PCR PROTOCOLS

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Strategy 1: Gene extraction, PCRs

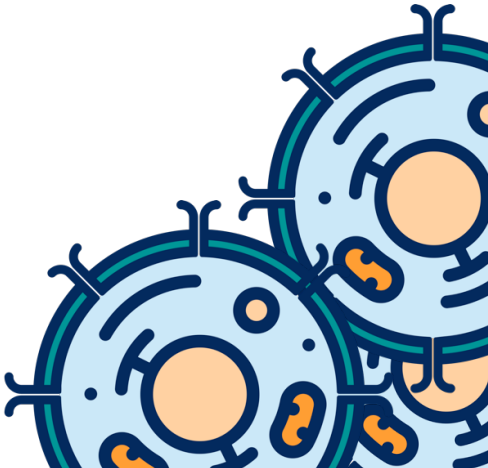
In this strategy, we considered obtaining the HLA-DQ from scratch. Based on former studies, only the exons 2 and 3 form each chain (α and β) codify for the extracellular domain of the HLA-DQ that interacts with gluten epitopes. With this in mind, we designed a robust model for the extraction of the α and β chains of the HLA-DQ from the genomic DNA of a celiac patient. A set of primers were designed to conduct 3 different PCRS (including 10 reactions) to obtain the α and β chains flanked with restriction sites for further cloning.

Strategy 2: Synthetic genes

As one of the main objectives is to express the human HLA in a bacterial cell host, several approaches to conduct this were considered. Firstly, a structural analysis of the protein was made in order to visualize the different protein domains. Based on this analysis, we decided to express only the extracellular domain which was encoded by the exons 2 and 3 of the HLA gene. Next, as an attempt to improve the protein stability, we chose to add Leucine Zippers to the constructs. And finally, specific tags were added at the C-terminus of the gene sequences to enable the protein purification.

With these ideas, our team designed different synthetic genes following three strategies for the heterologous expression of the human HLA in *E.coli.*

- Expression of both α chain and β chain as a unique gene by using a fusion linker.
- Expression of the α and β chain as two separate genes in the same constructs by establishing a RBS for each gene.
- Expression of the α and β chain as two separate genes but adding the Leucine Zippers to increase its stability.



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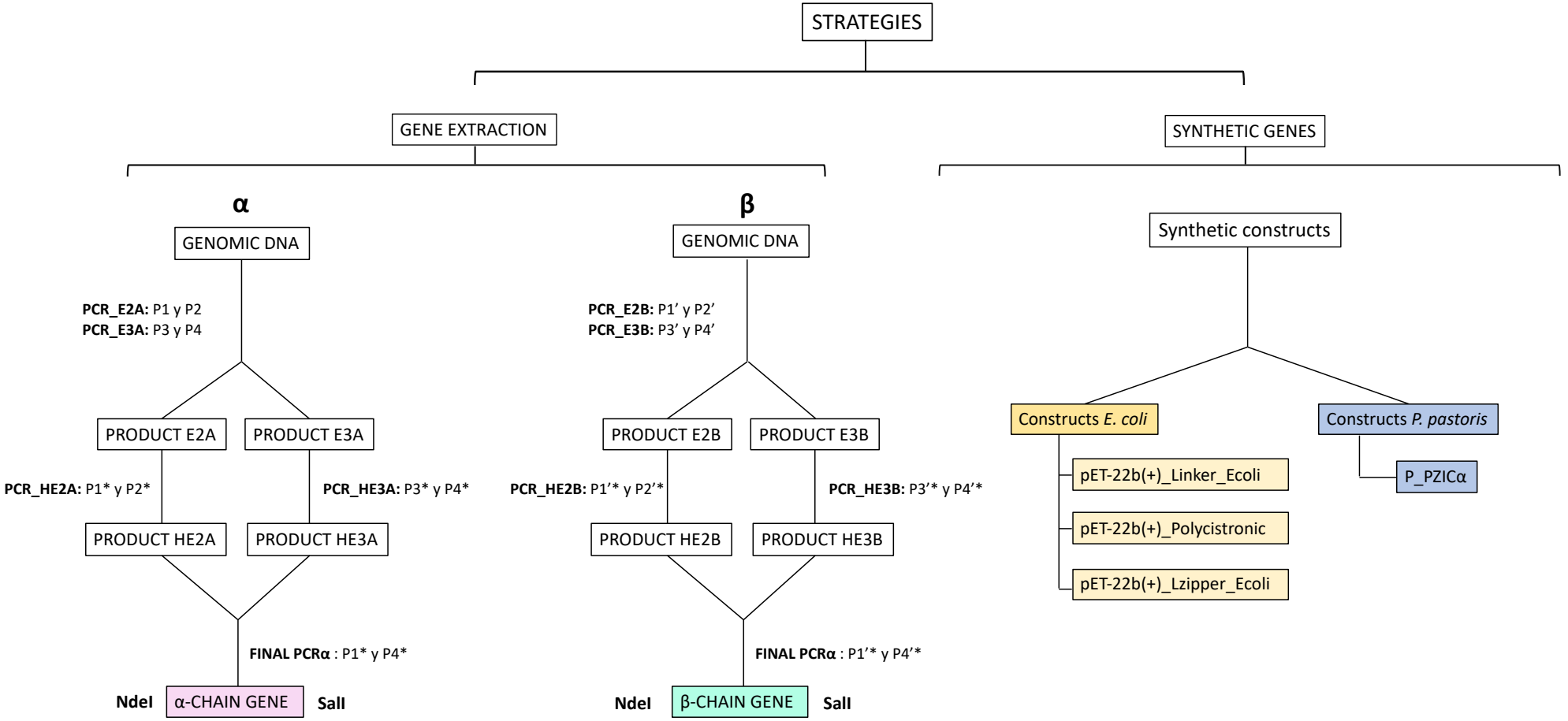
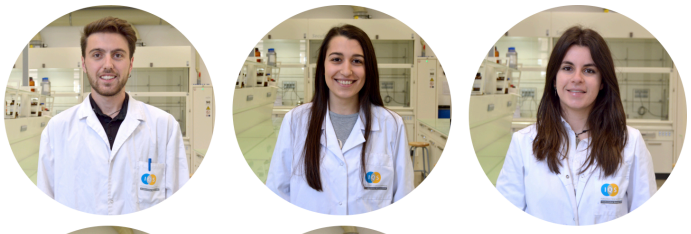
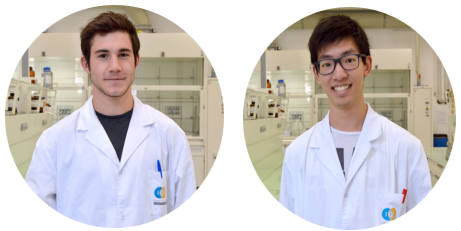






