CHARACTERIZATION OF AN ABNORMAL [redacted] PHENOTYPE

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BIOTECHNOLOGY BACHELOR'S DEGREE FINAL PROJECT

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I, Enric Bonay Giner, with DNI “41601856-P”, am aware of the guide to plagiarism prevention at the URV Prevention, detection and treatment of plagiarism in teaching: a guide for students (approved in July 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formacio-competencies-nuclears/plagi/) and I affirm that this TFG does not constitute any of the conducts considered like plagiarism by the URV.

Tarragona, June 7, 2021

Confidentiality disclaimer

Acknowledgements

Firstly, I would like to show my gratitude to Eva Martínez Caceres, head of Immunology department at the Germans Trias I Pujol University Hospital and director of the immunopathology group who despite the current situation, has given me the opportunity to do my degree final project in the immunopathology group. Also, despite being really busy Eva always took care of me. With her kind attitude she constantly gave me tips and answered any doubt I might have. That's why I wanted to thank her for that.

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Contents

About the centre

This biotechnology bachelor's degree final project has been carried out during the research stay that I made in the Immunopathology group at IGTP (Germans Trias i Pujol Research Institute, Barcelona, Spain) from October 2020 to June 2021.

IGTP is a public research center in Catalonia dedicated to increasing scientific knowledge and transferring it to improve the care and lives of patients. The institute is located at the biomedical campus of Can Ruti and is attached to Germans Trias University Hospital. Scientist caring out research in any of the nine areas present at the institute, that collectively publish over 500 papers a year, have access to several core facilities like High performance computing, Cryobiology, Cytometry, Microscopy, Proteomics and metabolomics, Genomics and more.

The Immunopathology group, who's responsible is Eva Martinez Caceres, focuses on multiple sclerosis, developing new immune tolerance-inducing therapies, and outcome predictive biomarkers on lung cancer patients.

The present research has been carried out purely on the scientific interest of identifying an abnormal immune condition. With the future expectation of getting an insight on the innerworkings of the [redacted].

Abstract

Background: A patient was detected with and abnormal [redacted] phenotype. [redacted], is a [redacted] expressed [redacted] on [redacted] and [redacted] used to [redacted] and [redacted].

Methods: Cryopreserved cells from the patient and the control were stained for flow cytometry for [redacted]. Moreover, gDNA was extracted from patient and control cells to sequence [redacted] and [redacted].

Results: Results suggest that [redacted] from the patient samples. Also, [redacted], [redacted] and that [redacted].

Conclusions: Despite our patient was [redacted], the [redacted] is [redacted]. This [redacted]**.** However, this result has to yet be confirmed by the sequencing of the [redacted] and [redacted].

Keywords: Human [redacted] cells; [redacted] ; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; Flow cytometry.

1. Introduction

Immune cells are classified based on the expression of cell markers. Cell markers are specifically expressed in all cells and reflect both growth and state of differentiation of cells. These markers can be molecules within the cell's plasma membrane (antigens) or intracellular molecules. These molecules not only serve as markers, but also have a functional role and it's possible to use the presence of them to diagnose or direct treatment to a disease.

Flow Cytometry's technic uses light to identify and measure these markers which allows us to characterize different cell populations. By using the properties of fluid dynamics, a sample that contains the cells suspended in a fluid is injected into the instrument where the cells will flow individually, and they would be identified both by their physical characteristics and by the fluorescent markers used to label them.

Flow cytometry raw data is represented in histograms or dot-plots. Histograms represent the number of events detected versus the intensity detected in a single channel (detector). Dot-plots represent the intensity of 2 different channels along the corresponding axes (events with similar intensities will cluster together in the same region), which allows us to group them in a selection, known as gate.

During the research stay that I have made in the Immunopathology group at IGTP, while I was analysing flow cytometry data for an unrelated study, I stumbled upon a patient with an unexpected [redacted] profile.

[redacted]

[redacted][2]. [redacted]

[redacted].

[redacted], [redacted], [redacted]. [redacted]

[redacted][3]. [redacted]. [redacted], [redacted] (Figure 1-2).

[redacted], [redacted], [redacted], [redacted], [redacted] [4] (Figure 1-2).

[redacted], [redacted], [redacted][4] (Figure 1-2). [redacted]

[redacted].

[redacted]

Figure 1. [redacted] ***representation.***

Extracted from reference 24.

[redacted]

[redacted]

[redacted].

[redacted]

[redacted]

[redacted]

[redacted]. [redacted],

[redacted]

[redacted].

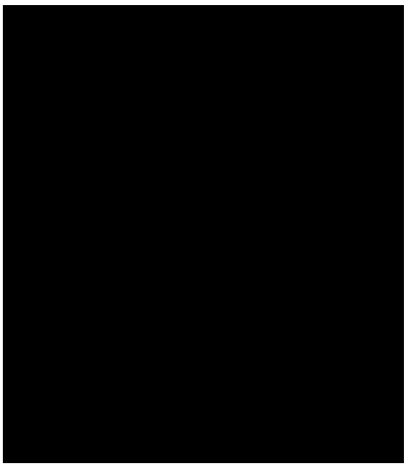


Figure 2. ***representation.***

.

.

. , , [23].

. . , , [4]. , , , [4].

. (Figure 3), [6] (Figure 4). , , , .

Figure 3. ***.*** *Extracted from reference 6.* *, from the reference 6,* .

. , , , .[14]

Figure 4. *Extracted from reference 6.*

(Supplementary figure 1)[7].

[8].

[9].

[10].

[9].

[8].

[11].

[redacted], [redacted]. [redacted]. [redacted], [redacted] (Figure 3), [redacted] (Figure 4) [6]. [redacted], [redacted].

[redacted] ([redacted], Table 1) [redacted] (Figure 1-2) [redacted] [4,12] (Figure 5). [redacted] [4] [redacted] (Figure 6) [4,12,13]. [redacted], [redacted], [13], [redacted] [14].

