

UNIVERSIDAD CARLOS III
BIOMEDICAL ENGINEERING

TISSUE ENGINEERING
TEACHER : MRS. SARA HERRAIZ GIL

Biomolecular characterization of tissues

Student :
Sophie OMS



Contents

1 Abstract	3
2 Introduction	3
3 Objectives	3
4 RNA extraction	3
5 Reverse transcription of RNA to form cDNA	6
6 DNA extraction	8
7 Polymerase Chain Reaction	12
8 Gel electrophoresis	17
9 Conclusion	28
10 Acknowledgments	28
11 References	28

List of Figures

1	Biomolecular characterization today	3
2	Summary of the strategy for RNA extraction	5
3	RNA solution (three phases)	6
4	RNA concentration after extraction in $ng/\mu l$	6
5	Steps leading to cDNA synthesis	7
6	Strategy to extract DNA	9
7	DNA extraction set-up	11
8	Buccal cell DNA samples	11
9	Centrifuge containing the DNA samples	11
10	Elements needed for a PCR reaction	12
11	Tubes containing the buccal cells extracts	15
12	DNA samples in the centrifuge	15
13	DNA samples in the incubator	16
14	Absorbance results for the samples	16
15	Balance	18
16	Trays for the gel	19
17	Loaded tray	19
18	Electrophoresis chamber	20
19	Gel appearance after migration	20
20	Gel in the UV transilluminator	20
21	Electrophoresis	21
22	RNA results A	21
23	Gel obtained for the GFP and the FN	22
24	DNA migration results	22
25	Fibroblast migration results	22
26	Distances migrated (d_image) for each band of the marker, knowing their size in bp	23
27	Distance measurements for RNA	23
28	Curve of the migrated distance depending on the log(size) for the black electrophoresis	23
29	Curve of the migrated distance depending on the log(size) for the gray electrophoresis	24
30	Deduction of the size depending on the migrated distance by the band for the black electrophoresis	24
31	Deduction of the size depending on the migrated distance by the band for the gray electrophoresis	25
32	VNTR according to the amplicon size	25
33	Classification of the results: homozygous or heterozygous	25
34	Deduction of the amplicons size from the migrated distance	26
35	Sequence of the GADPH gene pasted from NCBI database	26
36	Primers of the GADPH gene	27
37	Sequence of the GFP gene	27
38	Primers of the GFP gene	27

1 Abstract

2 Introduction

Deoxyribonucleic acid (DNA) is the molecule that encodes the genetic instructions used in the development and functioning of all known living organisms. It stores this information in the nucleus of all the cells of an organism.

Meanwhile, ribonucleic acid (RNA) is involved mainly in the regulation and expression of genes. Therefore, DNA is the same in all the cells of an organism, whilst the RNA profile varies from one cell to another, and from one condition to another.

In order to fully understand the various states of an organism we have to consider both the general, individual-related information (DNA) as well as the more specific, tissue and environment related information (RNA).

Here we are going to learn some of the basic biomolecular characterization techniques that help us understand the variations between individuals as well as the differences in various conditions (or various tissues) within one individual.

3 Objectives

- Understand and manage basic biomolecular techniques that are used for the characterization of tissues and cell cultures.
- Characterize the expression of GFP gene in a human fibroblast cell line.
- Isolation, amplification and characterization of your own D1S80 variable number tandem repeat (VNTR) locus



Figure 1: Biomolecular characterization today

4 RNA extraction

Introduction

Human fibroblasts play an important role in maintaining the structural integrity of connective tissue and in the synthesis of extracellular matrix proteins such as collagens, and glycoproteins. After tissue injury, fibroblasts are stimulated to produce wound healing proteins.

On the other hand, the green fluorescent protein (GFP) is composed of 238 aminoacid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range.

In cell and molecular biology, the GFP gene is frequently used as a reporter of expression. It can be introduced into animals or other species through genetic engineering techniques, and maintained in their genome and that of their offspring.

In order to verify the expression of the GFP in the fibroblast cell line, the first step is the extraction of the RNA in both the modified cultured cells (Fibros GFP) and the normal ones, as a negative control of the experiments (Fibros FN). The process of RNA extraction is complicated due to the ubiquitous presence of RNase enzymes in cells and tissues, which can rapidly degrade RNA. To prevent this degradation, equipment used for RNA extraction is usually cleaned thoroughly with ethanol, kept separate from common lab equipment and treated with various chemicals that destroy RNases.

Objective

Extract the RNA from a Fibros GFP and a Fibros FN in a Petri dish.

Materials

- 1,5 mL and 2 mL sterile eppendorfs
- Micropipettes and filtered pipette tip boxes
- Isopropanol
- PBS 1x
- Trizol
- Chloroform
- 70 percent Ethanol
- Distilled water
- Personal protective equipment (sterile gloves, laboratory coat)
- Thermoblock
- Microcentrifuge
- Vacuum system

Methods

At the beginning, there are two Petri dishes: one is labelled FH and contains normal fibroblasts sample, and the other is labelled FH-GFP and contains fibroblasts injected with a Green Fluorescence Protein.

In the fumehood of the tissue lab:

- Remove the DMEM solution by vacuum
- Add 3 mL of PBS, move around the dish and remove the PBS by vacuum.
- Add 750 μ L of Tryzol
- Scrape with a loop
- Collect the trizol with the cells in solution in a 2 mL Eppendorf tube and put it in ice for 5 min
- Add 150 μ L of chloroform
- Vortex for 15 minutes

In the molecular biology lab:

- Incubate for 3 minutes
- Centrifuge for 15 minutes at 12000 rotations/minute and 4°C
- Get the upper phase (that contains RNA) in a new tube
- Add 375 μ L of isopropanol
- Vortex then incubate at 4°C for 5 minutes
- Centrifuge 10 minutes at 14000 rotations/minute and 4°C
- Discard supernatant
- Add 500 μ L of EtOH 70 percent
- Wash the pellet
- Centrifuge 5 minutes at 14000 rotations/minute and 4°C
- Remove EtOH
- Dry the pellet at 37°C for 15 minutes
- Add 30 μ L of H2O

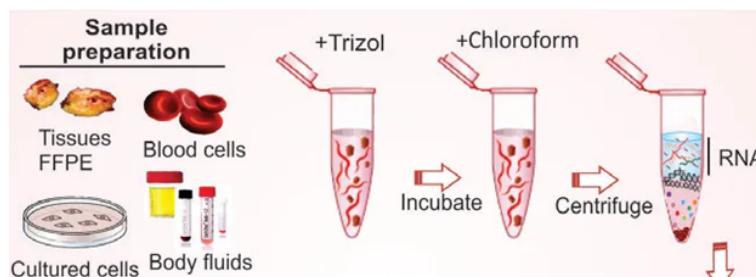


Figure 2: Summary of the strategy for RNA extraction

Results

After some time in the centrifuge, the final solution presents 3 phases : the upper phase is the aqueous phase, which has a lower density than the others and which contains RNA.

Below it, the white inter-phase phase can be found; it contains DNA. Below is the organic phase, which contains proteins and lipids.