List of materials and reagents

2 x iProof High Fidelity Master Mix (Biorad)

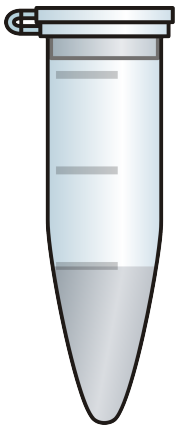
http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10002299.pdf

iProof High-Fidelity PCR Master Mixes are 2x concentrated, ready-to-use supermixes containing all components for high- fidelity, long, or fast PCR for amplicons up to 37 kb. Simply add your template and primers to start your reactions. The error rate of the iProof Polymerase is 50- fold lower than that of *Thermus aquaticus* DNA polymerase and sixfold lower than that of *Pyrococcus furiosus* DNA polymerase. iProof DNA Polymerase exhibits 5' —> 3' polymerase activity and 3' —> 5' exonuclease activity and produces blunt-end amplification products.

Genomic DNA from a celiac patient

You will be provided with genomic DNA from one of the members of the Bio IQS team, who has recently been diagnosed with celiac disease. We have developed a robust protocol for the obtention of the HLA-DQ of every patient. Therefore, if anyone in your group has been diagnosed with celiac disease, you can obtain her/his HLA-DQ!

Follow the protocol form Page 20 to extract her/his genomic DNA and proceed with the PCR protocols.