[redacted]; [redacted], [redacted], [redacted], [redacted] [4,12,13]. [redacted].

In this project we inquire into the [redacted] phenotype detected, on a deceased 68-year-old man [redacted] whose clinical history [redacted].

Table 1. [redacted] ***aminoacidic sequence.*** *Extracted from reference 3*

[redacted].

Figure 5. [redacted]**.** *Extracted from reference 4.*

[redacted].

Figure 6. [redacted]**.** *Extracted from reference 4.*

[redacted].

2. Objective

The main goal of this project is to characterize the abnormal [redacted] phenotype.

To reach this objective we marked two specific goals. First, to determine if [redacted]. Second, to sequence [redacted].´

3. Methodologies

3.1. Thawing cryopreserved cells for immunophenotyping

[redacted], had been preserved in cryopreservation medium composed of RPMI medium, 20% of Fetal Bovine Serum (FBS) and 20% of DMSO (cryoprotectant that reduces ice formation preventing cell bursting (death) during the freezing process) for the unrelated study previously mentioned.

To thaw cryopreserved cells, the tawing process must be done quickly to prevent cell death. Once the cryovial is removed from the liquid nitrogen it has to be speedily placed in a 37ºC water bath (if needed, for a short period of time, it can be maintained at -80ºC). Once thawed, due to the high toxicity of DMSO, the medium has to be replaced rapidly, but without rushing, with complete RPMI medium (10% FBS, 2% Penicillin and streptomycin and 1% L-Glutamine). Cryopreservation medium is salt rich which could cause an osmotic shock if not done carefully[15].

The protocol used was adapted form reference 15 (Protocol 1 A, Annex 5.3).

3.2. Viability stain, cell count and flow cytometry

Once cells were thawed, 20 µL of the cell suspension were reverse pipetted and stained with 7-AAD (7-Aminoactinomycin V) and annexin for 20 minutes in darkness at 4ºC. After the incubation was completed, 20 µL of Perfect-count microspheres from cytogonos were added by reverse pipetting. The sample was collected using the BD FACS Canto II cytometer (Protocol 1 B, Annex 5.3).

7-AAD binds to DNA, by this means excluding cells that are alive and maintain the membrane integrity. [16]

Annexin binds to the anionic phospholipid phosphatidylserine (PS). In viable cells, PS is located on the cytoplasmic side of the plasma membrane. However, during apoptosis, the plasma membrane structure changes and PS is translocated to the extracellular side.[17]

3.3. Cell resting

We tested if an overnight resting period was required to improve cell quality for immunophenotyping [15]. Once cells were thawed, counted and cryopreservation medium was removed, they were plated on a 24 well plate at $1 \cdot 10^6$ cells/mL with complete RPMI medium and rested for 18 hours on a CO_2 water jacketed incubator (37ºC, 5%CO_2, 95% humidity).

3.4. Cell surface staining and flow cytometry

Once cells were thawed and rested if required [redacted] were stained for [redacted]. This allowed us to check if [redacted] (Figure 12).

[redacted] were defined as [redacted], [redacted], [redacted] and [redacted].

[redacted], [redacted], [redacted], [redacted] and [redacted].

After cell staining was completed, cells were fixed with 300µL of PFA 1% in PBS for biosafety reasons and collected using the Cytek Aurora cytometer (Protocol 2, Annex 5.4).

Cytek Aurora cytometer is a full spectrum cytometer, where each fluorophore is identified by their distinct spectral signature (supplementary figure 2). On conventional cytometry each detector (channel) is assigned to a fluorophore which limits the quantity of fluorophores and their combinations. Also, due to a fluorophore light emission range, might present several peaks along the spectrum (Supplementary Figure 2), it makes compensating for spill over required.

Prior the cell collection, each fluorophore unique spectral signature was identified (Supplementary Figure 2).

3.5. DNA extraction

DNA was extracted utilizing Qiagen DNeasy Blood & Tissue Kit. Samples (thawed cells, $1'3 \cdot 10^6$ for the patient) were lysed utilizing the provided Proteinase K eliminating the need for mechanical disruption. Lysate was loaded onto a spin column with a silica-membrane. During centrifugation, DNA is selectively bound to the membrane as contaminants pass through. At the end DNA is eluted and ready for use (Protocol 3, Annex 5.5).

3.6. DNA quantify and quality control

DNA was first quantified using a Nanodrop, an advanced spectrophotometer where the sample is placed directly on top of the detection surface, so, there is no need for cuvettes. Measuring the absorbance at 260nm allows the nanodrop to calculate the DNA concentration in the sample. Meanwhile, the absorbance at 230nm and 280nm gives a rough idea of DNA purity and salts presence. 2 µL of DNA elution buffer (Qiagen DNeasy Blood & Tissue Kit; composed of 10mM Tris-Cl and 0,5mM EDTA; pH 9) were used as "Blank" prior to sample measurement.

Once this rough DNA quantification and quality check was done, samples were diluted to 40ng/µL and using a 2200 Tapestation bioanalyzer system, an integrity and quantitative analysis was performed. This is an automated analysis system based on capillary electrophoresis (gDNA ScreenTape assay), that is significantly faster than a "traditional" gel electrophoresis (less than 2 minutes per sample) and only requires 2 µL of the sample.

3.7. Polymerase Chain Reaction (PCR)

Once DNA quality was check and quantified, [redacted] and GAPDH (Housekeeping gene, as a control) were amplified by 2-step PCR. Amplification reactions were performed by the "Translational Genomics" platform in IGTP following primers and Taq polymerase manufacture recommendations.

Annealing temperature was calculated as the lowest melting temperature of the pair +4ºC.

