SENSOPAD

SENSING ENDOMETRIOSIS ON PORTABLE AUXILIARY DEVICES

D1.2

Test Antibodies

NATIONAL TECHNICAL UNIVERSITY OF ATHENS (NTUA)

28/02/2025

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Project Information

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WEBSITE	www.sensopad.eu
COORDINATOR	National Technical University of Athens (NTUA)
PROJECT OVERVIEW	To significantly reduce the diagnosis time of endometriosis (ED) through innovative, non-invasive detection technologies, thereby empowering women with early, accurate, and accessible diagnostics that improve health outcomes and reduce healthcare costs.

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Abbreviations

PCR	Polymerase Chain Reaction
IL	Interleukin
PAGE	Polyacrylamide Gel Electrophoresis

Table 1: Abbreviations

Executive Summary

SENSOPAD develops two revolutionary devices: Sensopad and sensoMF-gFET. Sensopad is a menstrual pad functionalized with plastic antibodies that bind pro-anti- and dual-inflammatory cytokines potentially correlated to endometriosis. Before developing plastic antibodies, we plan to test previously validated antibodies against the same proteins as positive controls for the performance of the plastic antibodies. SensoMFgFET receives menses, isolates Mesenchymal Stem cells (MSC), breaks MSC apart to release genomic MSC DNA, fractionates DNA, and delivers DNA onto the gFET. The gFET is functionalized with the Cas9 protein programmed via sgRNA to detect DNA Single Nucleotide Polymorphisms (SNP) correlated with endometriosis. Before functionalizing gFET with the complex Cas9-sgRN, we will show in vitro that the complex can bind and cleave the DNA sequence of interest.

In-vitro cleavage of mutant 2 template with the Cas9-sgRNA complex

In Vitro DNA Cleavage Reaction before developing sensoMFgFET

The in vitro cleavage reaction can reveal the ability of the Cas9-sgRNA to cleave the target sequence, including our chosen SNPs, before functionalizing the graphene surface. We reasoned that normal Cas9 can bind to target DNA sequences and generate an electric charge on the surface of gFET before cleaving the template. We selected Cas9 and not defective Cas9 (dCas9) since Cas9 is more validated and affordable than dCas9. Cas9 protein was bought from Genscript (Z03386-50). We examined the SNPs (rs1971256, rs71575922, rs2206949) of the genes *CCDC170, SYNE1, and ESR1,* respectively, with three criteria: First, the SNP should reside in the PAMer sequence recognized by the Cas9-sgRNA complex[1,2]. Second, the PAMer should contain the mutated nucleotide. Third, the mutation should be a G on the last position with the 5'-3' orientation. Only rs1971256 of the *CCDC170* satisfied all criteria. Two distinct DNA templates were designed for sgRNA-mediated cleavage reactions to evaluate the binding and activity of Cas9-sgRNA in vitro for mutants one and two (table 1). The Cas9-sgRNA is predicted to cleave mutant two but not mutant one. Therefore, mutant one is the negative control, and mutant two is the positive control. The wt sequence behaves similarly to the mutant one because it is predicted not to be cleaved by the Cas9-sgRNA complex. In both wt and mutant one sequence, the PAMer sequence does not conform to the NGG pattern, where N stands for every base, and thus, it is not recognized by the Cas9-sgRNA complex. The DNA templates were received as lyophilized plasmids, which will be transformed into *DH5a* bacteria and inoculated. The bacterial cultures were used for (Midi) plasmid DNA isolation. The plasmid DNA were submitted to PCR reactions with 2X KAPA HiFi HotStart ReadyMix to generate the mutant one and two PCR products.

1.1 Verification of negative control (mutant 1)

1.1.1 PCR amplification of negative control (mutant 1)

Performed PCR for negative control (mutant 1) with 2X KAPA HiFi HotStart ReadyMix to amplify and verify the DNA sequence.

