BAŞKENT UNIVERSITY
INSTITUTE OF SCIENCE AND ENGINEERING
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND GENETICS

DEVELOPMENT OF LOW-COST, FAST AND LOCAL *CHLAMYDIA TRACHOMATIS* REAL-TIME PCR DIAGNOSTIC KITS

BY

SELİM EMRE ERGÜL

MASTER OF SCIENCE THESIS

ANKARA-2024

BAŞKENT UNIVERSITY
INSTITUTE OF SCIENCE AND ENGINEERING
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS
MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND GENETICS

DEVELOPMENT OF LOW-COST, FAST AND LOCAL *CHLAMYDIA TRACHOMATIS* REAL-TIME PCR DIAGNOSTIC KITS

BY

SELİM EMRE ERGÜL

MASTER OF SCIENCE THESIS

ADVISOR

ASST. PROF. DR. OĞUZ BALCI

ANKARA-2024

BAŞKENT UNIVERSITY

INSTITUTE OF SCIENCES AND ENGINEERING

This study, which was prepared by Selim Emre ERGÜL, for the program of Molecular Biology and Genetics Master's Program with Thesis (English), has been approved in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Molecular Biology and Genetics Department by the following committee.

Date of Thesis Defense: 09 / 02 / 2024

Thesis Title: Development of Low-cost, Fast and Local *Chlamydia trachomatis* Real-Time PCR Diagnostic Kits

Examining Committee Members	**Signature**
Asst. Prof. Dr. Oğuz BALCI, Başkent University	……...
Prof. Dr. Veli Cengiz ÖZALP, Atılım University	……...
Asst. Prof. Dr. Aliye Ezgi GÜLEÇ TAŞKIRAN, Başkent University	……..…….

APPROVAL

Prof Dr. Ömer Faruk ELALDI

Director, Institute of Science and Engineering

Date: … / … / ……..

BAŞKENT ÜNİVERSİTESİ

FEN BİLİMLERİ ENSTİTÜSÜ

YÜKSEK LİSANS TEZ ÇALIŞMASI ORİJİNALLİK RAPORU

Tarih: 22 / 02 / 2024

Öğrencinin Adı, Soyadı : Selim Emre ERGÜL

Öğrencinin Numarası : 22010435

Anabilim Dalı : Moleküler Biyoloji ve Genetik

Programı : Moleküler Biyoloji ve Genetik Tezli Yüksek Lisans Programı (İngilizce)

Danışmanın Unvanı/Adı, Soyadı : Dr. Öğr. Üyesi Oğuz Balcı

Tez Başlığı : Development of Low-cost, Fast and Local *Chlamydia trachomatis* Real-Time PCR Diagnostic Kits

Yukarıda başlığı belirtilen Yüksek Lisans tez çalışmamın; Giriş, Ana Bölümler ve Sonuç Bölümünden oluşan, toplam 37 sayfalık kısmına ilişkin, 22 / 02 / 2024 tarihinde şahsım/tez danışmanım tarafından turnitin adlı intihal tespit programından aşağıda belirtilen filtrelemeler uygulanarak alınmış olan orijinallik raporuna göre, tezimin benzerlik oranı %9'dur. Uygulanan filtrelemeler:

1. Kaynakça hariç
2. Alıntılar hariç
3. Beş (5) kelimeden daha az örtüşme içeren metin kısımları hariç

"Başkent Üniversitesi Enstitüleri Tez Çalışması Orijinallik Raporu Alınması ve Kullanılması Usul ve Esaslarını" inceledim ve bu uygulama esaslarında belirtilen azami benzerlik oranlarına tez çalışmamın herhangi bir intihal içermediğini; aksinin tespit edileceği muhtemel durumda doğabilecek her türlü hukuki sorumluluğu kabul ettiğimi ve yukarıda vermiş olduğum bilgilerin doğru olduğunu beyan ederim.

Öğrenci İmzası:…….

ONAY

Tarih: … / … / 20…

Öğrenci Danışmanı

Dr. Öğr. Üyesi Oğuz Balcı

……

I would like to dedicate this work to my mother, Elmas ERGÜL, and my father, Hakan ERGÜL, who have supported me in every aspect throughout my life. Your belief in me has been a constant source of motivation and I am grateful for your presence in my life. I am grateful to my dear grandmother Suna UZALP, who is very precious to me, for her existence. I'm glad i have someone like you in my life.