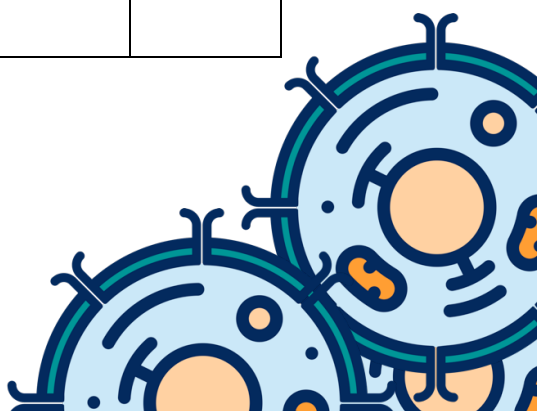


Primers

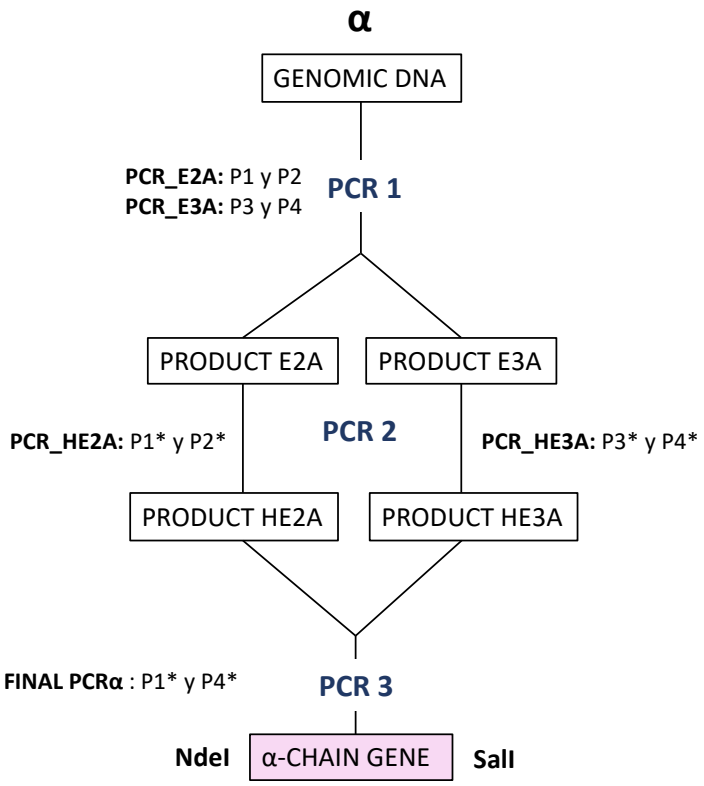
As before mentioned, a set of primers were designed to conduct the different PCRs. A rational design was done to check hairpin and heterodimer formation. Among the 300 different HLA-DQ haplotypes discovered to date, the primers are expected to efficiently hybridize to up to 90% of them.

List of primers

Primer	Sequence	Stock
P1	5’ GCTGACCACGTCGCCTCTTATG 3’	5 µM (10X)
P2	5’ CATTGGTAGCAGCGGTAGAGTTG 3’	
P3	5’ AGGTTCCTGAGGTCACAGTGTTTTC 3’	
P4	5’ CCCAGTGTTTCAGAAGAGGCTTG 3’	
P1*	5’ CATATGGCTGACCACGTCGCCTCTTATG 3’	
P2*	5’ CCTCAGGAACCTCATTGGTAGCAGCGGTAGAGTTG 3’	
P3*	5’ CTGCTACCAATGAGGTTCCTGAGGTCACAGTG 3’	
P4*	5’ GAGCTCCCCAGTGTTTCAGAAGAGGCTTG 3’	
Primer	Sequence	Stock
P1’	5’ GAGGATTTCGTGTACCAGTTTAAGGG 3’	5 µM (10X)
P2’	5’ TCCTCTGGGGTGGAACAAACG 3’	
P3’	5’ CCTATATCTTTCCCTGTCTGTTACTGCC 3’	
P4’	5’ CAATATCCCCTTACGCCACTCCAC 3’	
P1’*	5’ CATATGGAGGATTTCGTGTACCAGTTTAAGGG 3’	
P2’*	5’ CTGTGGGCTCCACTCGCCGCTGCAAGGTCGT 3’	
P3*	5’ GCAGCGGCGAGTGGAGCCCACAGTGAC 3’	
P4*	5’ GAGCTCGCCACTCCACGGTGATG 3’	



OBTENTION OF THE α CHAIN



In an attempt to extract the exons 2 and 3, which codify for the α chain, 3 PCRs have been designed. In the PCR1, amplification of the exons 2 and 3 is carried out by using primers P1-P2 and P3-P4 respectively in two separated reactions, called PCR_E2A and PCR_E3A.

The products of the beforementioned reactions are to be the template of the following PCR2. In this step, the restriction sites NdeI at 5' (in the case of exon 2) and SalI at 3' (for exon 3) are introduced for further cloning in the vector pET22b(+). Besides, a complementary region between exon 2 (3' end) and 3 (5' end) for further assembling is also introduced. To accomplish this, two separated reactions called PCR_HE2A and PCR_HE3A are carried out using primers P1*-P2* and P3*-P4* respectively.

Finally, in the PCR3, assembling of the exons 2 and 3 is done in one single reaction (Final PCR α), using a mix of the products from PCR_HE2A and PCR_HE3A and the flanking primers P1*-P4*.