DNA sequence	Forward Primer	Reverse Primer
TGTTCATCAGGTGCAATAAGTTACAGTAAGGAGTTACCAGGTCTGACAACGTTG ACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAGGA ACTAAACATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCAT CCTGGTCAAGAAGATCTGGCTGGCTCAGCATAACTTCGTATAGCATACATTATA CGAACGGTAGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGC AGGCATGCAAGCTTGGGAAGTTCCTATTCTCTAGAAAGAATAGGAACTTCTACC GTTCGTATAGCATACATTATACGAAGTTATATGCTCCTCTTACTCTTT	5'-TGTTCATCAGGTGCAATAAGTTACA-3'	5'-AAAGAGTAAGAGGAGCATATAACTT-3'

[1] Balderston, Sarah, Jeffrey J. Taulbee, Elizabeth Celaya, Kandace Fung, Amanda Jiao, Kasey Smith, Reza Hajian, et al. 2021. "Discrimination of Single-Point Mutations in Unamplified Genomic DNA via Cas9 Immobilized on a Graphene Field-Effect Transistor." Nature Biomedical Engineering 5 (7): 713–25.

[2] Sapkota, Yadav, Valgerdur Steinthorsdottir, Andrew P. Morris, Amelie Fassbender, Nilufer Rahmioglu, Immaculata De Vivo, Julie E. Buring, et al. 2017. "Meta-Analysis Identifies Five Novel Loci Associated with Endometriosis Highlighting Key Genes Involved in Hormone Metabolism." Nature Communications 8 (1): 15539.

Table 2: DNA sequence and primers used for mutant 1 PCR amplification

The final reaction volume of the PCR was 25μL. The reaction volumes used:

Component	Volume(μL)
KAPA HiFi HotStart ReadyMix(2X)	12.5
Forward primer (10μM)	0.75
Reverse primer (10μM)	0.75
Nuclease-free water	10
Template DNA(1ng/μL)	1
Total volume	25

Table 3: Volumes of the PCR components.

The following cycling protocol was followed for the PCR:

Step	Temperature(°C)	Duration of cycles	Number of cycles
Initial Denaturation	95	3 minutes	1 cycle
Denaturation	98	20 sec	35 cycles
Annealing	61	15 sec	35 cycles
Extension	72	5.5 sec	35 cycles
Final Extension	72	22 sec	1 cycle

Table 4: Cycling conditions implemented for mutant 1 PCR amplification.

The annealing temperature was adjusted to the primers' melting temperature (Tm). The duration of the extension cycle was modified according to the size of the PCR insert using the analogy of 15 seconds per 1000 bp. Since the expected product is 367 bp long, the duration of the extension is 5.5 seconds. The duration of the final extension was also altered according to the insert size (1 minute per 1kbp). The final extension time is 22 seconds.

1.1.2 Verification of the PCR product of the negative control (mutant 1) through 2% DNA agarose gel electrophoresis

Once the PCR was concluded, we conducted 2% DNA agarose gel electrophoresis to verify the size of the received fragment (Figure 1).

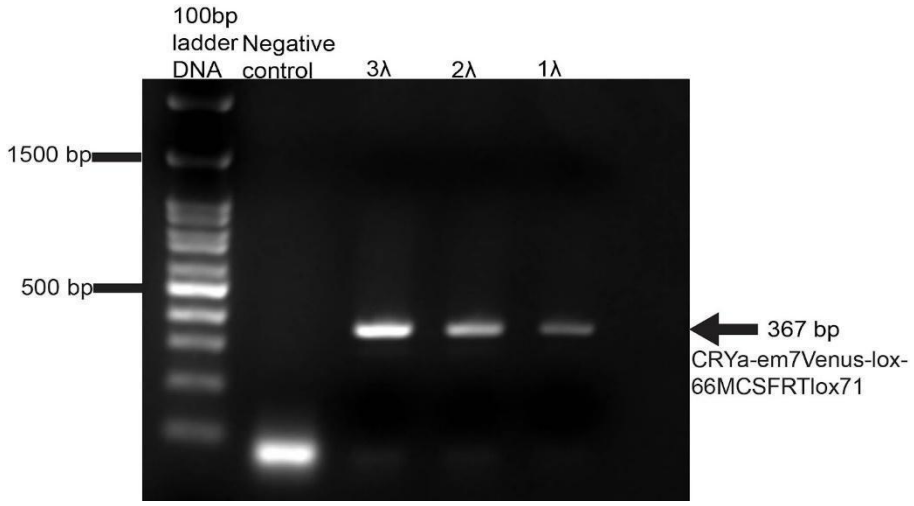

Figure 1: DNA electrophoresis of the PCR-amplified negative control (mutant 1) on a 2% agarose gel. Lane 1 contains the 100 bp DNA ladder from Nippon Genetics. Lane 2 shows the negative control of the PCR, while lanes 3, 4, and 5 contain 3 λ, 2 λ, and 1 λ of the PCR product, respectively. The arrow denotes the bands observed in the DNA electrophoresis and their corresponding base pairs.

