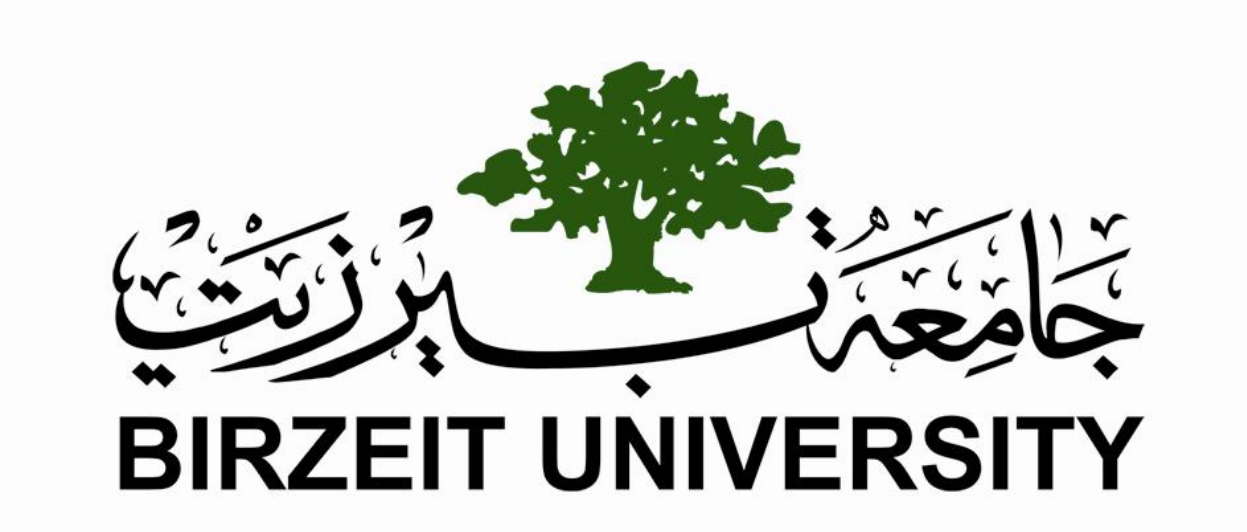

Master Program in Clinical Laboratory Science

Master Thesis

Mutational Spectrum of Cystic Fibrosis among the Palestinian Population

By
Osama H. Essawi

Supervisors

Dr. Tamer Essawi
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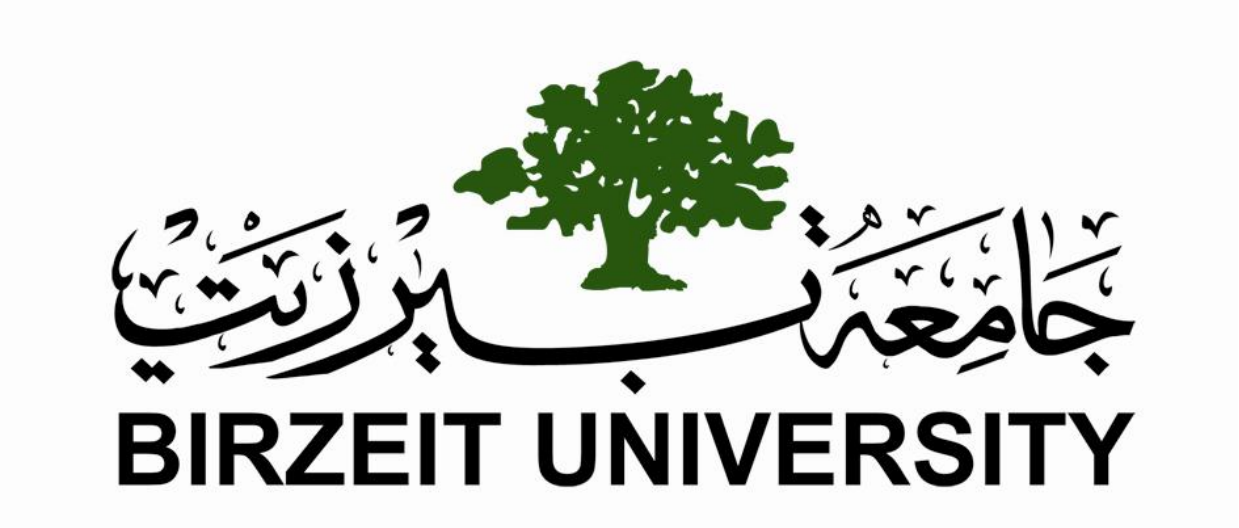
Birzeit University, Palestine.

July 2013



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This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Clinical laboratory Science, from the Faculty of Graduate Studies at Birzeit University, Palestine.

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July 2013

Mutational Spectrum of Cystic Fibrosis among the Palestinian Population

مجموعة الطفرات التي تسبب التليف الكيسي بين السكان الفلسطينيين

By

Osama H. Essawi

This Thesis was successfully defended and approved _____

Committee Members

Tamer Essawi, PhD, Supervisor _____

Paul Couke, PhD, Supervisor _____

Wail Hammoudeh, MD _____

Mohammad A. Farraj, PhD _____

To those children who are suffering from this dreadful disease and to their families who as well are suffering in silence but showing patience and understanding for their children. They were kind to support this important research. All the knowledge and experience I gained throughout this research project, I owe it them. To those smiles that made my way to their genes I dedicate the first step of research in the road to cure this disease and relief their suffering.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

CF	**Cystic Fibrosis**
CFTR	**Cystic Fibrosis Trans Membrane Conductance Regulator**
CFGAC	**Cystic Fibrosis Genetic Analysis Consortium**
ATP	**Adenosine Triphosphate**
ABC	**ATP Binding Cassette**
NBDs	**Nucleotide-Binding Domains**
PI	**Pancreatic Insufficiency**
PS	**Pancreatic Sufficiency**
UV	**Ultra Violet**
PCR	**Polymerase Chain Reaction**
NGS	**Next Generation Sequencing**
dNTPs	**Deoxy Nucleotides**
ddNTPs	**Dideoxy Nucleotides**
MLPA	**Multiple Ligation-dependent Probe Amplification**
ASMA	**Allele Specific Mutation Analysis**

Abstract

Cystic Fibrosis (CF) is a well-known life threatening inherited disease caused by mutations in the Cystic Fibrosis Trans Membrane Conductance Regulator gene (CFTR gene). Those mutations produce a defect in the encoded CFTR protein that functions mainly as a chloride channel and as regulator of other channels. CF patients are characterized by progressive lung disease, pancreatic dysfunction, elevated sweat electrolytes and male infertility. This study included a total of 73 patients (43 Males / 30 Females) (60 unrelated) residing in Palestine (West Bank and Gaza) who were clinically diagnosed with cystic fibrosis. The aim of the study was to determine the types and rates of mutations present in the CFTR gene and to develop allele specific mutation analysis test for the most common mutations among Palestinians. This test will make molecular testing for CF in Palestine possible and will have a direct impact on a better treatment of these patients. Whole blood was collected and DNA from these samples was extracted by automated methods. For each patient, PCR amplifications were performed for the coding region of the CF gene. Consequently, sequencing by Next Generation Sequencing was performed which enabled us to identify the CF mutations present in this cohort. After validating these mutations by different methods like Sanger sequencing, we defined a set of 18 mutations present in the Palestinian population. In a next step, we designed an easy and fast allele specific diagnostic test for 8 of the identified mutations that represent more than 80% of the CF mutations found in this population.

ملخص

التليف الكيسي (CF) مرض وراثي شائع و خطير يهدد حياة المرضى، و تسببه طفرات في جين التليف الكيسي CFTR. تلك الطفرات تنتج عن خلل في البروتين الناتج و الذي يعمل بشكل رئيسي كقناة للكلور و منظم لقنوات أخرى. يتميز مرضى التليف الكيسي بامراض مزمنة تصيب الرئة، خلل في وظيفة البنكرياس، ارتفاع كمية الاملاح في العرق و العقم لدى الذكور.

شملت الدراسة 73 مريضاً فلسطينياً ممن يقيمون في الضفة الغربية وقطاع غزة (43 ذكور / 30 إناث، 60 منهم لا تربطهم قرابة) ممن شخصوا سريرياً بمرض التليف الكيسي. تهدف الدراسة الى تحديد أنواع ومعدلات الطفرات الموجودة في الجين المسبب للمرض وتطوير اختبارات جزيئية لمعرفة الطفرات الأكثر شيوعاً لدى الفلسطينيين. وهذا سيؤدي الى تحسين العلاج عن طريق جعل هذه الاختبارات جزءاً من الاختبارات المتبعة للتشخيص الصحيح لهذا المرض في فلسطين.

تم جمع عينات الدم اللازمة من المرضى واستخراج الحمض النووي من هذه العينات بطرق آلية. ثم أجريت عملية PCR على الجين المسبب للمرض. وبعد ذلك تم تحديد ترتيب تسلسل القواعد الامينية مما مكننا من التعرف على الطفرات الموجودة لدى هؤلاء المرضى. وبعدها تم استخدم وسائل مختلفة للتحقق من صحة هذه الطفرات مثل استخدام طريقة سانجر وغيرها، وكنتيجة لذلك تم التعرف على 18 من الطفرات الموجودة لدى المرضى الفلسطينيين. و في خطوة اخيرة قمنا بتصميم اختبارات جزيئية سهلة وسريعة لتشخيص 8 من الطفرات التي تم تحديدها والتي تشكل أكثر من 80٪ من الطفرات الموجودة لدى المرضى الفلسطينيين.

Chapter 1
Introduction

Cystic Fibrosis (CF) is believed to be one of the most common genetic diseases among Caucasians with an incidence ranging from 1 in 2500 to 1 in 3600 [1].This severe and life threatening disease is inherited in an autosomal recessive manner and is caused by mutations in the Cystic Fibrosis Trans Membrane Conductance Regulator gene (CFTR gene) located on chromosomal region 7q31.2 [2]. A CF patient always carries two mutations on both CFTR alleles. The CF patient can carry either an identical CFTR mutation (homozygous patient) or two different CFTR mutations (compound heterozygous patient). If the mutation is found on one CFTR allele, the individual is referred to as CF carrier. So far, more than 1900 different CFTR mutations have been reported to the Cystic Fibrosis Genetic Analysis Consortium (CFGAC)[3] since it was isolated and cloned in 1989 [4]. Those mutations produce a defect in the encoded CFTR protein that functions as a chloride channel and as regulator of other channels across the epithelial cell membrane. Such mutations impair water movement across epithelia leading to formation of viscous mucus that obstructs the airways of the lungs and ducts of the pancreas. CF is characterized by progressive lung disease, pancreatic dysfunction, elevated sweat electrolytes and male infertility [5]. The severity and symptoms of the disease vary considerably due to different mutations but also modifier genes will play a role.

The most common mutation which affects about 70% of Caucasians is a three base-pair deletion mutation resulting in the deletion of the amino acid phenylalanine at position 508 causing a defective intracellular processing of the CFTR protein. This mutation is designated ΔF508 or delta-F508. Although the ΔF508 is the most common mutation, all other mutations are less frequent. There is also considerable variation of mutation frequencies between different populations worldwide [3].

1.1 Historical Background

Cystic Fibrosis was first described and named as “Celiac Syndrome” by the Swiss pediatrician Dr. Guido Fanconi in 1936 describing changes in the pancreas seen in children. The first definitive description of this disease was given in 1938 by Dr. Dorothy Anderson who called it “Cystic Fibrosis” during her work at the Babies’ Hospital in New York. Later in 1949, Dr. Dorothy Anderson discovered that CF was caused by a recessive mutant gene.

In 1949, Dr. Charles Lowe reported that CF was a genetic disorder due to its autosomal recessive pattern of inheritance assuming that the cause of this disease must be a gene defect [6]. Later on, in 1986 Quinton showed that the transportation of fluid and electrolyte in CF patient's sweat glands are not normal suggesting CF patients sweat ducts are impermeable to chloride and this explained the elevated levels of salt in the sweat of these patients [7]. Soon afterwards in 1989 the gene responsible for CF disease was identified by Tsui and Collins and colleagues. They named it the Cystic Fibrosis Trans Membrane Conductance Regulator gene (CFTR gene), although they didn’t have any idea about the protein structure [8]. This new insight in the molecular mechanisms involved lead to an improvement in nutritional therapy, antibiotics, chest physiotherapy, and earlier diagnosis which have a great effect on raising the median survival age of the CF patients who usually die in infancy or early childhood in the 1950's to 37.4 years in 2007 [9].

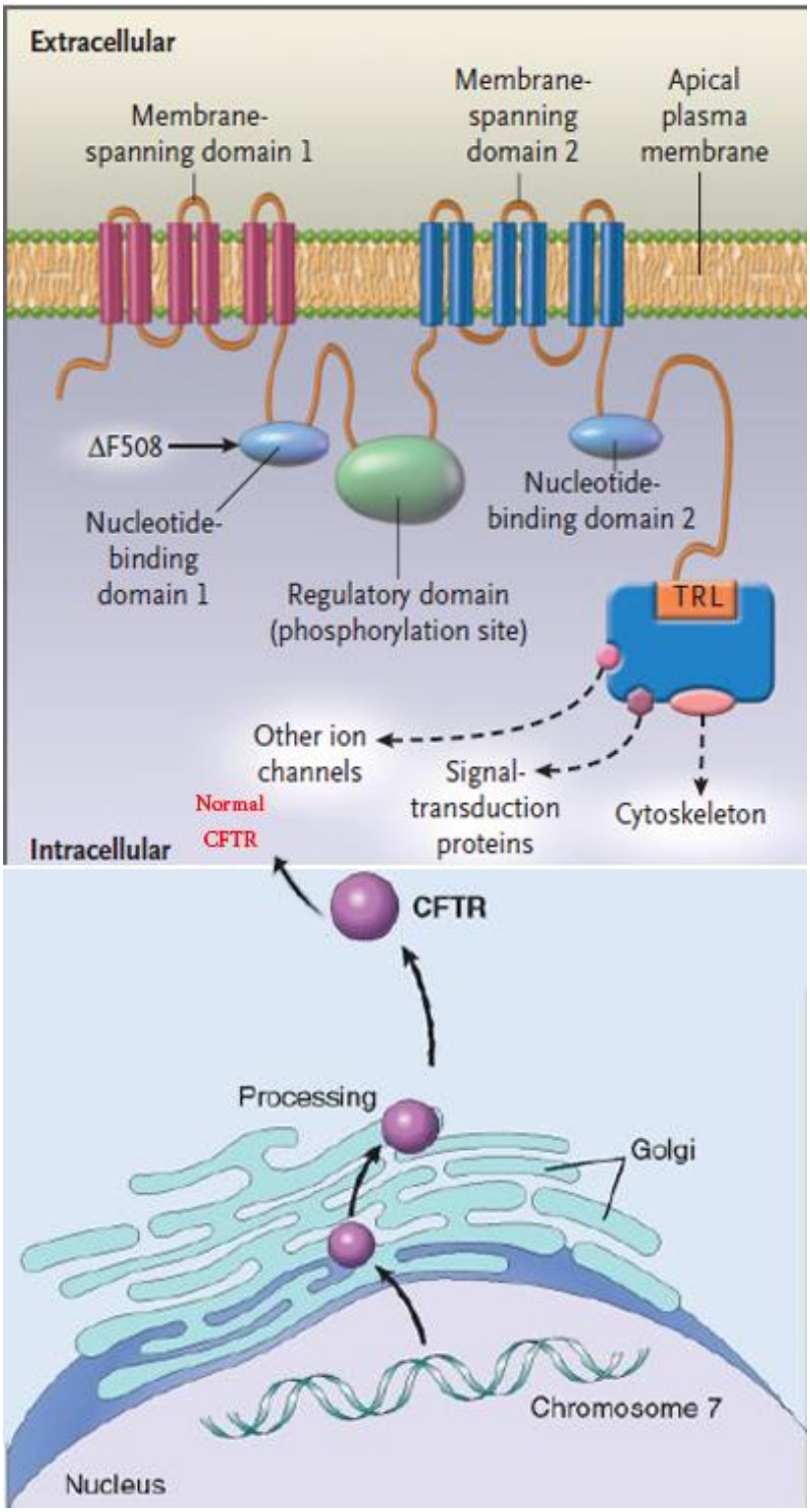
1.2 CFTR Structure and Function

The main defect in CF is due to an abnormal function of chloride channel protein found in epithelial cells. This 1480 amino acid CFTR protein structure suggests that the gene is a part of the ATP binding cassette (ABC) gene family which encodes proteins called trans-membrane proteins or ABC transporters that get energy from the hydrolysis of adenosine triphosphate (ATP) to carry out many different biological processes. For example CFTR down regulates trans-epithelial sodium (sodium channel) [10], it has also a role in regulating multiple ion channels and different cellular processes like potassium and calcium- activated chloride channels, gap junction channels, cellular processes involved in mucus secretion and ATP transport, and it has an important role in the formation of the plasma membrane [11].

The most common mutation in the CFTR gene is ΔF508 which results in degradation of CFTR before it reaches the cell surface, and improper folding of the protein in the endoplasmic reticulum. This mutation affects the interaction between the first nucleotide binding domain and the first membrane spanning domain regulating CFTR channel gating [12, 13]. The synthesis of CFTR, NBDs, R domains, and the membrane-spanning domains may be affected by other mutations [14].

Although CFTR was initially recognized as a chloride-conductance channel, it is well known that the absence of CFTR influences the expression of several other proteins, including important proteins in ion transport, cell signaling, and inflammatory responses. These proteins may explain the differences in clinical severity among patients with the same mutations since they may be included as potential modifiers of the CF phenotype.

Figure 1: CFTR Proposed Structure



Bottom: CFTR from gene to protein. The most prevalent mutation in the *CFTR* gene (ΔF508) a part of Class II mutations results in defective protein folding in the endoplasmic reticulum and premature degradation of the protein before reaching the cell surface. Mutations of other classes may affect the synthesis of CFTR, membrane-spanning domains, NBDs and R domains.

Top: CFTR structure and activation. CFTR consists of two nucleotide-binding domains (NBDs), two membrane spanning- domains, and a regulatory domain.

Activation of CFTR depends on phosphorylating of it at the Regulatory domain, this occurs after through the activation of protein kinase A.

The two nucleotide-binding domains regulate the channel activity.

The membrane-spanning domains consist of six membrane-spanning alpha helixes which form the chloride-conductance channel.

ΔF508 mutation occurs on the surface of nucleotide-binding domain

1.3 Cystic Fibrosis Mutations

More than 1900 mutations were reported to be associated with the disease; these mutations were described in different regions of the gene and in the messenger RNA. CF mutations are classified according to the mechanism used to causes the disease as designated in Figure 2 below.

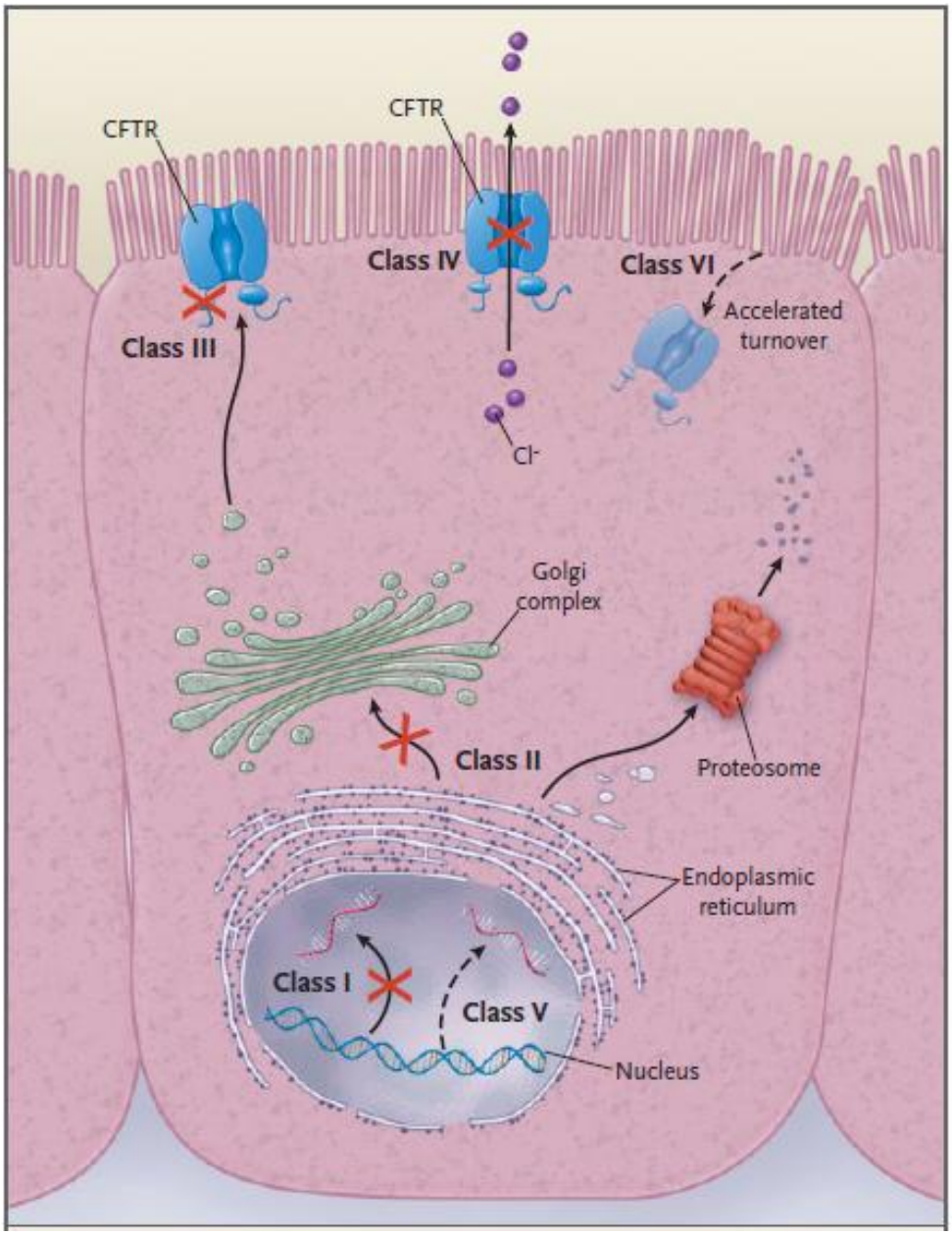


Figure 2: Categories of CFTR Mutations.

Classes of defects in the CFTR gene include the absence of synthesis (class I); defective protein maturation and premature degradation (class II); disordered regulation, such as diminished ATP binding and hydrolysis (class III); defective chloride conductance or channel gating (class IV); a reduced number of CFTR transcripts due to a promoter or splicing abnormality (class V); and accelerated turnover from the cell surface (class VI).

Six classes of mutations have been described with classes I, II, and V cause insufficient production of the protein, whereas III and IV produce sufficient protein with impaired chloride translocation. Class VI provides sufficient protein at the apical membrane, but the protein degrades before functioning appropriately.

Class I, II, III and VI are associated with pancreatic insufficiency (PI) and “classic CF” or “severe CF” while Classes IV and V are associated with pancreatic sufficiency (PS) and “mild CF”. This clinical distinction based on pancreatic function is somewhat misleading as people with classic (PI) CF may have good lung function while those with mild (PS) CF may have dreadful lung disease [15].

The most prevalent mutation ΔF508 (deletion mutation lacking a phenylalanine at position 508) is considered to be a class II defect, for this mutation the protein is rapidly misfolded after synthesis then it is degraded before reaching its site of action at the cell surface. Other well-known mutations and important mutations such as N1303K and G85E are considered to be from class II which means that they have defective protein maturation (misfolding) and then premature degradation. G542X is recognized as class I mutation because there is no protein synthesis, this kind of mutations are designated by "X" to say that they have a stopping codon.