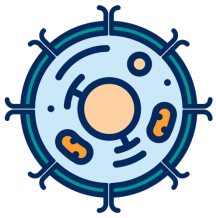
Table 1. Brief summary of the PCR protocol to obtain α chain.

Stage	PCR Name	DNA Template	Primers	Tm (°C)	Elongation (s)
PCR 1	PCR_E2A	Genomic DNA	P1 – P2	60	15
	PCR_E3A	Genomic DNA	P3 – P4	60	15
PCR 2	PCR_HE2A	PCR_E2A product	P1* – P2*	60	15
	PCR_HE3A	PCR_E3A product	P3* – P4*	60	15
PCR3	FINAL PCRα	PCR_HE2A + PCR_HE3A product mix	P1* – P4*	60	25

Preparing the reaction

PCR 1 comprises two reactions, PCR_E2A and PCR_E3A. iProof HF is an excellent DNA polymerase to amplify genes that are to be cloned in an expression vector. Besides, iProof HF comes together with dNTPs and the PCR buffer required for the reaction. Therefore, preparing the reaction is straightforward, as you only need to mix the Master mix, primers, DNA and H_2O.

Reactions: PCR_E2A and PCR_E3A	
Reagents	Volume (µl)
H_2O Milli-Q	2
Mix iProof (2X)	10
Primer P1 or P3 (10X)	2
Primer P2 or P4 (10X)	2
Genomic DNA	4
Final volume: 20 µl	



Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Let's set the PCR Program

Every PCR involves a total of 5 steps. A pre-denaturation step, followed by denaturation, annealing, and elongation. Being the expected PCR products no longer than 250 bp and considering that the iProof HF amplifies 1kb/min, the elongation time is set at 15 seconds.

Reactions: PCR_E2A and PCR_E3A		
Process	Temperature (°C)	Time (min)
Pre-denaturation	98	3:00
Denaturation	98	00:10
Annealing	60	00:20
Elongation	72	00:15
Final elongation	72	10:00
Storage	4	O/N
Denaturation – Annealing- Elongation (30 cycles)		

Remember to set the hotlid temperature at 105 ℃

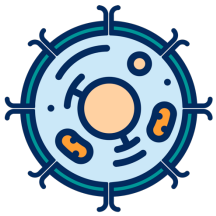
Expected results

PCR products from the reactions PCR_E2A and PCR_E3A are to be analyzed in an agarose gel. Since the expected product size is no longer than 250 bp, it is recommended to prepare a 2% agarose gel for a better resolution. It is also suggested to use a DNA Marker with a molecular range comprising 0.1-1 kb.

Reaction	Product	Expected Size (bp)
PCR_E2A	Exon 2, α chain	243
PCR_E3A	Exon 3, α chain	263

DNA sample analysis in an agarose gel requires a step of sample preparation. Therefore, the following mixture needs to be prepared before charging the DNA sample into the agarose gel.

Reactions: PCR_E2A and PCR_E3A	
Reagents	Volume (µl)
H_2O Milli-Q	8
DNA loading buffer (6X)	2
DNA sample	2
Final volume: 12 µl	



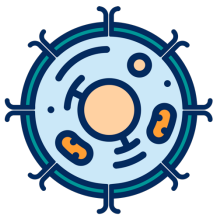
Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Congratulations! You have successfully amplified the exons 2 and 3 of the HLA-DQ α chain of a celiac patient!

Preparing the reaction

PCR 2 also comprises two reactions, PCR_HE2A and PCR_HE3A. The setup for these reactions bears a close resemblance to the PCR1. This time, the amplification of the exon 2 proceeds by using the primers P1* and P2*, which contain the restriction site NdeI and the homologous region of the 5' end of the exon 3, respectively. Being the amount of DNA a limited resource for the following PCR, a preparative PCR2 has been designed. The adjusted volumes are described in the following table:

Reactions: PCR_HE2A and PCR_HE3A	
Reagents	Volume (µl)
H_2O Milli-Q	18
Mix iProof (2X)	25
Primer P1* or P3* (10X)	2
Primer P2* or P4* (10X)	2
PCR1 product	3
Final volume: 50 µl	



Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Let's set the PCR Program

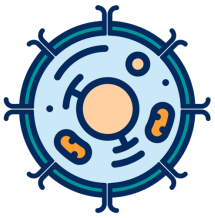
The PCR2 reactions proceed very much in the same way as the ones from PCR1. After screening different temperatures of annealing, the one that resulted in a higher specificity was 58°C.