Primers obtained from Invitrogen were as follows:

- [redacted]
    - Forward 5’- [redacted] -3’
    - Reverse 5’- [redacted] -3’
- GAPDH
    - Forward 5’- TGC TAC ATG GTG AGC CCC AAA G -3’
    - Reverse 5’- AGC AGA GAA GCA GAC AGT TAT GAA C -3’

Annealing temperature for [redacted] pair was calculated to be [redacted]ºC and 64ºC for GAPDH pair. Due to this disparity, the PCR was performed in a gradient thermocycler that allowed using different temperatures at the same time.

3.8. Amplicon control

The following parts were performed by the “Translational Genomics” platform in IGTP. After DNA amplification, a small sample was run in the bioanalyzer to perform a capillary electrophoresis in order to check the length of the amplicons (in both cases about 500bp).

3.9. Clean up and cycle sequencing

Once the PCR size is checked, we proceed to it’s clean up with an enzymatic treatment that degrades the unused primers and unwanted material.

After this clean up process, the BigDye Terminator Cycle Sequencing Kit is used. Two sequence reactions are performed for each gene ([redacted] and GAPDH) using in each case only one of the primers (Forward or reverse). After the reactions are completed another clean up is performed.

Once the cycle sequencing reactions are purified, a capillary electrophoresis is performed on a sanger sequencer.

3.10. Samples

This study was conducted as a part of an oncology study with approval from the investigation ethical committee.

Accordingly, the control samples were obtained from cryopreserved cells of a healthy donor who had signed an informed consent when samples were obtained. Alike the patient samples were obtained as part of an oncologic study at “Hospital Universitari Germans Trias I Pujol” and signed an informed consent that allowed this kind of study to take place.

3.11. Patient demographics and clinical symptoms

The clinical history and the physicians reported that the patient, a deceased 68-year-old man with 3 children, had the following clinical conditions:

- [redacted].
- [redacted].
- At age 60, [redacted].
- At age 61 [redacted].
- At age 67 [redacted].
- Patient was [redacted]. From age 67 onwards [redacted].
- Patient usually reported [redacted].

No reports were available from the childhood of the patient and there were no reports about [redacted]. As previously stated, we speculated that [redacted].

4. Results and Discussion

4.1. Initial detection

As previously said, while I was analysing flow cytometry data for an unrelated study during the research stay that I made in the Immunopathology group at IGTP, I stumbled upon a patient with an unexpected [redacted] profile. Defined according to section 3.4.

[redacted], [redacted] [redacted]. [redacted], [redacted], which is an unusual fact (Figure 7-8). To make sure it wasn't a fluke of the sample, we check other timepoint samples we had available from the same patient (0-month, 3-month and 6-month of treatment) and the profile was consistent. Moreover, [redacted], [redacted], [redacted] (Figure 9).

Figure 7. Dot-plot comparison of "[redacted]" on the left vs "[redacted]" on the right. [redacted].

[redacted]. [redacted] "[redacted]" [redacted].

Figure 8. Dot-plot comparison of "[redacted]" on the left vs "[redacted]" on the right. [redacted].

[redacted].

[redacted].

[redacted].

[redacted].

Figure 9. Dot-plots with contour. Comparison of "[redacted]" on the left vs the "[redacted]" on the right. [redacted].

[redacted].

4.2. Thawed cells viability

BD FACS Canto II cytometer was used to collect the sample of thawed cells that were stained according to section 3.2.

Cells were gated using 7-AAD, as a live exclusion marker, and Annexin, as an apoptotic exclusion marker. When plotted, the double negative population (bottom left) represents the viable cells (Figure 10).

On the control, we got about 85% of viable cells, whereas on the patient we got about 70% of viable cells (Figure 10). It's important to note that a higher viability isn't inherently proof of a better thawing process. During thawing cells are susceptible and things like excessive pipetting or localized ice recrystallization (caused by removing the cryovial too early from the water bath) can disrupt the cells or cause cellular damage [15,18.] This can result in the lysis of the more susceptible cells (the ones that are slightly apoptotic) giving the impression of higher viability. Of course, this variance could also be attributed to sample differences or differences in the cryopreservation.

The patient cryovial contained $4 \cdot 10^6$ cells and we recovered about $3 \cdot 10^6$, of which $2{,}1 \cdot 10^6$ were viable cells. Meanwhile, the control cryovial contained $10 \cdot 10^6$ cells and we recovered about $6 \cdot 10^6$, of which $5{,}1 \cdot 10^6$ were viable cells.

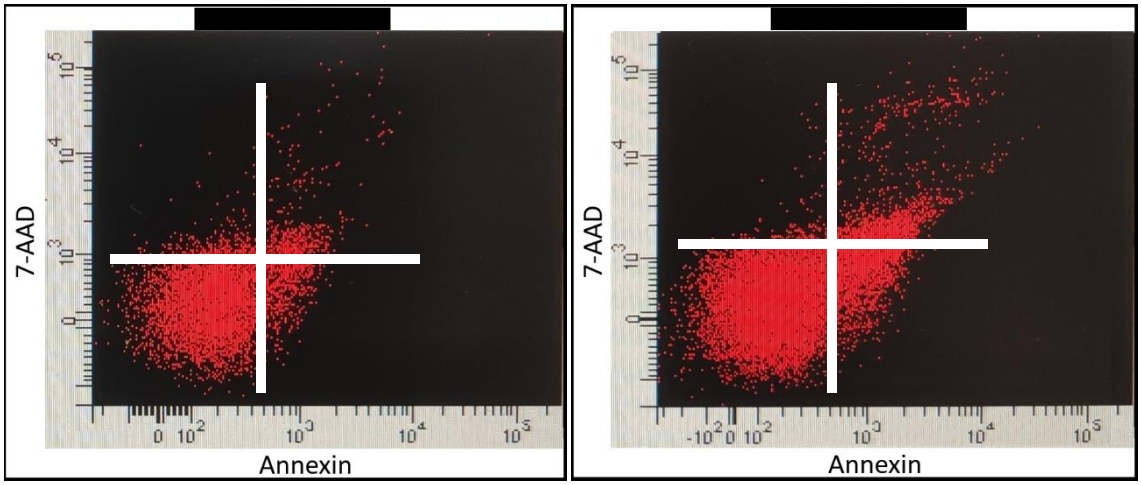


Figure 10. Dot-plots. Viability of the cells from the control on the left and viability of the cells of the patient on the right.