CFTR mutations in the other classes encode full length and well processed proteins, but the problem is that these proteins lack normal ion-channel activity. Class III mutations have no or

little chloride channel function that result in an abnormal regulation. Class IV mutations are believed to show partial chloride channel activity and this explains the milder phenotypes[16], class V include mutations that cause reduced number of CFTR transcripts, and class VI include mutations that cause unstable CFTR at the cell surface [17, 18].

Table 1: Classes of Different Mutations

Class of Mutation	Molecular defect of CFTR Protein	Examples
I	Defective synthesis	1525-2A → G, 1717-1G → A, 1898+1G → A, 2184delA, 4010del4,G542X, Q552X, W1282X
II	Defective processing and maturation	A559T, D979A, ΔF508, ΔI507, G480C, G85E, N1303K, S549I, S549N, S549R
III	Defective regulation	G1244E, G1349D, G551D, G551S, G85E, H199R, I1072T, I48T, L1077P, R560T, S1255P, S549(R75Q)
IV	Defective conductance	A800G, D1152H,G314E, G576A, G622D, G85E, H620Q,I1139V, I1234V, R117H R347H, R347P
V	Reduced function / synthesis	2789+5G → A, 3120G → A, 3849+10kbC → T, 5T variant, 711+3A → G, A455E, IVS8 poly T
VI	Accelerated turnover from cell surface	Q1412X

1.4 CF in the Arab World

Whereas in Europe and the US CF has been clinically diagnosed since more than 50 years, CF has been increasingly diagnosed in the Middle East during the last two decades, , revealing various mutation rates according to the ethnic origin of populations[19]. In addition, Middle Eastern societies, particularly Arabs are characterized by close family relationships. The tendency for marrying relatives is a common cultural practice despite discouragement of consanguineous marriage by major religions. In these societies genetic disorders are relatively frequent particularly autosomal recessive diseases [20]. Population-based surveys in the Middle East have found total consanguinity rates between 25% – 65 % and these rates indicate increased risk of congenital disorders and recessive inherited diseases. In Palestine, the rate of consanguineous marriages was found to be 45% in 2004 [21], indicating that Palestine has a very high risk of congenital disorders including CF, but there are no accurate epidemiological data on CF. The general impression has been that the disease is rare, but this is most likely the result of under-diagnosis or misdiagnosis due to limited awareness of the condition in the region.

Despite that CF used to be considered very rare among Arabs, several research projects have documented many Arab families with the classical form of the disease, indicating that CF alleles are present in this ethnic group [22-29].

Limited mutation analysis studies suggest that the distribution of CF mutations in Arabs differs from that in Caucasians and that ΔF508 might not be the most frequent CF allele among Arabs as is in other populations [22-24].

Other studies showed that Arabs carry CF mutations that have never been identified in Caucasian CF patients, and there were characteristic mutations among different Arab subgroups mainly Muslims, Christians, Druze, Bedouins and minority groups such as Armenians.

Concerning Palestinian Arabs, limited number of investigations were done by the Israeli's on Palestinians who live in "Israel", and the findings of such investigations reflect the coexistence of numerous communities, which have their own range of mutations, showing little mixing between the different groups [28].

Table 2 below shows the major mutations in CFTR gene that appear in the Middle East.

Table 2: Major Mutations in CFTR gene in the Middle East.

Country	Year of Study	Tested Patients	Major Mutations
Lebanon	2010[1]	221[1]	ΔF508, N1303K, W1282XSX4
UAE	1994[24]	17	S549R, ΔF508
Jordan	2007	120	ΔF508, W1282X
Saudi Arabia	1997[3]	15	3120+1G→A,N1303K, 1548delG, ΔF508, I1234V
	1998[25]	70	N1303K, R553X,
	1999[26]	70	3120+1G→A, S549R
Israeli Arabs	1999[28]	42	ΔF508, N1303K, 3120+1Kbdel8.6Kb, W1282X G85E

Cited references are shown.

There are no published data about the mutations that cause CF nor their frequencies among Palestinian CF patients who are living in Palestine. Therefore, the main objective of this study was to detect and identify the various mutations among Palestinian CF patients. This will provide the information needed for diagnosing CF and later in establishing a prevention program for it.

Chapter 2

Objectives

Reviewing the history of CF in the Middle East revealed that little is known about this life threatening disease in this region. Furthermore, less information is known about CF among the Palestinian population. There is no reliable clinical database registering all CF cases and no effective clinical treatment program has been developed. Molecular diagnosis is not available for CF patients. Nevertheless, it is extremely important for this region to develop a program to face the different risks related to this dangerous disease. Such program will be significant for the Palestinian population who has a very high risk to develop autosomal recessive diseases including CF due to the high consanguinity rates.

Therefore, this study has been designed to investigate the nature of pathogenic mutations in the CFTR gene and determine their frequencies in the Palestinian population. The identification of the mutation spectrum in Palestinian CF patients is essential for the development of reliable genetic testing. To achieve this goal, the following objectives have been followed:

1. Samples from non-related CF patients will be collected from all regions throughout Palestine West Bank and Gaza.
2. Molecular characterization and identification of mutations will be performed for all patients.
3. A mutation spectrum will be defined for most mutations (80%) present in the tested CF patients.
4. Rapid and comprehensive molecular testing will be developed and implemented to detect the majority of mutations in CF patients and to be used for screening the population to identify carriers.

Chapter 3

Materials and Methods

3.1 Patients and Sampling

3.1.1 Patients Selection Criteria

A total of 73 cystic fibrosis patients (60 unrelated) have participated in this study. There were 59 % (43/73) male patients and 41 % (30/73) females. Most of the patients were children (< 18 years old) as shown in Figure 3 below. The patients were distributed throughout the different regions of the West Bank and Gaza, Palestine as shown in Table 3. The criteria for inclusion of participants in this study were based on previous diagnosis of patients, typical pulmonary and / or gastrointestinal tract manifestations and elevated sweat chloride concentrations (> 60 mmol/L).

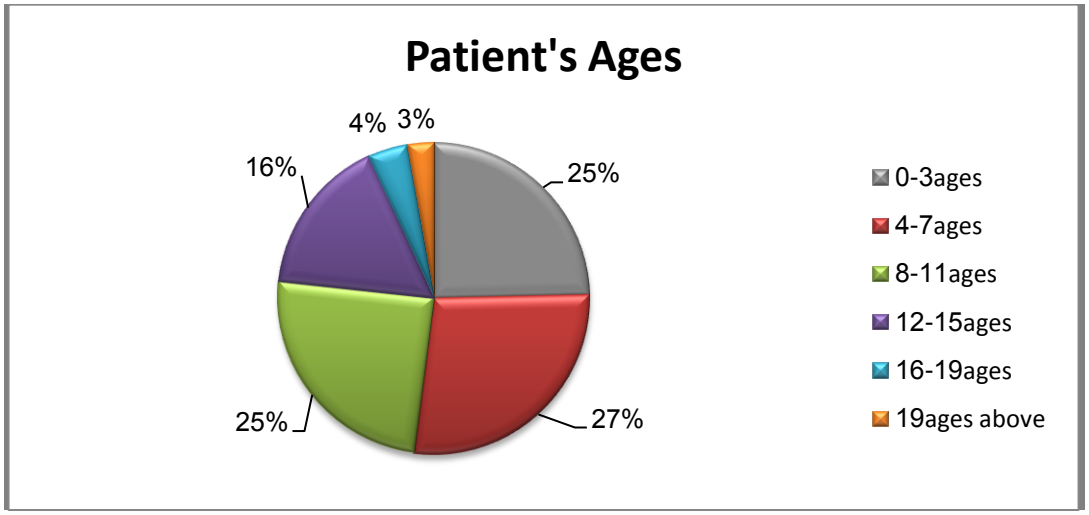


Figure 3: Pie chart showing the age distribution of patients participated in the study.

Table 3: Geographical Distribution of the Patients

City	No. of Patients	No. of non-related Patients
Gaza	49	42
Hebron	18	14
Qalqilia	1	1
Jenin	5	3
Total	73	60

3.1.2 Sample Collection and Processing

Whole blood (3 ml) was collected in EDTA vacutainer tubes (BD) following aseptic technique. Samples collected from Gaza in December 2012 were transported immediately to the laboratory and stored at -80^{o}C to ensure good yield, while samples collected from the West Bank between January to February 2013 were stored at -20 ^{o}C. A total of 73 samples were collected from cystic fibrosis patients. The ethical guidelines put by Birzeit University were implemented. In addition, participation in the project was based on the free will of the participants. A signed consent was obtained from each participant and/or the guardian.

3.2 DNA Extraction

Genomic DNA for all patients was extracted and purified from whole blood by automated extraction methods mainly using AUTOPURE LS "Large Sample Nucleic Acid Purification" since there was a sufficient quantity of blood (usually more than 1 ml), except for three patients the quantity of blood was insufficient to use this method so there DNA was extracted using the

QIAamp DNA extraction method that deals with small amounts of blood samples (~0.2 ml of blood).

For 13 of the patients; DNA was extracted using both methods since the quantity of their blood samples were about ~ 1 ml. Therefore, the quantity was divided to make sure that DNA will be extracted by one of these two procedures.

3.2.1 AUTOPURE L S "Large Sample Nucleic Acid Purification"

The AUTOPURE LS is an automated method for the purification of the DNA from 1-10 ml of blood and other DNA consisting samples using the PUREGENE(R) purification chemistry which was found by Gentra / Qiagen. This method contains two sets of 50 ml centrifugation tubes which can handle up to 16 different samples in each run. The extraction procedure used in this research began by adding about 1 ml of each of the blood samples to a 50 ml centrifugation tube from the first set of tubes that are distinguished from the other set of tubes by the cab color, then 40 µl of glycogen is added to a tube from the other set of the 50 ml centrifugation tubes. For each tube of blood there should be a tube of Glycogen which is added for its important role in increasing the yield of DNA precipitate by alcohol. After adding the samples to the tubes they should be introduced to the extraction machine using a barcode system. The automated extraction process that is performed by the AUTOPURE system is similar to the manual DNA extraction process which consist of several steps that begin by lysing the cell to expose the DNA, the next step is removing the unwanted cell contents like membrane lipids, proteins, and RNA by adding detergents and other specific enzymes like protease, and finally precipitating the DNA with an alcohol. These steps include several times of washing and centrifugation as in manual extraction process. The running time of this technique varies according to the program used for

extraction, the sample size and kind. In our case using the software method for frozen samples the running time of the machine was about 80 minutes for every 16 different samples.

At the end of each run a DNA pellet is found in each one of the introduced tubes, it should be dried for about 10 minutes then dissolved in about 100 µl of DNA hydration solution using a hot water path (65 ºC) for 60 minutes and finally the solution should be centrifuged for 3 minutes at 2000 rpm and 18 ºC to be ready for use.

3.2.2 QIAamp DNA Mini Kit

DNA of 16 patients was extracted using the QIAamp DNA Mini Kit assay. It is a simple, rapid and an easy to follow assay for the extraction and purification of DNA from small quantities of blood or body fluids (up to 200 µl) by enzymatic lyses of cells using Proteinase K and then passing the lysate through silica-membrane column to purify DNA from other molecules. This assay can be automated on the QIAcube which can handle up to 12 samples per run.

3.3 Determination of DNA Quality

DNA quality for each sample was determined by different methods; these methods are discussed below:

3.3.1 DNA Concentration

DNA concentration was measured using DropSense96 (Trinean) which can read up to 96 samples in 5 minutes. DropSense96 is a state of the art spectrophotometer that determines the concentration of DNA after absorbing ultraviolet light at 260 nm wavelength.

The DNA concentration and purity of all the patients were measured except for three patients who had little volume of blood and there DNA was extracted using QIAamp procedure. The

concentration of DNA extracted by QIAamp method is usually not measured since it is very low to be detected, and it is always considered to be about 25 ng / µl so when used it is not diluted. The measurement of the concentration and purity of a DNA sample is very simple and easy, it just need the loading of a special plastic chip which can handle 16 samples with a 2 µl of each DNA sample then introduce the special plastic chip to the DropSense96 equipment which analyze the samples as described previously.

3.3.2 DNA Purity

DNA Purity was also measured using spectrophotometric analysis. Absorbance measurements made on the Trinean DropSense96 calculates the ratio of UV light absorbance at 260 nm and 280 nm wavelengths to determine the purity of DNA, since proteins and other contaminants absorbs uv light at or near the 280 nm wavelength range, it is easy to realize from the A260/A280 ratio the presence of several contaminants especially proteins.

"Pure" DNA is indicated by the A_{260}/A_{280} ratio of ~1.8, and A_{260}/A_{280} ratio range of 1.6 – 2.0 indicates a good quality DNA, lower ratios indicates the presence of more contaminants, but this does not refer that the DNA is unsuitable for any application. Purity and concentration are measured simultaneously, and both of them are measured using the same procedure.

3.3.3 DNA Quality Test by PCR

The DNA quality was checked by amplifying DNA fragment using PCR "polymerase chain reaction"**.** Amplification was performed with 2.5 µl of the purified DNA template in a total of 10 µl reaction mixture. The complete mix constituted of the following components: 5 µl of 2X KAPA2G Robust Hot Start ready Mix, 1.25 µl upstream primer (0.1µM), and 1.25 µl

downstream primer (0.1μM), and Primers used for PCR amplification are CFTR – 13 Assay primers listed in Table 1 - Appendix I.

The amplification conditions were as described in Table 4for FORD PCR program. The LabChip GX (PerkinElmer) capillary electrophoresis was used to assess PCR product which is CFTR – 13 Assay Amplicon.

3.4 Polymerase Chain Reaction (PCR) of the CFTR Gene Coding Region

PCR reactions consisted of 20 – 40 thermal cycles to amplify DNA fragments of 0.1 – 10000 base pairs length, each cycle consisted usually of three different temperature steps. First was the Denaturation step where the reaction is heated to 95 ˚C for 20-30 seconds, this high temperature caused melting of the DNA template by breaking the hydrogen bonds between the complementary bases, forming single stranded DNA. The second step was the Annealing step in which the temperature is lowered to around 60 ˚C for 20 – 40 seconds allowing the attachment of the primers to the single stranded DNA template forming new stable hydrogen bonds between the primers sequence and the DNA template sequence, the DNA polymerase binds to the new formed hybrid and initiate the DNA synthesis. The third step was the Elongation step in which the temperature depends on the optimum temperature for the DNA polymerase activity, between 70-80 ˚C. At this step the DNA polymerase adds new nucleotides to the newly synthesized DNA. Elongation time depends on the type of DNA polymerase used and the length of the amplified DNA fragment.

There final step of the PCR is two step procedure, the first one is an elongation for 5-15 minutes to make sure that all the single stranded DNA are elongated. The second step is the final hold, in which the temperature is lowered to 4 – 15 ˚C for an indefinite time, and this step can be considered as short term storage for the reaction.

The PCR machine that used in this project was the 2720 Thermal cycler (Applied Biosystems). Different PCR programs were used in this research and they are all shown in Table 4 below. The CFTR gene was divided according to the transcript (ENST00000003084) to 27 exons. This transcript was used to form 28 different assays used to amplify each exon of the CFTR gene. For each patient 28 different PCR reactions were performed using FORD program to amplify all the exons as a preparative step prior to the DNA sequencing. The details for each assay are found in Table 1- Appendix I, and the amplification procedure is the same as the one used previously to test for DNA quality. PCR products were electrophoresed using the capillary electrophoresis instrument the LabChip GX (PerkinElmer – USA).

Table 4: PCR Programs

	Program					
	Ford (35 cycles)		**CF62 (34 cycles)**		**CF55 (34 cycles)**	
Step	Time	Temp.	Time	Temp.	Time	Temp.
Initial Denaturation	3 min	95^{o} C	5 min	95^{o} C	5 min	95^{o} C
Denaturation	15 sec	95^{o} C	30 sec	95^{o} C	30 sec	95^{o} C
Annealing	10 sec	60^{o} C	30 sec	62^{o} C	30 sec	55^{o} C
Elongation	15 sec	72^{o} C	30 sec	72^{o} C	30 sec	72^{o} C
Terminal elongation	1 min	72^{o} C	10 min	72^{o} C	10 min	72^{o} C

3.5 INNO-LIPA CFTR

INNO-LIPA CFTR (Innogenetics, Ghent, Belgium) is the most comprehensive multi-parameter assay that provides simultaneous detection and identification of 36 of the most frequent CFTR mutations and their wild-type sequences in the Caucasian population. This assay is based on the reverse hybridization technology in which probes are coated on strips of a nitrocellulose membrane as parallel lines, and these probes are ready to hybridize specifically with their complimentary sequences. This leads to the detection of point or multiple base pairs mutations, deletions or insertions. The advantage of this assay is that it is rapidly performed and easily processed in a manual or an automatic way. This method is sensitive and highly specific.

The INNO-LIPA assay consists of three steps:

1- DNA extraction and purification.

2- Amplification of target DNA by PCR.

3- Detection of the specific hybrids on the reverse hybridization strips.

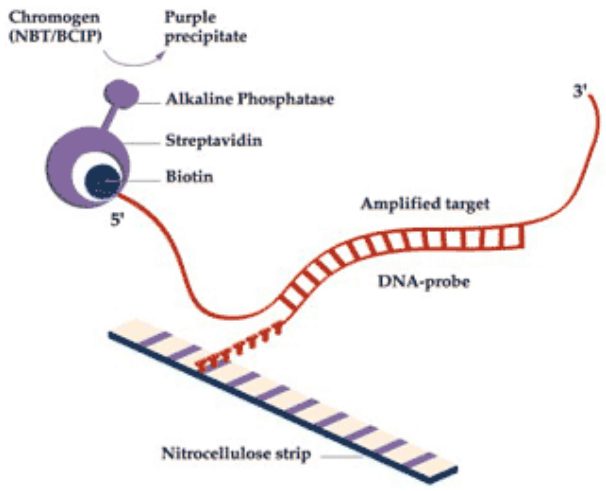


Figure 4: Principle of reverse hybridization with the probe firmly anchored to the membrane

The interpretation of the results is easy and based on a visible hybridization pattern. The analysis of both the wild-type and the mutant sequences identifies heterozygosity or homozygosity of each tested mutation, and thus differentiates between carriers and patients. Figure 5 demonstrates how the strips of the assay appear and it shows the two kinds of strips (CFTR 17 + Tn & CFTR 19) which together detects 36 different CF causing mutations. The first two strips in the figure indicate "No Mutation" so the bands appear on the strips are the wild type bands. The second two strips indicates a homozygous ΔF508 patient and it is apparent that the CFTR 19 strip has a band for the mutation in the upper side of the strip (mutant side) and this band disappeared from the lower part of the strip (wild type side). The third and fourth sets of strips show a compound heterozygote mutation in two different patients. In these mutations two bands appear for each mutation on both sides of the strip (mutant and wild type).

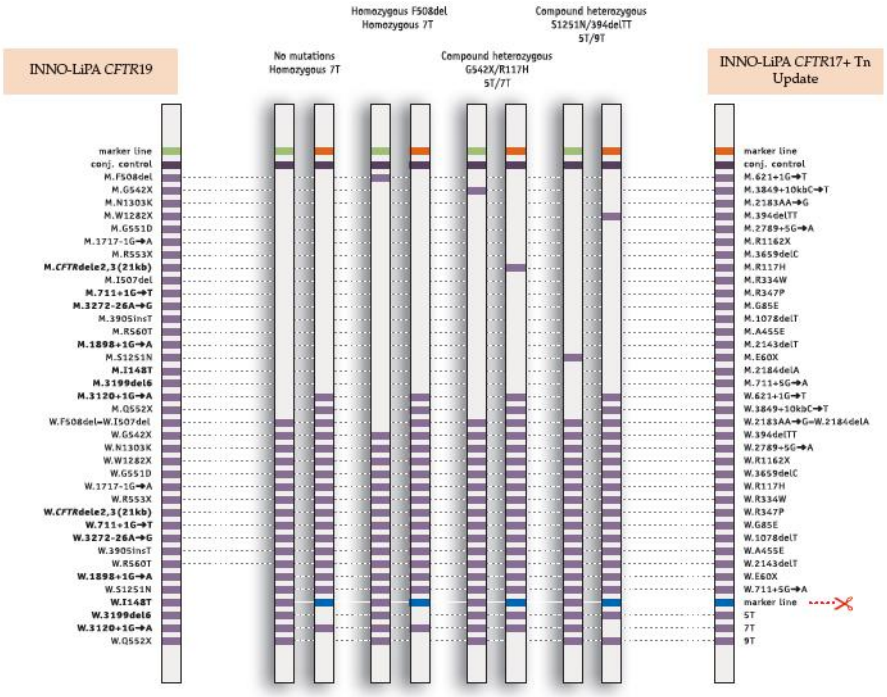


Figure 5: INNO-LIPA CFTR Strips.

In this project the INNO-LIPA CFTR assay was performed for the15 patients whose CF mutations were previously determined as shown in Table 5. DNA of these patients was amplified according to the instructions of the INNO-LIPA CFTR kit and processed automatically using the *AUTO*-LIPA 48.

Number	Patient	CF Mutation 1st allele	CF Mutation 2nd allele	Region
1	OE - 3	c.1209+1G→A	c.1209+1G→A	Gaza
2	OE - 4	Δ F508	Δ F508	Gaza
3	OE - 10	Δ F508	Δ F508	Gaza
4	OE - 13	Δ F508	Δ F508	Gaza
5	OE - 18	W1282x	Unknown	Gaza
6	OE - 19	Δ F508	Δ F508	Gaza
7	OE - 21	Δ F508	Δ F508	Gaza
8	OE - 25	Δ F508	W1282X	Gaza
9	OE - 31	Δ F508	Unknown	Gaza
10	OE - 32	Δ F508	G542X	Gaza
11	OE - 33	Δ F508	Δ F508	Gaza
12	OE - 37	W1282X	Unknown	Gaza
13	OE - 44	W1282X	Unknown	Gaza
14	OE - 50	N1303K	N1303K	West Bank
15	OE - 53	G85E	N1303K	West Bank

Table 5: Patients with previously determined CF Mutations

3.6 ΔF508 Mutation Detection by Heteroduplex Analysis

The ΔF508 mutation located in exon 11, is a three bp deletion between nucleotides 1652 and1655 - see Figure 7. These nucleotides constitute the codon for phenylalanine (F) at position 508. This kind of mutation can be detected by several methods. However, one of the easiest methods to analyze this mutation is the heteroduplex analysis method which is based on the formation of a heteroduplex DNA from complementary but non identical DNA strands. This occurs when the DNA double helix denatures and anneals again with another complementary DNA strand. When the heteroduplex DNA contains a mutation, a mismatch will be generated at one or more base pair positions as shown in Figure 6. This mismatch will appear as a kink or a bend in the DNA structure that will have reduced mobility during electrophoresis. This method allows the detection of mismatches as heteroduplex DNA which migrates slower than homoduplex DNA.