Reactions: PCR_HE2A and PCR_HE3A		
Process	Temperature (°C)	Time (min)
Pre-denaturation	98	3:00
Denaturation	98	00:10
Annealing	60	00:20
Elongation	72	00:15
Final elongation	72	10:00
Storage	4	O/N
Denaturation – Annealing- Elongation (30 cycles)		

Remember to set the hotlid at 105 ℃. To avoid non-especific amplifications, place the samples in the PCR once the hotlid has reached the temperature (HOT STAR).

Expected results

PCR products from the preparative reactions PCR_HE2A and PCR_HE3A are to be run in an agarose gel. Expected size bands (see table) are to be purified using Qiagen QIAquick Gel extraction kit (www.qiagen.com/us), see the protocol on page X.



Don't worry if non-specific amplifications appear. HOT STAR PCR is recommended to avoid the presence of non-desired products.

Reaction	Product	Expected Size (bp)
PCR_HE2A	Exon 2, α chain + NdeI	264
PCR_HE3A	Exon 3, α chain + SalI	292

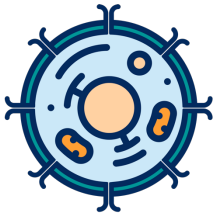
Purified samples must be analyzed in an analytical agarose gel to test purification efficiency.

Well done! Exons 2 and 3 of the HLA-DQ α are ready to be assembled and cloned!

Preparing the reaction

The assembling of exons 2 and 3 is done in PCR 3, which involves one single reaction (FINAL_PCRα). To do so, primers P1* and P4* flanking the 5' and 3' ends of the final construct (exon2 + exon3) are used. The adjusted volumes for FINAL_PCRα are described in the following table:

Reaction: FINAL_PCRα	
Reagents	Volume (µl)
H_2O Milli-Q	4
Mix iProof (2X)	10
Primer P1* (10X)	2
Primer P4* (10X)	2
Mix of products (PCR2_HE2A + PCR2_HE3A)	2
Final volume: 20 µl	

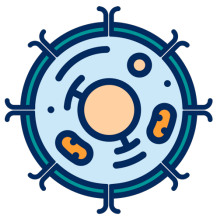


Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Let's set the PCR Program

In contrast to PCRs 1 and 2, the elongation time of FINAL_PCRα is higher. Considering that the expected product size is 556 bp and that the iPRoof amplifies at 1kb/min, the elongation time to set is 25 seconds.

Reaction: FINAL_PCRα		
Process	Temperature (°C)	Time (min)
Pre-denaturation	98	3:00
Denaturation	98	00:10
Annealing	61	00:20
Elongation	72	00:25
Final elongation	72	10:00
Storage	4	O/N
Denaturation – Annealing- Elongation (30 cycles)		



Remember to set the hotlid at 105 ℃. To avoid non-especific amplifications, place the samples in the PCR once the hotlid has reached the temperature (HOT STAR).

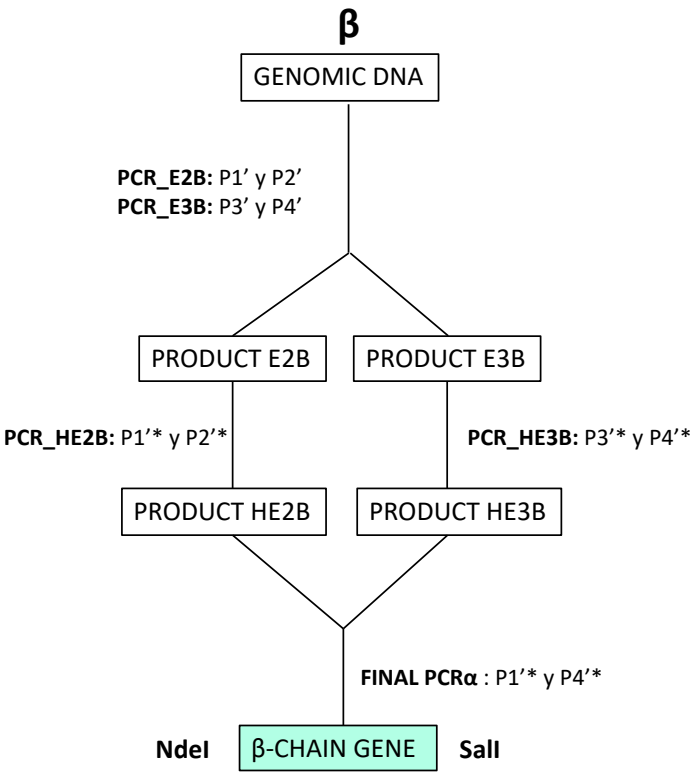
Expected results

Note that non-specific amplifications may appear. One intense band at 556 bp should be present. This band is expected to belong to the assembling of exons 2 and 3 containing the restriction sites necessaries for further cloning.