The white lines mark from which point onwards 7-AAD or annexin positivity was considered. Double negative (bottom left) quadrant represents viable cells. Bottom right quadrant represents early apoptotic cells. Top right quadrant represents late apoptotic cells. Top left quadrant represents dead cells.

As it's appreciable, the viability of cells of the patient is lower than the control, this can be attributed to many reasons, it could be that the more susceptible cells in the control were disrupted giving the impression of higher viability, having a lower recovery rate.

4.3. Cell resting

To test if an overnight resting period was required to improve cell quality for immunophenotyping, we collected cells from the control, both rested and non-rested, according to section 3.4.

We noticed that cells were in a slightly better shape just after thawing. So, no resting period was required. This was shown in the cytometry as a reduction on the cellular size, that was a tell-tale sign that, despite cells were alive, they were slightly more apoptotic (Figure 11). Despite not being a huge difference, this meant that, due to the stress suffered during the thawing process, some cells were dying during the overnight resting.

Figure 11. Comparison between rested and non-rested cells.

FSC, also forward scatter, represents the size of the cell. Meanwhile SSC, side scatter, represents granularity.

[redacted]

If a closer look is taken on [redacted] area is possible to distinguish that a part of the rested cells appear to be slightly lower on the FSC channel. Therefore, they are smaller and thus slightly apoptotic. So as said, non-rested cells are in slightly better shape.

4.4. Cell surface staining and flow cytometry

To test [redacted], we stained and collected the cells according to section 3.4.

On the cytometry data of the control, [redacted]. (Figure 12. A).

On the patient sample, the same procedure would produce [redacted] (Figure 12. B.) or [redacted] (Figure 12. C).

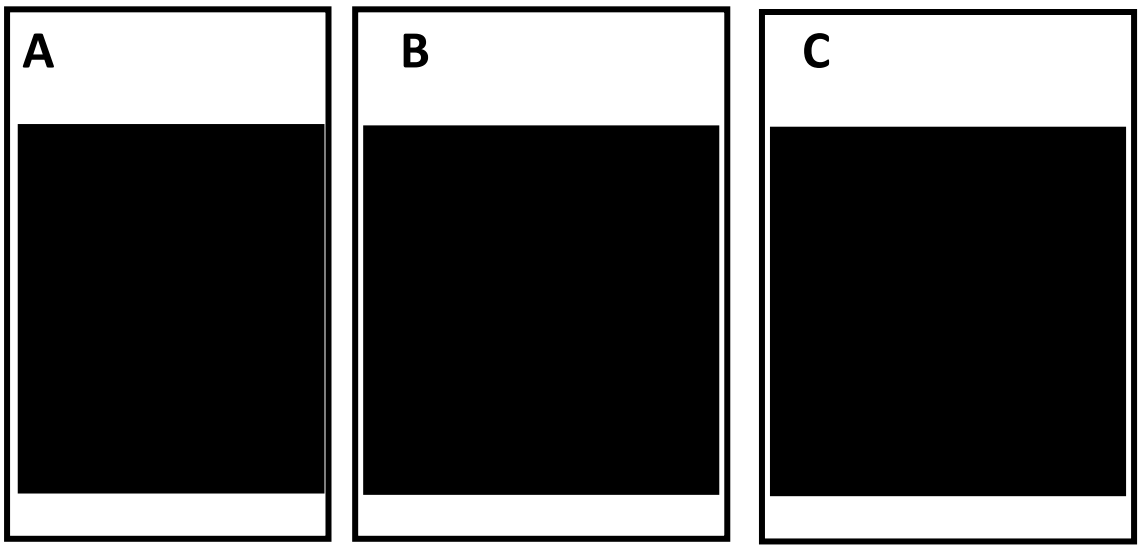


Figure 12. [redacted] ***labelled by*** [redacted]***.***

The three possible outcomes of the assay are represented.