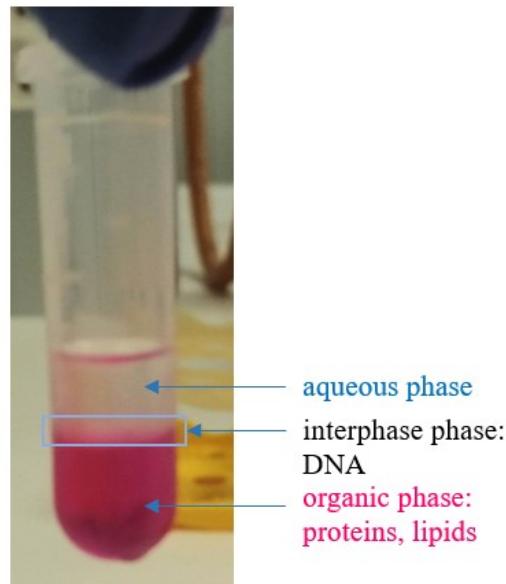


Figure 3: RNA solution (three phases)

We only keep the upper phase.

Sample	[RNA]
CATA FH	557.92
RAA FH GFP	616.12
MARCOS STM GFP	116.68
SO GFP	151.4

Figure 4: RNA concentration after extraction in $ng/\mu l$

For the Fibros GFP sample, the obtained concentration of RNA amounted to $151.4 \text{ ng}/\mu \text{l}$.

5 Reverse transcription of RNA to form cDNA

Introduction

In order to have double stranded DNA for the PCR step, we must perform a reverse transcription, as illustrated below.

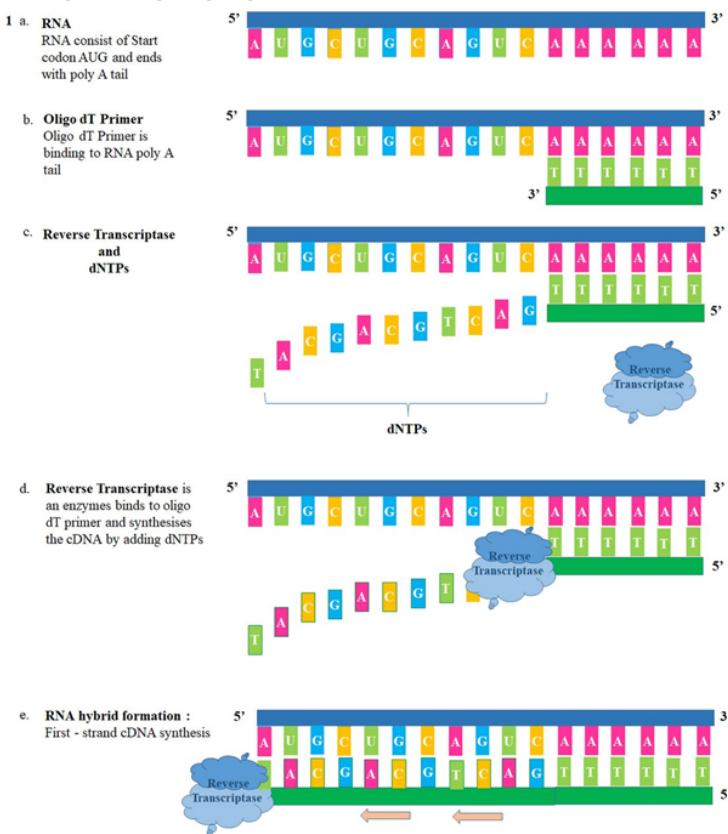


Figure 5: Steps leading to cDNA synthesis

In this scheme, we observe that the third step (c) is **reverse transcription**: complementary nucleotides of the RNA strand are produced. dNTP stands for deoxyribose nucleotide triphosphate : it is employed in PCR to expand the growing DNA strand.

The RT-PCR, reverse transcription reaction together with polymerase chain reaction (RT-PCR) is a technique commonly used in molecular biology to detect RNA expression. In RT-PCR, the RNA template is first converted into a complementary DNA (cDNA) using an enzyme called reverse transcriptase.

cDNA is DNA synthesized from a messenger RNA (mRNA) template in a reaction catalyzed by the enzyme reverse transcriptase. Apart from the analysis of RNA expression, the cDNA has other applications, like the cloning of eukaryotic genes in prokaryotes, or the expression of a specific protein in a cell that does not normally express that protein (heterologous expression).

Objective

Perform a reverse transcription for both RNA extracts (Fibros FN and Fibros GFP samples).

Materials

- 1,5 ml sterile eppendorfs and 200 µL PCR tubes

- Micropipettes and filtered pipette tip boxes
- Marker pen
- NZYRT Enzyme Mix, NZYRT 2x Master Mix and NZY RNase H (E. coli)
- DEPC-treated H₂O
- Personal protective equipment (sterile gloves, laboratory coat)
- Thermoblock
- Thermalcycler

Methods

1. On ice, add the following reaction components into a sterile, nuclease-free micro-centrifuge tube (for multiple reactions a Master Mix without the RNA may be prepared). Prepare all the reagents except RNA in one tube, and mix. After, divide the mixture in the necessary tubes, and add the appropriate amount of RNA to each tube: NZYRT 2X Master Mix 10 µL, NZYRT Enzyme Mix 2 µL, RNA (up to 5 µg) N µL, DEPC treated water Up to final volume 20 µL
2. In the thermocycler, mix gently and incubate at 25°C for 10 minutes
3. Incubate at 50°C for 30 minutes
4. Inactivate the reaction by heating at 98°C for 5 minutes, and then chill on ice
5. Add 1 µL of NZY RNase H and incubate at 37°C for 20 minutes
6. Store the cDNA in the fridge (4°C) until PCR is performed.

Here is the calculus to determine the quantity N of RNA from Fibros FN and Fibros GFP that we must add:

$$N(FH) = 5.10 - 6 * 1.10 - 6 / 702.88 * 10 - 9N = 7\mu L$$

$$N(GFP) = 5.10 - 6 * 1.10 - 6 / 151.4 * 10 - 9 = 33\mu L$$

6 DNA extraction

Introduction

To set up a different PCR reaction that is specific for the D1S80 locus within the human genome, we will use our buccal cell DNA. This locus is polymorphic (highly variable) within the human population.

The variability at D1S80 is caused by the presence of Variable Numbers of Tandem Repeats (VNTRs), which are polymorphic DNA sequences composed of different numbers of a repeated “core” DNA sequence arranged in tandem. The size of the core sequence can vary from 7 to 100 bp in different VNTRs.

The number of repeats present at a VNTR locus can also vary widely depending on which allele you have inherited. The D1S80 locus is located on chromosome 1, the largest human

chromosome, and the repeating sequence at D1S80 is 16 bp in length, repeated in tandem from 14 to 41 times ranging in size from 300 to 801 bp respectively.

Each of us has two copies of the D1S80 locus, one inherited from each of our parents. Approximately 86 percents of the population is heterozygous at this particular locus because a different VNTR allele has been inherited from each parent. PCR is so powerful that it is possible to derive enough DNA for amplification and analysis from your own oral mucosa cells. After collecting and lysing cheek cells, separation of DNA from other cell components (proteins, fats and carbohydrates) and precipitation will be carried out. These DNA molecules may be stored for short times at -20° C or at -70° C for long-term storage.