Reaction	Product	Expected Size (bp)
FINAL_PCRα	NdeI + Exon 2 + Exon 3+ SalI	556

Well done! You have successfully obtained a ready-to-clone α chain of the HLA-DQ gene.

OBTENTION OF THE β CHAIN



In an attempt to extract the exons 2 and 3, which codify for the β chain, 3 PCRs have been designed. In the PCR1, amplification of the exons 2 and 3 is carried out by using primers P1'-P2' and P3'-P4' respectively in two separated reactions, called PCR_E2A and PCR_E3A.

The products of the beforementioned reactions are to be the template of the following PCR2. In this step, the restriction sites NdeI at 5' (in the case of exon 2) and SalI at 3' (for exon 3) are introduced for further cloning in the vector pET22b(+). Besides, a complementary region between exon 2 (3' end) and 3 (5' end) for further assembling is also introduced. To accomplish this, two separated reactions called PCR_HE2B and PCR_HE3B are carried out using primers P1'*-P2'* and P3'*-P4'* respectively.

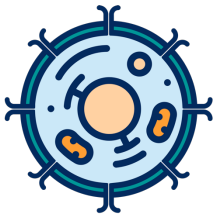
Finally, in the PCR3, assembling of the exons 2 and 3 is done in one single reaction (Final PCR β), using a mix of the products from PCR_HE2B and PCR_HE3B and the flanking primers P1'*-P4'*.

Stage	PCR Name	DNA Template	Primers	Tm (°C)	Elongation (s)
PCR 1	PCR_E2B	Genomic DNA	P1' – P2'	60	15
	PCR_E3B	Genomic DNA	P3' – P4'	60	15
PCR 2	PCR_HE2B	PCR_E2B product	P1'* – P2'*	60	15
	PCR_HE3B	PCR_E3B product	P3'* – P4'*	60	15
PCR3	FINAL PCRβ	PCR_HE2B + PCR_HE3B product mix	P1'* – P4'*	60/57	25

Preparing the reaction

PCR 1 comprises two reactions, PCR_E2B and PCR_E3B. iProof HF is an excellent DNA polymerase to amplify genes that are to be cloned in an expression vector. Besides, iProof HF comes together with dNTPs and the PCR buffer required for the reaction. Therefore, preparing the reaction is straightforward, as you only need to mix the Master mix, primers, DNA and H_2O.

Reactions: PCR_E2B and PCR_E3B	
Reagents	Volume (µl)
H_2O Milli-Q	2
Mix iProof (2X)	10
Primer P1' or P3' (10X)	2
Primer P2' or P4' (10X)	2
Genomic DNA	4
Final volume: 20 µl	



Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Let's set the PCR Program

Every PCR involves a total of 5 steps. A pre-denaturation step, followed by denaturation, annealing, and elongation. Being the expected PCR products no longer than 250 bp and considering that the iProof HF amplifies 2kb/min, the elongation time is set at 15 seconds.

Reactions: PCR_E2B and PCR_E3B		
Process	Temperature (°C)	Time (min)
Pre-denaturation	98	3:00
Denaturation	98	00:10
Annealing	60	00:20
Elongation	72	00:15
Final elongation	72	10:00
Storage	4	O/N
Denaturation – Annealing- Elongation (30 cycles)		

Remember to set the hotlid temperature at 105 ℃

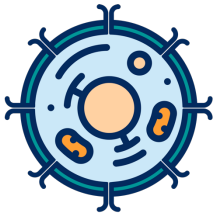
Expected results

PCR products from the reactions PCR_E2B and PCR_E3B are to be analyzed in an agarose gel. Since the expected product size is no longer than 270 bp, it is recommended to prepare a 2% agarose gel for a better resolution. It is also suggested to use a DNA Marker with a molecular range comprising 0.1-1 kb.

Reaction	Product	Expected Size (bp)
PCR_E2B	Exon 2, β chain	391
PCR_E3B	Exon 3, β chain	328

DNA sample analysis in an agarose gel requires a step of sample preparation. Therefore, the following mixture needs to be prepared before charging the DNA sample into the agarose gel.

Reactions: PCR_E2B and PCR_E3B	
Reagents	Volume (µl)
H_2O Milli-Q	8
DNA loading buffer (6X)	2
DNA sample	2
Final volume: 12 µl	



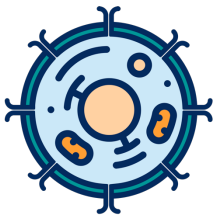
Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Congratulations! You have successfully amplified the exons 2 and 3 of the HLA-DQ β chain of a celiac patient!