A. [redacted].

B. [redacted].

C. [redacted].

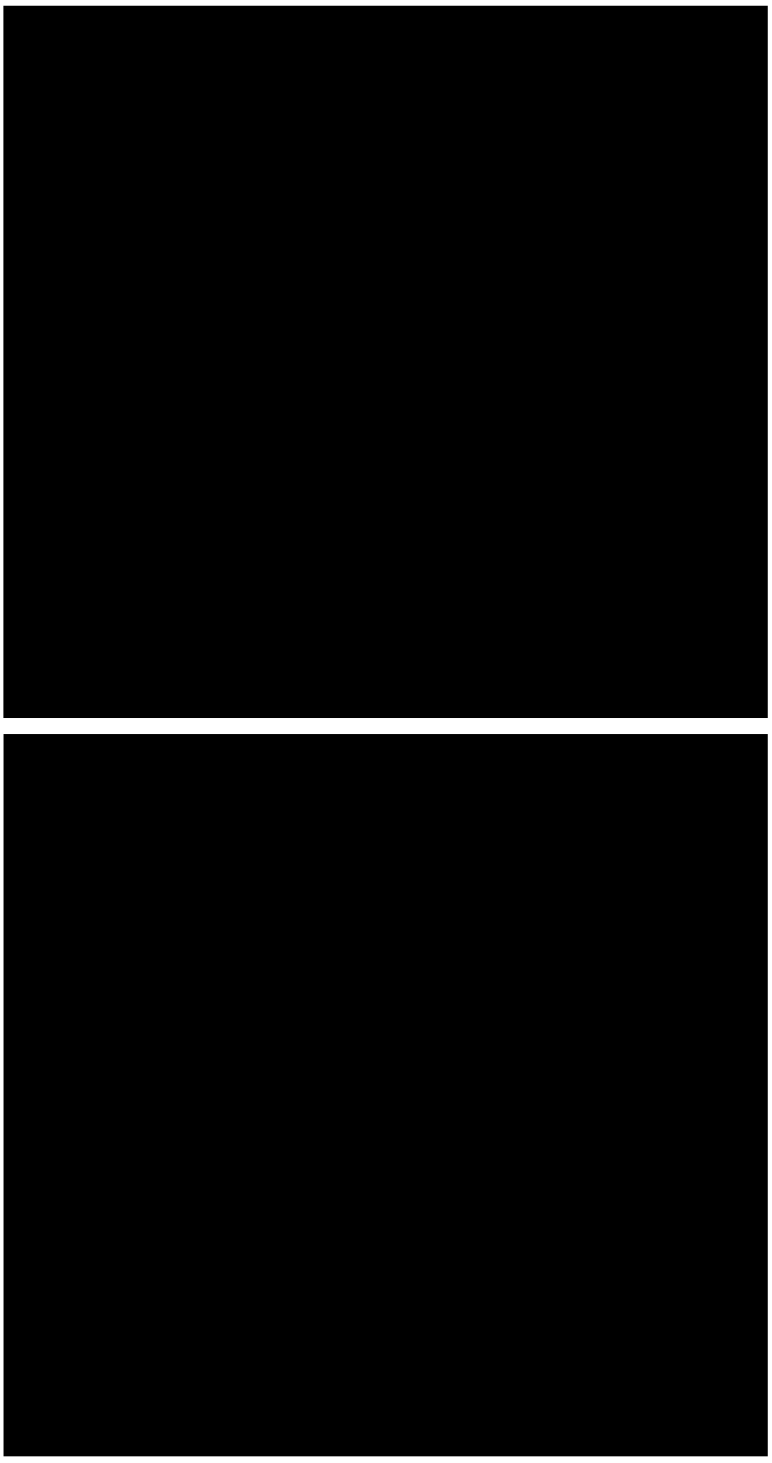


Figure 13. [redacted].

[redacted].

In the control (image on the top) [redacted].

Meanwhile, in the patient (image on the bottom), [redacted].

The cytometry data revealed that, [redacted], [redacted] (Figure 13).

4.5. DNA quantification and quality control with Nanodrop

Once DNA extraction was completed, it was quantified using an advanced spectrophotometer (Nanodrop) that automatically quantifies DNA using the Lambert-Beer equation. We quantified about 45ng/µL from the patient sample and 75ng/µL from the control sample. This disparity is due to we used substantially more cells for the DNA extraction on the control.

Nucleic acids have the maxim absorbance at 260nm, meanwhile maxim protein absorbance is at 280nm. A ratio A260/A280 of 1'8 is generally accepted as "pure" for DNA [19] which is spot one what we obtained (Figure 14-15).

Similarly, absorbance at 230nm is accepted as being the result of other contamination. A ratio A260/230 of 2 or 2.2 is generally accepted as "pure"[19]. In this regard on the patient both measurements were slightly lower, about 1,85-1,95. On the control, the first measurement was 2,6 and the second measurement 1,5. While taking the reading we didn't notice it, however this can be attributed to an improper cleaning of the pedestal.

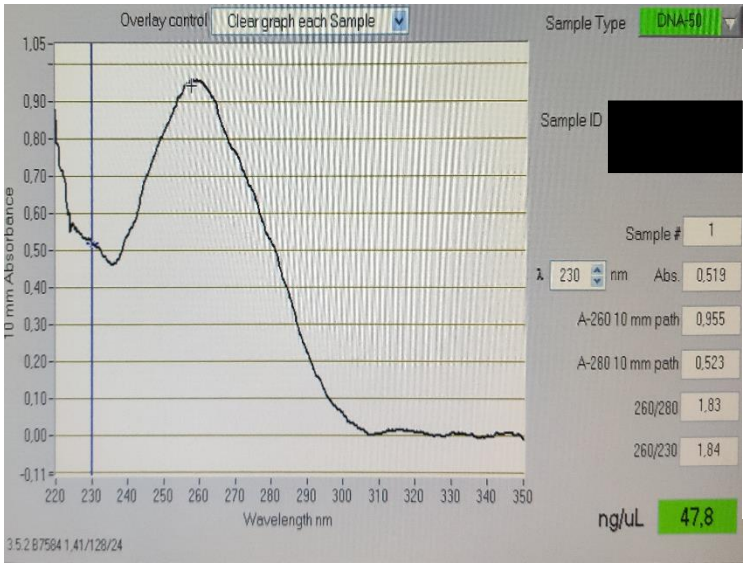


Figure 14. NanoDrop Patient.

We quantified about 45ng/µL on the patient. Moreover 260/280 ratio was perfect (1,8) meanwhile 260/230 was slightly low (~ 1,9).

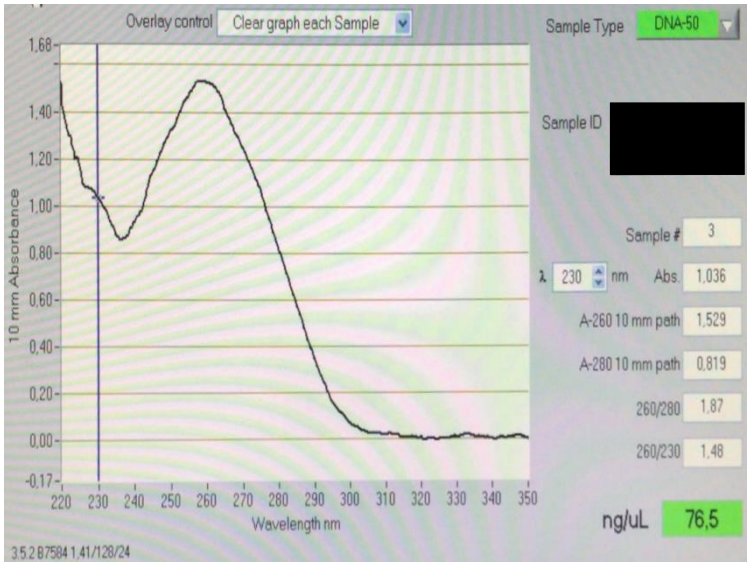


Figure 15. NanoDrop Control.