The procedure begins with genomic DNA extraction from your oral mucosa cells and DNA precipitation as floating white strands in solution. The DNA strands are then easily collected and transferred to a glass vial.

Objective Extract DNA from buccal cells.

Materials

- 1,5 mL and 2 mL sterile eppendorffs tubes
- Micropipettes and filter pipette tip boxes
- DNA lysis buffer
- 50mM Tris-HCl pH 8, 100 mM EDTA pH 8, 100 mM NaCl, 1X SDS
- Saline solution: 0,9 percent NaCl, NaCl 5M
- Proteinase K (20 mg/ml)
- 96 and 70 percent EtOH
- Distilled water
- Personal protective equipment (sterile gloves, laboratory coat)
- Thermoblock
- Microcentrifuge
- Vacuum system

Methods



Figure 6: Strategy to extract DNA

- Rinse mouth with a saline solution to obtain mucosa cells for DNA extraction

- Expel 3 times into a 50 ml conical tube.
- Transfer all the volume into 6 eppendorf tubes (2ml) with the same volume.
- Centrifuge at 13.000rpm for 2 minutes at 4ºC.
- Remove the supernatant by vacuum aspiration.
- Add 600 µL of lysis buffer to one cell pellet and recollect all the pellets in the same 2 ml tube
- Add 17,5 µL of proteinase K to your sample.Add 17,5 µL of proteinase K to your sample.
- Incubate at 65ºC for 10 minutes to digest all cellular proteins.
- Add 167 µl of NaCl 5M and gently invert tube to rupture cell and nuclear membranes.
- Centrifuge the tube at 13.000rpm for 10 minutes at 4ºC.
- Transfer the supernatant to a new 1,5-ml eppendorf tube using a micropipette and keep the tube on ice.
- Add 600 µl of ice-cold ethanol 96 percent and mix by inversion.
- Let the tube for 5 minutes on ice: the DNA precipitates.
- Centrifuge the tube at 13.000 rpm for 10 minutes at 4ºC.
- Remove the supernatant using a micropipette and add 700 µl of ice-cold ethanol 70 percent to wash the DNA.
- Centrifuge the tube at 13.000 rpm for 10 minutes at 4ºC.
- Remove supernatant using a micropipette.
- Dry the DNA at 37°C in the thermoblock for 10 min to evaporate residual ethanol.
- Re-suspend the pellet on 100 µl of distilled water.
- Let dissolve DNA over night at room temperature.

Results

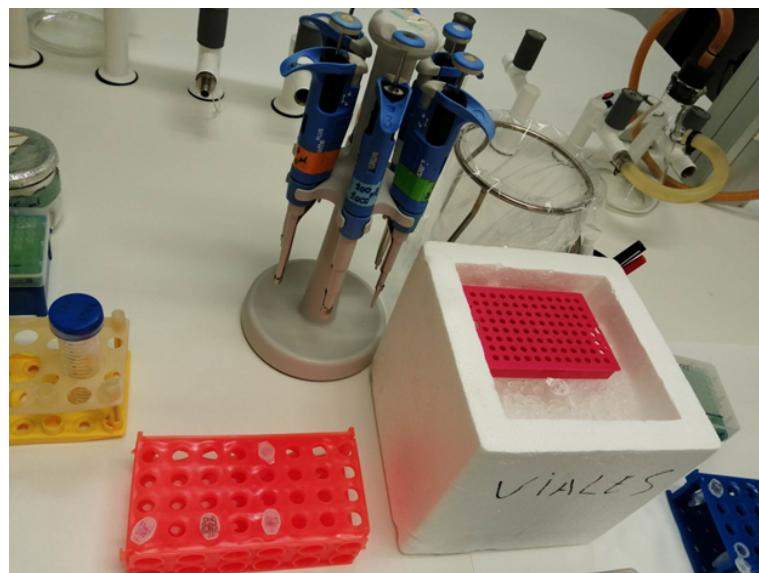


Figure 7: DNA extraction set-up

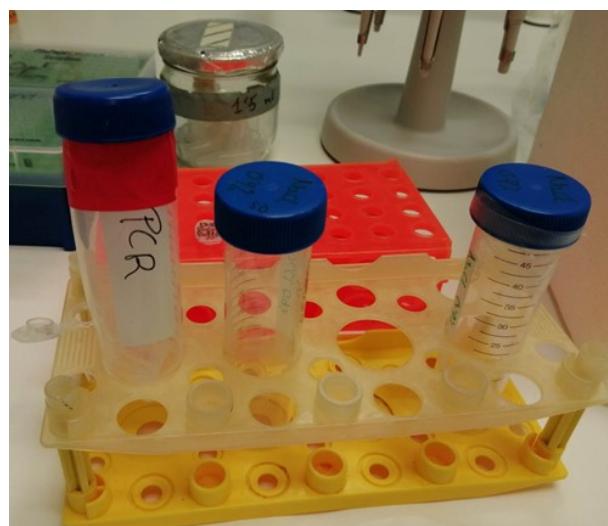


Figure 8: Buccal cell DNA samples

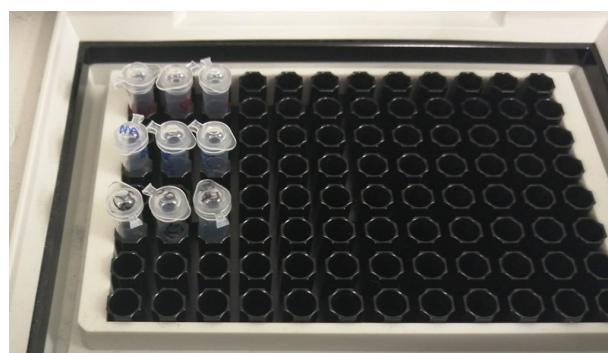


Figure 9: Centrifuge containing the DNA samples

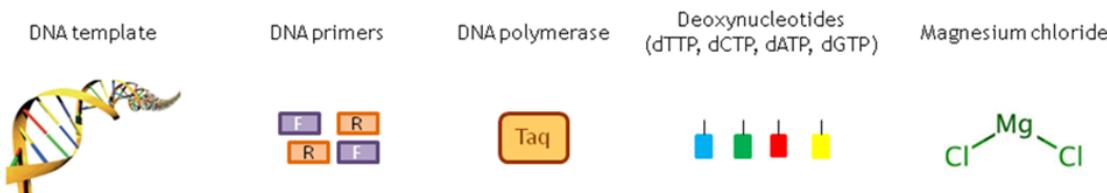


Figure 10: Elements needed for a PCR reaction

7 Polymerase Chain Reaction

Introduction

Polarized by the Covid detection, the polymerase chain reaction (PCR) is a relatively simple and inexpensive tool that allows to synthesize a defined region of DNA from a minute amount (as low as one molecule) to yield quantities sufficient for detailed analysis. This technique has become widely used not only for several medical applications, including genetic diagnosis, forensics and detection of infectious organisms, but also for innumerable basic research applications.

It is necessary to have a sample of DNA containing the segment you wish to amplify (here the D1S80 locus). This DNA is called the template because it provides the pattern of base sequence to be duplicated during the PCR process.

Along with template DNA, PCR requires two short singlestranded pieces of DNA called primers. These are usually about 20 bases in length and are complementary to opposite strands of the template at the ends of the target DNA segment being amplified.