Preparing the reaction

PCR 2 also comprises two reactions, PCR_HE2B and PCR_HE3B. The setup for these reactions bears a close resemblance to the PCR1. This time, the amplification of the exon 2 proceeds by using the primers P1'* and P2'*, which contain the restriction site NdeI and the homologous region of the 5' end of the exon 3, respectively. Being the amount of DNA a limited resource for the following PCR, a preparative PCR2 has been designed. The adjusted volumes are described in the following table:

Reactions: PCR_HE2B and PCR_HE3B	
Reagents	Volume (µl)
H_2O Milli-Q	15
Mix iProof (2X)	25
Primer P1* or P3* (10X)	2
Primer P2* or P4* (10X)	2
PCR1 product	6
Final volume: 50 µl	



Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Let's set the PCR Program

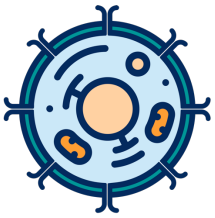
The PCR2 reactions proceed very much in the same way as the ones from PCR1. After screening different temperatures of annealing, the one that resulted in a higher specificity was 58°C.

Reactions: PCR_HE2B and PCR_HE3B		
Process	Temperature (°C)	Time (min)
Pre-denaturation	98	3:00
Denaturation	98	00:10
Annealing	58	00:20
Elongation	72	00:15
Final elongation	72	10:00
Storage	4	O/N
Denaturation – Annealing- Elongation (30 cycles)		

Remember to set the hotlid at 105 ℃. To avoid non-especific amplifications, place the samples in the PCR once the hotlid has reached the temperature (HOT STAR).

Expected results

PCR products from the preparative reactions PCR_HE2B and PCR_HE3B are to be run in an agarose gel. Expected size bands (see table) are to be purified using Qiagen QIAquick Gel extraction kit (www.qiagen.com/us), see the protocol on page X.



Don't worry if non-specific amplifications appear. HOT STAR PCR is recommended to avoid the presence of non-desired products. Besides, although restriction sites are added at the 5' and 3' end of each exon, the resulting amplification shows lower size than the products from PCR1. The reason for this is that in PCR1 the 3'end of the product contains part of the intron surrounding the gene. In PCR2 (nested PCR), the intron of the PCR1 product is not amplified.

Reaction	Product	Expected Size (bp)
PCR_HE2B	Exon 2, β chain + NdeI	289
PCR_HE3B	Exon 3, β chain + SalI	328

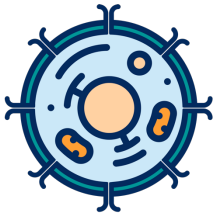
Purified samples must be analyzed in an analytical agarose gel to test purification efficiency.

Well done! Exons 2 and 3 of the HLA-DQ β are ready to be assembled and cloned!

Preparing the reaction

The assembling of exons 2 and 3 is done in PCR 3, which involves one single reaction (FINAL_PCRβ). To do so, primers P1'* and P4'* flanking the 5' and 3' ends of the final construct (exon2 + exon3) are used. The adjusted volumes for FINAL_PCRβ are described in the following table:

Reaction: FINAL_PCRβ	
Reagents	Volume (µl)
H_2O Milli-Q	4
Mix iProof (2X)	10
Primer P1'* (10X)	2
Primer P4'* (10X)	2
Mix of products (PCR2_HE2A + PCR2_HE3A)	2
Final volume: 20 µl	

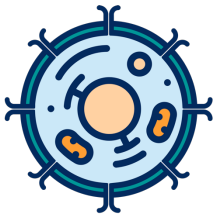


Remember to use DNAsa-free H_2O Milli-Q, tips and eppis.

Let's set the PCR Program

In contrast to PCRs 1 and 2, the elongation time of FINAL_PCRβ is higher. Considering that the expected product size is 617 bp and that the iPRoof amplifies at 1kb/min, the elongation time to set is 25 seconds.

Reaction: FINAL_PCRβ		
Process	Temperature (°C)	Time (min)
Pre-denaturation	98	3:00
Denaturation	98	00:10
Annealing	60/57	00:20
Elongation	72	00:25
Final elongation	72	10:00
Storage	4	O/N
Denaturation – Annealing- Elongation (30 cycles)		



Remember to set the hotlid at 105 ℃. To avoid non-especific amplifications, place the samples in the PCR once the hotlid has reached the temperature (HOT STAR).