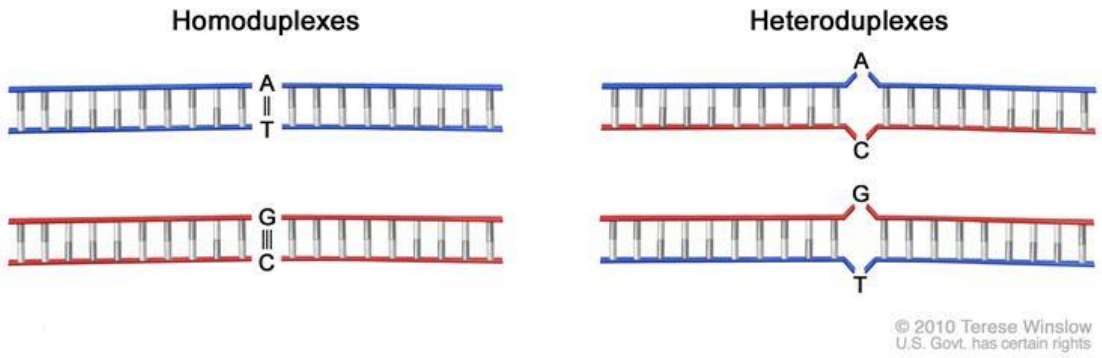


Figure 6: Heteroduplex Analysis. Annealing mutant and wild type DNA generate heteroduplexes with one or more mismatched bases (heteroduplexes), mismatching causes the double helix to have conformation change that hinders its mobility during electrophoresis.

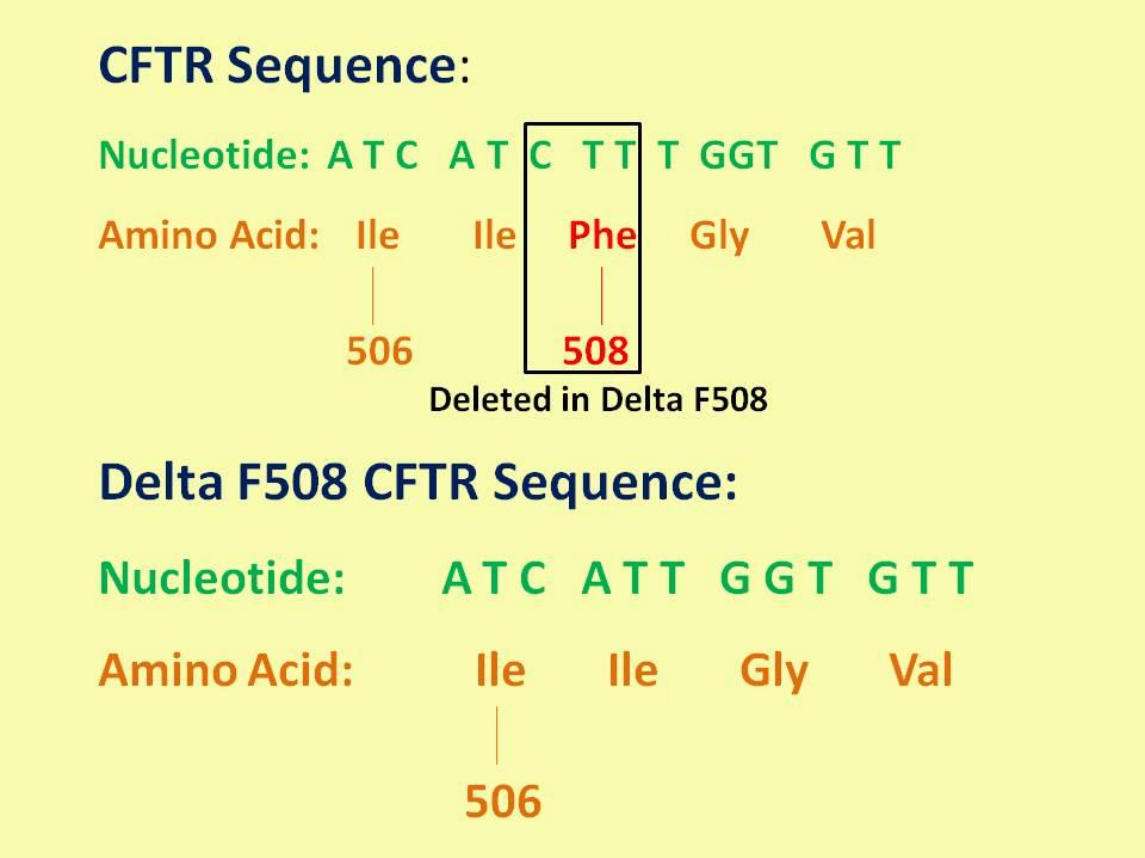


Figure 7: ΔF508: A deletion mutation within CFTR gene.

3.6.1 Heterozygote Mutation Analysis

Mutation analysis based on heteroduplex formation requires first a PCR reaction performed using the FORD program (Table 4) to amplify exon 11(Table 1 – Appendix I) for all patients. The amplification procedure is described previously (testing DNA quality). As a result of this amplification heteroduplexes are detected in patients who are heterozygous carriers of theΔF508 mutation.

3.6.2 Homozygote Mutation Analysis

For the detection of homozygote mutations, analysis can be performed as described for heterozygote mutation analysis. However to detect these homozygous mutations forΔF508, the DNA of all patients needs to be mixed with wild type DNA to form a heteroduplex between the wild type DNA and the homozygous mutant DNA. Then capillary electrophoresis analysis by LabChip GX (PerkinElmer) is performed to visualize the homozygote mutations.

3.7 Next Generation Sequencing (NGS) of the PCR Products

Next generation sequencing is a new developed sophisticated method to sequence DNA in an easy, automated and rapid manner that is relatively cost effective.

In this project, NGS sequencing was used to identify the mutations in the CFTR gene in a set of 74 CF patients (60 unrelated).The sequencing machine that was used is the "MiSeq" (Illumina).This massively parallel sequencing technology based on a reversible dye-terminators technology [30].

The main concept of this method is the formation of "DNA Clusters". This involves the attachment of DNA molecules and primers on a slide and then amplified with polymerase. This is followed by the determination of the sequence in which four different types of reversible terminators bases are used. Nucleotides that are incorporated are washed away.

The fluorescence generated by excitation with a LASER is captured by a camera and then the DNA is removed from the dye and the terminal 3' blocker to allow the next cycle to begin. In this process DNA chains are extended one nucleotide at a time in conjunction with imaging of the nucleotide addition which can be performed at a delayed moment, and this allows the capture of successive images for very large arrays of DNA colonies from a single camera. The instrument

throughput depends on the number of cameras used and the camera conversion rate. Nowadays the throughput of this instrument can be multiplies of one million nucleotides per second, this means that one human genome equivalent can be sequenced within one hour using one instrument equipped with one camera [31].

To perform the NGS for all patients, the PCR products of the 28 CFTR assays (27 exons) were pooled together by taking 2 µl from each PCR product to have a total of 56 µl. Then the PCR pools were checked by performing capillary electrophoresis using LabChip GX (PerkinElmer). Since the electrophoresis results were adequate, calculations were performed to dilute the samples so that they can be introduced to the "MiSeq" for Next Generation Sequencing (Illumina). Data interpretation is carried out by comparing all the detected variants in the CFTR gene with an up-to-date database, and then a human interpretation is performed to decide whether the detected variants can be considered as disease causing or not. Figures 12, 13 and 14 – Appendix II show some examples of "Miseq" results.

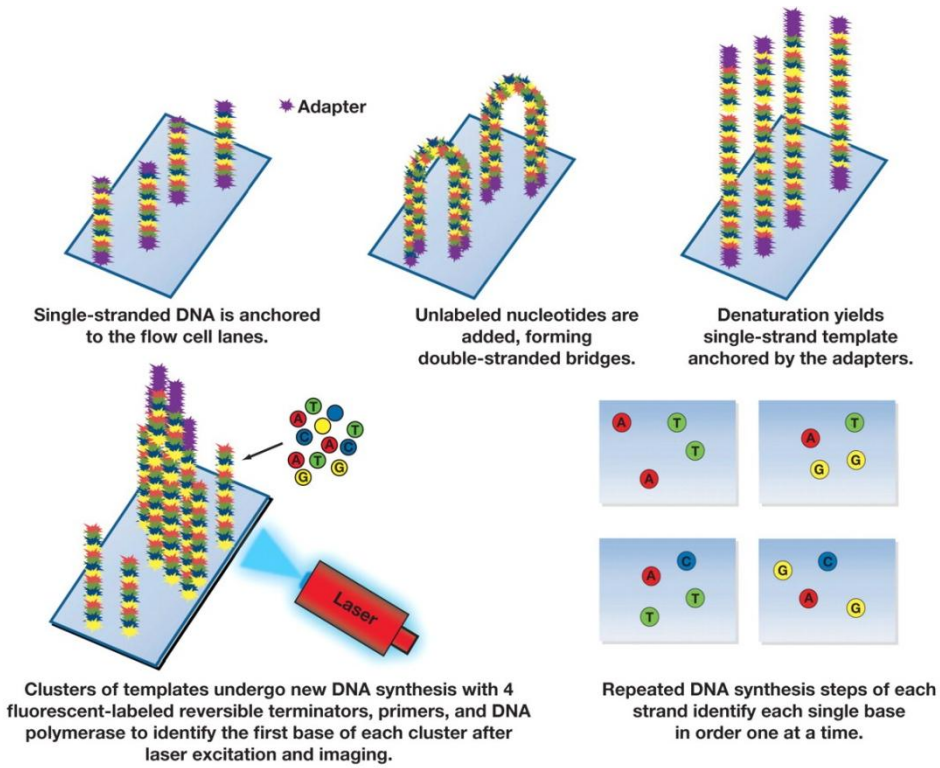


Figure 8: A diagram of the Illumina sequence by synthesis next-generation sequencing platform.

3.8 Confirmation of Identified Mutations by Sanger Sequencing

Sanger sequencing is a DNA sequencing method in which DNA is replicated by selectively adding chain terminating dideoxy-nucleotides by DNA polymerase [32]. This sequencing method was the most widely used method for about 25 years. In spite of the new developments in sequencing technology, Sanger sequencing is still widely used especially for small scale projects and to confirm results obtained by NGS analysis.

To perform sequencing using Sanger method, in addition to the normal requirements of any PCR reaction (DNA primer, DNA polymerase, normal nucleotides; deoxy nucleotide triphosphates dNTPs, Single stranded DNA template), modified dideoxy nucleotides (ddNTPs) are essential to terminate the DNA strand elongation. These modified nucleotides can't form a phospho-diester bond between two nucleotides because they lack a hydroxyl group (OH) on their 3' position where the addition of the next nucleotide occurs. This fact stops the DNA elongation process because DNA polymerase can't function any more in the presence of such an incorporated nucleotide.

In order to sequence DNA in a sample using this method, the sample should be divided into four separate sequencing reactions, each reaction should contain DNA polymerase, DNA primer, the four normal dNTPs (dATP, dGTP, dCTP and dTTP) and only one of the four dideoxy-nucleotides (ddATP, ddGTP, ddCTP, or ddTTP). After several rounds of DNA template extension, the DNA fragments resulted from the reaction are denatured and separated according to their size using gel electrophoresis for each one of the four different reactions, the different lengths of the DNA fragments appear as dark bands in one of the four lanes which indicates one of the ddNTPs reactions, the length of the DNA fragment will depend on the position where the

dideoxy nucleotide (ddNTP) binds and terminates the chain. DNA sequence is obtained by reading the relative position for the different bands from bottom to top among the four lanes.

The ddNTPs may be tagged using a fluorescent or radiolabeling, this labeling allowed to automate the sequencing process and made it easier, faster, and cost effective.

In this study, most of the mutations that were determined using NGS method were verified and validated by the Sanger Sequencing method. To interpret the data obtained from Sanger sequencing, the sequence was introduced to a program called "SeqScape" which helps to locate any variant in the sequenced DNA fragment after comparing it with a reference sequence.
Some examples of these interpretations can be found in Appendix II – Figures 8-11.

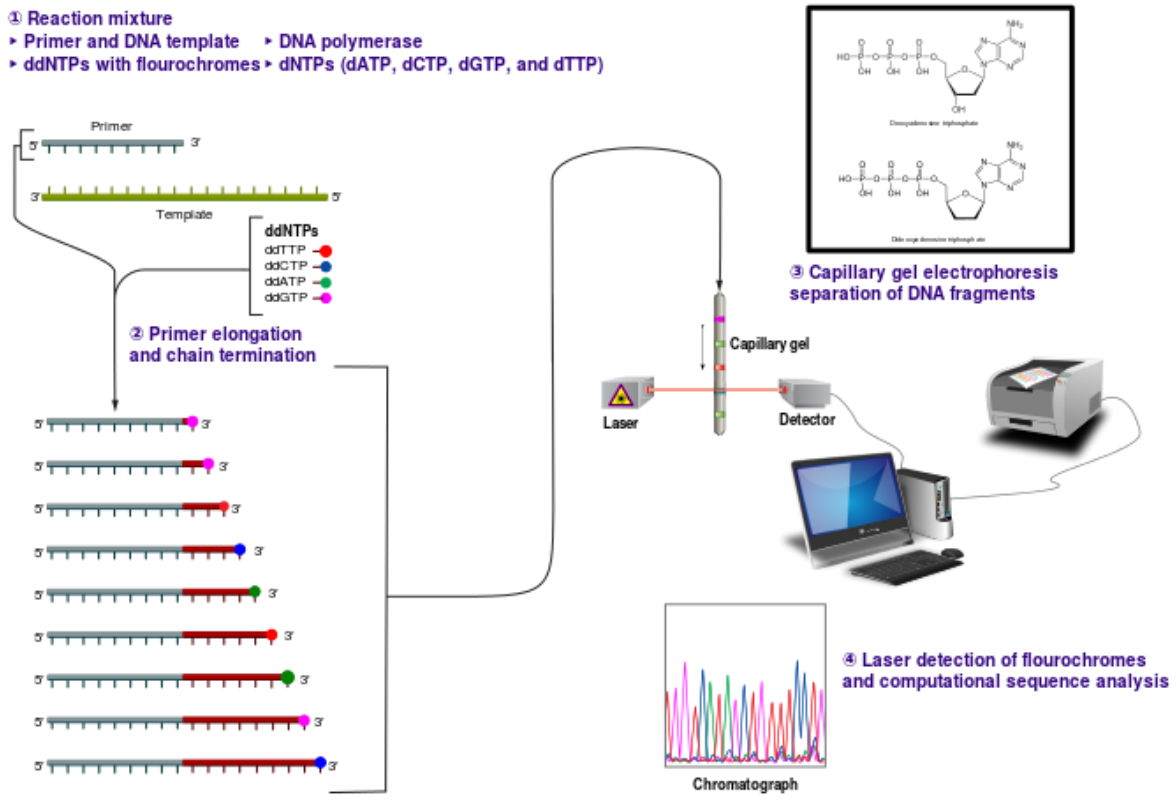


Figure 9: Schematic diagram of Sanger Sequencing Method

3.9 Allele Specific Mutation Analysis (ASMA)

Allele specific mutation analysis is a diagnostic technique used to detect single nucleotide variants. This technique requires previous knowledge of the tested DNA sequence. To detect the single nucleotide variations, primers are designed and amplified with the suspected DNA under suitable conditions. If amplification with the mutant primer is performed and result in the expected length of the DNA band on gel (if gel electrophoresis is used) the mutation is present, whereas in case the wild-type primer is used, no band should be observed for patients with a homozygous mutation. Figure 10 shows the two allele specific primers that are used to detect a mutation. If an amplified product is observed with the mutant primer as well as with the normal primer, the mutation is present as a heterozygous state.

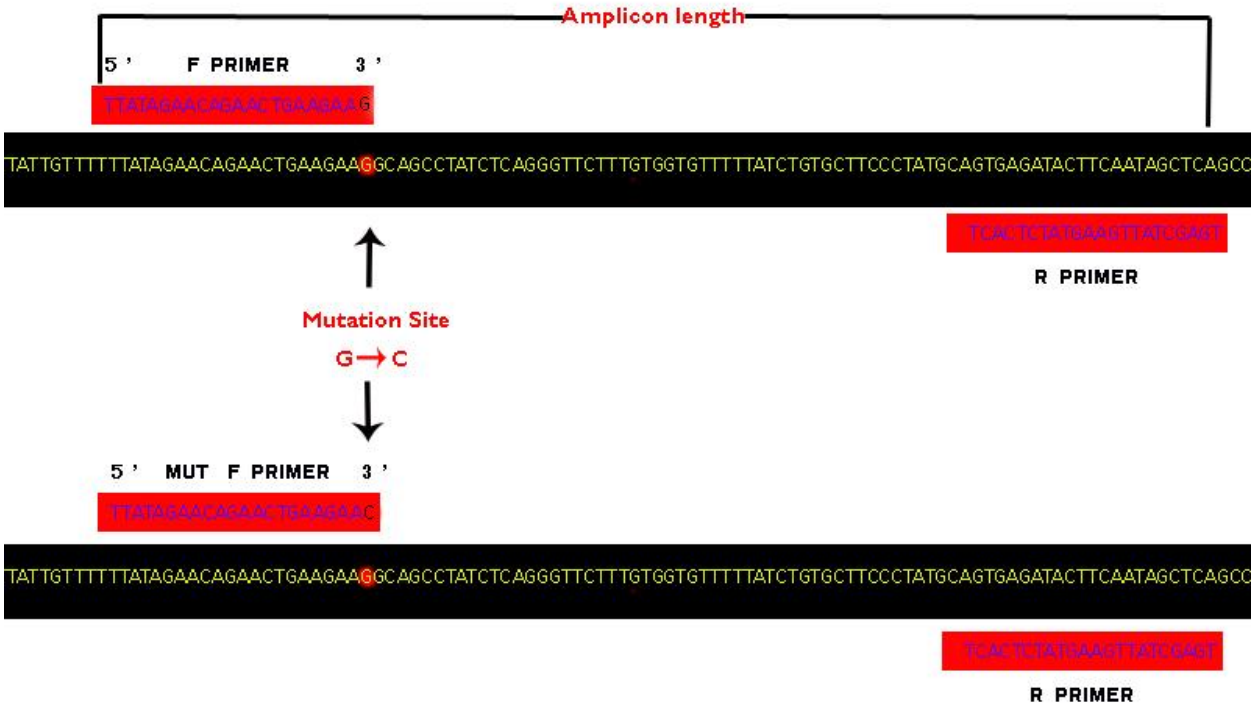


Figure 10: Allele Specific Mutation Analysis

This allele specific mutation analysis was developed to be used as a diagnostic method to detect the most prevalent mutations among Palestinians. All these mutations were identified by NGS sequencing. An example of the results is shown in Figure 15 – Appendix II. Table 6 below shows the primers designed to detect each of the mutations**.**

Mutation	Forward Primer	Reverse Primer	PCR Prog.
R347P	TCATTCTGCATTGTTCTGCG TCATTCTGCATTGTTCTGCC	GCACATTTTTGCAAAGTTCA	Ford
2183A A →G	CTCCTGGACAGAAACAAAAAAA CTCCTGGACAGAAACAAAAAG	CTCTTCGATGCCATTCATTTGTAA	CF 62
W1282X	TGTGCAACAAGGTTTGAATGA	TCACTCCAAAGGCTTTCCTC TCACTCCAAAGGCTTTCCTT	CF 55
R75X	ACTCATTAATGCCCTTCGGC AACTCATTAATGCCCTTCGGT	TTTGGAGTTGGATTCATCCTTT	CF 62
N1303K	AGAACTTGATGGTAAGTACATG	ACTGTTCATAGGGATCCAAG ACTGTTCATAGGGATCCAAC	Ford
G85E	GAAATAGGACAACTAAAATATTTGCAC	CTTACCCCTAAATATAAAAAGATTC CTTACCCCTAAATATAAAAAGATTT	Ford
1525-1G→A	TAATAATGATGGGTTTTATTTCCAG TAATAATGATGGGTTTTATTTCCAA	GTGAAGGGTTCATATGCATAATCAA	CF 62
3120+1Kbdel8.6Kb[33]	AACCAGACTGTCAGTTTGCCTCAT	AATGCCCATAAACACCCAGGAAAG	CF 62

Table 6: Primers and PCR programs used to detect different CFTR mutations

For exon 19, 20 and 21 deletion mutation (3120+1Kbdel8.6Kb) see reference [33]

For exons 19, 20 and the 21 deletion mutation (3120+1Kbdel8.6Kb), the primers used were obtained from published literature [33]. PCR amplification can detect the deletions only when the breakpoint sites of these deletions are known.

3.10 Multiple Ligation-dependent Probe Amplification (MLPA)

MLPA is a multiplex PCR method used to detect abnormal copy numbers of genomic DNA sequences [30]. This technique is one of the easiest and fastest techniques performed to detect whole gene deletions/duplications but also partial gene deletions or duplications can be detected. This technology requires a PCR machine and capillary electrophoresis equipment. SALSA MLPA Kit from MRC-Holland is used for this technique. The type of mutations detected by this technique are usually missed with other techniques such as conventional DNA sequencing.

There are four major steps in the MLPA reaction; the first step is the denaturation of the DNA by overnight incubation with a mixture of MLPA probes that consist of two separate oligonucleotides (each contains one of the PCR primers). The overnight incubation allows the two probes to hybridize to adjacent target sequences (Figure 11 - step 1). The second step is the ligation reaction in which the two probe oligonucleotides are ligated after being hybridized to their adjacent targets (Figure 11 - step 2).The third step is a PCR reaction in which ligated probes from the previous step will be amplified exponentially and the number of the target sequences in the sample is measured by the number of probe ligation products because any probe that is not-ligated in the second step will not amplify in this step and therefore the removal of these probes is unnecessary because they will not generate a signal in the separation step (Figure 11 - step 3).The fourth step is the separation of the PCR products by capillary electrophoresis (Figure 11step 4). Finally, data is analyzed by comparing the peak pattern obtained by electrophoreses with reference samples and thus difference can be detected.

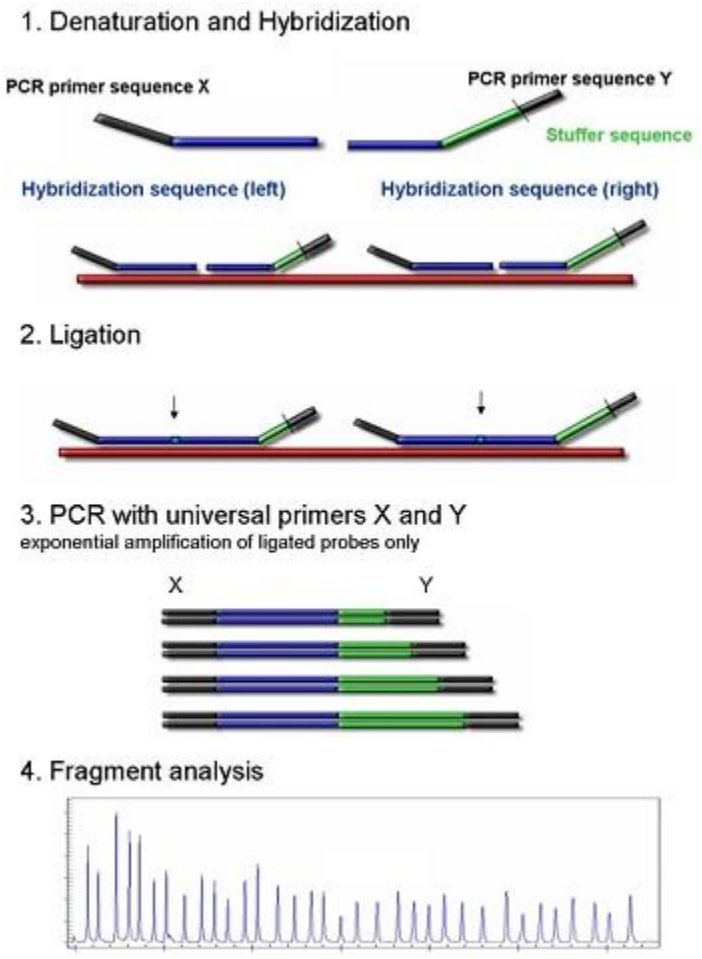


Figure 11: MLPA reaction; the MLPA probes should be hybridized to the target sequence. Then a single pair PCR primers is used for MLPA amplification in a multiplex PCR. The resulting PCR products of the MLPA kit range between 130 - 480 nucleotides in length and they can be analyzed by capillary electrophoresis.