Primers attach (during annealing phase) to their complementary sites on the template and are used as initiation sites for synthesis of new DNA strands. Deoxynucleotides containing the bases A, C, G, and T are also added to the reaction. The enzyme DNA polymerase binds to one end of each annealed primer and strings the deoxynucleotides together to form new DNA chains complementary to the template.

The DNA polymerase enzyme absolutely requires the metal ion magnesium (Mg⁺⁺) for its activity. It is supplied to the reaction in the form of MgCl₂ salt. A buffer is used to maintain an optimal pH level.

MgCl₂ is an important cofactor, because the RNA polymerase is not working without it.

Tube 1 : amplified GHDP-F Tube 2: amplify GFP-F Tube 3: negative control to check that no contamination occurred.

The control tube, labelled C-, serves as negative control to check the absence of contamination.

PCR procedure PCR is accomplished by cycling a reaction through several temperature steps. In the first step, the two strands of the template DNA molecule are separated by exposure to a high temperature (usually 94° to 96°C). This process is called DNA denaturing or melting. Once in a single-stranded form, the bases of the template DNA are exposed and are free to interact with the primers.

In the second step of PCR, called annealing, the reaction is brought down to a temperature usually between 50°C to 65 °C. At this lower temperature, stable hydrogen bonds can form between the complementary bases of the primers and template. Although human genomic DNA is billions of base pairs in length, the primers require only seconds to locate and anneal to their complementary sites.

In the third step of PCR, called **extension**, the reaction temperature is raised to an intermediate level (65°C to 72°C). During this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs to the ends of the annealed primers. These three phases are repeated over and over again, doubling the number of DNA molecules with each cycle. After 25 to 40 cycles, millions of copies of DNA are produced. The PCR process taken through four cycles is illustrated on the below image:

Objective

Perform a PCR.

Materials

- Thin-walled PCR tube
- Micropipettes
- Filter pipette tip boxes
- Ice bucket
- Marker pen
- Buccal cell DNA sample and the cDNA sample from the RNA Fibros GFP and FN extract
- Buffer (10x), 15mM MgCl₂
- Deoxinucleotides (dNTPs) 10mM
- Primers: Forward and reverse D1S80-primer (25 μ M), forward and reverse GFP-primer (25 μ M), Forward and reverse GAPDH-primer (25 μ M)
- Taq DNA polymerase
- Distillated water
- Personal protective equipment (sterile gloves, laboratory coat)
- Thermalcycler

Methods

Procedure for the buccal cell DNA:

1. Measure the DNA concentration and quality, using the spectrophotometer, for a 1/100 DNA dilution in water.
2. Prepare the PCR-mix reaction in a thin-walled PCR tube. Prepare a Mastermix: Calculate the amount of reagents for 2 tubes (negative control and sample) and do a mix with all of them except the DNA.

3. After that pipette in the PCR tubes the amount of mix necessary for each of them, and add the DNA or water for the negative control. Keep the PCR tubes cold in ice.

In each tube there has to be the following:

- dH₂O: 16.2 μ l
- Buffer containing 15mM MgCl₂ (10X): 2.5 μ l
- dNTPs 10mM: 2 μ l
- Forward D1S80-primer (25 μ M): 0.5 μ l
- Reverse D1S80-primer (25 μ M): 0.5 μ l
- Sigma Taq DNA polymerase: 0.3 μ l
- DNA sample (or water in the negative control): 3 μ l

Final volume: 25 μ l

Then put the PCR tubes into the thermalcycler that has been programmed for the following conditions:

1. Denaturation 94°C for 1 min, to bring the samples to the correct temperature
2. 30 cycles at 94°C for 15 sec (**Denaturing**)
3. 68°C for 15 sec (**Annealing**)
4. 72°C for 15 sec (**DNA Synthesis**)
5. Termination: 72°C
6. 10 min 4°C Until sample rescue (The low temperature ensures the stability of the PCR product)

Procedure for the Fibros GFP and FN cDNA

1. Measure the cDNA concentration (diluted 1/100 in water). Dilute the cDNA 1/5 in water for the PCR (approx, depending on the concentration). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is amplified in all samples as a positive control, but can also be used to normalize gene expression for sample-to-sample differences in RNA input, RNA quality and reverse transcription efficiency (what is called a housekeeping gene).
2. Prepare the PCR-mix reaction in a thin-walled PCR tube. Prepare a mastermix.
3. Pipette in the PCR tubes the amount of mix necessary for each of them, and add to each tube the appropriate amount of DNA or the water for the negative control.

Results

PCR consists of denaturing, annealing, and extension. After 30 cycles, there are $2^{30} = 10^9$ copies, so the amplification of the fragments is successful.

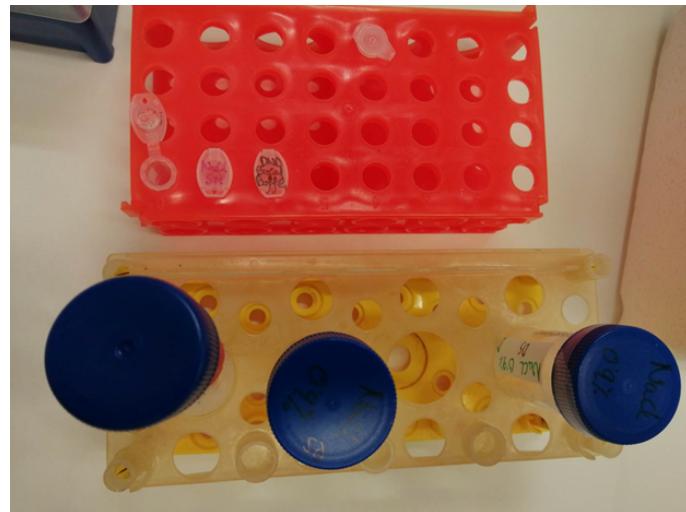


Figure 11: Tubes containing the buccal cells extracts

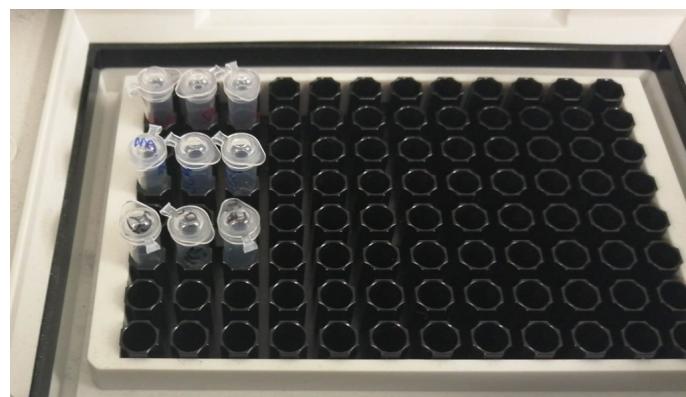


Figure 12: DNA samples in the centrifuge



Figure 13: DNA samples in the incubator

Sample	A ₂₆₀
CIG FH normal	8.377
CIG GFP	24.97
FH Coronado/Corral	11.16
GFP Coronado/Corral	0.564
Fibro Normal AFAM	7.851
GFP AFAM	53.96
INTA FH normal	0.760
INTA GFP	3.281
YL FH	26.08
YL GFP	10.13
PI FH	70.01
PI GFP	3.203
RHINO FH (blue label)	9.35
RHINO (red label)	10.75
FHRF GFP ↓	8.958
FHAf C-	14.02

Figure 14: Absorbance results for the samples

For low concentrations, the absorbance measurement is governed by Beer's Law: $A = \epsilon * l * c$. A is absorbance, ϵ is the molar extinction coefficient, l is the path length, and c is the analyte concentration (here it is the DNA). When the molar coefficient and path length are constant, absorbance is proportional to the concentration of RNA in the cuve. The path length is usually defined as 1 centimeter.