The sample of the voluntary donor was quantified at about 75ng/µL on the control. Despite 260/230 ratio looks a bit low is important to note that a previous measurement of the sample reported a ratio of 2,6. So we attribute this as an error cleaning the nanodrop between measurements. It must be noted that DNA concentration and 260/280 values were consistent between the measurements (~1,8).

Once the samples were inspected with the Nanodrop, the concentration was adjusted to 40 ng/µL and the samples were brought to the “Translational Genomics” platform in IGTP. There they did a capillary electrophoresis according to section 3.6 to further evaluate the quality.

As reported by them (Figure 16), being B1 our control sample and C1 our patient sample, gDNA integrity (DIN) was 8.2 for the control and 8.3 for the patient. A high DIN indicates highly intact gDNA and a low DIN a strongly degraded gDNA (scale 1-10). So, our gDNA integrity was great.

Regarding quantification, we were aiming for 40 ng/µL. However, on the patient either we diluted in excess our sample or the nanodrop measurement was not accurate due to RNA in the sample because the quantification by capillary electrophoresis was 32 ng/µL.

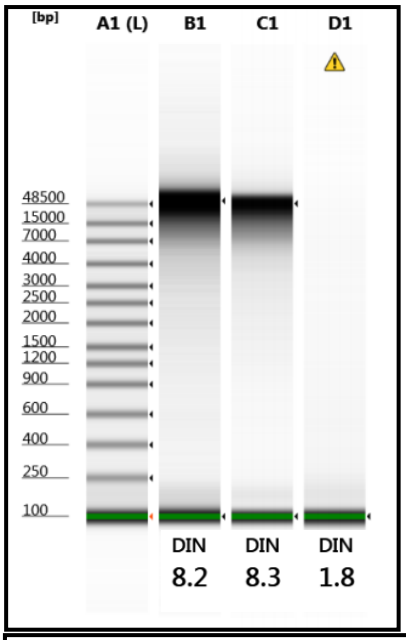


Figure 16. TapeStation 2200, genomic DNA Screen Tape assay results.

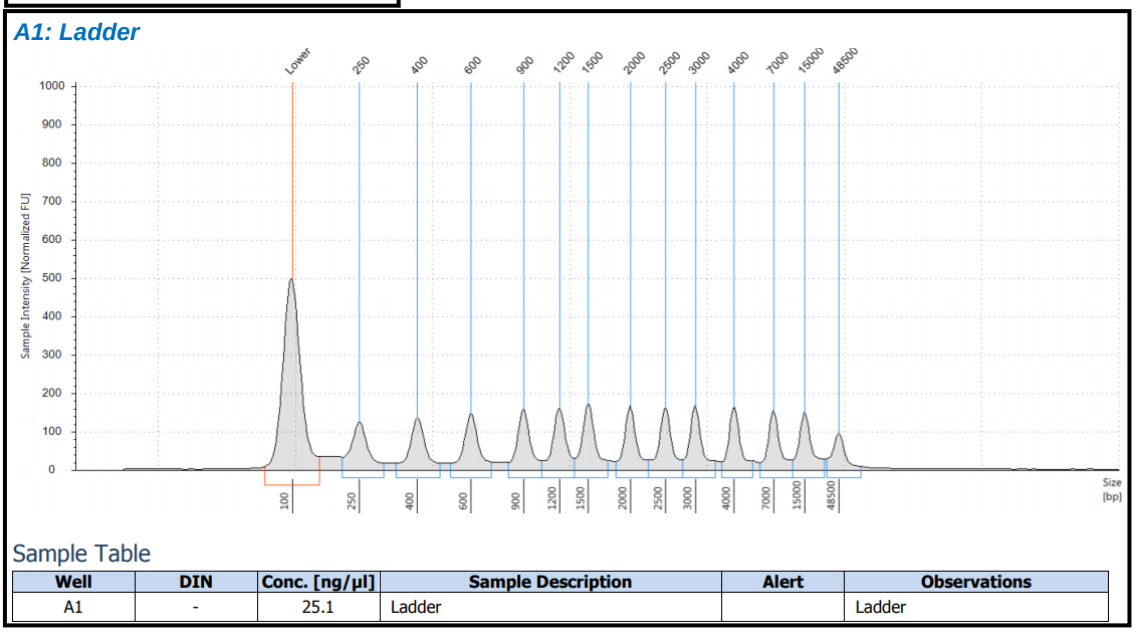
A1. Is the Ladder

B1. Our control sample

C1. Our patient sample

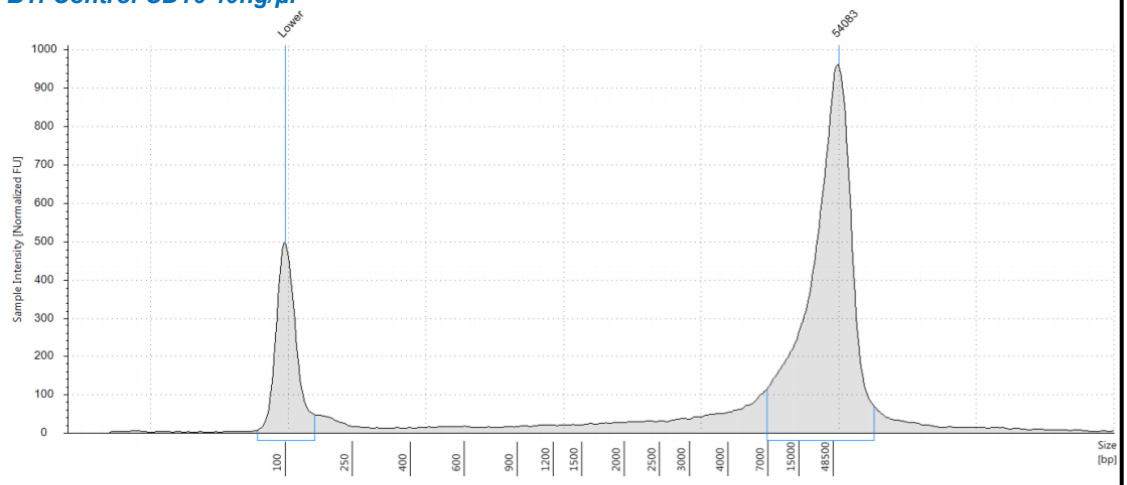
D1. A negative control (H_2O)