Chapter 4

Results

4.1 DNA Quantity and Quality

The quantity and the quality of extracted DNA were determined by Dropsense 96 (Trinean). Although we obtained for all samples a sufficient yield of DNA, our results showed that the amount of DNA recovered from samples stored at -80 ºC was larger (mean = 517.1ng/µl) than those stored at -20 ºC (mean = 189.3ng/µl) respectively. For the purity of the extracted DNA, most of the samples have good quality DNA since the ratio ranged between an OD of 1.6 – 2.0. On the other hand, some DNA samples (OE-28, 32, 43, and 55) gave results lower than 1.6 indicating the presence of contaminants. However, this didn't indicate that those DNA samples are unsuitable for use. The results of quantity and quality measurements are shown in Table 2 in Appendix I.

The quality of the DNA was also assessed by PCR (for the exons of the CFTR gene) and the amplicons were checked by capillary electrophoresis. The results showed that the DNA for all samples tested have good quality indicated by sharp bands at 436bp as shown in Appendix II Figure1.

4.2 INNO-LIPA CFTR

The INNO-LIPA test is a genetic method that can detect 36 mutations on the CFTR gene and wild type simultaneously by reverse hybridization. The DNA probes for the 36 mutations are coated on 2 strips. The results of the INNO-LIPA test are interpreted according to the appearance and/or absence of bands on the test strips. For homozygous mutations, a band appears in the mutation area but disappears from the normal (wild type) non-mutated area. For heterozygous mutations, the band will appear in both areas; the mutated and the wild type. The results of INNO-LIPA are shown in Figures 2 and 3 (Appendix II). The purpose of using this method was to confirm previously diagnosed patients on one hand and to assess the possibility of adopting this technique to identify the mutations that may be present on the CFTR gene of CF patients in Palestine.

Table 7: INNO-LIPPA Test Results.

Patient number	Mutation Identified in Palestine	INNO-LIPPA	Sequencing
OE-3[1]	c.1209+1G→A/ c.1209+1G→A	Not detected	c.1209+1G→A/ c.1209+1G→A
OE-4	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-9[2]	Δ F508/ Δ F508	2183AA→G/2183AA→G	2183AA→G/2183AA→G
OE-13	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-18[2]	W1282X/???	Not detected	c.3793G→A/ c.3793G→A
OE-19	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-21	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-25[2]	Δ F508/W1282X	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-31[2]	Δ F508/???	Not detected	Not detected
OE-32	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-33	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508
OE-37[2]	W1282X/???	R347P/R347P	R347P/R347P
OE-44[2]	W1282X/???	Not detected	Not detected
OE-53[1]	G85E/N1303K	N1303K / G85E	N1303K / G85E
OE-56[1]	Δ F508/ Δ F508	Δ F508/ Δ F508	Δ F508/ Δ F508

1. Tests done at foreign labs.
2. Indicate discrepancies between the results

Table 7 summarizes the type of mutation detected for each patient as compared with tests conducted in Palestine and the reference results by sequencing. The foreign laboratories are mostly Israeli's, these labs performed molecular diagnostic tests to identify some CF mutations.

It is remarkable that for the 12 patients tests performed in local laboratories only 20 mutations were identified. Inaccurate results have been reported in 40% of the cases (8/20) reflecting a considerable rate of errors. It was found that INNO-LIPPA test can detect 8 of the 18 mutations identified among the Palestinian population which represent 66 % of the identified mutations as shown in Table 4 – Appendix I.

4.3 ΔF508 Mutation Detection by Heteroduplex Analysis

Heterozygote and homozygote mutation analysis

During electrophoresis, a patient heterozygote ΔF508 will form a heteroduplex between the wild-type strand and the mutant strand. This DNA fragment will migrate slowly and will show a different band as shown for patients OE 24, OE 26, OE 32, and OE 59 in Figure 4 (Appendix II) and Table 3 (Appendix I).

To detect the homozygote ΔF508 patients, PCR products were amplified using CFTR 13 assay primers, after mixing their entire DNA with wild type DNA to artificially form a heteroduplex. The same visualization method can be used as described above. Homozygous mutations were identified for patients (OE1, OE2, OE 4, OE 11,OE 13, OE 19, OE 21, OE 25, OE 33, OE 56, OE 58, OE60, and OE 62). Figure 5 (Appendix II) shows all the heteroduplexes formed (Homozygous and heterozygous ΔF508 carriers).

4.4 Polymerase Chain Reaction (PCR) of the Coding Region of the CFTR Gene

For each patient 28 assays were used to amplify the 27 exons of the CFTR gene; these assays were designated as CFTR – 3 to CFTR – 30. To check the quality of these assays, capillary electrophoresis was performed on two assays randomly chosen for each patient.

The electrophoresis results showed that assays 16, 18 and 22 were not acceptable. Therefore, the PCR for these assays was repeated and optimized using different DNA concentrations. Subsequent capillary electrophoresis showed that the results were acceptable. The results are shown in Figures 6.1, 6.2, and 7 (Appendix II)

4. 5 Next Generation sequencing (NGS) of the PCR Products

The Next Generation Sequencing method (MiSeq System, Illumina) was used to sequence all 27 exons of the CFTR gene. This method is automated and rapid. It can simultaneously analyze and interpret the data by comparing all detected variants in the gene with an up-to-date database. Subsequently, human interpretation is required to check the variants detected by the instrument to decide whether these variants are CF causing mutations or not. Since this method gives rise to a very huge amount of data, many different programs are used to help in interpreting this data. One of the used programs is "Alamut" program which helps in recognizing the influence and the pathogenicity of any mutation.

Most of the exons for all patients had enough coverage to do a reliable interpretation of the sequencing data. Sequencing of CFTR genes of all patients and identifying the deletion regions of this gene allowed us to detect 16 different mutations distributed over 81alleles (41patients); these mutations are shown in Table 8 below. All mutations in the different patients identified by

NGS are listed in table 3 (APPENDIX I). Several mutations (for examples ΔF508) were confirming the INNO-LIPA results or the heteroduplex analysis, where one of these 16 mutations was not identified before. 66 alleles (33 patients) were homozygous, 14 alleles (7 patients) were compound heterozygous, and 1 allele (1 patient) was even heterozygous. Remarkable was the fact that for patient OE51 and OE52 no reads for exon 2 were identified, whereas for all other patients this exon was nicely covered. As no mutations in the other exons were found in those two family-members, this result was very suggestive for a deletion of exon2 and this was confirmed by MLPA. Further PCR analysis revealed also a deletion of other exons in the CFTR gene, which were identified by designing primers flanking the deletion mutation 3120+1Kbdel8.6Kb (deletion of exons 19, 20 and 21); we were able to visualize this mutation after PCR amplification and electrophoresis. We were able to detect the deletion in 9 alleles. Figures 16, 18, and 21 - Appendix II show capillary electrophoresis and MLPA results for exons 19,20 and 21deletion detected by these methods, and Table 3 - Appendix I shows all the results derived from these methods.

Table 8: Detected Mutations

Legacy Name	c.DNA Name	Exon or Intron #	Class
ΔF508	c.1521_1523delCTT	Exon 11	2
3120+1Kbdel8.6Kb*	-----	Del exons 19,20,21	
1525- 1G →A	c.1393-1G→A	Intron 10 - Exon 11	
G85E	c.254G→A	Exon 3	2
2183AA→G	c.2051_2052delAAinsG	Exon 14	1
1717-1G→A	c.1585-1G→A	Intron 11 - Exon 12	1
N1303K	c.3909C→G	Exon 24	2
W1282X	c.3846G→A	Exon 23	1
Exon 2 Del*	-----	Del exon 2	
1341+ 1G→A	c.1209+1G→A	Intron 9 - Exon 10	
4382delA	c.4251delA	Exon 27	
R75X	c.223C→T	Exon 3	1
G1265R	c.3793G→A	Exon 23	
R347P	c.1040G→C	Exon 8	4
D1270N	c.3808G→A	Exon 23	
2221insA	c.2089_2090insA	Exon 14	
G542X	c.1624G→T	Exon 12	1
Q1100P	c.3299A→C	Exon 20	

Mutations designated by * were not identified by this method. For the NGS results Table 3-Appendix I shows them and show the other tests to confirm them.

4.6 Confirmation of Identified Mutations by Sanger Sequencing

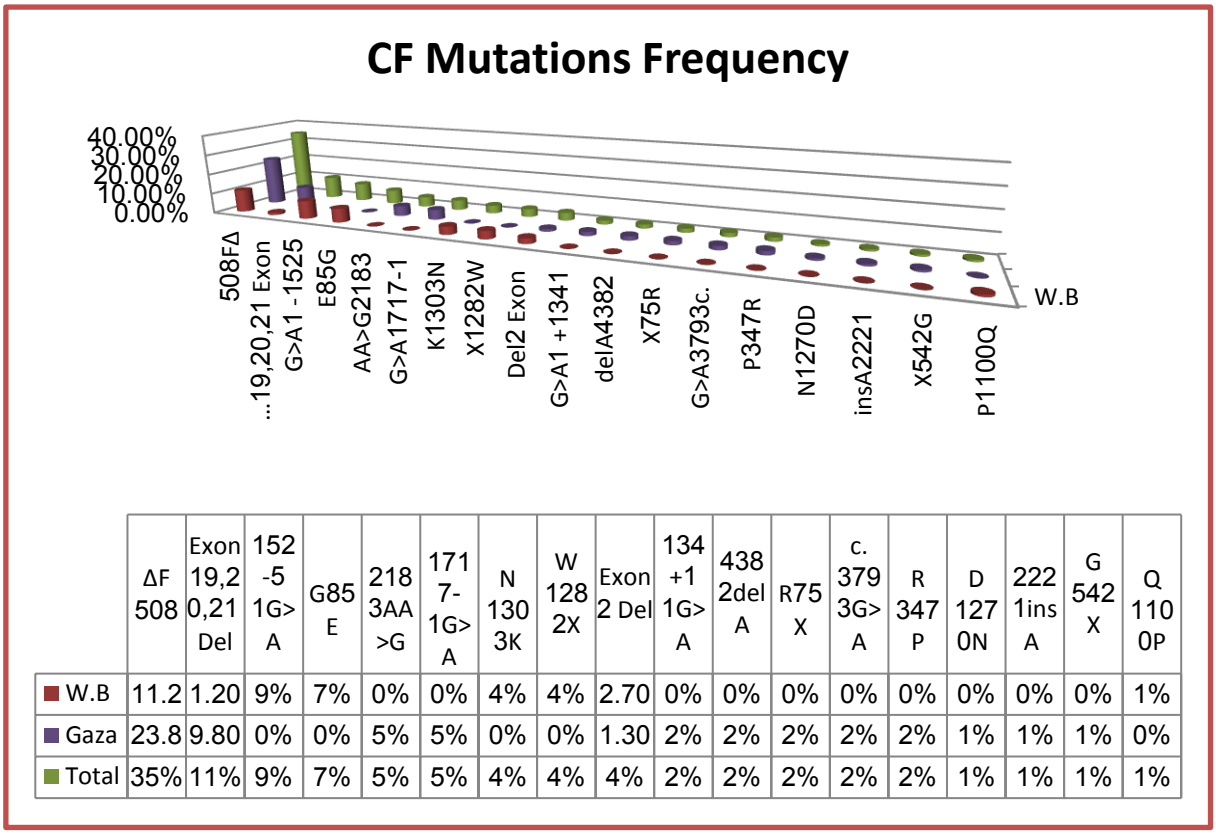
Sanger sequencing was used to confirm the CFTR mutations identified by the NGS method. All the results obtained by the Sanger sequencing method were identical to the results of the NGS method. The sequences were analyzed using "SeqScape" program to identify the mutation site and type of mutation, some representative results are shown in Figures 8-11 Appendix II and all the confirmed mutations by Sanger method are shown in Table3 - Appendix I.

4.7 Multiple Ligation-dependent Probe Amplification (MLPA)

This method was used to detect any deletion in the CFTR gene especially to verify if this mutations occurs among the tested population, and also because deletions and insertions are often not detected by DNA sequencing (12/120 alleles).Twelve alleles with 2 different kinds of deletion mutations (exon 2 deletions and 3120+1Kbdel8.6Kb{deletion of exons 19, 20 and 21}) were detected which proves that this test is really relevant. All results are shown in Table 3 - Appendix I, and some of the interpretations of the results are shown in figures 18-21 - Appendix II.

4.8 Mutational Spectrum of Cystic Fibrosis

As a result of this research and from all the data gathered by the previous methods, Table 9 shows the frequency of different mutations among Palestinians residing in Gaza and West Bank, other graphs to clarify the difference between the patients originated from Gaza and those from the West Bank are shown in Figure 12 below and Figures 22 and 23 - Appendix II.



	ΔF 508	Exon 19,20,21 Del	152-5 1G>A	G85E	2183AA>G	1717-1G>A	N1303K	W1282X	Exon 2 Del	1341G>A	4382delA	R75X	c.3793G>A	R347P	D1270N	2221insA	G542X	Q1100P
W.B	11.2	1.20	9%	7%	0%	0%	4%	4%	2.70	0%	0%	0%	0%	0%	0%	0%	0%	1%
Gaza	23.8	9.80	0%	0%	5%	5%	0%	0%	1.30	2%	2%	2%	2%	2%	1%	1%	1%	0%
Total	35%	11%	9%	7%	5%	5%	4%	4%	4%	2%	2%	2%	2%	2%	1%	1%	1%	1%

Figure 12: A chart showing the prevalence rates of different mutations in Palestine

Table 9: Frequency of CF mutations among Palestinians in Gaza and West Bank.

No.	Mutation	Mutated Alleles			Percentage			Total %
		W.B	Gaza	Total	W.B	Gaza	Total	
1	ΔF508	9	19	28	11.2%	23.8%	35%	**35 %**
2	Exon 19,20,21 Del	1	8	9	1.2%	9.8%	11%	**46 %**
3	1525- 1G→A	7	0	7	9%	0%	9%	**55 %**
4	G85E	6	0	6	7%	0%	7%	**62 %**
5	2183AA→G	0	4	4	0%	5%	5%	**67 %**
6	1717-1G→A	0	4	4	0%	5%	5%	**72 %**
7	N1303K	3	0	3	4%	0%	4%	**76 %**
8	W1282X	3	0	3	4%	0%	4%	**80 %**
9	Exon 2 Del	2	1	3	2.7%	1.3%	4%	**84 %**
10	1341+ 1G→A	0	2	2	0%	2%	2%	**86 %**
11	4382delA	0	2	2	0%	2%	2%	**88 %**
12	R75X	0	2	2	0%	2%	2%	**90 %**
13	c.3793G→A	0	2	2	0%	2%	2%	**92 %**
14	R347P	0	2	2	0%	2%	2%	**94 %**
15	D1270N	0	1	1	0%	1%	1%	**95 %**
16	2221insA	0	1	1	0%	1%	1%	**96 %**
17	G542X	0	1	1	0%	1%	1%	**97 %**
18	Q1100P	1	0	1	1%	0%	1%	**98 %**
	Total	**32**	**49**	**81**			**100%**	

4.9 Allele Specific Mutation Analysis (ASMA)

In order to identify the 8 most frequent CFTR mutations present in the Palestinian population representing 80% of the mutations present, we designed a relatively easy method using the ASMA technique (Table 6). This method allows us to have a simple and an easy to perform molecular diagnostic test for CF which can be adopted without the need for more sophisticated sequencing equipments. Figures 16 and 17 show the capillary electrophoresis results for two different mutations detected by this ASMA method, and Table 3 - Appendix I shows all the results derived from this method.

Chapter 5

Discussion

This study was conducted on 73 patients (60 unrelated) residing in the West Bank and Gaza, Palestine who were diagnosed with cystic fibrosis. The aim of this study was to determine the types and rates of mutations present in the CFTR gene and to develop allele specific mutation analysis method that can be useful in identifying the most common mutations among Palestinians and can be utilized for proper and rapid diagnosis of CF.

The nature and distribution of the CFTR gene mutations among the Palestinian population is different from mutations reported in neighboring and regional countries such as Jordan, Lebanon, Egypt, Tunis, Algeria, Turkey, Iran and Israel [35-44]. It is apparent that the spectrum and rate of the various CFTR gene mutations varies significantly between these countries.

The most common five mutations identified in the tested samples represent 72% of all mutations detected. Specifically, ΔF508 (28/81) at a rate of 34.6%, followed by the deletion of exons 19, 20 and 21 (3120+1Kbdel8.6Kb), 1525- 1G→A, G85E, 2183AA→ G and 1717-1G→ A at rates of 11.1% (9/81), 8.6 % (7/81), 7.4 % (6/81), 4.9% (4/81) and 4.9% (4/81) respectively.

The types of the CFTR gene mutations detected among Palestinian population were; missense mutations 33 % (6/18), non-sense mutations 16.5% (3/18) and splicing error mutations 16.5 % (3/18). Comparing our results with the most common mutations reported worldwide where missense mutations were higher than ours (48.7 %), while the nonsense mutations and splicing error mutations were (12.9%) and (15.7%) respectively similar to the results obtained by us[14].

This is the first study to address the prevalence of CFTR gene mutations in Palestine. There is a lack of published literature regarding this subject. However, a study conducted in Israel by Laufer-Cahana et al on mutations among Israeli Arab patients showed similar rates for mutations G85E (8%), 2183AA→ G (8%), deletion of exons 19,20 and 21(3120+1Kbdel8.6Kb) (13%). These results are in agreement with results obtained in our study. Interestingly, the deletion of exons19, 20 and 21(3120+1Kbdel8.6Kb) was only found among Palestinian Arabs which may indicate that this mutation is a founder mutation among this population [33,45].The rate of ΔF508 in the Israeli study was much lower (23.5%) than our results although it was the most common mutation in both studies [28].

A study conducted in Israel by Quint et al (2005) on Jewish CF patients living in Israel reported that W1282X mutation was the most common (43%) compared to (4%) among Palestinian population tested in this study. However, ΔF508 was the second common mutation among Jewish CF patients (33.5 %) similar to the rate identified in this study among Palestinians (35%). Moreover, the G542X mutation was more common among Jewish CF patients (10 %) as compared to (1%) among Palestinian CF patients while1717-1G → A mutation was more common among Palestinians (5%) as compared to (1%) among Jewish CF patients. The rate of N1303K mutation was about the same in both populations (5%). Interestingly the G85E mutation was only among Balkan Jews (Turkish and Greeks) at a rate of (9.5 %) which is similar to the rate found in this study (7 %) [44].

It was notable the presence of differences in the rates of mutations identified in patients residing in the West Bank versus those residing in Gaza. For example mutations 1525- 1G→A, G85E, W1282X and N1303K were only found (100%) among Palestinians residing in the West Bank, while mutations 2183AA→ G, 1717-1G→ A were only found among Palestinians residing in

Gaza. It was interesting to observe that ΔF508 mutation was primarily prevalent among patients living in Gaza (68%) as compared to patients living in the West Bank (32%). This may be due to heterogeneous Arab population screened and /or geographical distribution of the patients tested, and the limitations of freedom of movement due to Israeli occupation.

Interestingly, we identified a new CF mutation for the first time in the CFTR gene in an 8 years old male child from Gaza. This mutation has not been reported in the literature and hence can be considered to be the first ever reported. This is a homozygous missense mutation located in exon 23 in which the nucleotide Guanine is replaced by Adenine (c.3793G→A), and on the protein level it is altering the amino acid Glycine to Arginine (p.Gly1265Arg). More investigations should be held to understand the influence and the class of this mutation to recognize its pathogenicity.

It was also significant that our findings indicated the presence of mutations in 81 alleles for the 60 patients tested. It is expected to find a total of120 mutations corresponding to the 120 alleles present in the tested patients. Interpreting our results indicates that there are 40 patients with two identified mutations (80 alleles), 33 of these patients had homozygous mutations on both alleles while the remaining 7 patients were compound heterozygous, and one patient had only one mutation on one allele (even heterozygous). Since we tested all CFTR gene exons with more than one method including sequencing, and ultimately confirmed the findings with various methods that are highly sensitive and specific, this may lead us to presume the presence of misdiagnosis. The rate of patients who had no mutations detected was about 32.5 % (19.5/60). This can be partially explained by the lack of precision and / or quality control measures followed by local laboratories in the West Bank and Gaza. Additionally, the physicians here may rely only on the results of the sweat test to diagnose this disease. We compared known results

reported for 15 patients with INNO-LIPA and sequencing and found an error rate of 40 % (8/20) as shown in Table 7. Such high rate of errors confirms our doubts of the presence of misdiagnosis on one hand and the erroneous reporting of mutations on the other. However, at the beginning of this project it was not clear if the 36 mutations detected by this test are representative for the CF mutations in the Palestinian population. It was found that 8 of the 18 mutations identified among the Palestinian population can be detected using INNO-LIPA test, and these 8 mutations represent 66 % of the mutations identified among this population as shown in Table 4 – Appendix I.

This may explain the controversies encountered between our results and those conducted by local laboratories as well as the absence of mutations in many of the CF patients tested.

We observed the presence of significant difference in the amount of DNA extracted from samples stored at -80 ºC as compared to those stored at -20 ºC by independent 2-tailed T – test (SPSS 17) at 95% level of significance (P value< 0.05).

In conclusion, this study indicated that the most common mutation encountered among Palestinian people is the ΔF508. In addition, the deletion mutation of exons 19, 20 and 21(3120+1Kbdel8.6Kb) appears to be an unusual mutation that seems to be a founder mutation among Palestinian population. Furthermore, a new mutation has been identified on exon 23 (G1265R) of the CFTR gene in an 8 year old child from Gaza.

It was apparent in this study the presence of a considerable misdiagnosis among CF population in the West Bank in general and Gaza in particular. A high rate of errors has been encountered throughout this study due to inaccurate screening tests performed by local laboratories. These discrepancies in the identification of the mutations on the CFTR gene could be attributed to

misdiagnoses of patients. Molecular characterization of the CF patients in Palestine was unsuccessful. Therefore, the data obtained failed to contribute to the establishment of a comprehensive database for all the mutations found in the CFTR gene among this population. INNO-LIPA was an alternative molecular testing method used to identify the existing mutations in the CFTR gene. This molecular diagnostic method was partially successful since it can identify about half (8/18) the mutations that occur among this population. To get a more comprehensive spectrum of the predominant CF mutations present in the Palestinian population, allele specific mutation analysis method was modified to identify about 80% of the mutations.

Chapter 6

Recommendations

1- The Palestinian Ministry of Health must implement specific criteria to be followed by physicians in the diagnosis of CF patients.

2- The criteria for CF diagnosis must not relay on symptoms and sweat test only.

3- Screening tests should be performed especially for families with CF history.

4- Molecular characterization and mutation analysis should be implemented in the diagnosis procedure.

5- Extensive molecular techniques must be used to identify all possible mutations that could be present in the Palestinian CF patients.

6- A comprehensive database must be established to hereditary diseases commonly found among Palestinian population including CF.

7- The relationship between the CFTR gene mutations and the course of the disease should be evaluated in order to provide better management and treatment for the CF patients.