8 Gel electrophoresis

Introduction

Following PCR amplification, alleles are separated according to their size in bp thanks to the agarose gel electrophoresis. After staining with Gel Red, the DNA bands amplified are visible in each lane.

Different DNA fragments appear as distinct bands, each composed of several billion copies of the amplified sequence. A band position on the gel indicates the size of the amplified sequence: smaller sizes move a longer distance from their origin, while larger move a shorter distance.

To determine the sizes of DNA fragments, a marker containing DNAs of known size should be also loaded on the gel. In this case DNA Molecular Weight Marker IX will be used.

Objective

Read the results from the PCR, by evaluating band migrations on the gel electrophoresis.

Materials

- Micropipettes and filter pipette tip boxes
- 1.5 ml sterile eppendorfs and 100 mL flasks
- The amplified DNA fragments.
- Agarose, GelRed
- Tris-acetate-EDTA (TAE) electrophoresis buffer
- 40mM Tris, 20mM acetic acid and 1mM EDTA pH 8
- Loading dye 6X
- 0.25 percent bromophenol blue, 0.25 percent xylene cyanol FF, 30 percent glycerol in water
- DNA Molecular Weight Marker IX
- Personal protective equipment (sterile gloves, laboratory coat)
- Gel electrophoresis chamber
- Power supply
- UV transilluminator

Methods

Preparation of a 1.5 percent agarose gel.

1. Prepare the gel tray with the appropriate combs.
2. Weight the correct amount of agarose for a 50 mL gel. Do it in the fumehood.



Figure 15: Balance

1. Dissolve the agarose in 50 mL TAE buffer. Use the microwave to boil the solution until it is totally clear.
2. Cool the solution using cold water before adding 2 uL of Gel Red solution for each 50mL of TAE.
3. Place the solution in the tray and wait until it jellifies (about 20-30 minutes).

The agarose solution was poured in the tray on the right, then it jellified after some time.

Preparation of the samples to load them in the gel:

1. Transfer 12 μ L of your PCR sample to a 1,5-ml eppendorf.
2. Add 2 ul of loading dye to the PCR sample.
3. Pour TAE electrophoresis buffer into the electrophoresis chamber containing the 1,5 percent agarose gel to level that just covers gel surface.
4. Load 12 μ l of DNA Molecular Weight Marker IX into one lane next to your PCR sample.
5. Carefully load 15 μ l of your sample into assigned well of a 1.5 percent agarose gel.
6. Load by starting at bottom of the well and slowly raising tip during loading process.
7. When all samples are loaded, close the lid on the gel box and connect the electrical leads to the power supply.

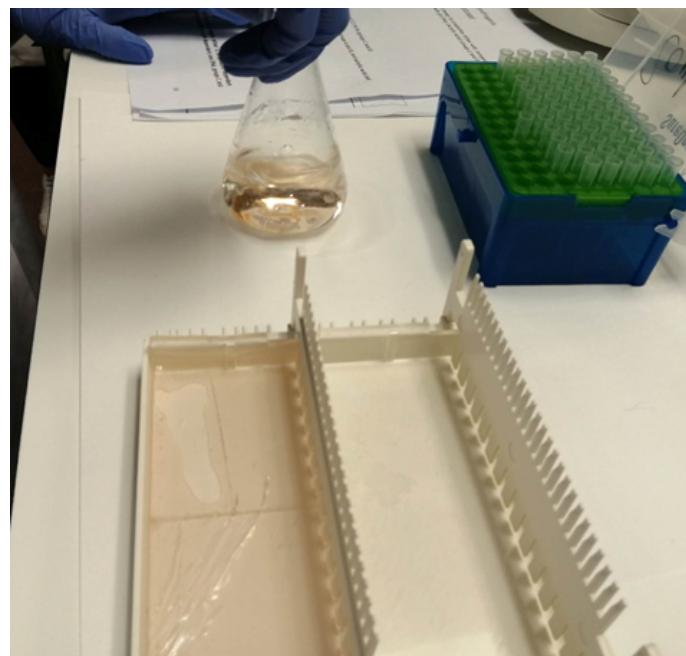


Figure 16: Trays for the gel

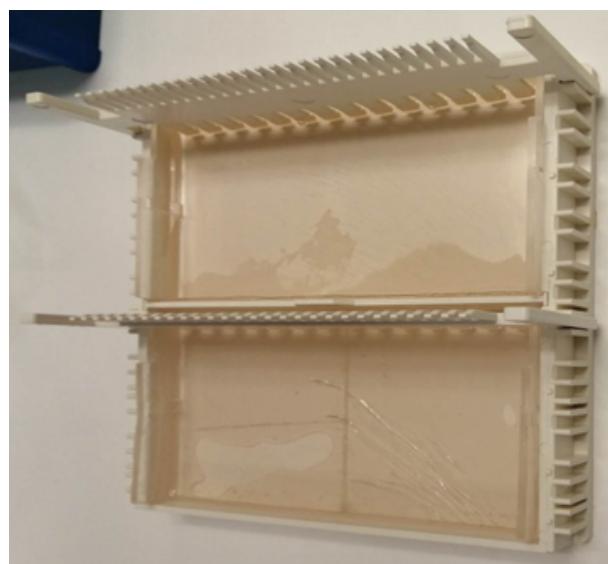


Figure 17: Loaded tray

8. Run the gel at 100 mV until the front dye has migrated close to the gel border (40 minutes).
9. Turn off the power supply, disconnect the electrode leads, and remove the chamber lid.
10. Remove the gel from the electrophoresis chamber and place it on the UV transilluminator.
11. Examine the gel by UV light and take a photograph of your gel.