Selim Emre ERGÜL

Ankara-2024

ACKNOWLEDGMENT

I would like to express my sincere gratitude and heartfelt thanks to my supervisor, Asst. Prof. Dr. Oğuz BALCI “Başkent University”, for making this thesis project possible. His guidance and advice have been invaluable throughout all stages of writing my thesis project. I would also like to extend my appreciation to Denovo Biotechnology Inc., a significant supporter of this thesis project, for its investment and provision of the experimental environment.

My deepest appreciation goes to Prof. Dr. Veli Cengiz ÖZALP “Atılım University” and Asst. Prof. Dr. Aliye Ezgi GÜLEÇ TAŞKIRAN “Başkent University” for their valuable consultancy support.

I would like to acknowledge the Turkish Scientific and Technical Research Council, under Grant No. 1170446, the project's funding agency, for their investment and support.

I would like to thank my dear grandfather, Hüseyin Dilli, and my dear grandmother, Gülüzar Dilli, who made great efforts for me. I would like to thank my dear uncle Fatih ERGÜL, who helped me make the right decision at one of the turning points that would have a significant impact on my education and life. Finally, I would like to thank my beloved dogs Zuzu&Migi for giving me peace and happiness.

ABSTRACT

Selim Emre ERGÜL

DEVELOPMENT OF LOW-COST, FAST AND LOCAL *CHLAMYDIA TRACHOMATIS* REAL-TIME PCR DIAGNOSTIC KITS

Başkent University Institute of Sciences

Molecular Biology and Genetics Program

2024

Chlamydia trachomatis is a STI (Sexually Transmitted Infection) that infects more than 100 million people every year and is most common in the young population. This infection causes many diseases, so a diagnostic kit that is easily accessible, fast, economical and provides accurate results is serviceable. Along with the PCR method, the most commonly used methods are ELISA and culture methods. The risk of contamination of culture and the inability to reach results quickly, and the low accuracy of results in ELISA tests, have been the reasons for choosing the PCR method. In the study where primer and probe designs were made, an end-point PCR device was used in order to reach the results quickly. Experiments were performed with 22 negative and 29 positive samples. Validation studies were carried out according to the results obtained from the experiments. The validation test results obtained as a result of these studies are as follows; the cut-off score was calculated as (31,055), the limit of detection was found to be 242 copies/ml, the kit tested against cross-contamination with 18 reference infections for analytical specificity gave 18 negative results, trend line values of linear range were found as y = 0,2933x – 66,143 and R^2 = (0,993), coefficient of variation value was calculated as 2,27%, trend line result values based on reference system and prototype system bacterial load result comparison were found as y=0,9305x+106,54 and R^2=0,8973, diagnostic specificity and diagnostic sensitivity values were found to be 95.5% and 96.6%. When the results are analyzed, it is seen that the study is quite successful in the subjects it focuses on. Diagnostic specificity and sensitivity rates are above the gold standard of 95%. Following these promising results, a study with a larger sample group and other commercial kits can be designed to take the study to the next level. After these repeated studies, comparisons can be made with other commercial kits, advantageous and disadvantageous points can be seen, and improvements can be made accordingly.

KEYWORDS: Molecular diagnostics, *Chlamydia trachomatis*, uantitati ve PCR, microparticles, validation.

Tubitak 1170446 Support Program, Denovo Biotechnology Company, Atilim University, Başkent University

