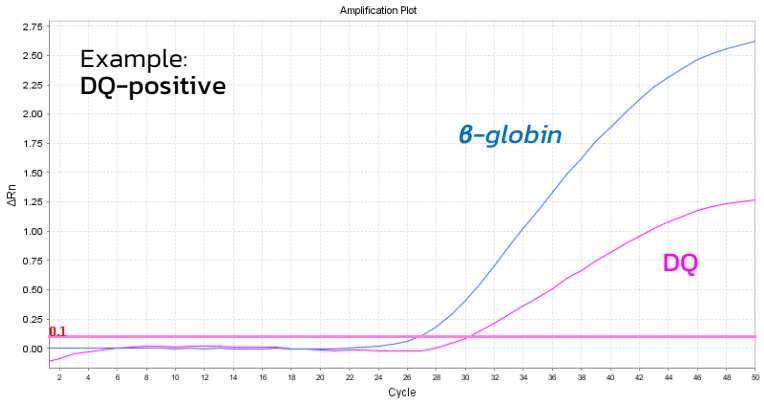
Figure 8. Expected result for a positive sample for allele HLA-DQA1:03. Amplification is only detected in the VIC channel.

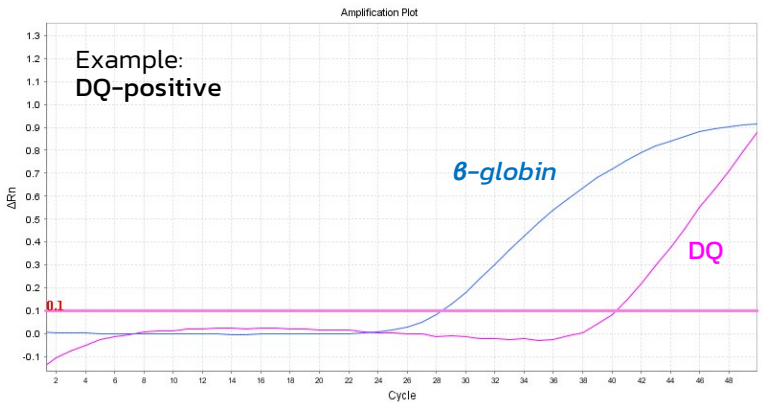
NOTE: We have not found any sample with presence of the *HLA-DQB1*03:02* allele in the absence of the *HLA-DQA1*03* allele.

Recommendations for the interpretation of coeliac disease

Establishing a cut-off value is recommended based on the results obtained for the endogenous gene, β-globin. The obtained results will be considered:

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





The risk of developing coeliac disease varies according to the HLA-DQ genotype present. Based on this, a degree of risk can be determined for each individual.

Haplotypes	DQ2		DQ7.5	DQ8	Other
	DQ2.5	DQ2.2			
	HLA-DQB1*02 HLA-DQA1*05	HLA-DQB1*02 HLA-DQA1*02	HLA-DQB1*03:01 HLA-DQA1*05	HLA-DQB1*03:02 HLA-DQA1*03	
DQ2.5	Very high	Very high	High	High	High
DQ2.2		Moderate	High	Moderate	Moderate
DQ7.5			Low	Moderate	Low
DQ8				High	Moderate
Other					No risk

Table 5. Risks attributed to the different haplotype combinations.

Recommendations for the interpretation of genetic reports in coeliac disease

A genetic report must include the following information:

- Indicate whether the individual carries the HLA-DQ2 heterodymer, in reference to DQ2.5 (presence of alleles *HLA-DQA1*05* and *HLA-DQB1*02*), and/or *HLA-DQ8* (presence of alleles *HLA-DQA1*03* and *HLA-DQB1*03:02*).
- If the individual is not a carrier of either HLA-DQ2 (DQ2.5) or DQ8, it must indicate whether they carry any of the alleles that encode DQ2.5: *HLA-DQA1*05* or *HLA-DQB1*02*.

References

- *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.*
- *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.*

09 Troubleshooting

The following table lists the results that can be obtained from the analysis of the different controls and a test sample, as well as their interpretation:

Sample	HLA alleles	β-Globin	Cause
Positive control	+	+	Expected result
	–	–	PCR amplification failure [1]
	+	–	
	–	+	
Sample	+	+	Expected result
	–	+	
	+	–	PCR amplification failure [1]
	–	–	Samples amplification failure [2]
Negative PCR control	–	–	Expected result
	+	+	Contamination of PCR with human DNA[3]

Table 6. Interpretation the possible results obtained using the Imegen® Coeliac

(1) PCR amplification failure: Check the amplification program and the fluorescence capture settings. Amplification failure may be due to a technical issue while setting up the PCR program.

(2) Sample amplification failure: Check that sample quantification corresponds to the recommended values; if so, the specified result can be due to high degradation of the sample.

(3) PCR contamination with human DNA: PCR contamination may be due to mishandling of the sample, 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 as the last step to prevent cross-contamination. In this case, it is recommended to repeat the assay.

10 Limitations

10.1 | Equipment

Imegen® Coeliac has been validated for use with the following PCR thermal cyclers:

- *7500 FAST Real-Time PCR System* (Thermo Fisher Scientific)
- *StepOne Real-Time PCR System* (Thermo Fisher Scientific)

Technically, this kit is compatible with any real-time PCR system capable of detecting 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 service.

10.2 | Reagents

Imegen® Coeliac 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

The optimal performance of this product is achieved when the specified recommended storage conditions are applied, within the product expiration date associated with each 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

healthincode