Expected results

Note that non-specific amplifications may appear. One intense band at 617 bp should be present. This band is expected to belong to the assembling of exons 2 and 3 containing the restriction sites necessaries for further cloning.

Reaction	Product	Expected Size (bp)
FINAL_PCRβ	NdeI + Exon 2 + Exon 3+ SalI	617

Well done! You have successfully obtained a ready-to-clone β chain of the HLA-DQ gene.

Genomic DNA extraction from saliva

Reagents

- Sterile saline solution (0.17 M).
- Sterile PBS: 0.01M phosphate buffer, 0.15M NaCl at pH 7.4.
- Stock Proteinasa K at 1mg/mL in PBS.
- Depressor.
- Conical tubes (15 mL).
- Sterile Eppendorfs (1.5 mL).

Steps

1. Perform a wash out of the mouth with sterile saline solution and discard the liquid.
2. Scratch lightly the mucous membrane with a depressor.
3. Perform a vigorous wash out of the mouth with 10 mL of sterile saline solution and transfer the volume to a sterile 15 mL conical tube. Repeat to obtain 2 samples of 10 mL.
4. Centrifuge the samples 10 min at 3000 rpm. Discard the supernatant carefully by decantation. Avoid pellet detachment.
5. Resuspend the pellet with 1 mL of DNAsa-free PBS. Transfer the volume to a clean Eppendorf.
6. Centrifuge 10 min at 6000 rpm.
7. Discard the supernatant and resuspend with 1 mL of DNAsa-free PBS.
8. Centrifuge again 10 min at 6000 rpm.
9. Resuspend the last pellet with 100-400 μl of PBS (assess the amount of pellet).
10. Transfer 90 μl of the last solution to a new clean Eppendorf and add 10 μl of proteinase K solution of 1 mg/mL to a final concentration of 0.1 mg/mL.
11. Incubate the sample 1 h to 55 °C in a bath.
12. Then move it to another bath at 100 °C and incubate 10 min.
13. Cool down the sample with ice and then store it at – 20 °C till use.

Gel extraction kit

Protocol: QIAquick Gel Extraction Kit using a Microcentrifuge

This protocol is designed to extract and purify DNA of 70 bp to 10 kb from standard or low-melt agarose gels in TAE or TBE buffer. Up to 400 mg agarose can be processed per spin column. This kit can also be used for DNA cleanup from enzymatic reactions (see page 7). For DNA cleanup from enzymatic reactions using this protocol, add 3 volumes of Buffer QG and 1 volume of isopropanol to the reaction, mix, and proceed with step 6 of the protocol. Alternatively, use the MinElute Reaction Cleanup Kit.

Important points before starting

- The yellow color of Buffer QG indicates a pH ≤7.5.
- Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume).
- All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at temperature (15–25°C).

Gel Extraction Spin Protocol

Procedure

1. **Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.**

   Minimize the size of the gel slice by removing extra agarose.

2. **Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).**

   For example, add 300 µl of Buffer QG to each 100 mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400 mg; for gel slices >400 mg use more than one QIAquick column.

3. **Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.**

   IMPORTANT: Solubilize agarose completely. For >2% gels, increase incubation time.

4. **After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).**

   If the color of the mixture is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn to yellow.

   The adsorption of DNA to the QIAquick membrane is efficient only at pH ≤7.5. Buffer QG contains a pH Indicator which is yellow at pH ≤7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.

5. **Add 1 gel volume of isopropanol to the sample and mix.**

   For example, if the agarose gel slice is 100 mg, add 100 µl isopropanol. This step increases the yield of DNA fragments <500 bp and >4 kb. For DNA fragments between 500 bp and 4 kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. **Place a QIAquick spin column in a provided 2 ml collection tube.**
7. **To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.**

   The maximum volume of the column reservoir is 800 µl. For sample volumes of more than 800 µl, simply load and spin again.
8. **Discard flow-through and place QIAquick column back in the same collection tube.**

   Collection tubes are reused to reduce plastic waste.
9. **Recommended: Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.**

   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, in vitro transcription, or microinjection.
10. **To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.**

    Note: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2–5 min after addition of Buffer PE, before centrifuging.
11. **Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 17,900 x *g* (13,000 rpm).**

    IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
12. **Place QIAquick column into a clean 1.5 ml microcentrifuge tube.**
13. **To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min. After the addition of Buffer EB to the QIAquick membrane, increasing the incubation time to up to 4 min can increase the yield of purified DNA.**

    IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl.

    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.
14. **If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.**

    Loading Dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimization of agarose gel run time. Refer to Table 2 (page 14) to identify the dyes according to migration distance and agarose gel percentage and type.