In Figure 1, lane 1 presents the 100-bp DNA ladder from Nippon Genetics, while lane 2 contains the PCR negative control. Under 100 bp, a bright band is presented; this band is explained to be primer dimers. Lanes 3, 4, and 5 show three linear fragments at approximately 367 bp, a fragment size corresponding to the PCR sequence's size, the plasmid was correct.

1.1.3 Verification of negative control (mutant 1) using diagnostic digestion

Diagnostic digestion was performed to verify the amplified fragment further. When designing the cassette, we added a multiple cloning site, a multicloning site.

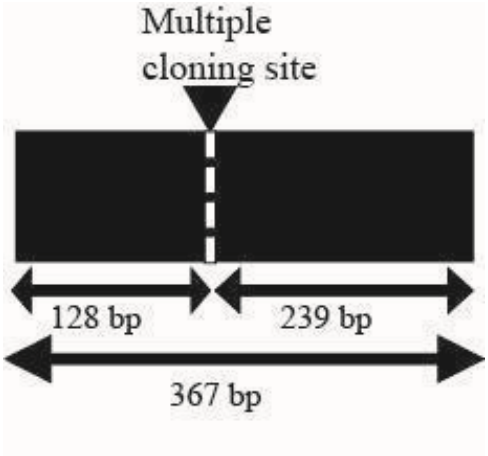

Figure 2: Visual representation of a multiple cloning site.

Specifically, the fragment was digested using the restriction enzymes EcoRI, XmaI, SmaI, KpnI, and BamHI from Nippon Genetics. Once the digestion was concluded, it was anticipated to receive two DNA fragments of 239 bp and 128bp, with their combined length equaling the total base pair length of the

non-digested sequence. 2% DNA agarose gel electrophoresis was conducted to verify the digestion results.

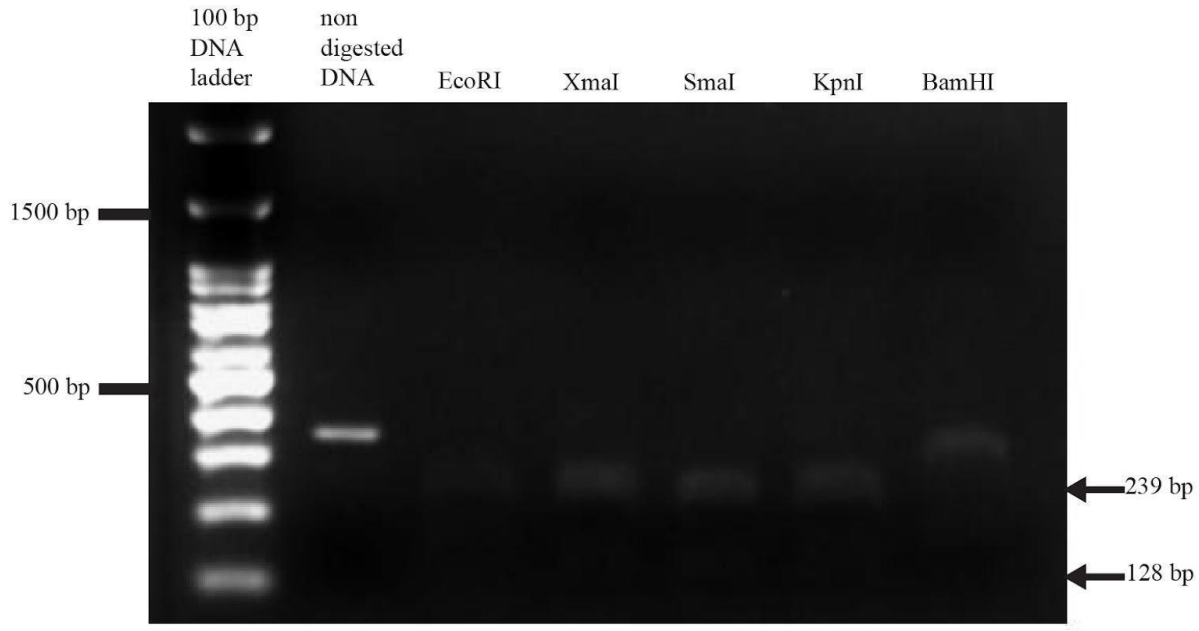

Figure 3: 2% DNA agarose gel electrophoresis of post-digested negative control (mutant 1). Lane 1 shows the 100bp DNA ladder from Nippon Genetics. Lane 2 presents the non-digested DNA. Lanes 3, 4, 5, 6, and 7 express the digested DNA with the restriction enzymes EcoRI, XmaI, SmaI, KpnI, and BamH1, respectively. The two arrows denote the anticipated digestion products.