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Appendix I

Table 1: Primers used in CFTR Assays

CFTR Assay #	Exon #	Forward Primer	Reverse Primer	Amplicon Length
3	1	GAAAGCCGCTAGAGCAAA	CCAAACCCAACCCATACA	419
4	2	GCCTGTAAGAGATGAAGCCT	CCACCATACTTGGCTCCTAT	435
5	3	TCTGGCTGAGTGTTTGGT	TGGTTTCTTAGTGTTTGGAGTTG	448
6	4	TTGTGTTGAAATTCTCAGGGTAT	CTTGTACCAGCTCACTACCTA	414
7	5	CAACTAGAAGCATGCCAGTAT	TAATTACTATTATCTGACCCAGGAA	428
8	6	AGAACCACGAAGTGTTTGA	CACTGAAGATCACTGTTCTATGC	427
9	7	AGGCTGTCATAAGGGATAGAG	AGGTGGAAGTCTACCATGA	481
10	8	TCCATTCCAAGATCCCTGATA	CCATCATACTGTCCAGAGAAA	550
11	9	AGATGTAGCACAATGAGAGTAT	TGGCCATTCCTCTACTTCTTA	388
12	10	TCCTCTAGAAACCGTATGCT	CTTCCAGCACTACAAACTAGA	483
13	11	CTTCTGCTTAGGATGATAATTGG	GCTTACCCATAGAGGAAACA	436
14	12	TCAACTGTGGTTAAAGCAATAG	GATTCTTAACCCACTAGCCATA	391
15	13	TCTACACTAGATGACCAGGAA	GAGAAACTGGTTTAGCATGAG	410
16	14	CTATCAGAATTCACAAGGTACCAAT	AGAGTTGATTGGATTGAGAATAGAA	573
17	14	GTCTCCTGGACAGAAACAA	TTTAAGATACACCTTATCCTAATCCT	616
18	15	CCACAATGGTGGCATGAAA	AGTAGTGGTTCTACTTGTTGATT	480
19	16	GGGAGGAATAGGTGAAGATG	CTGCACATGCTCACAATTTA	486
20	17	AAGGGTGCATGCTCTTCTA	TGATGGTGGATCAGCAGTT	459
21	18	TTCTAAGTCTATCTGATTCTATTTG	GGGATTGCCTCAGGTTTG	477
22	19	CACTGACACACTTTGTCCAC	CCATGTGTACTTTGTAATATAGTTTCCT	436
23	20	AGAATGGCACCAGTGTGAA	GGAAATTCAAAGAAATCACTTGTTC	572
24	21	GCCCTAGGAGAAGTGTGAATA	GAATGCTCACTGCAGTATTAGAT	438
25	22	GTGAAATTGTCTGCCATTCTTAAA	GGTTCAGGACTCTGCAAATTAAA	527
26	23	TCCACTGGTGACAGGATAAA	AAAGACAGCAATGCATAACAAAT	432
27	24	CAAGGGACTCCAAATATTGCT	AGCCATTTGTGTTGGTATGAG	405
28	25	TTCAAATGGTGGCAGGTAGT	TCTGTTCCCACTGTGCTATT	395
29	26	AAGAAGTACTGGTGATTCTACAT	AGAATTACAAGGGCAATGAGAT	456
30	27	GTCTGACCTGCCTTCTGTC	AGACCCATATCAGTGTCCTC	466

Table 2: Extraction & DNA Quality Data

Sample #	Extraction method	Blood Sample (ml)	DHS added (µl)	DNA Conc. (ng/µl)	DNA Weight (µg)	Purity A260/A280
OE - 1	1	1	100	790	79	1.79
OE - 2	1	1	100	640	64	1.77
OE - 3	1	1	100	432	43.2	1.73
OE - 4	1	1	100	504	50.4	1.69
OE - 5	1	1	100	851	85.1	1.79
OE - 6	1	1	100	961	96.1	1.75
OE - 7	1	1	100	366	36.6	1.75
OE - 8	1	1	100	143	14.3	1.71
OE - 9	1	1	100	443	44.3	1.78
OE - 10	1	1	100	730	73	1.8
OE - 11	1	1	100	537	53.7	1.67
OE - 12	1	0.8	100	1046	104.6	1.8
OE - 13	1	1	100	339	33.9	1.69
OE - 14	1	1	100	763	76.3	1.81
OE - 15	1	1	100	836	83.6	1.81
OE - 16	1	1.4	100	1409	140.9	1.89
OE - 17	1	1	100	571	57.1	1.79
OE - 18	1	1	100	790	79	1.76
OE - 19	1	1	100	1167	116.7	1.73
OE - 20	1	1	100	443	44.3	1.79
OE - 21	1	1	100	262	26.2	1.65
OE - 22	1	1	100	738	73.8	1.82
OE - 23	1	1	100	1266	126.6	1.9
OE - 24	1	1	100	667	66.7	1.72
OE - 25	1	1	100	410	41	1.69
OE - 26	1 + 2	1 + 0.2	100	549	54.9	1.69
OE - 27	1 + 2	0.7 + 0.2	100	495	49.5	1.69
OE - 28	1	1	100	2131	213.1	1.45
OE - 29	1	1	100	90	9	1.71
OE - 30	1	1	100	279	27.9	1.7
OE - 31	1	2	35	87	3.045	1.61
OE - 32	1 + 2	0.4 + 0.2	35	142	4.97	1.54
OE - 33	1 + 2	0.8 + 0.2		201	0	1.66
OE - 34	2	0.2	40	Q A	0	0
OE - 35	1 + 2	0.8 + 0.2	35	114	3.99	1.73
OE - 36	1 + 2	1.1 + 0.2	35	158	5.53	1.66
OE - 37	1 + 2	1 + 0.2	40	199	7.96	1.6
OE - 38	1 + 2	1 + 0.2		145	0	1.73
OE - 39	2	0.2	100	QA	0	

Sample #	Extraction method	Blood Sample (ml)	DHS added (µl)	DNA Conc. (ng/µl)	DNA Weight (µg)	Purity A260/A280
OE - 40	1 + 2	0.7 + 0.2	80	98	7.84	1.62
OE - 41	1 + 2	0.8 + 0.2		100	0	1.61
OE - 42	2	0.2	80	QA	0	0
OE - 43	1 + 2	0.4 + 0.2	100	70	7	1.54
OE - 44	1	1	100	177	17.7	1.62
OE - 45	1	1	80	591	47.28	1.72
OE - 46	1 + 2	0.8 + 0.2	100	131	13.1	1.65
OE - 47	1	1	40	755	30.2	1.79
OE - 48	1 + 2	0.6 + 0.2	80	118	9.44	1.7
OE - 49	1	1	100	54	5.4	1.76
OE - 50	1	2	200	599	119.8	1.61
OE - 51	1	2	200	139	27.8	1.8
OE - 52	1	2	100	107	10.7	1.8
OE - 53	1	2.2	100	564	56.4	1.72
OE - 54	1	1	200	76	15.2	1.73
OE - 55	1	2	100	214	21.4	1.46
OE - 56	1	2.5	200	569	113.8	1.66
OE - 57	1	2	220	77	16.94	1.76
OE - 58	1	2	200	61	12.2	1.81
OE - 59	1	2	200	96	19.2	1.8
OE - 60	1	2	200	28	5.6	1.66
OE - 61	1	2	100	40	4	1.75
OE - 62	1	1	200	147	29.4	1.69
OE - 63	1	2	250	435	108.75	1.49
OE - 64	1	2	250	48	12	1.76
OE - 65	1	2	200	108	21.6	1.82
OE - 66	1	2	30	171	5.13	1.81
OE - 67	1	2	100	320	32	1.63
OE - 68	1	2	100	64	6.4	1.76
OE - 69	1	2	100	36	3.6	1.72
OE - 70	1	2	100	38	3.8	1.69
OE - 71	1	1	100	200	20	1.81
OE - 72	1	2	30	157	4.71	1.81
OE - 73	1	2	30	248	7.44	1.48

Table 3: Identified and Confirmed Mutation by Different Methods

Sample	NGS	InnoLipa	Heteroduplex	ASMA	Sanger Seq.	MLPA
OE – 1*	ΔF508		ΔF508		ΔF508	
OE – 2*	ΔF508		ΔF508			
OE – 3	c.1209+1G→A	N.D			c.1209+1G→A	
OE – 4	ΔF508	ΔF508	ΔF508			
OE – 5	N.D		N.D			N.D
OE – 6	N.D		N.D			N.D
OE – 7	4382delA				4382delA	
OE – 8	N.D					
OE – 9*	2183AA→G	2183AA→G		2183AA→G		
OE – 10*	2183AA→G			2183AA→G	2183AA→G	
OE – 11	ΔF508		ΔF508		ΔF508	
OE – 12	Del exons 19,20,21			Del ex 19,20,21		Del ex19,20,21
OE – 13	ΔF508	ΔF508	ΔF508			
OE – 14	N.D		N.D			N.D
OE – 15	R75X			R75X	R75X	
OE – 16	N.D		N.D			N.D
OE – 17	N.D		N.D			N.D
OE – 18	c.3793G→A	N.D			c.3793G→A	
OE – 19	ΔF508	ΔF508	ΔF508		ΔF508	
OE – 20[1]	D1270N				D1270N	N.D.
OE – 21	ΔF508	ΔF508	ΔF508		ΔF508	
OE – 22	Del exons 19,20,21			Del ex 19,20,21		Del ex 19,20,21
OE – 23	Del exons 19,20,21			Del ex 19,20,21		Del ex 19,20,21
OE – 24[2]	ΔF508/2221insA		ΔF508		ΔF508/2221insA	
OE – 25	ΔF508		ΔF508			
OE – 26[2]	ΔF508		ΔF508			Del ex 2/ΔF508
OE – 27*	2183AA→G			2183AA→G	2183AA→G	
OE – 28*	2183AA→G			2183AA→G	2183AA→G	
OE – 29*	1717- 1G→A				1717- 1G→A	
OE – 30*	1717- 1G→A					
OE – 31	N.D		N.D			N.D
OE – 32[2]	ΔF508/G542X	ΔF508/G542X	ΔF508			
OE – 33	ΔF508	ΔF508	ΔF508			
OE – 34	N.D		N.D			N.D
OE – 35	1717- 1G→A				1717- 1G→A	
OE – 36	N.D.		N.D			N.D
OE – 37	R347P	R347P		R347P	R347P	
OE – 38	N.D.		N.D			N.D

Sample	NGS	InnoLipa	Heteroduplex	ASMA	Sanger Seq.	MLPA
OE – 39	N.D.		N.D			N.D
OE – 40	N.D.		N.D			N.D
OE – 41	Del ex 19,20,21			Del ex 19,20,21		Del ex 19,20,21
OE – 42	N.D.		N.D			N.D
OE – 43	N.D.		N.D			N.D
OE – 44	N.D.		N.D			N.D
OE – 45	N.D.		N.D			N.D
OE – 46	N.D.		N.D			N.D
OE – 47	N.D.		N.D			N.D
OE – 48	N.D.		N.D			N.D
OE – 49	N.D.		N.D			N.D
OE – 50	1525- 1G→A	N.D.			1525- 1G→A	
OE – 51*	N.D.					Del ex 2
OE – 52*	N.D.					Del ex 2
OE – 53	N1303K / G85E	N1303K/G85E		N1303K / G85E		
OE – 54	N.D		N.D			N.D
OE – 55	N.D		N.D			N.D
OE – 56	ΔF508	ΔF508	ΔF508		ΔF508	
OE – 57	1525- 1G→A			1525- 1G→A	1525- 1G→A	
OE – 58	ΔF508		ΔF508			
OE – 59[2]	Del19,20,21/F508		ΔF508	Del ex 19,20,21		Del19,20,21/F508
OE – 60	ΔF508		ΔF508			
OE – 61	N1303K			N1303K	N1303K	
OE – 62	ΔF508		ΔF508			
OE – 63	N.D		N.D			N.D
OE – 64*	1525- 1G→A				1525- 1G→A	
OE – 65*	1525- 1G→A			1525- 1G→A		
OE – 66[2]	W1282X /1525- 1G→A			W1282X /1525- 1G	W1282X /1525- 1G	
OE – 67*	G85E			G85E	G85E	
OE – 68*	G85E			G85E		
OE – 69*	G85E			G85E		
OE – 70[2]	G85E / Q1100P			G85E	G85E / Q1100P	
OE – 71	G85E			G85E		
OE – 72	N.D.		N.D			N.D
OE – 73	W1282X			W1282X	W1282X	

N.D Means a Negative Test

Samples indicated by * are Family members

Sample indicated by [1] is the even Heterozygote

Samples indicated by [2] are Heterozygote CF Patients

Table 4: Mutations detected by INNO-LIPA CFTR 19 & 17 (36 Mutations)

INNO-LiPA *CFTR*19	**INNO-LiPA *CFTR*17+ Tn**
ΔF508*	621 + 1 G → T
G542X	3849 + 10Kb C → T
N1303K*	2183 AA → G*
W1282X*	394del TT
G551D	2789 + 5 G → A
1717 – 1 G → A*	R1162X
R553X	3659del C
CFTR dele 2,3*	R117H
I507del	R334W
711 + 1 G → T	R347P*
3272 – 26 A → G	G85E*
3905 insT	1078 del T
R560T	A445E
1898 + 1G → A	2143 del T
S1251N	E60X
I148T	2184 del A
3199del6	711 + 5 G → A
3120 + 1G → A	
Q552X	

Mutations indicated by * are those that were identified among the Palestinian population and can be detected by the INNO-LIPA

Appendix II

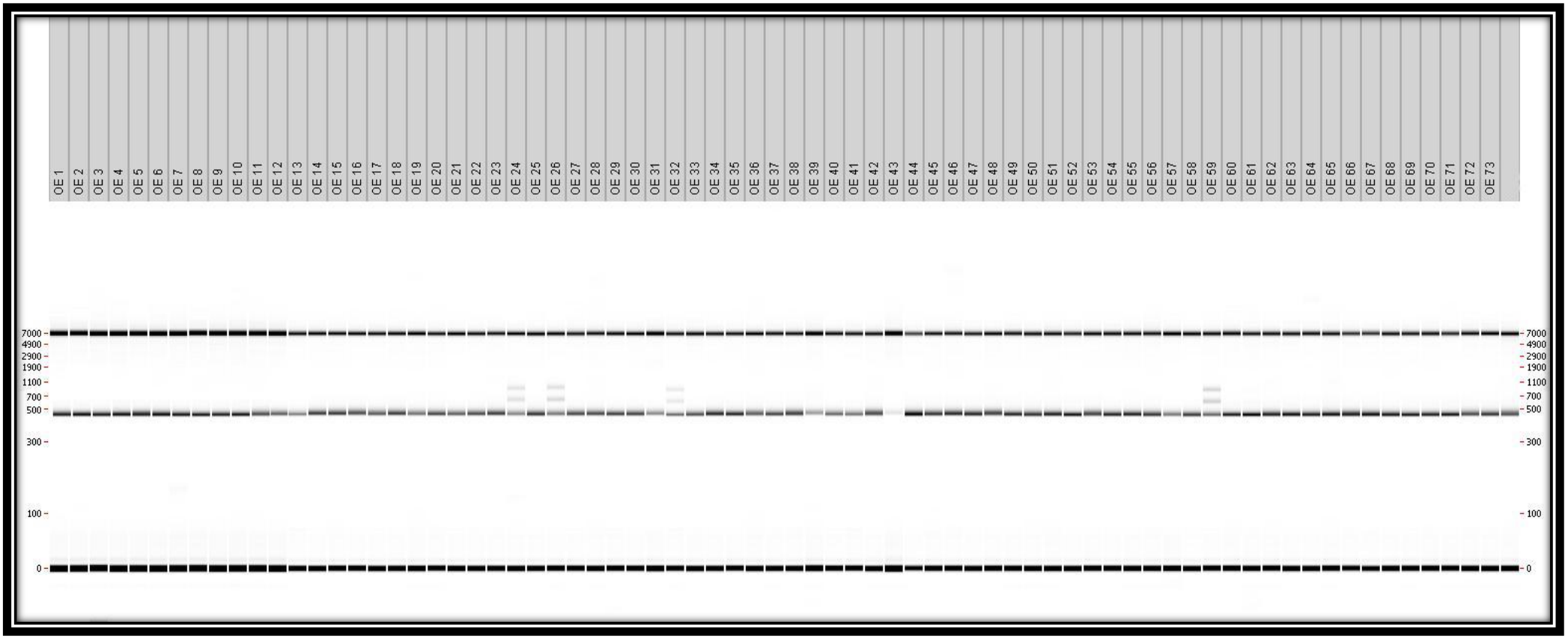

Figure 1: DNA Quality Test

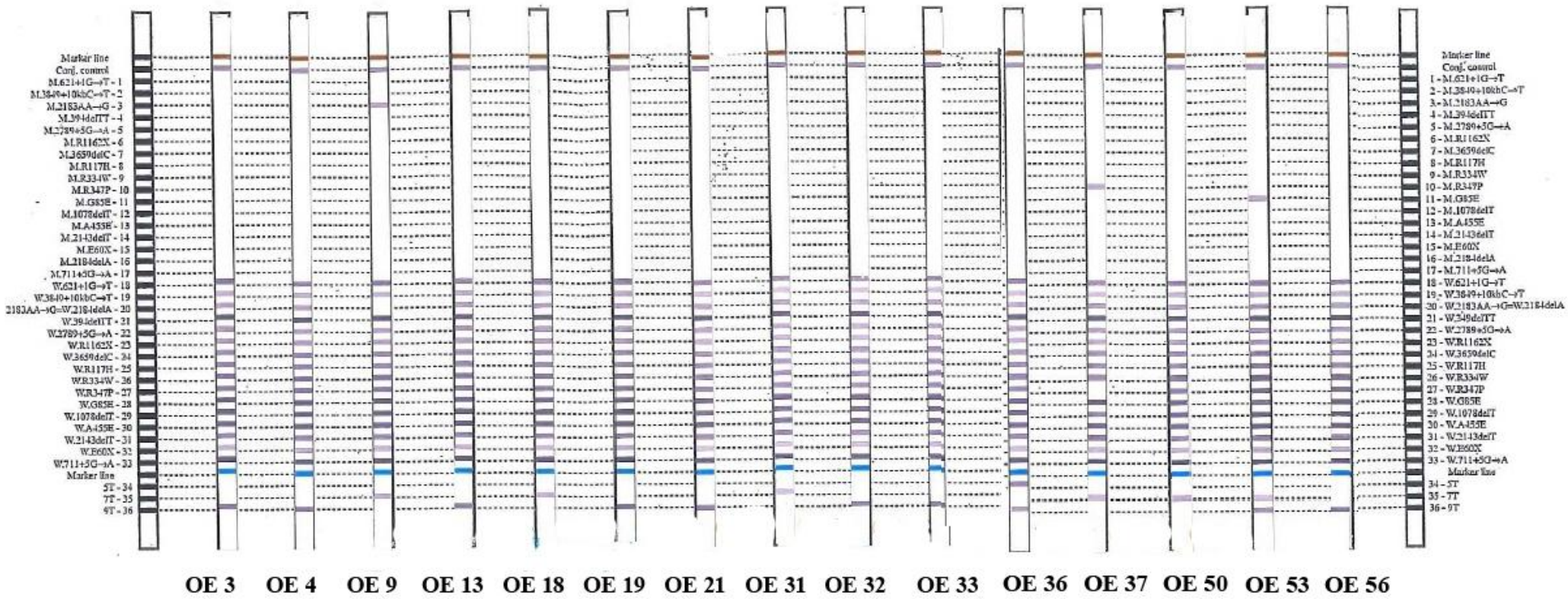

Figure 2: INNO-LIPA CFTR 17 + Tn Strips.

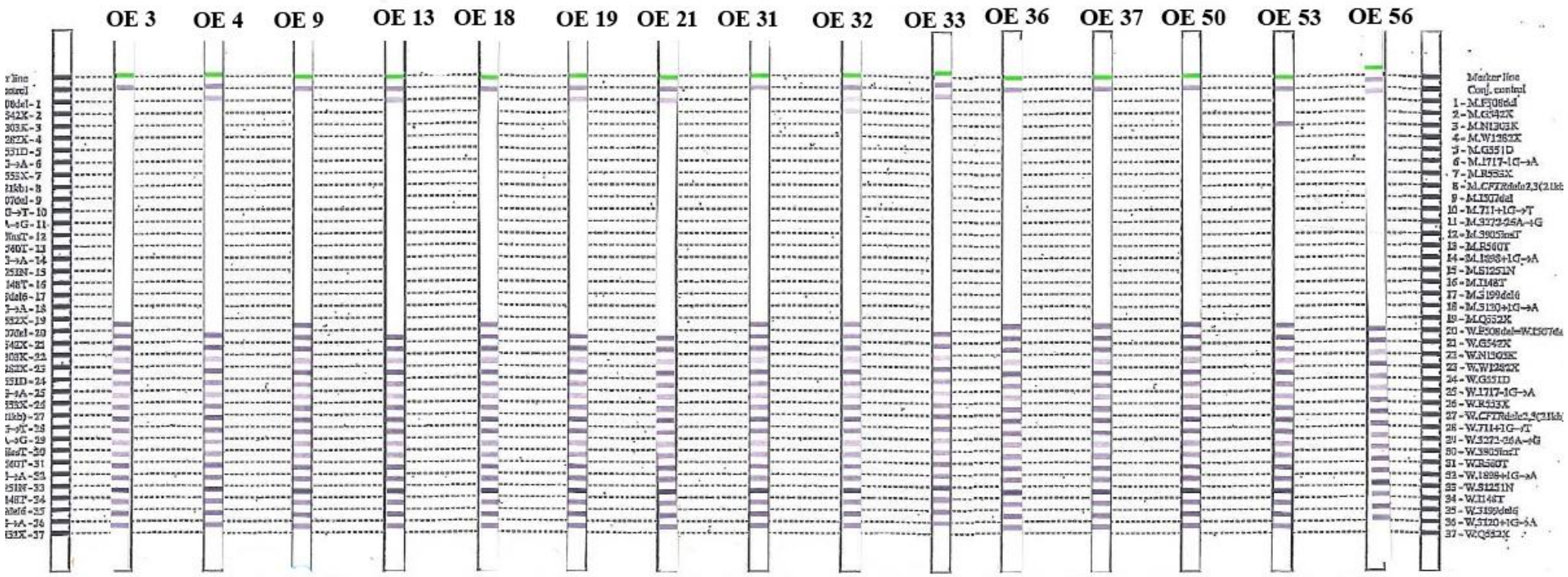

Figure 3: INNO-LIPA CFTR 19 Strips.