ÖZET

Selim Emre ERGÜL

DÜŞÜK MALİYETLİ, HIZLI VE YERLİ CHLAMYDIA TRACHOMATIS REAL TIME PCR TEŞHİS KİTLERİNİN GELİŞTİRİLMESİ

Başkent Üniversitesi Fen Bilimleri Enstitüsü

Moleküler Biyoloji ve Genetik Programı

2024

Chlamydia trachomatis her yıl 100 milyondan fazla kişiyi enfekte eden CYBE'dir (Cinsel Yolla Bulaşan Enfeksiyon) ve genç nüfusta çokca görülmektedir. Bu enfeksiyon birçok hastalığa sebebiyet vermektedir bu yüzden kolay ulaşılabilen, hızlı, ekonomik ve doğru sonuç veren tanı kiti önem teşkil etmektedir. PCR yöteminiyle beraber en çok kullanılan yötemler ELISA ve kültür yöntemidir. Kültürünün kontaminasyon riski ve sonuca hızlı ulaşılamaması, ELISA testlerinde ise sonucun düşük doğruluk oranına sahip olması, PCR yöntemi için tercih sebebi olmuştur. Primer ve prob tasarımları yapılan çalışmada sonuca hızlı ulaşabilmek amacıyla end-point özellikli PCR cihazı kullanılmıştır. Deneyler 22 negatif ve 29 pozitif örnekle yapıldı. Deneylerden elde edilen sonuçlara göre validasyon çalışmaları yapıldı. Bu çalışmalar sonucunda elde edilen validasyon testi sonuçları aşağıdaki gibidir: kesme puanı: 31,055 olarak belirlendi, algılama limiti 242 kopya/ml olarak bulundu, analitik özgüllük açısından 18 referans enfeksiyonla çapraz-kontaminasyona karşı test edilen kit 18 negatif sonuç vermiştir, doğrusal aralığın eğilim çizgisi değerleri y = 0,2933x - 66,143 ve R^2 = 0,993 olarak bulundu, değişim katsayısı ise %2,27 olarak bulundu, referans sistem ve prototip sistem bakteriyel yük sonuç karşılaştırmasına dayalı eğilim çizgisi sonucu y=0,9305x+106,54 ve R^2=0,8973 olarak bulunmuştur, tanısal özgüllük ve tanısal duyarlılık değerleri %95,5 ve %96,6 olarak bulundu. Çıkan sonuçlar analiz edildiğinde çalışmanın odaklandığı konularda oldukça başarılı olduğu görülmektedir. Tanısal özgüllük ve duyarlılık oranı altın standart olan %95'in üstündedir. Bu umut verici sonuçlar sonrasında çalışmanın bir ileri seviyeye taşınması için daha geniş çaplı örneklem grubu ile diğer ticari kitlerin olduğu bir çalışma dizayn edilebilir. Tekrarlı yapılan bu çalışmalar sonrasında diğer ticari kitlerle karşılaştırma yapılıp avantajlı ve dezavantajlı noktalar görülüp bu doğrultularda iyileştirmeler de yapılabilir.

ANAHTAR KELİMELER: Moleküler teşhis, *Chlamydia trachomatis*, kantitatif PCR, mikropartiküller, doğrulama.

Tübitak 1170446 Destek Programı, Denovo Biyoteknoloji Şirketi, Atılım Üniversitesi, Başkent Üniversitesi

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS AND ABBREVIATIONS

CMV	Cytomegalovirus
CoV	Coefficient of Variation
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
E. coli	Escherichia coli
EBV	Epstein-Barr Virus
ELISA	Enzyme-Linked Immunosorbent Assay
FVU	First Void Urine
H. pylori	Helicobacter pylori
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HHV6	Human Herpesvirus 6
HHV8	Human Herpesvirus 8
HIV	Human Immunodeficiency Virus
HPV16	Human Papillomavirus
HSV1	Herpes Simplex Virus Type 1
HSV2	Herpes Simplex Virus Type 2
LGV	Lymphogranuloma Venereum
LoD	Limit of Detection
MRSA	Methicillin-resistant Staphylococcus aureus
NAATs	Nucleic Acid Amplification Tests
nvCT	new variant Chlamydia trachomatis
PCR	Polymerase Chain Reaction
PID	Pelvic Inflammatory Disease
RT-PCR	Real Time Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
SD	Standard Deviation
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
TOXO	Toxoplasmosis
TTV	Torque Teno Virus
WHO	World Health Organization

1. INTRODUCTION

Increasing epidemics and infections in recent years have had a global impact due to the increasing population and lack of precaution. For this reason, early and rapid diagnosisis critical for these infections, which affects millions of people every year. It is of critical importance that people who think they are infected have easy access to diagnostic kits, that it does not burden the person financially, that they get results quickly, and that the results are reliable. The consequences of an incorrect diagnosis can be life-affecting. Early and accurate diagnosis ensures that the person receives appropriate treatment with minimal damage from the infection. It prevents the infection from passing on to other people. In addition, treatment costs will be high as serious health problems will occur in the later stages of the infection.

Chlamydia trachomatis infection is one of them. According to WHO reports, *Chlamydia trachomatis* infects more than 100 million people every year. In the USA alone, the number of people infected in 2014 and 2015 is approximately 3 million. [1-3] *Chlamydia trachomatis* a gram-negative bacterium, is one of the most common causes of sexually transmitted diseases. [1,4] *Chlamydia trachomatis* is spherical and is an obligate intracellular parasite since it cannot synthesize ATP metabolically. [5-7]

It is the most common STI (Sexually Transmitted Infection) in the United Kingdom, and around 70% of applicants in 2013 were sexually active persons younger than 25 years of age. [8] Although it is distributed among all age groups, the *Chlamydia trachomatis* disease is most common between the ages of 15 and 24. [8-10] Because *Chlamydia trachomatis* is asymptomatic in most people, many people do not know they have it. [1,4,9] Asymptomatic cases it is seen at levels exceeding 70% in women and 50% in men, so it cannot be detected most of the time. [11,12] Although it progresses as low- grade or asymptomatic, it also causes serious diseases if the infection is not treated. [4]