As shown in Figure 3, lane 1 contains the 100bp DNA ladder from Nippon Genetics. Lane 2 displays the non-digested mutant 1 and shows one singular band at 367 bp, equal to the full length of mutant 1. Lanes 3, 4, 5, and 6 show a shift in the length of the PCR product towards 239 bp, while the intensity of the 128 bp band is below detection threshold and thus not visible. BamH1 digestion in lane 7 was not successful.

1.2 Verification of positive control (mutant 2)

1.2.1 PCR amplification of positive control (mutant 2)

PCR was conducted for the positive control (mutant 2) using 2X KAPA HiFi HotStart ReadyMix to amplify and validate the DNA sequence.

DNA sequence	Forward Primer	Reverse Primer

CGACTCACTATAGGGGAGAGACCACACCCAAGCTGTCTAGAGCCGCCACCG TTGACAATTAATCATCGGCATAGTATATCGGCATAGTATAATACGACAAGGTGAG GAACTAAACATGGTGTCAAAAGGGGAGGAATTGATCAAAGAGAACATGCATATG AAACTCAAGAAGATCTGGCTGGCTCAGGATGCACAGCTCAGCACTGCTCTGTT GCCTGGTCCTCCTGACTGGGGTGAGGGCCAGCCCAGGCCAGGGCACCCAG TCTGAGAACAGCTGCACCCACTTCCCAGGCAACCTGCCTAACATGCTTCGAG ATCTCCGAGATGCCTTCAGCAGAGTGAAGACTTTCTTTCAAATGAAGGATCAGC TGGACAACTTGTTGTTAAAGGAGTCCTTGCTGGAGGACTTTAAGGGTTACCTGG GTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACCTGGAGGAGGTGATGCCC CAAGCTGAGAACCAAGACCCAGACATCAAGGCGCATGTGAACTCCCTGGGG GAGAACCTGAAGACCCTCAGGCTGAGGCTACGGCGCTGTCATCGATTTCTTCC CTGTGAAAACAAGAGCAAGGCCGTGGAGCAGGTGAAGAATGCCTTTAATAAGC TCCAAGAGAAAGGCATCTACAAAGCCATGAGTGAGTTTGACATCTTCATCAACT ACATAGAAGCCTACATGACAATGAAGATACGAAACGACTACAAAGACCATGAC GGTGATTATAAAGATCATGATATCGATTACAAGGATGACGATGACAAGTGA	5'- CGACTC ACTATA GGGGA GAGACC AC-3'	5'- TCACTT GTCATC GTCATC CTTGTA A-3'

Table 5: DNA sequence and primers used for mutant 2 PCR amplification.

The final reaction volume of the PCR was 25 µL. The reaction volumes used:

Component	Volume(µL)
KAPA HiFi HotStart ReadyMix(2X)	12.5
Forward primer (10µM)	0.75
Reverse primer (10µM)	0.75
Nuclease-free water	10
Template DNA(1ng/µL)	1
Total volume	25

Table 6: Volumes of the PCR components.

The following cycling protocol was followed for the PCR:

Step	Temperature(°C)	Duration of cycles	Number of cycles
Initial Denaturation	95	3 minutes	1 cycle
Denaturation	98	20 sec	35 cycles
Annealing	61	15 sec	35 cycles
Extension	72	12 sec	35 cycles
Final Extension	72	47 sec	1 cycle

Table 7: Cycling conditions implemented for mutant 2 PCR amplification.

The annealing temperature was adjusted to the primers' melting temperature (Tm). The duration of the extension cycle was modified according to the size of the PCR insert using the analogy of 15 seconds per 1000 bp. Since the expected product is 791 bp long, the extension duration is 12 seconds. The duration of the final extension was also altered according to the insert size (1 minute per 1kbp). The final extension time is 47 seconds.

1.2.2 Verification of the PCR product of the positive control (mutant 2) through 2% DNA agarose gel electrophoresis

2% DNA agarose gel electrophoresis was performed to verify the PCR results. The results are depicted in Figure 4.