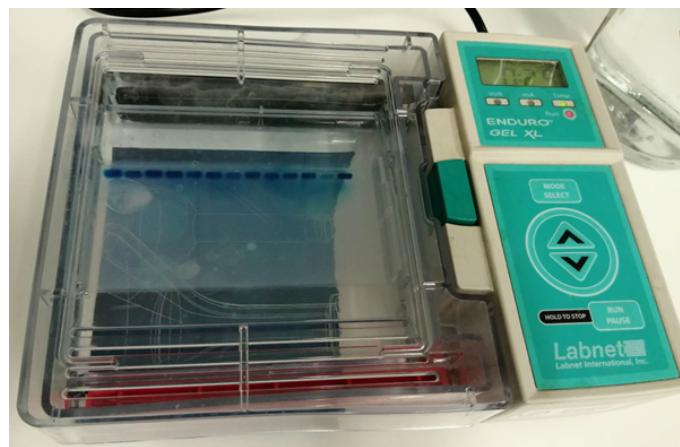


Figure 18: Electrophoresis chamber

Results

Here is the gel electrophoresis obtained: The bands that migrated appear as blue, but to

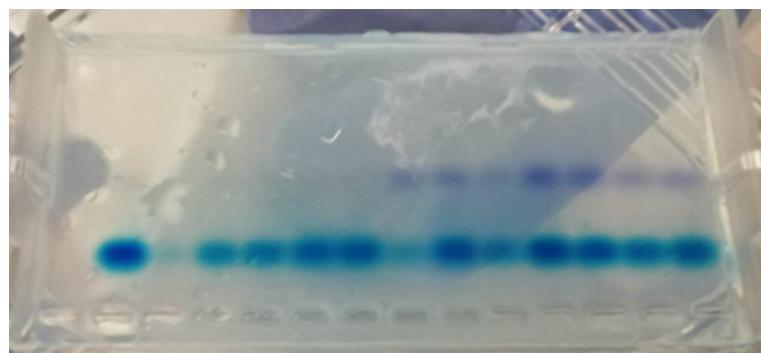


Figure 19: Gel appearance after migration

see them more distinctly, the UV transilluminator is needed.



Figure 20: Gel in the UV transilluminator

Bands can be observed for the marker and for fibroblasts on the right, but unfortunately, no bands can be observed for the buccal cells DNA extracts.

The marker presents several characteristic bands, which are extremely visible.



Figure 21: Electrophoresis

1. Measurements of the distances in mm that all fragments in the molecular weight marker have migrated from the loading well to the center of the band.

Since the DNA did not give exploitable results, I will apply the method to the electrophoresis in the following slides, provided by Mrs Herraiz Gil:

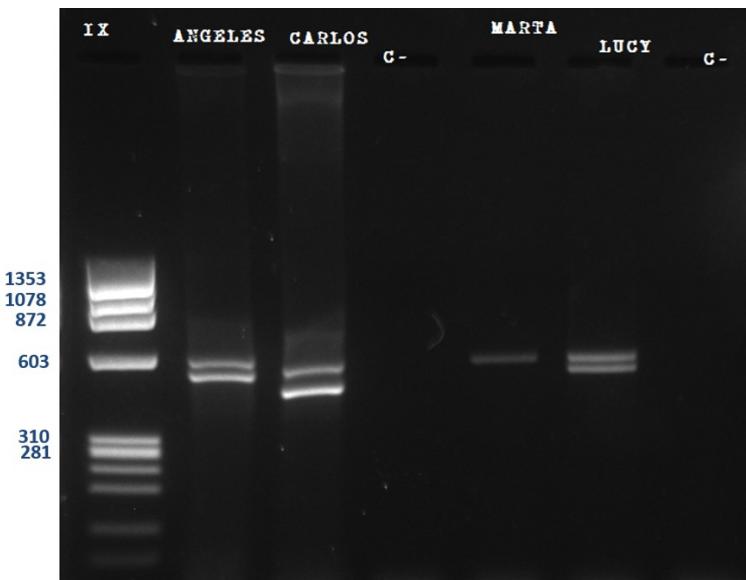


Figure 22: RNA results A

This is the results of an electrophoresis performed to compare diverse DNA amplified fragments for different people: Angeles, Carlos, Marta and Lucy.

IX and M refer to the molecular marker. Ctrl is the FN. GFP stands for the fibroblasts where the Green Fluorescent Protein was added.

The number of bands we see in each of them is an indicator. The FN sample presents only one band.

The GFP presents two bands, one at the same level than FN, and another below.

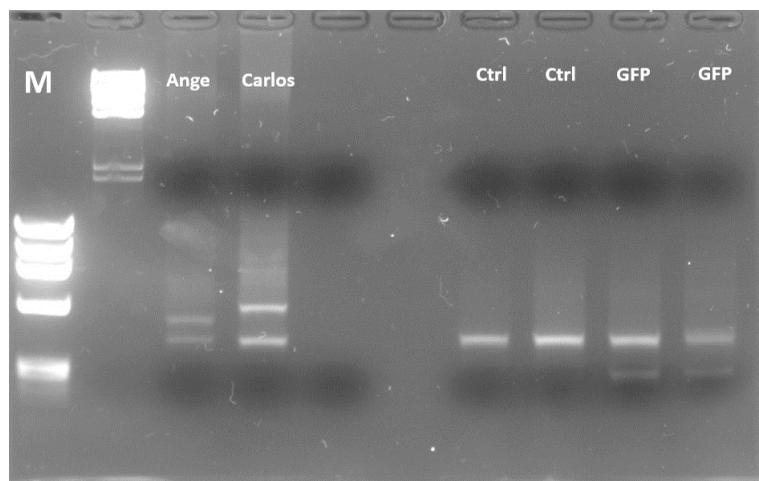


Figure 23: Gel obtained for the GFP and the FN

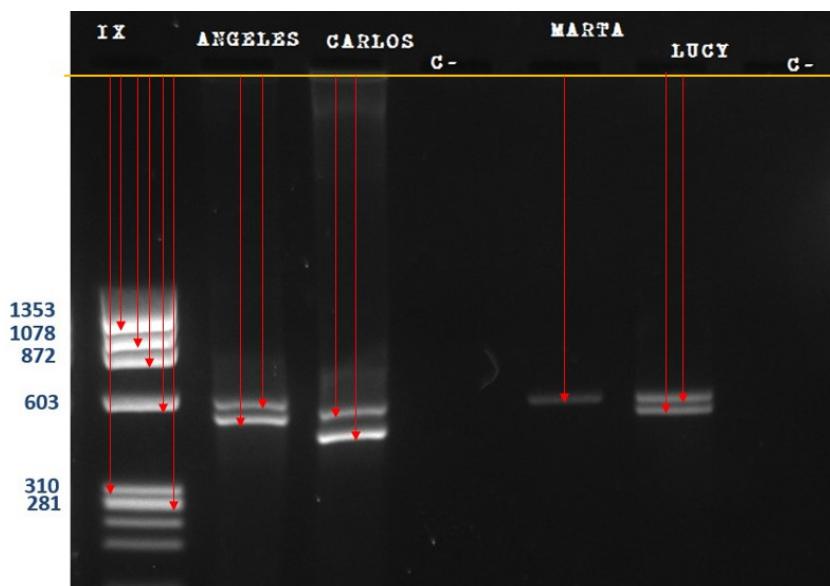


Figure 24: DNA migration results

We draw a yellow line of reference for the band migration.