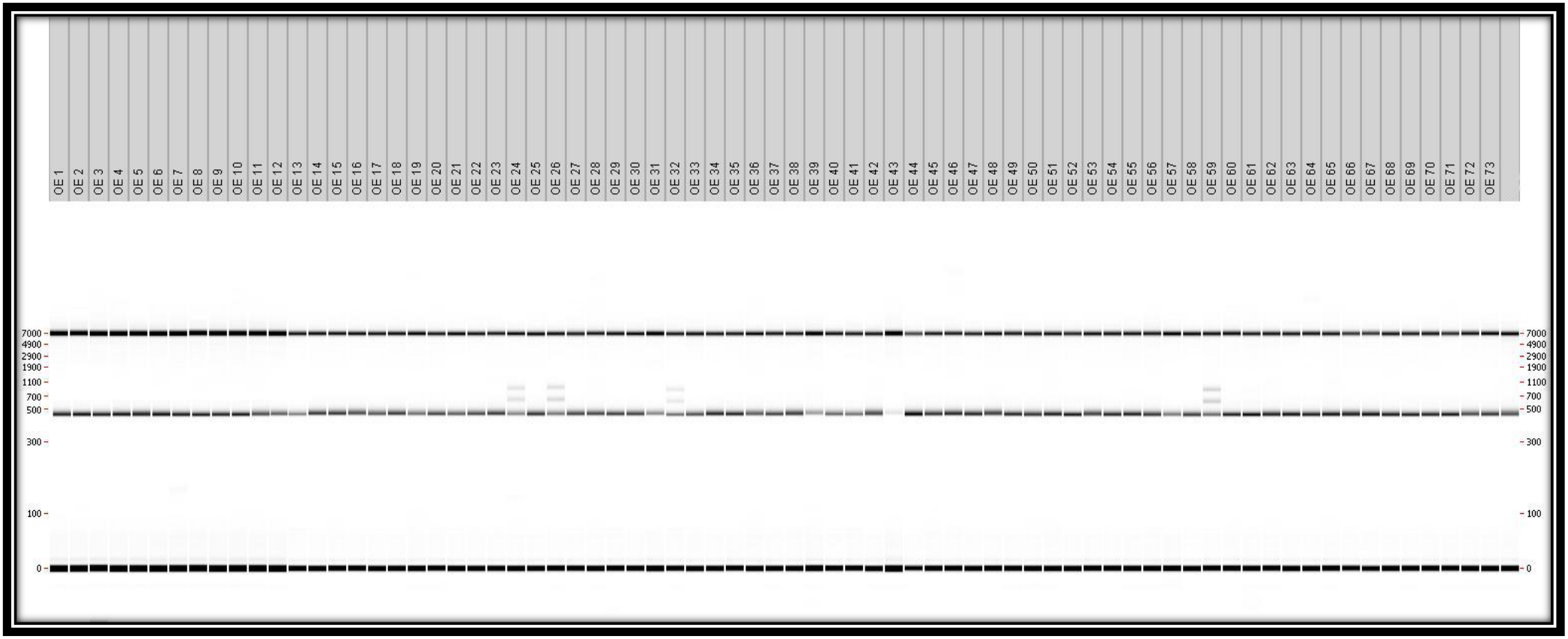

Figure 4: ΔF508 Heteroduplex Analysis

The bands in the figure above indicate the heterozygotes of ΔF508

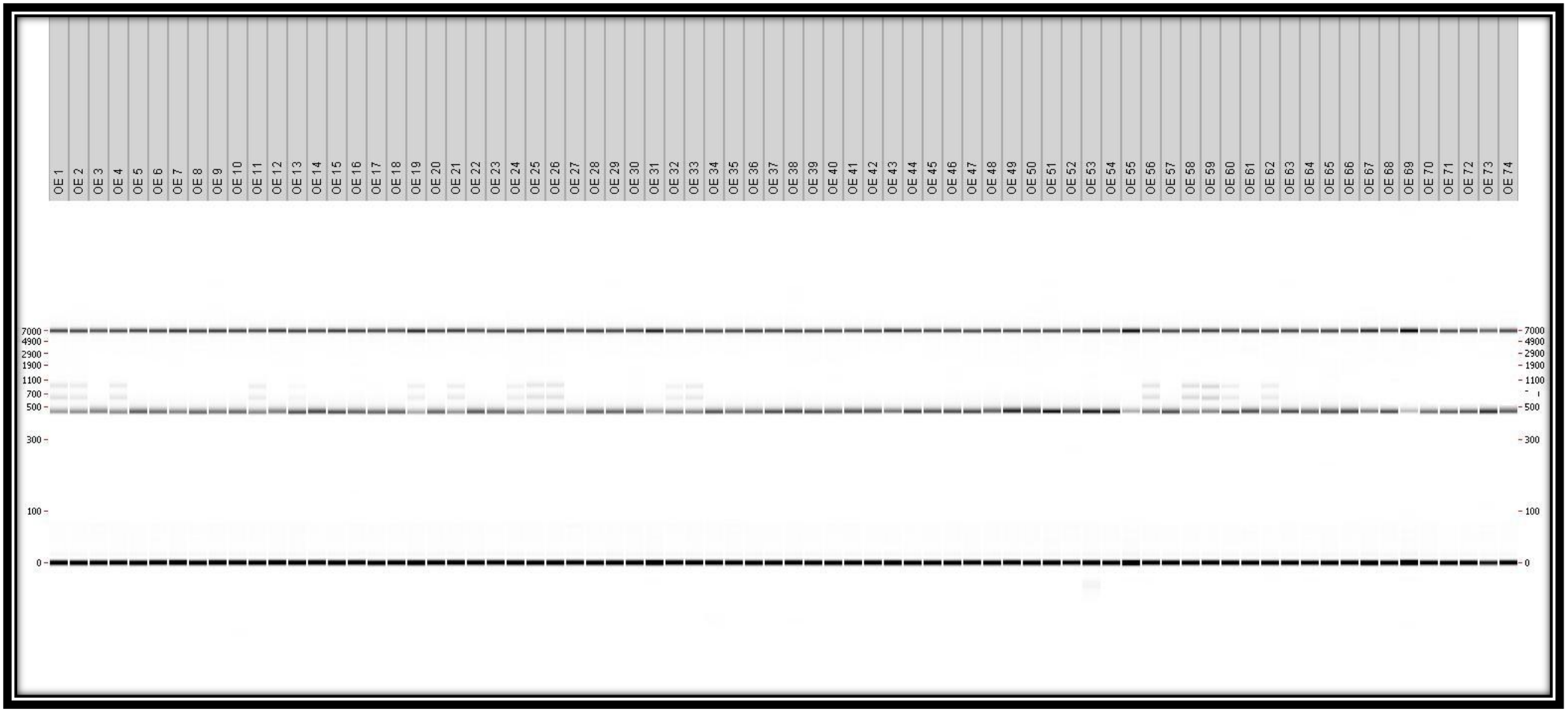

Figure 5: ΔF508 Heteroduplex Analysis

A mixture experiment to detect homozygotes for ΔF508, in the figure above the bands indicate the heterozygotes and homozygotes of ΔF508

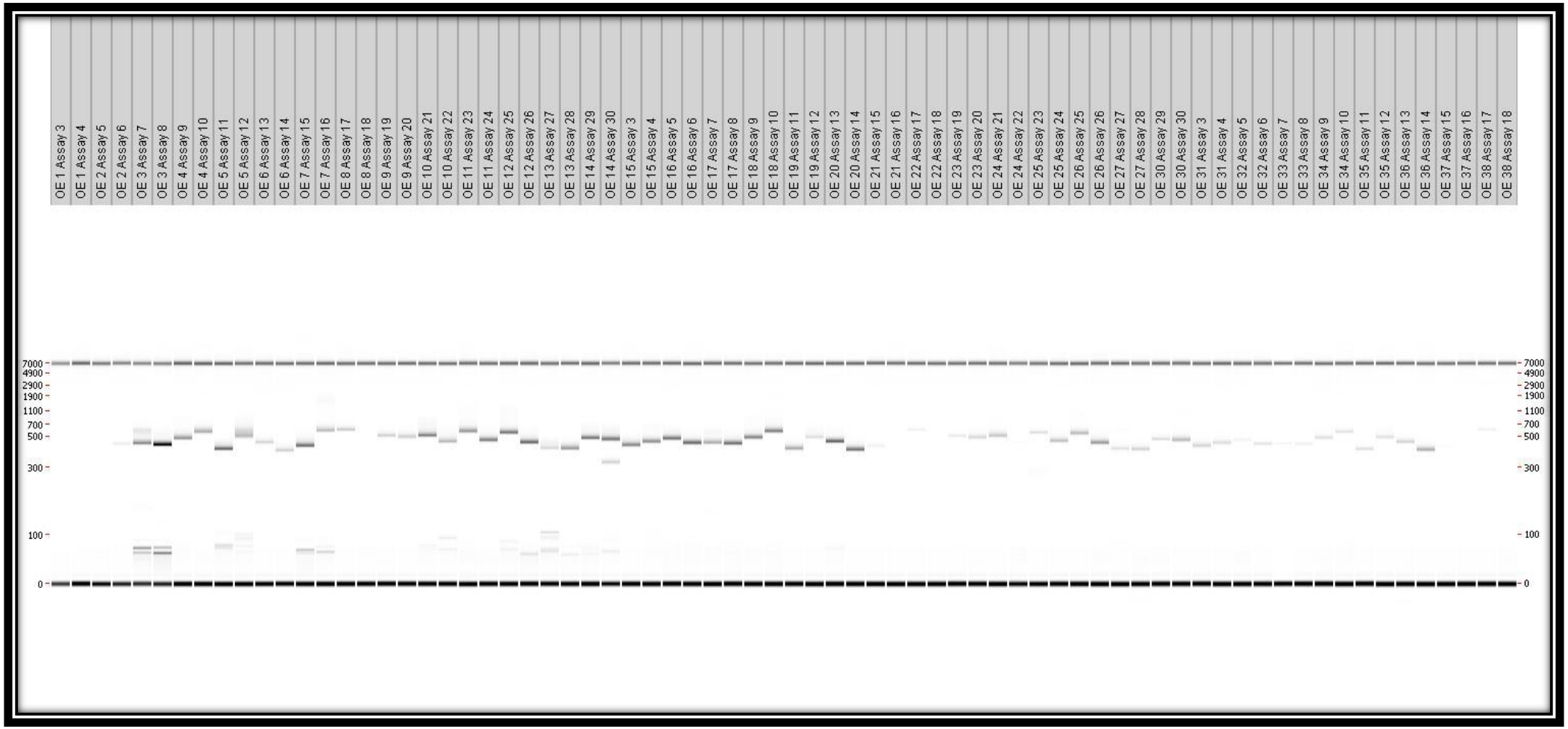

Figure 6.1: PCR of all assays

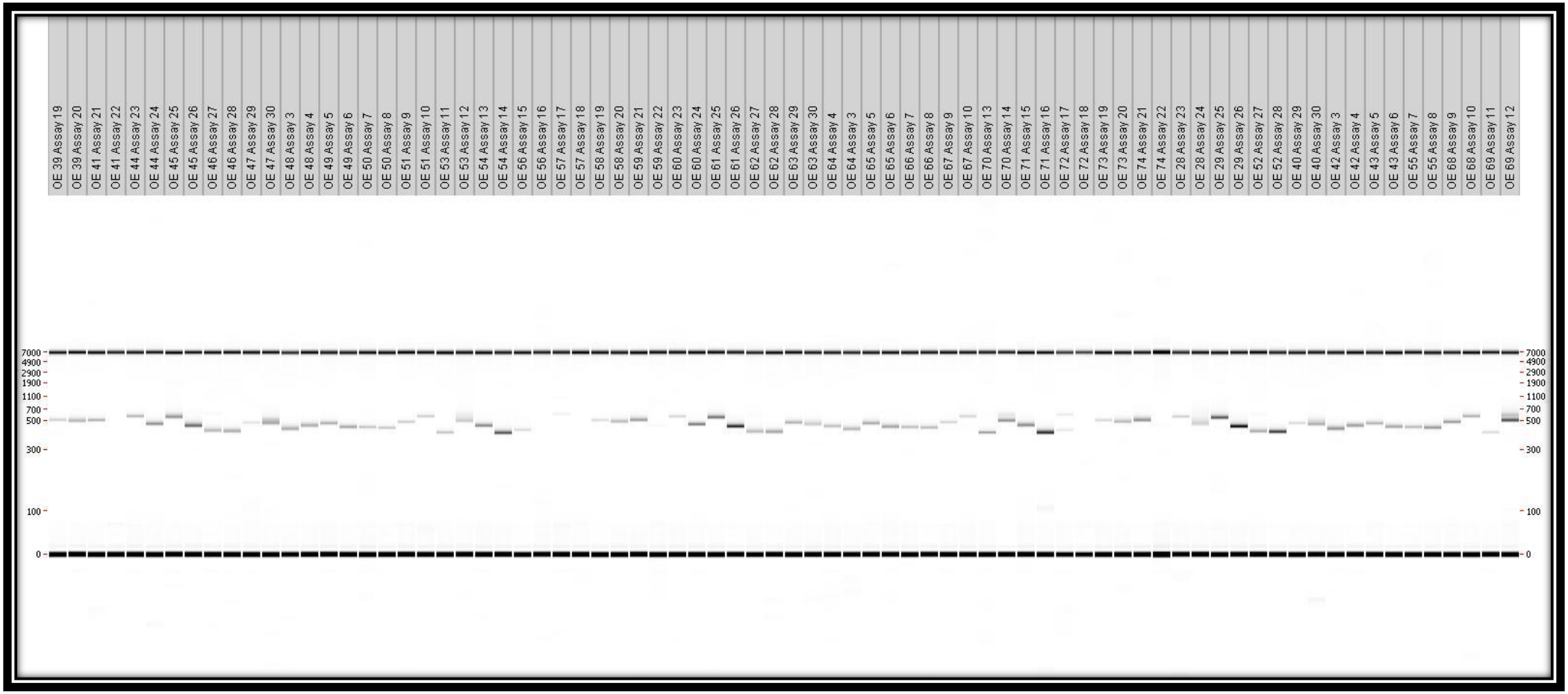

Figure 6.2: PCR of all assays

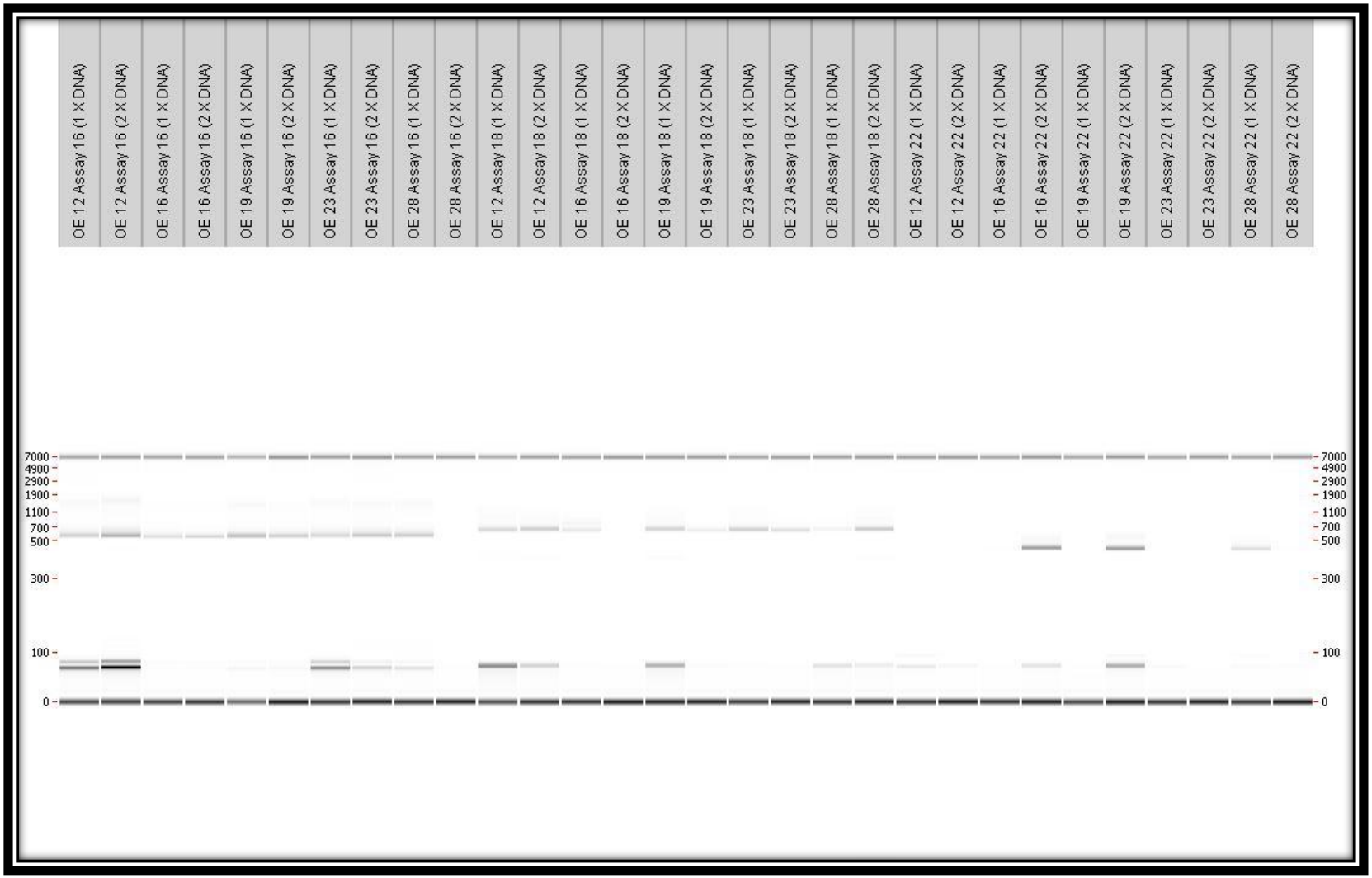

Figure 7: PCR optimizing for assays 16,18,22

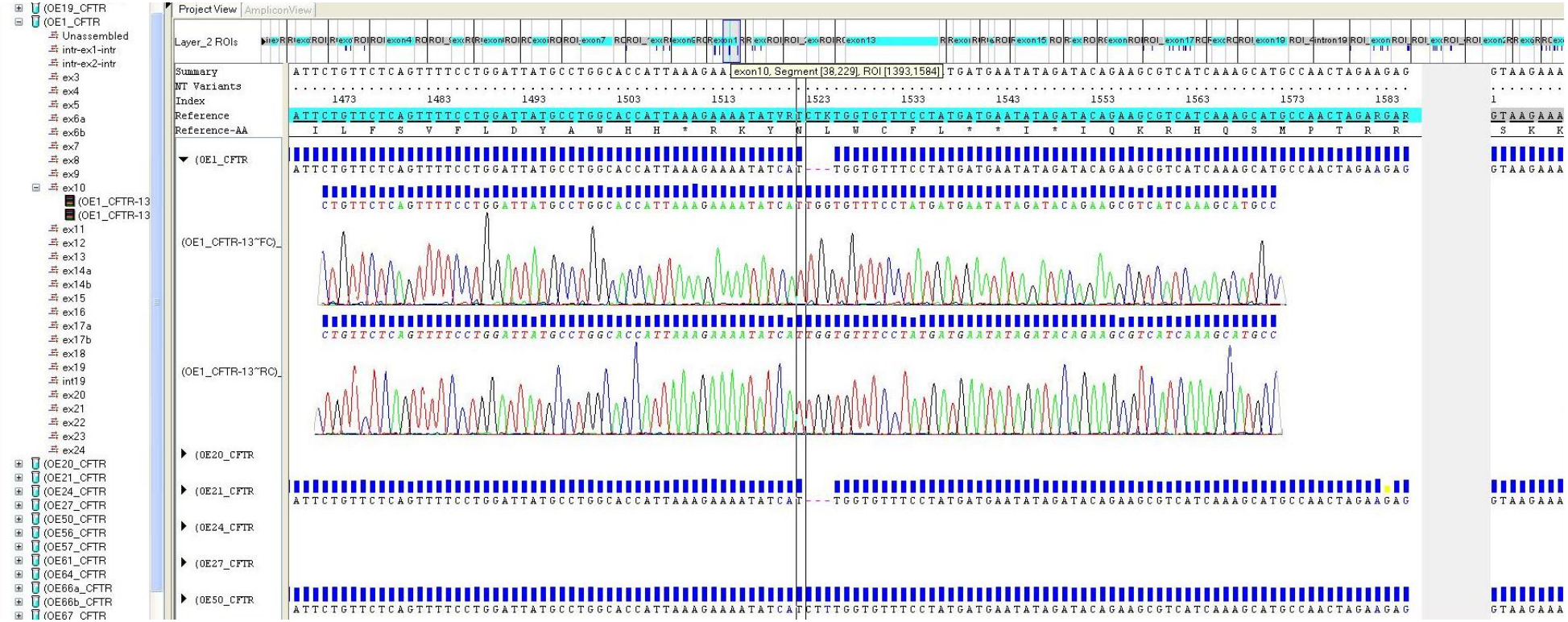

Figure 8: Print screen of "Seqscape" program confirming homozygous **F508** mutation on the patient OE - 1

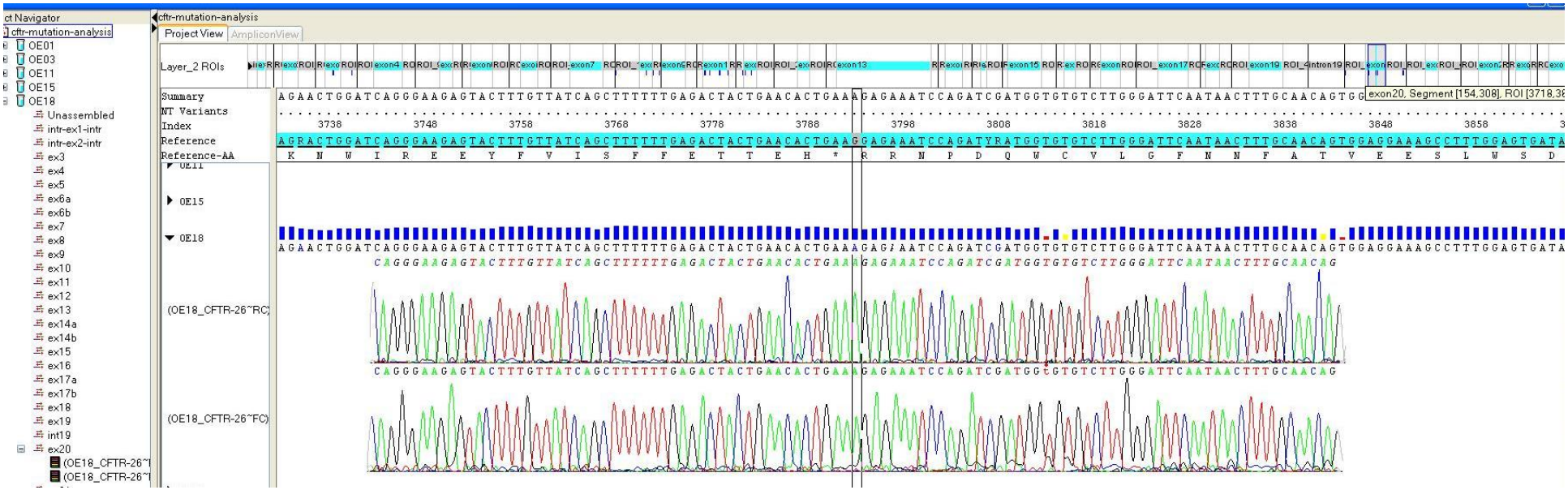

Figure 9: Print screen of "Seqscape" program confirming homozygous **c.3793G>A** mutation on the patient OE - 18