We observe good integrity of the extracted gDNA and a final quantification of 32 ng/µL for the patient and 43 ng/µL for the control.



Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
A1	-	25.1	Ladder		Ladder

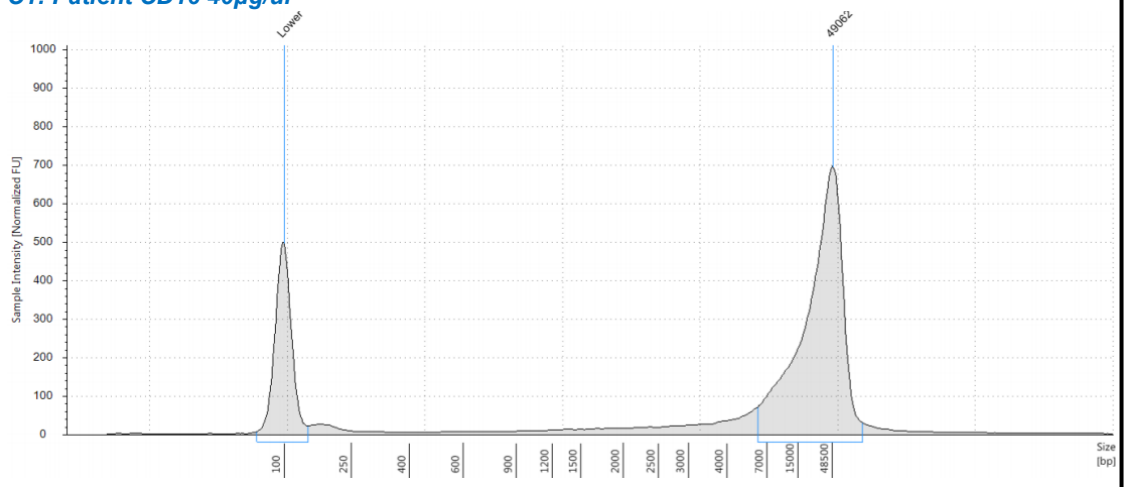
B1: Control CD16 40ng/µl



Sample Table

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
B1	8.2	42.8	[redacted]		

C1: Patient CD16 40µg/ul



Sample Table

Well	DIN	Conc. [ng/µl]	Sample Description	Alert	Observations
C1	8.3	32.4	[redacted]		

4.6. Amplicon control and sequencing

At the time of writing these steps are programmed but not yet conducted, I waited as long as possible but at the end it couldn't be made on time for this assay. However, [redacted], though we cannot secure this until the results of sequencing come back.

5. Conclusions

The results obtained in this study suggest [redacted].

[redacted]. However, this result needs to be confirmed by the sequencing [redacted].

Despite all this, study should be deepen in order to elucidate further points:

In the first place, [redacted] assay, despite we are short on samples of the patient and [redacted] [20,21]. [redacted].

In the second place, [redacted]. [redacted] [22]. [redacted].

In the third place, [redacted].

Finally, [redacted].

References

1. [redacted]
2. [redacted]
3. [redacted]
4. [redacted]
5. [redacted]
6. [redacted]
7. [redacted]
8. [redacted]
9. [redacted]
10. [redacted]
11. [redacted]
12. [redacted]

13. [redacted]

14. [redacted]

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19. Thermo Fisher Scientific, Matlock B. *Assessment of Nucleic Acid Purity*. Accessed June 2, 2021. www.thermoscientific.com

20. [redacted]

21. [redacted]

22. [redacted]

23. [redacted]

[redacted]

24. [redacted]

Self-evaluation

First, I would like to express how satisfied I am to have been able to carry out this project. I really felt like I was the lead researcher, investigating by myself, reading previous reports, making and presenting hypothesis to my supervisors, taking decisions and carrying out the different assays. That made me feel I was being trusted, respected and valued. But the best part, above all, was that I had been given the opportunity to put in practice the knowledge and skill acquired in the degree.

Secondly, this project has helped me to be more organize and to communicate better. It also has helped me to learn a lot about immunology and flow cytometry. Accordingly, my laboratory skills improved a lot as a result of being able to practice a lot of different technics.

Thirdly, It has been amazing to have “My project”. This is the way I felt it, like “My project”, I discovered it and I was in charge of conducting the research and realizing the appropriate assays. At the end, when sequencing and [redacted] assay that we intend to do are done, we intend to publish it. This makes this experience even more thrilling. I can’t wait for having the sequencing results and finally getting to know what’s the deal.

Fourthly, on a sad note, it’s a pity that we couldn’t do the sequencing in time for this work. But, we suffered a nearly 5-month delay from the primer ordering to delivery. It’s understandable that due to covid the supply chains where broken, but it was frustrating anyways. Primers were ordered late December 2020 and they were delivered late April - early May 2021.

To conclude, my whole internship and this project have been amazing, and it helped me to see how I wanted follow up with learning helping me decide which master I wanted to study.