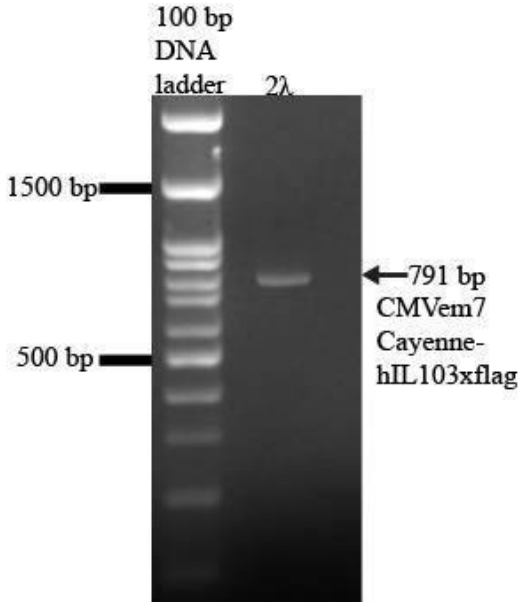

Figure 4: DNA electrophoresis of the PCR-amplified positive control (mutant 2) on a 2% agarose gel. Lane 1 contains the 100 bp DNA ladder from Nippon Genetics. Lane 2 shows the negative control of the PCR, while lanes 3, 4, and 5 contain 3 λ, 2 λ, and 1 λ of the PCR product, respectively. The arrow denotes the bands observed in the DNA electrophoresis and their corresponding base pairs.

In Figure 4, lane 1 contains the 100 bp DNA ladder from Nippon Genetics. Lane 2 contains 2λ from the PCR product. As anticipated, lane 2 displays a single linear band at 791 bp, confirming we have received the correct plasmid.

1.3 In vitro cleavage reaction using Cas9

Cas9, in the absence of sgRNA, cannot detect and cleave the mutant 2 template. On the contrary, in the presence of sgRNA, Cas9 successfully cleaves mutant 2 template producing two fragments. The in-vitro cleavage reaction was conducted using the GenScript Cas9 and sgRNA. The cleavage reaction was set up according to the Simple protocol for gene editing using GenCrisprTM Cas9 nuclease. To confirm the results of the in-vitro cleavage assay, 2% DNA agarose gel electrophoresis was performed.

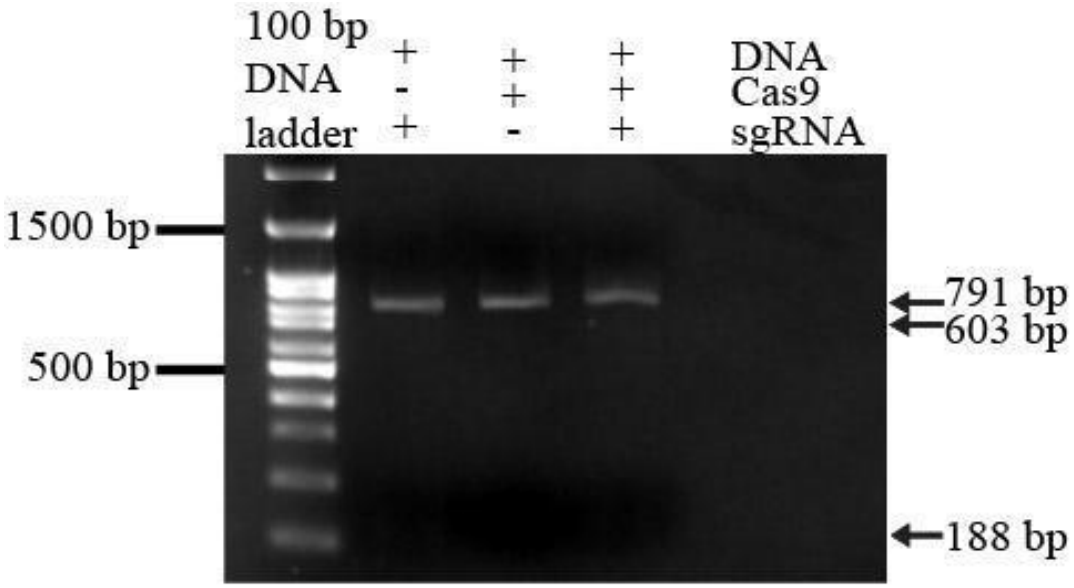

Figure 5: 2% DNA agarose gel electrophoresis of post-in-vitro cleavage using Cas9. Lane 1 shows the 100bp DNA ladder from Nippon Genetics. Lane 2 contains the template DNA and the sgRNA. Lane 3 displays the in-vitro cleavage of the DNA only with the Cas9. Lane 4 presents the in-vitro cleavage of the DNA with the Cas9-sgRNA complex. The first arrow denotes the non-digested DNA, while the second and third arrows indicate the anticipated in-vitro cleavage results.