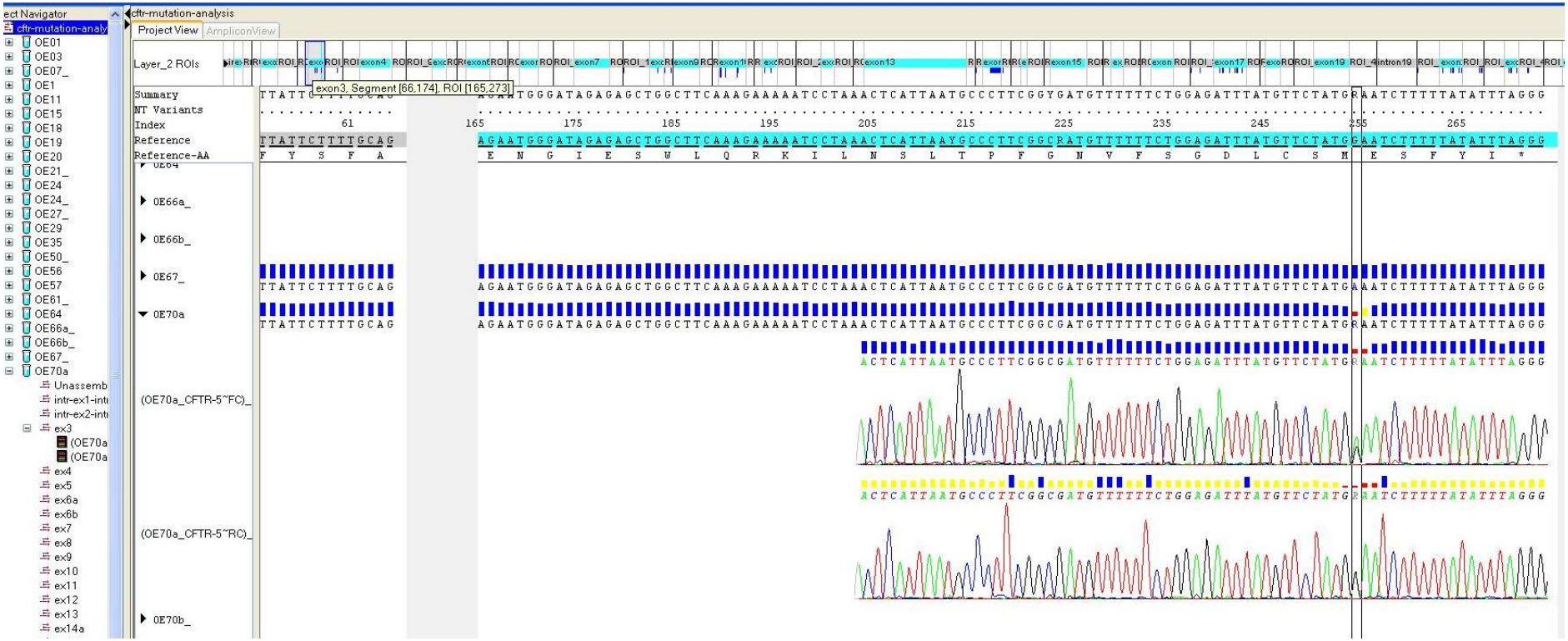

Figure 10: print screen of "Seqscape" program confirming heterozygous **G85E** mutation in patient OE - 70

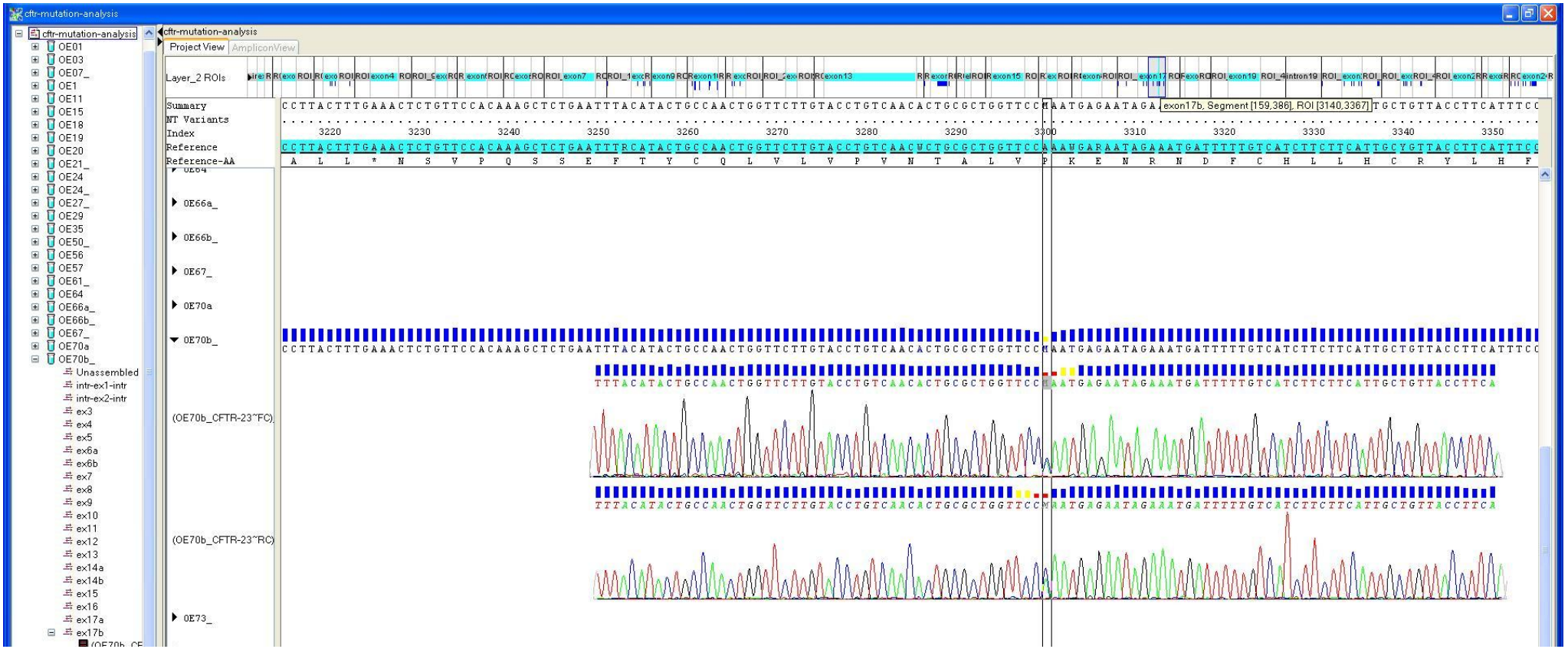

Figure 11: Print screen of "Seqscape" program confirming heterozygous **Q1100P** mutation in patient OE - 70

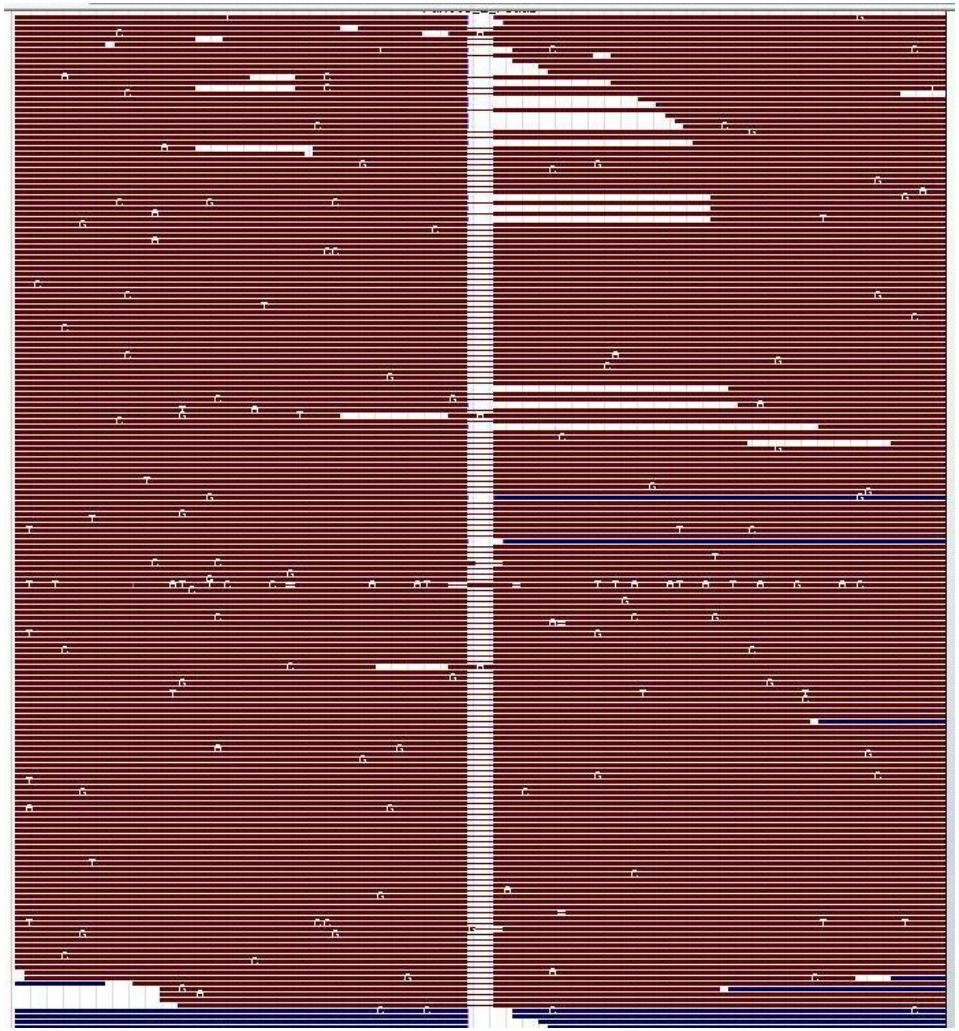

Figure 12: Print screen of "Miseq" result indicating homozygous ΔF508 mutation

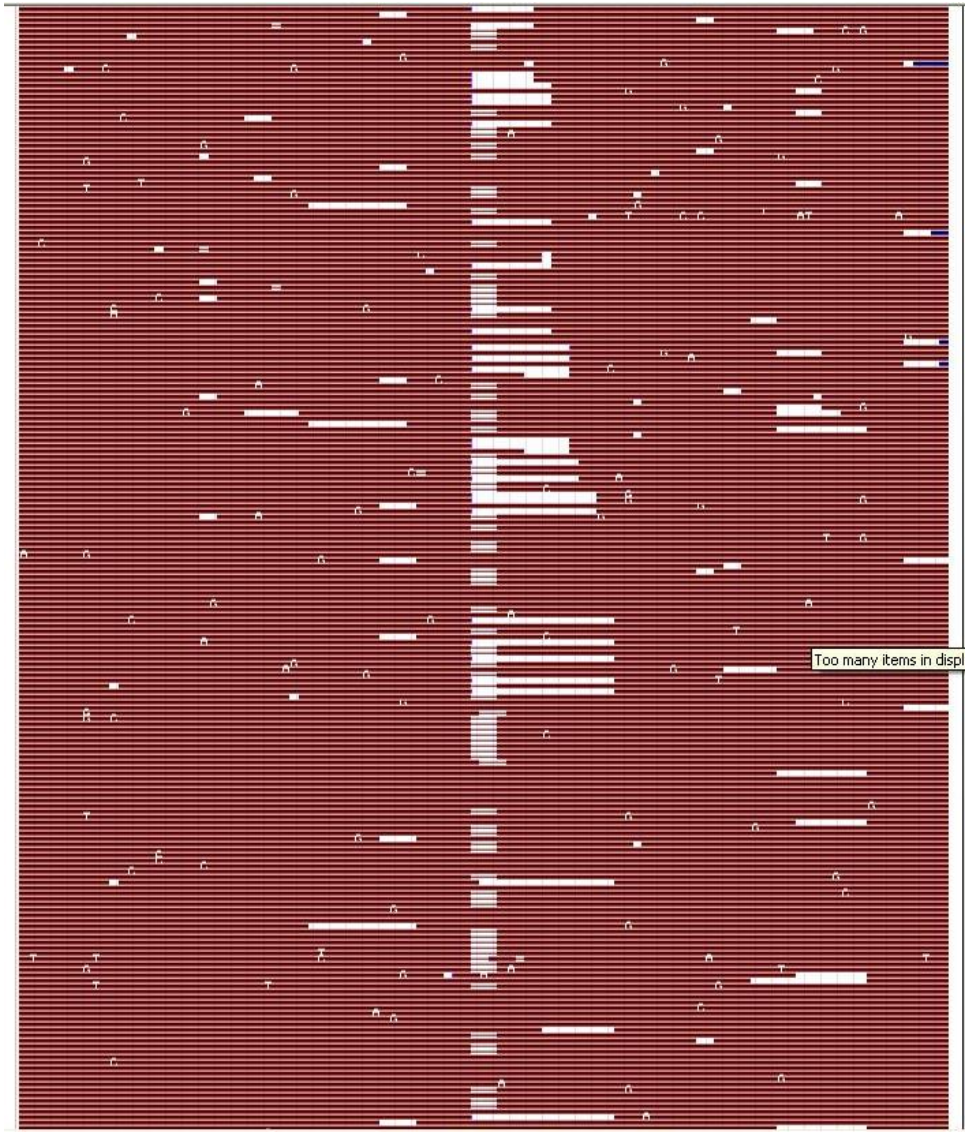

Figure 13: Print screen of "Miseq" result indicating heterozygous ΔF508 mutation

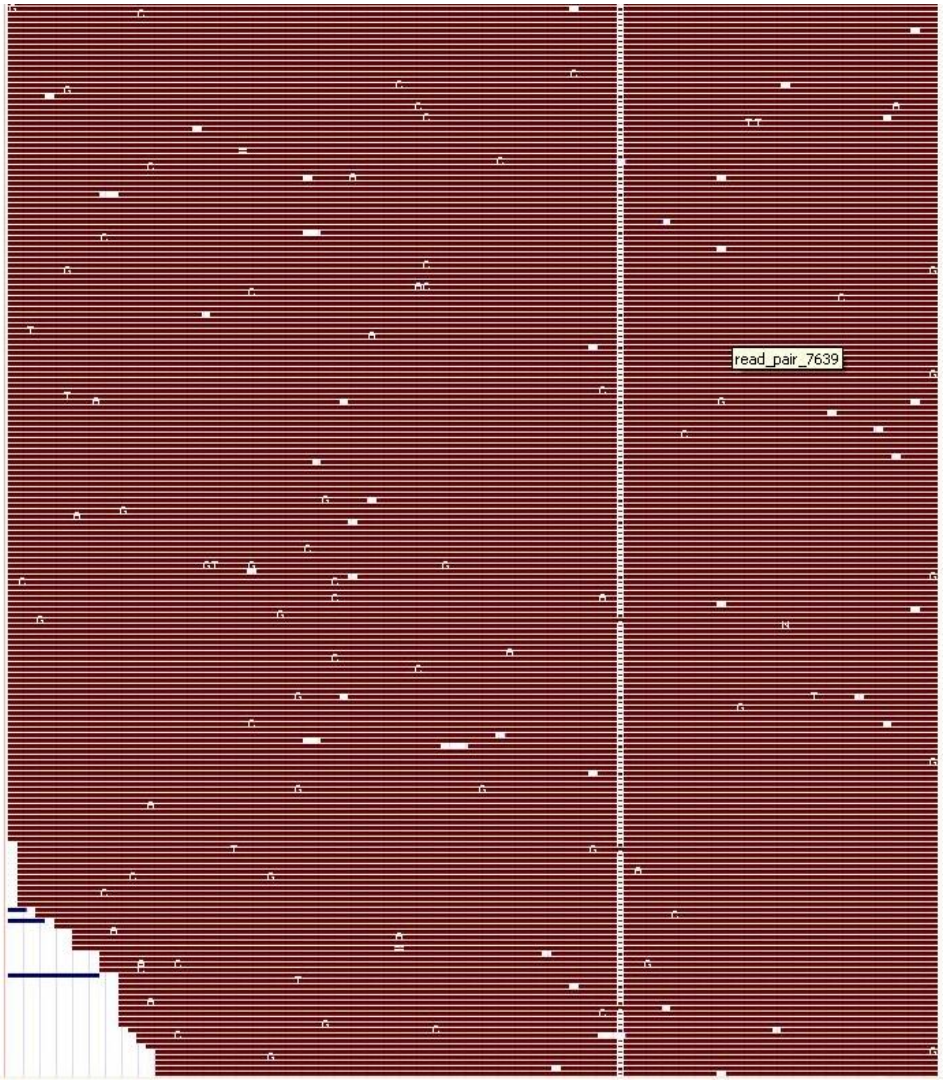

Figure 14: Print screen of "Miseq" result indicating homozygous 1525- 1G>A mutation

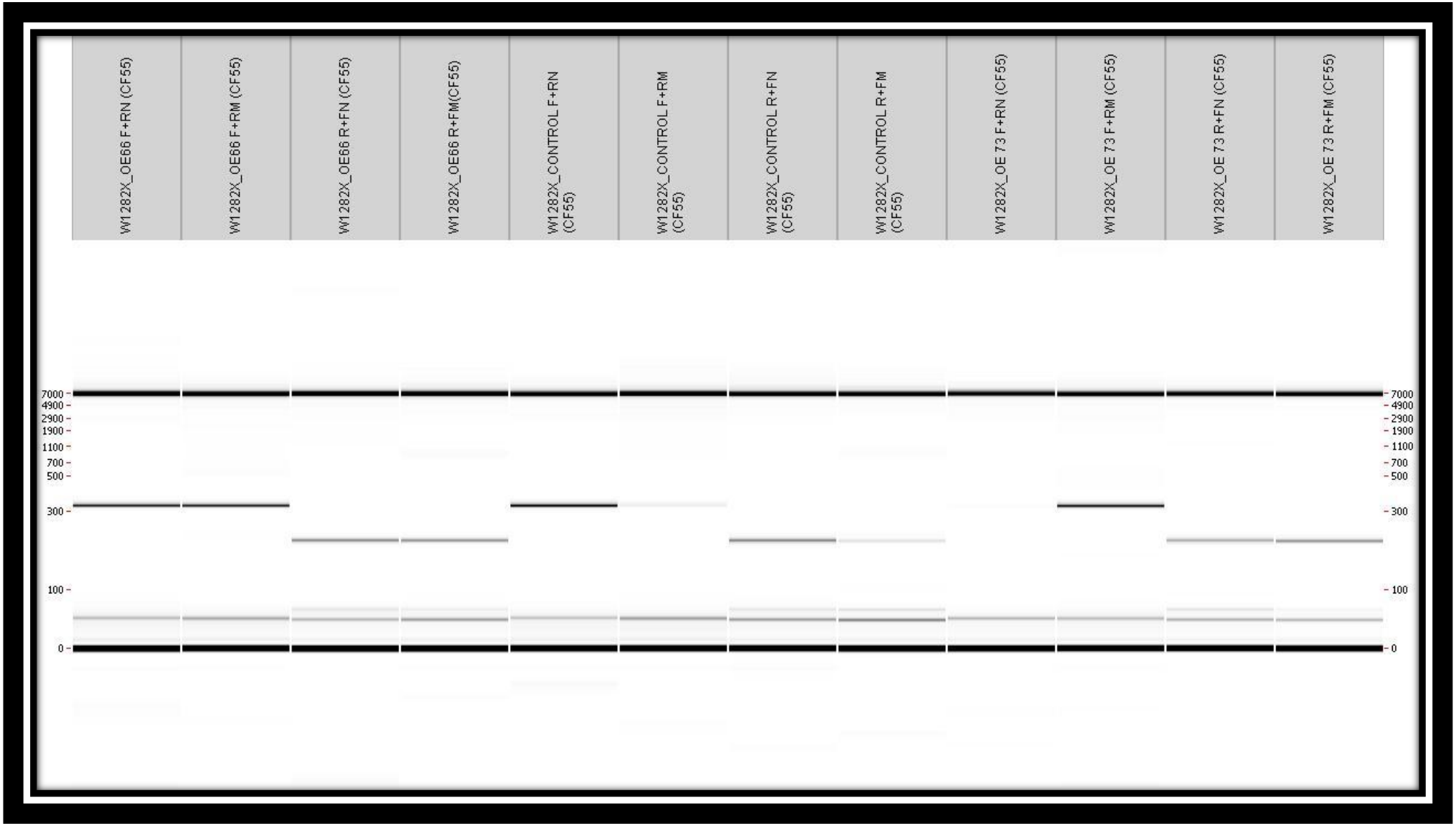

Figure 15: W1282X mutation; optimizing Allele specific mutation analysis conditions this bands were derived using CF 55 PCR program, form the figure it is obvious that using the Reverse primer to detect this mutation is better than using the Forward primer.

Figure 16: 3120+1Kbdel8.6Kb mutation ; this result shows that patients OE- 12, 22,23 and 41 have this deletion in their CFTR gene and the patient OE- 43 do not carry this mutation.

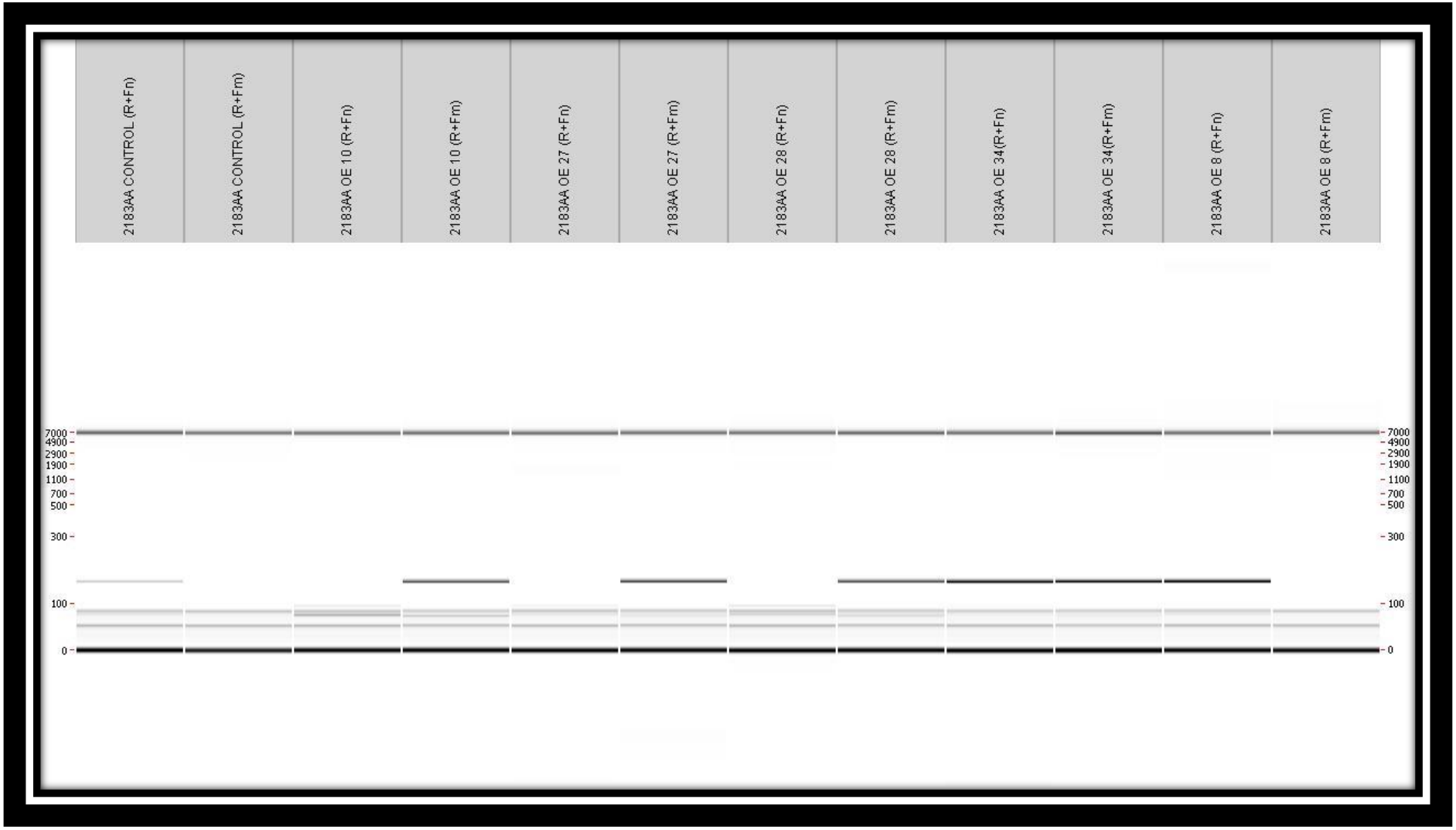

Figure 17: 2183AA>G mutation ; this result shows that patients OE- 10,27,28 are homozygous for this mutation and patient OE-34 is heterozygous for the mutation, and finally OE- 8 and the control do not have this mutation.