Annex

5.1. Supplementary figure 1

Supplementary figure 1. **.**

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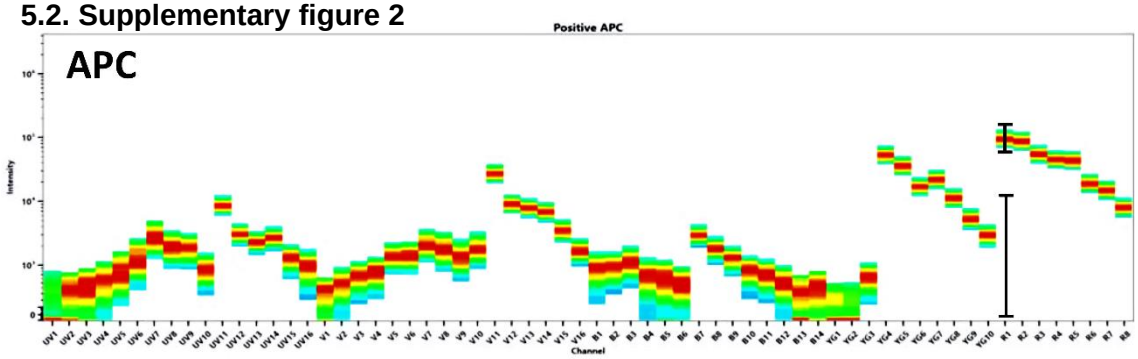
5.2. Supplementary figure 2



Supplemental figure 2. Example of APC fluorophore unique spectral signature. *Obtained from cytek spectral references.*

In conventional flow cytometry it would be read with a single detector at 660nm and the emission at other wavelength (spill over) would need to be corrected with compensation. In spectral flow cytometry, the full spectrum is detected, analysed and isolated from the rest of spectrums.

5.3. Protocol 1.

A. Thawing cryopreserved cells for immunophenotyping

1. Prepare 15mL of complete RPMI medium (10%FBS, 2% Penicillin and streptomycin and 1% L-Glutamine). Place 10mL on a 15mL falcon and 5 on another 15mL falcon.
2. Place the prepared medium on a 37ºC water bath.
3. Grab the cryovials that contain frozen cells from the liquid nitrogen tank and transport them in a small EPS container with dry ice.
4. Using tweezers partially submerge the cryovial on a 37ºC water bath and thaw for 60-120 seconds.
5. Slowly (dropwise) add 1mL of warmed (37ºC) complete RPMI medium against the side of the cryovial (If a resting has to be performed the following steps have to be performed inside a Biosafety cabinet).
6. Pour the cryovial contents to a falcon containing 5mL of warmed complete RPMI medium.
7. Rinse the cryovial with warmed complete RPMI twice and pour it to the cell mixture.
8. Incubate the cells for 5 minutes in the 37ºC water bath.
9. Centrifuge the cells for 10 minutes at 350G.
10. Remove the supernatant.
11. Resuspend the pellet with a final volume of 1mL of complete RPMI medium. Proceed with section B.

B. Viability staining and counting

1. Reverse pipet 20 µL of the cell suspension and place it in a cytometry tube.
2. Add 40 µL of Annexin Bufer, 2,3 µL of Annexin and 2,3 µL of 7-AAD
3. Incubate for 20 minutes in darkness at 4ºC
4. Add 100 µL of PBS 1X
5. Reverse pipet 20 µL of Beads (Perfect-count microspheres)
6. Collect using BD FACS Canto II cytometer
7. Calculate according to the collected results

$$Nº\ viable\ cells = \frac{Nº\ viable\ events \cdot Final\ volume\ of\ the\ sample \cdot Beads\ factor}{Nº\ Beads}$$

5.4. Protocol 2

1. Add the appropriate amount of cells per tube (Stain $1 \cdot 10^6$;Unstain $0{,}5 \cdot 10^6$)
2. Add 3ml of FACS Flow (stored in darkness) to the Stain tube. Add 3ml of Stain Buffer to the Unstain tube.
3. Centrifuge the stain tube at 400g for 5minutes
4. Decant the supernatant withowt disturbing the pellet (final volume ± 50 µL)
5. Add X µL of general mix (Stain tube). Mix well by pipetting
6. Incubate for 20 min at RT in the dark.
7. Add 3mL of FACSFlow (stored in darknes)
8. Vortex for 5 seconds
9. Centrifuge at 400g 5 minutes Stain and Unstain tubes
10. Decant the supernatant
11. Add 300 µL of PFA 1% in PBS. Mix by pippeting
12. Incubate for 10 min at RT protected from light
13. Repeat wash steps 7-10
14. Add FACSFlow stored in darkness for a final volume of 150 µL.
15. Vortex and verify if aggregates are present in suspension
16. Store protected from light until ready to run in spectral cytometer

5.5. Protocol 3

Reference protocol from Qiagen DNeasy Blood & Tissue Kit.

1. Centrifuge the appropriate number of cells for 5 min at 300 x g
2. Resuspend the pellet in 200 µL PBS. Add 20 µL Proteinase K
3. Add 200 µL Buffer AL. Mix thoroughly by vortexing, and incubate at 56ºC for 10 min
4. Add 200 µL ethanol (96-100%) to the sample, and mix thoroughly by vortexing
5. Pipet the mixture into the DNeasy Mini spin column placed in a 2mL collection tube.
6. Centrifuge at 6000g for 1 min and discard flow-through and collection tube
7. Place the DNeasy Mini spin column in a new 2mL collection tube and add 500 µL of Buffer AW1.
8. Centrifuge at 6000g for 1 min and discard flow-through and collection tube
9. Place the DNeasy Mini spin column in a new 2mL collection tube and add 500 µL of Buffer AW2.
10. Centrifuge at 20000g for 3 min and discard flow-through
11. Centrifuge again at 20000g for 1 min and discard flow-through and collection tube
12. Place the DNeasy Mini spin column in a clean microcentrifuge tube. Add 200 µL of Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000g to elute the DNA.