As shown in Figure 5, lane 1 contains the 100 bp DNA ladder from Nippon Genetics. For lanes 2 and 3 show a 791 bp. Lane 4 contains the DNA as well as the Cas9-sgRNA complex. While two fragments were expected at 603 bp and 188 bp, on lane 4, the gel shows a unique 791 bp band. **Conclusion**: Adjusting the reaction's stoichiometry will be necessary to obtain the expected results, before designing and testing an alternative sgRNA.

Testing antibodies against specific ED-related pro and anti-inflammatory proteins (sensoPAD)

1.4 Preparatory actions

Interleukins (IL) and transforming growth factor-beta 1 (TGF-β1) are crucial cytokines that regulate immune responses, inflammation, and cellular processes.

Interleukin-4 (IL-4) is a key cytokine in the Th2 immune response and plays a crucial role in allergic reactions and asthma, as well as in the suppression of inflammatory responses by inhibiting Th1 cytokines. Interleukin-6 (IL-6) is a pleiotropic cytokine involved in both pro-inflammatory and anti-inflammatory responses and plays a role in chronic inflammation, contributing to conditions like rheumatoid arthritis. Interleukin-8 (IL-8, CXCL8) is a pro-inflammatory chemokine primarily acting as a neutrophil chemoattractant. Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine that suppresses excessive immune activation and inhibits pro-inflammatory cytokine production (e.g., TNF-α, IL-1, IL-6). Transforming Growth Factor-Beta 1 (TGF-β1) is a multifunctional cytokine involved in immune regulation, wound healing, and fibrosis and inhibits T cell proliferation and suppresses immune responses, promoting immune tolerance. These cytokines are critical mediators of immune responses, inflammation, and tissue homeostasis. While IL-4, IL-6, and IL-8 primarily promote immune activation

and inflammation, IL-10 and TGF-β1 exert immunosuppressive effects. Their dysregulation is implicated in autoimmune diseases, chronic inflammatory conditions, and cancer progression.

Western Blots will be performed, once all necessary reagents have been procured to test multiple antibodies against pro (IL-6, IL-8) and anti-inflammatory proteins (IL-10), or proteins with dual role in inflammation (TGFB1), and to select the ones with the best affinity and avidity.

Protein (Uniprot entry)	AA / Molecular weight (Da)	Antibody to check
Interleukin-4 (P05112)	1 53 / 17.492	CSB-PA556277 (Cusabio) Rabbit anti-Homo sapiens (Human) IL4 Polyclonal antibody
Interleukin-6 (P05231)	212 / 23.718	CSB-PA06757A0Rb (Cusabio) Rabbit anti-Homo sapiens (Human) IL6 Polyclonal antibody
Interleukin-8 (P10145)	99 / 11.098	HPA057179 (Atlas Antibodies Sigma-Aldrich) Rabbit anti-Homo sapiens (Human) IL8 Polyclonal antibody
Interleukin-10 (P22301)	178 / 20.517	HPA071391 (Atlas Antibodies Sigma-Aldrich) Rabbit anti-Homo sapiens (Human) IL10 Polyclonal antibody
Transforming growth factor beta-1 (P01137)	390 / 44.325	HPA073356 (Atlas Antibodies Sigma-Aldrich) Rabbit anti-Homo sapiens (Human) TGFB1 Polyclonal antibody

Figure 7: Depiction of Azure 600 Imager.

Conclusions

To further enhance the credibility of the so far results, the procurement of suitable reagents is necessary. This action though already foreseen during the implementation of Task 1.2, has not been successfully addressed due to notable delivery delays from the respective provider. This issue has been communicated with the EC, kindly requesting an extension of the delivery deadline of the present deliverable, namely on the May 31st, 2025. NTUA is committed to provide high quality results in the frame of the present deliverable, considering the use of Western Blots which will be performed with the versatile Azure 600, a multichannel, multimodal imager, with IR, visible light, and UV excitation channels and Spectra Fluorescent Western Blotting Kits with fluorescent secondary antibodies.

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