The main causes of infection are; multiple partners, not using condoms and sexual activity. [4,10,13] The incidence of infection in the partners of infected people is also quite high and it is approximately 75%. [14,15] Mothers with infections are also more likely to infect their babies at birth. [16] After the treatment, there is a possibility of recurrence up to 30% in the young population. [17] In a study, it was seen that the interval with the

highest probability of recurrence of the infection was the first 5 months. [18]

Patients with *Chlamydia trachomatis* infection also have a high rate of carrying other sexually transmitted diseases, since the transmission routes are the same. In a study, 30% of individuals with *Chlamydia trachomatis* infection were HIV-positive (Human Immunodeficiency Virus), while 50% of HIV-positive individuals were found to carry *Chlamydia trachomatis* infection. [19]

The purpose of the diagnostic kit to be produced against such a common bacterium that has serious effects on human health is to reach results quickly and economically. Therefore, an endpoint-enabled PCR device was used in the study. Apart from the method we will use for the diagnosis of *Chlamydia trachomatis* infection, conventional PCR and Quantitative PCR methods are also used. Although the conventional PCR method is a relatively inexpensive method, it has disadvantages such as the risk of contamination, the need for qualified personnel, the length of the analysis period and the carcinogenicity of the chemicals used. Although the quantitative PCR method provides faster and more reliable results, it is quite costly due to the components it has. Culture and ELISA methods are also used in the diagnosis of *Chlamydia trachomatis*. However, the culture method, like conventional PCR, has a longer analysis time and risk of contamination, and ELISA tests have lower accuracy of the results than PCR tests.

For this infection that is so common and affects human life; It was aimed to create a *Chlamydia trachomatis* diagnostic kit that is easily accessible, affordable, provides fast results and provides high accuracy. In order to make the study economical, an end-point PCR (Polymerase Chain Reaction) device was used. *Chlamydia trachomatis* specific primer and probe design were carried out to produce an original and commercial study. Prototype experiments were carried out with known negative and positive samples and reference samples with known copies/ml ratio. To demonstrate the accuracy of the study, cut-off score, analytical sensitivity, analytical specificity, linear range, precision, diagnostic sensitivity and diagnostic specificity studies were performed. The cut-off score represents the lowest limit required for positive, successful, or valid results to emerge in the study or research. Analytical specificity is the ability of the test to detect very low concentrations of a particular substance in a biological sample. Analytical specificity refers to the ability of an assay to measure on particular organism or substance, rather than

others, in a sample. The linear range is the range that shows the relationship between the fluorescence intensity value obtained from the samples studied (via the fluorescent probe) and the recorded signal intensity. Precision is a measure of dispersion of results for a repeatedly tested sample. Reproducibility is the amount of agreement between the results of samples tested in different laboratories. Diagnostic sensitivity is the ratio of the samples that have positive results to those that also turn out to be positive as a result of the test. Diagnostic specificity is the rate at which the samples in the population with negative results are also negative as a result of the test. Nucleotide comparisons were also performed to avoid cross-contamination with other infections. In this way, it was aimed to produce a high-quality and competitive diagnostic kit that gives reliable results.

2. LITERATURE RESEARCH

Chlamydia trachomatis has 19 serovars. These serovars are determined according to the main outer membrane protein. [20] These serovars show different clinical effects. A-C serovars (A, B, Ba, C) are common in the Middle East, Asia, Africa, and South America. These serovars are active in ocular infections and cause trachoma. [4-7,20,21] Trachoma is an ocular infection that occurs in the eyelids and causes blindness. It can become chronic or can be seen as repeated infections in the person. [22] D-K serovars (D, Da, E, F, G, Ga, H, I, Ia, J, k) are sexually transmitted and cause infections of the urogenital system, rectum, and pharynx. [4,23,24] L serovars (L1, L2, L3) cause lymphogranuloma venereum (LGV). [4,23] Except for L serovars, the infectious effects of other serovars are limited to the mucosal epithelium, but L serovars can penetrate the epithelium, thus causing an invasive infection called LGV. This type of infection occurs in underdeveloped countries. [19]

Immune system disorders caused by *Chlamydia trachomatis* may also cause the infection to recur or persist. [19,26] Symptoms in infected people include: It is seen as dysuria and discharge in men, an increase in vaginal discharge in women, dysuria, pelvic tenderness, and bleeding. In addition, genital and rectal infections can be encountered. [23]