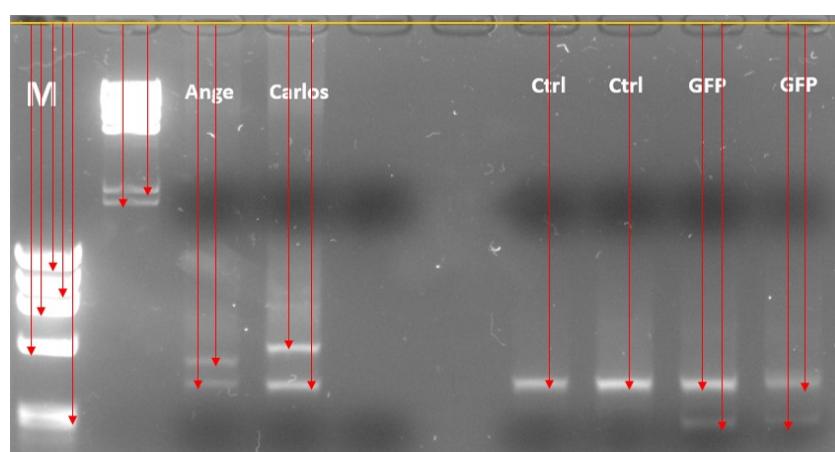


Figure 25: Fibroblast migration results

	X	Y
size (bp)	log(size)	d_image(mm)
1353	3,1312978	56
1078	3,03261876	59,7
872	2,94051648	64,1
603	2,78031731	73,5
310	2,49136169	92
281	2,44870632	95,5

Figure 26: Distances migrated (d_{image}) for each band of the marker, knowing their size in bp

For the gray gel electrophoresis, we infer that the same molecular marker IX was used.

	size(bp)	log(size)	d_image(cm)
M	1353	3,1312978	58,3
	1078	3,03261876	64,5
	872	2,94051648	68,8
	603	2,78031731	77,9
	310	2,49136169	94,4

Figure 27: Distance measurements for RNA

2. Calibration curves representing the distances migrated by the standards fragments (Y) versus the log10 of its size (in base pairs) (X).

$$d = f(\log(\text{size}))$$

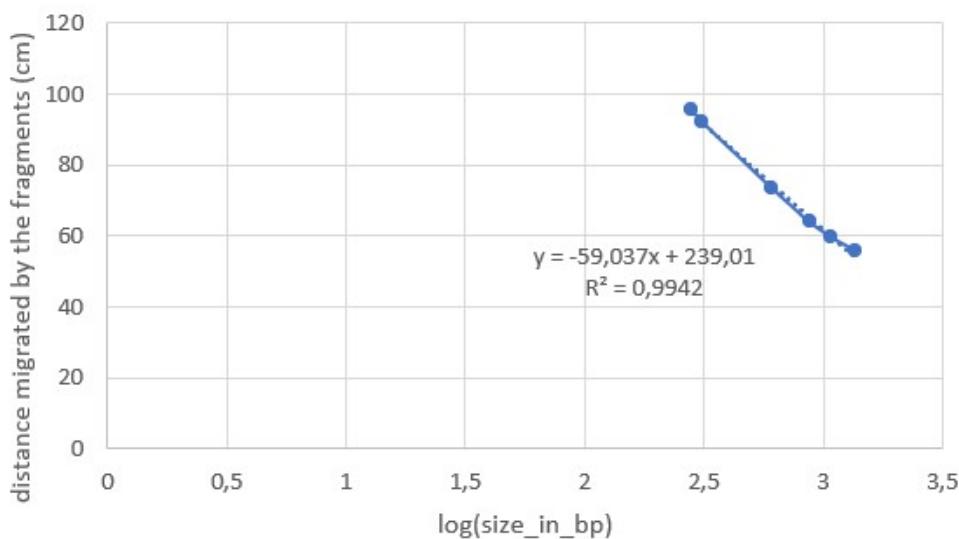


Figure 28: Curve of the migrated distance depending on the $\log(\text{size})$ for the black electrophoresis

The regression coefficient is strictly superior to 0.98, so the linear model fits the function $y = f(x)$.

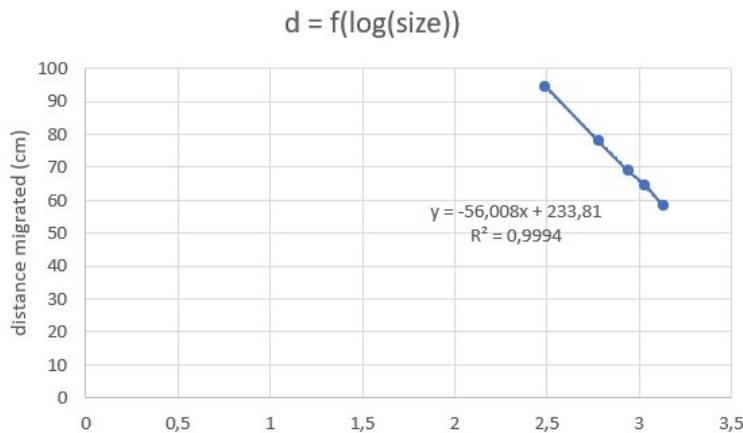


Figure 29: Curve of the migrated distance depending on the $\log(\text{size})$ for the gray electrophoresis

3. Interpolation of the distance migrated by the amplified sample and estimation of the fragment sizes of the D1S80 alleles in the DNA.

	size (bp)	$\log(\text{size})$	$d_{\text{image}}(\text{mm})$
angeles	646	-8,3335874	73,1
	553	-9,0111286	77,1
marta	680	-8,1133865	71,8
	680	-8,1133865	71,8
lucy	598	-8,672358	75,1
	598	-8,672358	75,1
carlos	490	-9,536223	80,2

Figure 30: Deduction of the size depending on the migrated distance by the band for the black electrophoresis

The linear regression gives the equation $y = -5.9037 * x + 23.901$.

Therefore we deduce that : $x = (y - 23.901)/(-5.9037)$.

x is the logarithm in base 10 of the size in bp. So:

$\text{size} = 10^{[y - 23.901]/(-5.9037)}$. We use this formula to calculate the size in bp of the DNA from Angeles, Marta, Lucy and Carlos (first column, in gray) in the Excel file.

For the gray gel: The linear regression gives the equation $y = -56.008 * x + 233.81$.

Therefore we deduce that : $x = (y - 233.81)/(-56.008)$.

We use this formula to find x , and then the size in bp can be deduced by calculating 10^x .

4. Number of the tandem repeats in one's D1S80 alleles.

First, let's interpolate with the distance of the fragments to calculate the size of each amplicon.

The number of VNTR corresponds to the size of the amplicon + 145 bp. These extra 145 bp correspond to the amplification boundaries.

	size(bp)	log(size)	d_image(mm)
Ange	551	2,74085845	80,3
	443	2,64622911	85,6
Carlos	652	2,81406228	76,2
	436	2,63908727	86
Ctrl	436	2,63908727	86
GFP	436	2,63908727	86
	291	2,46411227	95,8

Figure 31: Deduction of the size depending on the migrated distance by the band for the gray electrophoresis

Name	amplicon_size_bp	nb of VNTR
Angeles	553	698
	646	791
Carlos	598	743
	490	635
Marta	680	825
Lucy	680	825
	598	743

Figure 32: VNTR according to the amplicon size

This tab contains all the VNTR deductions depending on the amplicon size deduced at the previous question.

5. Homozygous or heterozygous for the D1S80 locus

Marta's sample has only one band in the electrophoresis. It means she is homozygous for this locus, meaning she has inherited the same versions (alleles) of a genomic marker from each biological parent. Angeles, Carlos, and Lucy's samples have two bands, therefore they are heterozygous for the D1S80 locus: they possess twice the same allele, so the bands get superposed on the gel electrophoresis.