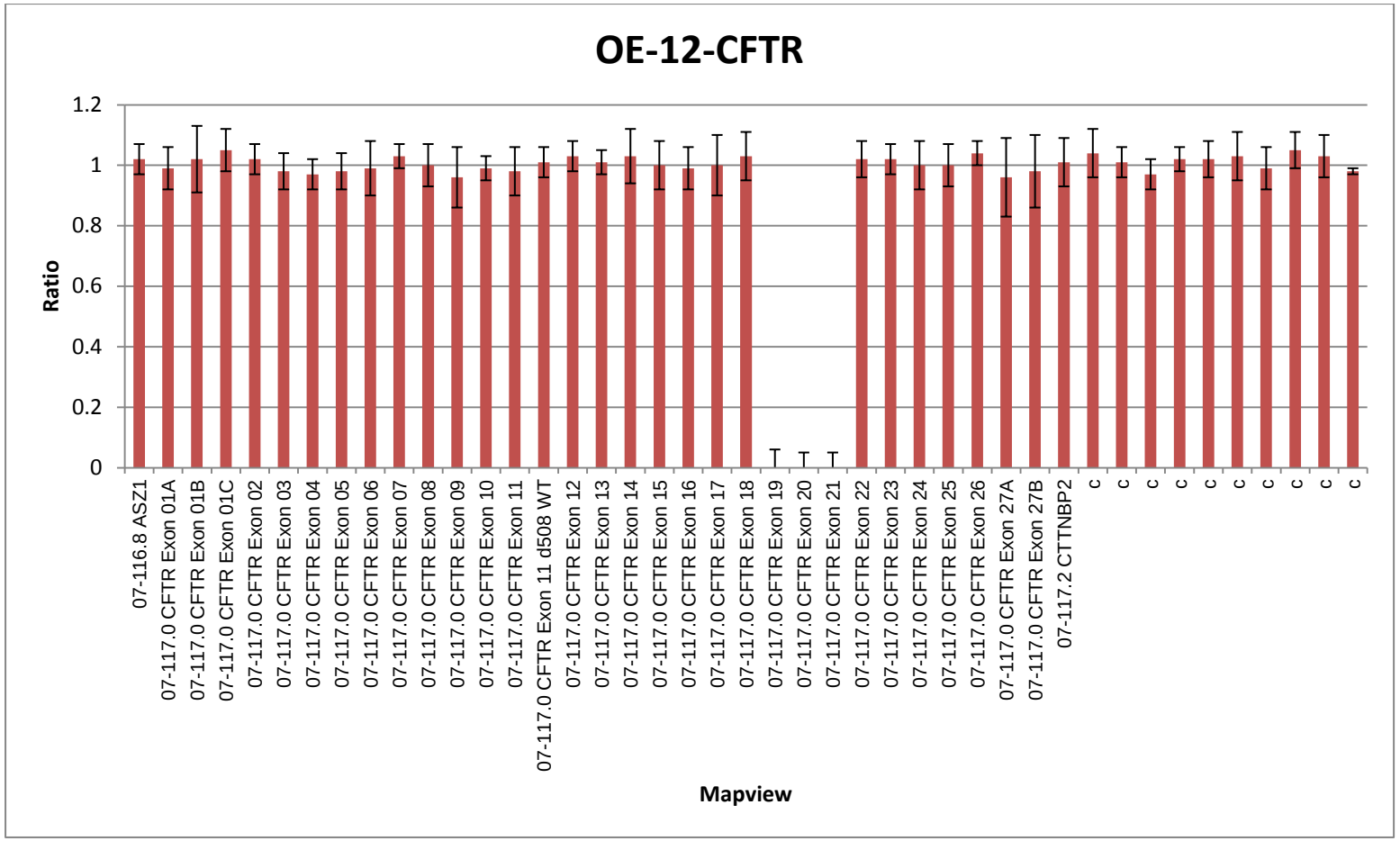

Figure 18: MLPA result for patient OE-12 showing a homozygous carrier of 3120+ 1Kbdel8.6Kb mutations (Del of exons 19, 20, 21).

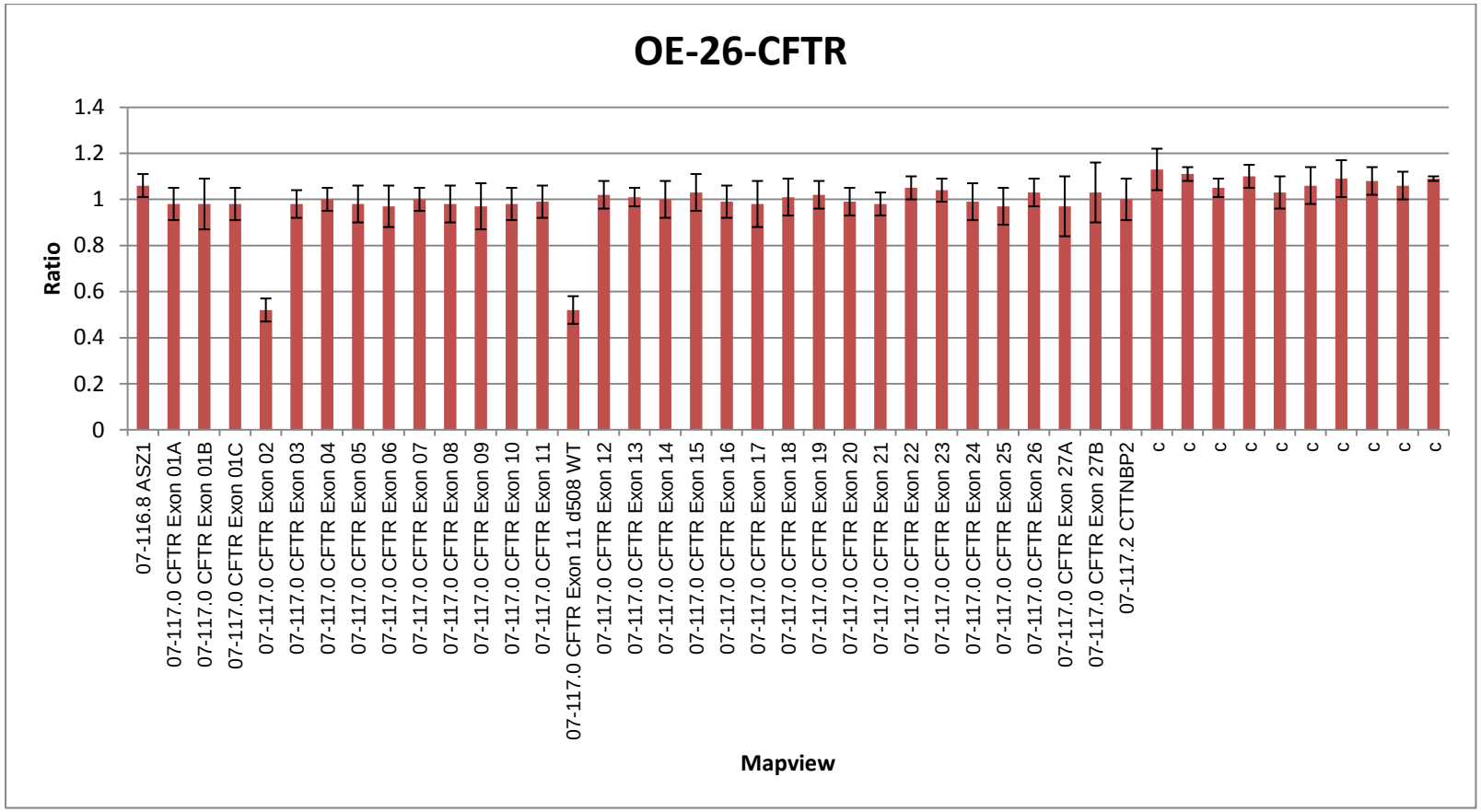

Figure 19: MLPA result for patient OE-26 showing a heterozygous carrier of Del 2 mutation (deletion of exon 2) and ΔF508.

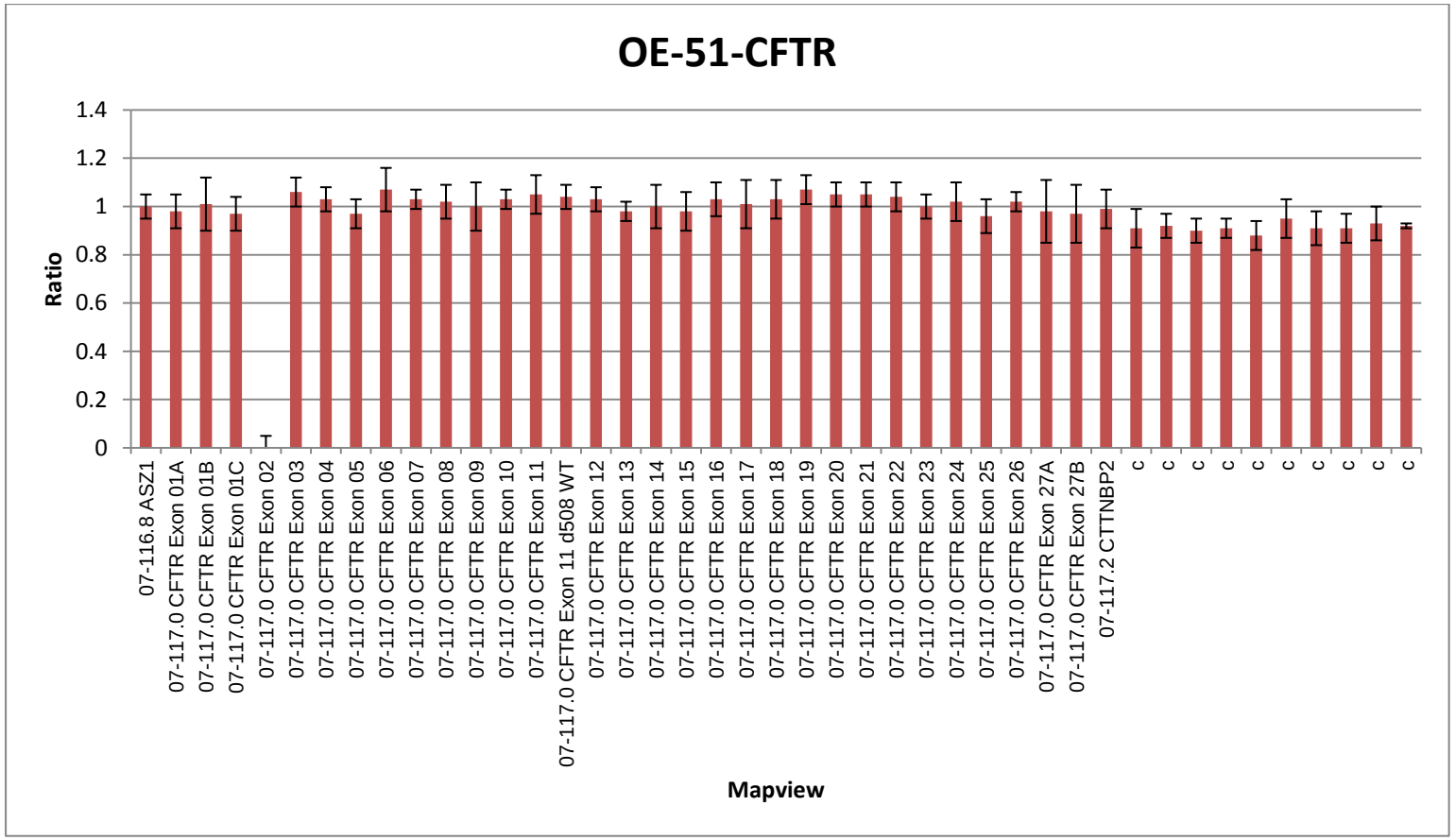

Figure 20: MLPA result for patient OE-51 showing a homozygous carrier of Del 2 mutation (deletion of exon 2).

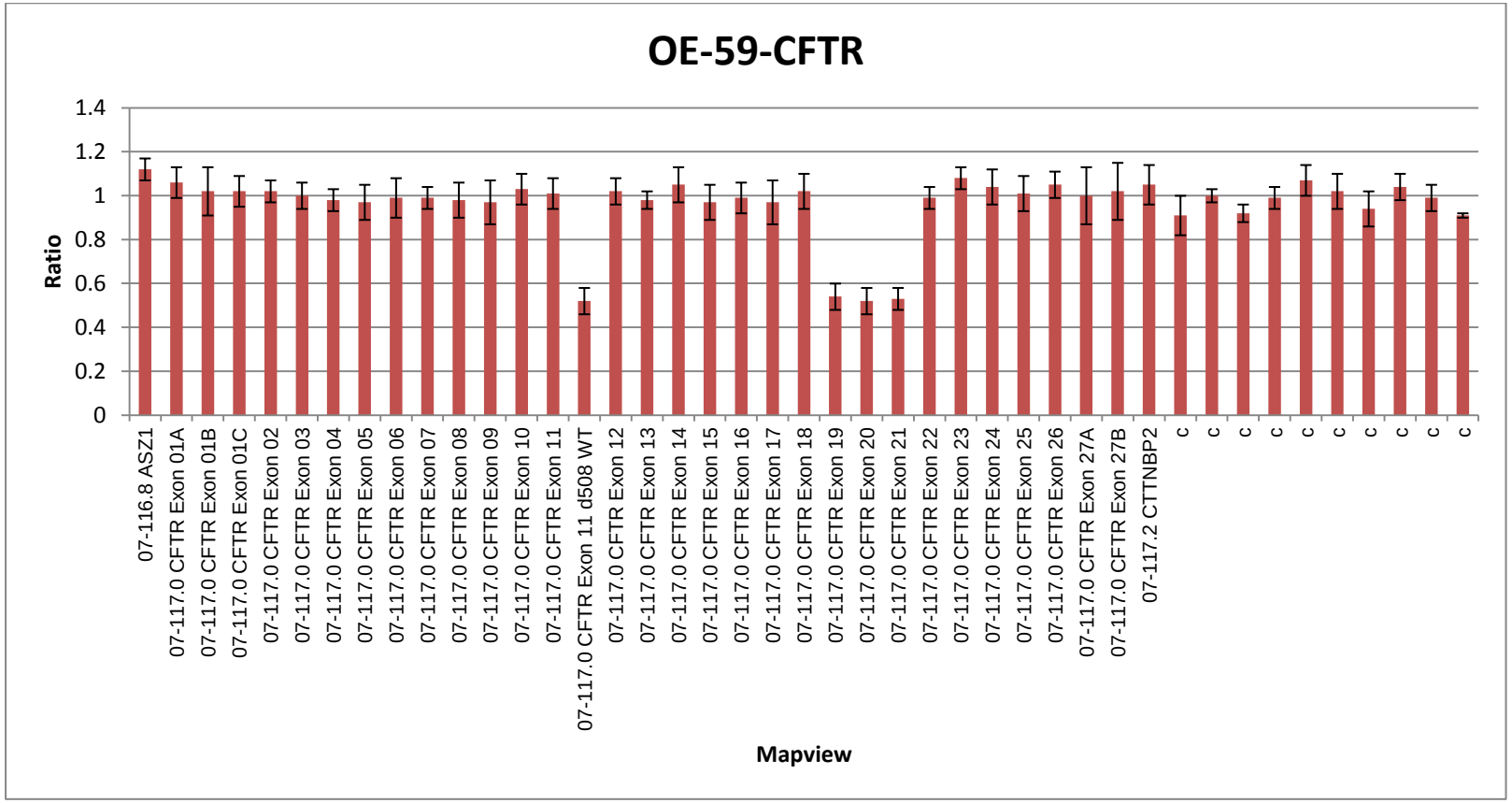

Figure 21: MLPA result for patient OE-59 showing a heterozygous carrier of 3120+ 1Kbdel8.6Kb mutations (Del of exons 19, 20, 21).

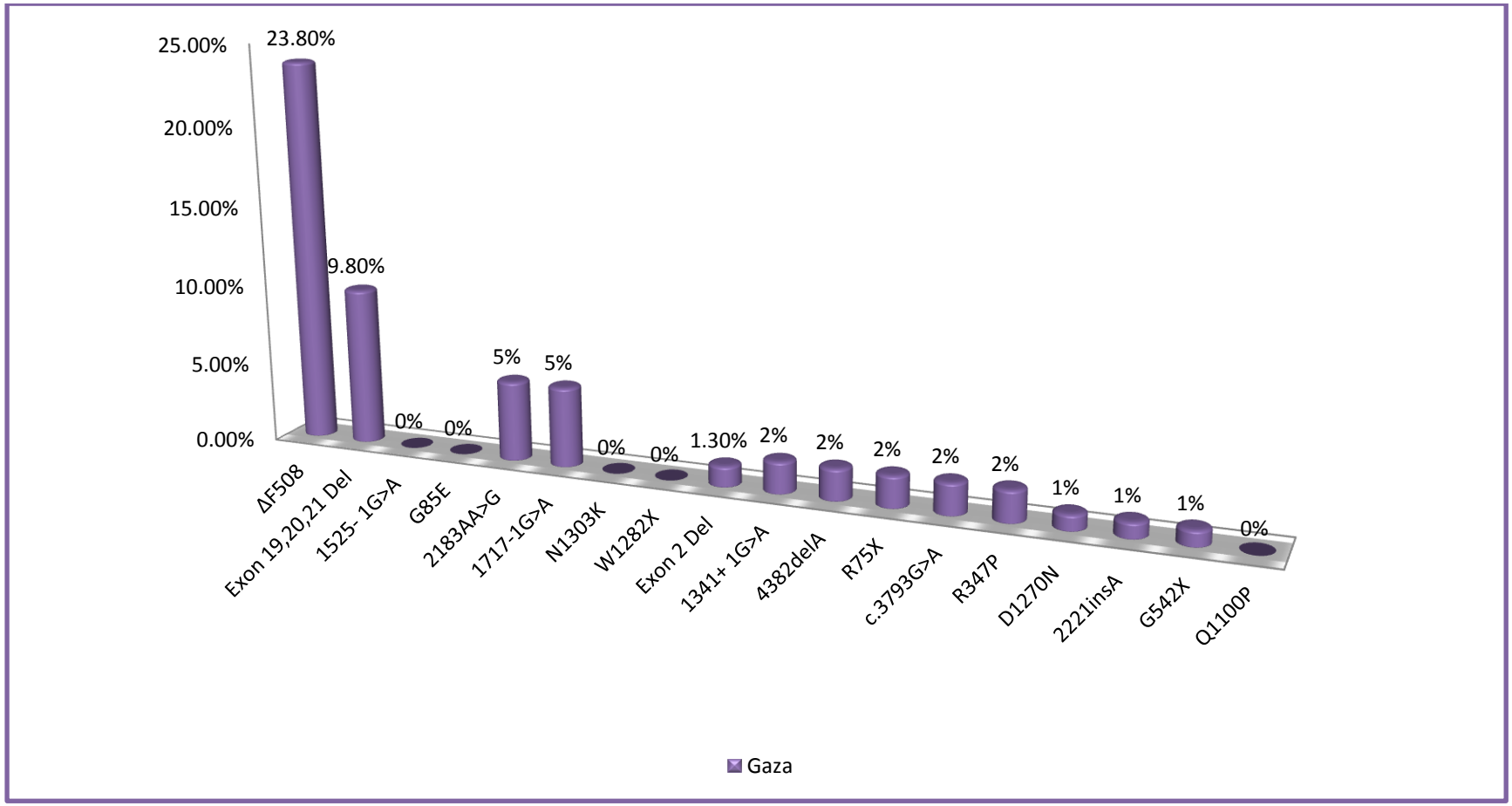

Figure 22: A chart showing the prevalence rates of different mutations in Gaza

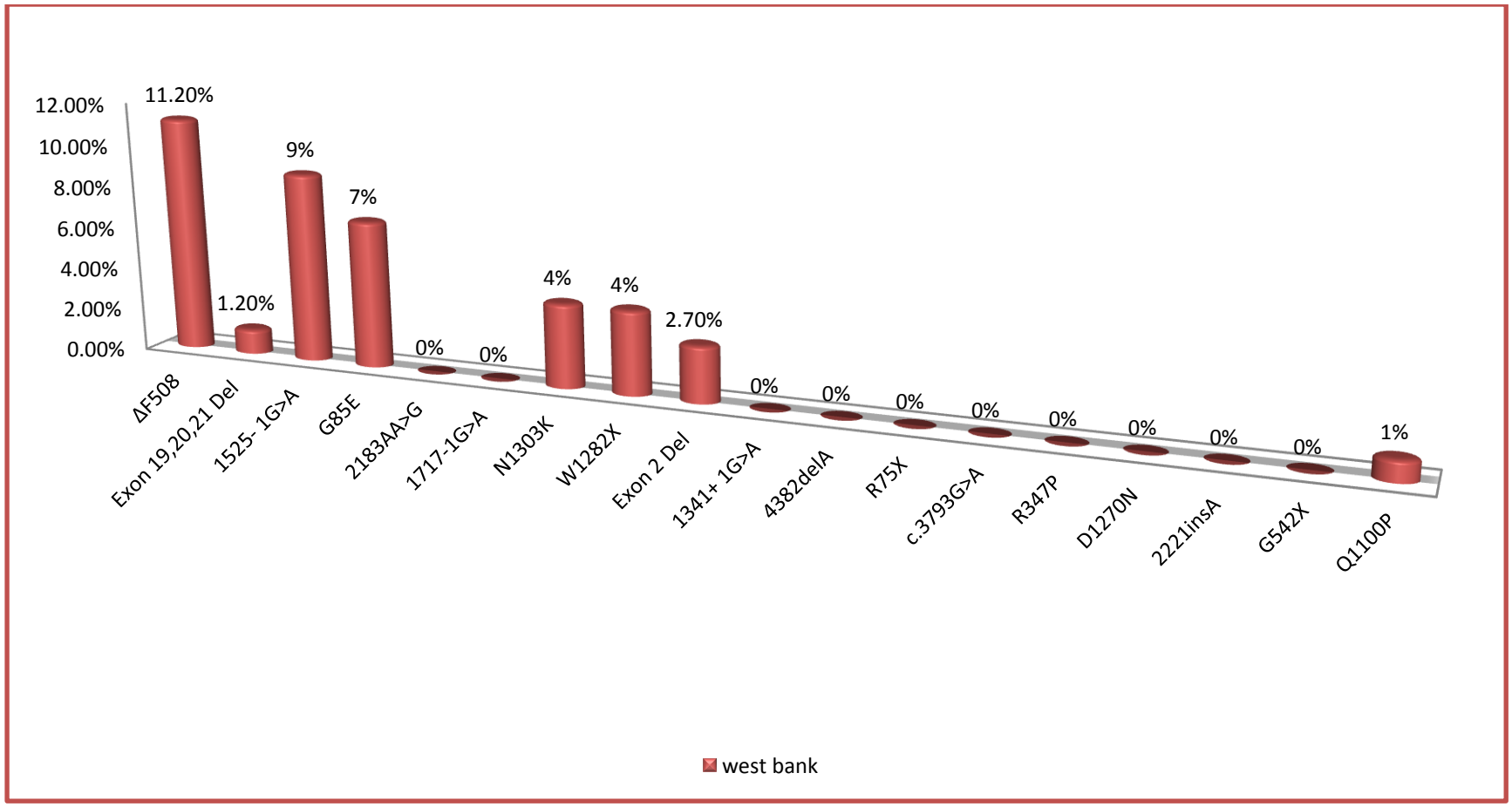

Figure 23: A chart showing the prevalence rates of different mutations in the West Bank