Name	Nb of bands	homozygous	heterozygous	amplicon_size_bp	nb of VNTR
Angeles	2		x	553	698
				646	791
Carlos	2		x	598	743
				490	635
Marta	1	x		680	825
Lucy	2		x	680	825
				598	743

Figure 33: Classification of the results: homozygous or heterozygous

6. Interpretation of the gel obtained for the Fibros GFP and the Fibros FN.

For the Fibros FN, only one band appears (size = 436 bp). It corresponds to the GAPDH housekeeping gene. For the Fibros GFP, two bands appear. One is at the same level (size = 436 bp) than for the Fibros FN, and there is an extra one, at size = 291 bp.

The first one was expected, since GFP-modified fibroblasts are still fibroblasts, and the housekeeping gene GAPDH is essential to these. The second band is characteristic of the GFP gene.

The gray electrophoresis results highlights that we are amplifying three genes: D1S80 (from DNA), and GFP and GAPDH (from RNA).

7. Theoretical size of the DNA amplified sequences and comparison with the one obtained from the gel.

The theoretical size of the amplified fragment can be determined by counting the number of base pairs between the first and the second primers. Both sequences were downloaded from the database NCBI, in FASTA format.

	size(bp)	log(size)	d_image(mm)
Ange	551	2,74085845	80,3
	443	2,64622911	85,6
Carlos	652	2,81406228	76,2
	436	2,63908727	86
Ctrl	436	2,63908727	86
GFP	436	2,63908727	86
	291	2,46411227	95,8

Figure 34: Deduction of the amplicons size from the migrated distance

For the housekeeping gene GADPH, the Forward GADPH primer is highlighted in yellow, and the second primer in blue (Reverse primer is the reverse complement sequence).

GenBank ▾ Send to: ▾

Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 4, mRNA

NCBI Reference Sequence: NM_001289746.2

[FASTA](#) [Graphics](#)

Go to:

```

LOCUS      NM_001289746          1525 bp   mRNA    linear    PRI 30-NOV-2022
DEFINITION Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH),
transcript variant 4, mRNA.
ACCESSION NM_001289746
VERSION   NM_001289746.2
KEYWORDS  RefSeq.
SOURCE    Homo sapiens (human)
ORGANISM  Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;

```

Figure 35: Sequence of the GADPH gene pasted from NCBI database

On Word, we go to Revisions > Statistics and view the number of bp between the two primers. There are 413 bp, so we should obtain this amplicon size for the Ctrl of RNA.

>NM_001289746.2 Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (GAPDH), transcript variant 4, mRNA

```
CCCATGTCATGGGTGAACCATGAGAAGTATGACAACAGCCTCAAGATCATCAGCAATGCCTCT
GCACCACCAACTGCTAGCACCCCTGGCAAGGTATCCATGACAACTTGGTATCGTGGAAAGGACTCAT
GACCACAGTCCATGCCATCACTGCCACCCAGAAGACTGTGGATGGCCCTCCGGAAACTGTGGCGTGT
GGCGCGGGGCTCTCCAGAACATCATCCCTGCCTACTGGCGCTGCCAAGGCTGGCAAGGTCATCC
CTGAGCTAACGGGAAGCTCACTGGCATGGCCTCCGTGTCCCCACTGCCAACGTGTAGTGGTGACCT
GACCTGCCGTAGAAAAACCTGCCAAATATGATGACATCAAGAAGGTGGTAAGCAGGCGTCGGAGGGC
CCCCTCAAGGGCATCTGGGTACACTGAGCACCGAGGTGGTCTCTCTGACTTCAACAGCAGACACCCACT
CCTCCACCTTGACGCTGGGTGGCATTGCCCTAACGACCACTTGTCAAGCTCATTCCTGGTATGA
CAACGAATTGGCTACAGCAACAGGGTGGTGGACCTATGGCCCATGGCCTCAAGGAGTAAGACCCC
```

Figure 36: Primers of the GADPH gene

For the GFP gene, I highlighted the Forward primer in yellow and the second primer in green.

[GenBank](#) ▾

Recombinant vector pTR-CB-GFP, complete sequence

GenBank: MK225672.1

[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	MK225672	5763 bp	DNA	circular	SYN 26-JUN-2019
DEFINITION	Recombinant vector pTR-CB-GFP, complete sequence.				
ACCESSION	MK225672				
VERSION	MK225672.1				
KEYWORDS	.				
SOURCE	Recombinant vector pTR-CB-GFP				
ORGANISM	Recombinant vector pTR-CB-GFP other sequences; artificial sequences; vectors.				

Figure 37: Sequence of the GFP gene

```
CAAGGGCGAGGAGCTTCACCGGGGTGGTGCCTCCTGGTCGAGCTGGACGGGACGTAAACGGGCCACAAGT
TCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCCTGAAGTTCATCTGCACCACGGCA
AGCTGCCGTGCCCTGGCCCACCCCTCGTACCACCCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCGACCA
CATGAAGCAGCACGACTTCAAGTCCGCCATGCCGAAGGCTACGTCCAGGGAGCGCACCATCTCTCAAGGAC
GACGGCAACTACAAGACCCGCCGAGGTGAAGTTGAGGGCGACACCTGGTGAACCGCATCGAGCTGAAGGG
CATCCACTCAACGCCAACCCCAACATCTCCCCCAACAACTCTCCACTACAACATAAACACCCACAAACCTCTATAT
```

Figure 38: Primers of the GFP gene

Once again, we go to Revisions > Statistics and view the number of base pairs between the two primers. There are 265 bp, so we should obtain this amplicon size for the GFP migration of the RNA electrophoresis.

However, the experiment reveals an amplicon size of 291 bp.

Therefore, for both samples (Fibros FN and Fibros GFP), there were differences between the theoretical and the empirical amplicon sizes.

We can infer that there was some degradation within the sample or problems linked to the fluorescence acquisition.

9 Conclusion

RNA was extracted from both human primary fibroblasts and GFP-modified human fibroblasts. Then a reverse transcription for both RNA extracts was performed. In parallel, DNA from buccal cells was extracted, to perform the isolation, amplification and characterization of our own D1S80 variable number tandem repeat (VNTR) locus.

The PCR in combination with prior reverse transcription (RT-PCR) of the mRNA of interest provided a mean for measuring gene expression using a little extract of buccal cells.

The lack of results for the DNA migration can be explained by the discovery of some laboratory practices. Alternative methods to quantify DNA than absorbance would be fluorescent techniques that are much more sensitive and specific for DNA.

Overall, a common gene expression was expressed in the fibroblasts extracts and our buccal cells extracts, which correspond to the housekeeping gene GAPDH, that all humans possess. Furthermore, common alleles for the D1S80 locus were identified respectively in Marta and Lucy then in Carlos and Lucy. The notions of homozygous a

10 Acknowledgments

I express my utmost gratitude to Angelica and Pablo for sharing their experience and knowledge with us and taking care of the experimenting conditions. I also want to thank Sara Herraiz Gil for teaching the PCR method to us and taking time to explain the steps to us.

11 References

- [1] Laboratory Practical Manual: Tissue/Organ Regeneration and Bioengineering: Biomolecular Characterization of Tissues, Ángeles Mencía Rodríguez, Carlos León Canseco, 2022-2023 [PDF]