Complications due to infection are ectopic pregnancy, tubal infertility, infertility, pelvic inflammatory disease (PID), perihepatitis, salpingitis, and endometritis in women. [23,27,28] In a study, *Chlamydia trachomatis* was detected in approximately 30% of infertile women. [29] It causes the onset of urethritis, SARS (Severe Acute Respiratory Syndrome) and epididymo-orchitis in men. [16,23,30,31] Previous studies have been associated with infertility, but no effect or relationship was found. [32-35] In the study conducted in 2020, it was seen that it had an effect on sperm motility and vitality and had a negative effect. [36] Long-term exposure to infection is thought to be one of the important factors in tissue damage. [37,38] *Chlamydia trachomatis* is thought to cause pneumonia in more than 10,000 newborns in the United States each year. There may be differences in symptoms and complications depending on the response of the immune system, the fact that people are asymptomatic, or the results of the tests. [23]

It is thought that people with *Chlamydia trachomatis* infection will catch HIV more

easily, and studies are in this direction. [39,40] One of these reasons is the similarity of the risk factors in the transmission routes of the disease. [39] In a study, HIV was found in 30% of patients with *Chlamydia trachomatis*, while *Chlamydia trachomatis* was observed in 50% of HIV-positive patients. [19]

Screening tests are recommended for pregnant women, young women (25 years and younger), and women at high risk of infection. [41] Although these procedures seem costly, the sooner the disease is detected and treated, the faster its spread will be reduced, and the higher costs that may occur later on will be prevented. [42] In a study conducted in London, it was revealed that approximately 160 pounds were spent for each PID treatment, and the cost of treatment in London in just one year was more than 1 million pounds. [28]

The preferred specimen samples for *Chlamydia trachomatis* infection are the vulvovaginal swab in women and the first voiding urine (FVU) in men. [43,44] In women, first voiding urine or endocervical examination samples can also be used. [45] Currently, nucleic acid amplification tests (NAATs) are the best method to identify this infection. It gives more sensitive and reliable results than other methods. [9,45,46] It makes NAAT advantageous as it offers a wide range of options for sample control. There are options such as swab samples and first urine. [2,47] PCR and RT-PCR technologies are used in NAATs. An example (Roche Diagnostics, Abbott, IL, USA) can be given. Inhibition test in NAATs is also important for reliability. [48] In 2006, a new variant *Chlamydia trachomatis* (nvCT) was isolated in Sweden and it has a 337 bp deletion in the region targeted by some of the commercial NAATs. [49] Such novel variants and target regions should be considered in commercial NAATs, as these tests can give us false-negative results, which can lead to worse results in the future.

This study focuses on the aim of creating an economical method that provides fast and accurate results with PCR. “DNA and protein analysis are used in similar methods. Although the use of SDS-page (Sodium dodecyl-sulfate polyacrylamide gel electrophoresis and electrophoresis has advantages, its use is limited due to heat treatment” (Ayaz et al. 2006). “In addition, time, cost and the use of qualified personnel create disadvantages. In addition, close infection or cross-reactivity between species is also observed” (Ayaz et al. 2006) (Violeta et al. 2010).

“The use of PCR methods in analyses based on DNA analysis is much more stable and sensitive” (Mafra et al., 2008). However, despite this reliable result and sensitivity; It has a high price and high priced components. This reveals the main reason that pushed us to do this study. A diagnostic kit that will work compatible with end-point PCR. Cutoff score, analytical sensitivity, analytical specificity, linear range, precision, diagnostic sensitivity and diagnostic specificity studies were performed to demonstrate the accuracy of the study.

“With developing technology, diagnostic tests began to give reliable results, and these features made the PCR test more useful compared to other traditional methods such as ELISA, DNA-based detection methods display several advantages such as an increased sensitivity and specificity, and the possibility for molecular typing” (Laude et al., 2016). “Recently, nucleic acid amplification and detection techniques have progressed based on advances in microfluidics, microelectronics, and optical systems. Nucleic acids amplification-based point-of-care test (POCT) in resource-limited settings requires simple visual detection methods. Several biosensing methods were previously used to visually detect nucleic acids. However, prolonged assay time, several washing steps, and a need for specific antibodies limited their use” (Hwang et al., 2016).

“Applications of PCR methods that require time and qualified personnel are disadvantageous in terms of their efficiency” (Morinha et al., 2012).

In the experiment conducted with 48 people for HBV (Hepatitis B Virus), ELISA and PCR methods were used to diagnose people as positivity or negativity. The results obtained were compared with each other. It has been observed that the PCR method gives more sensitive and accurate results than the ELISA test in terms of detecting positivity and viral load. [50]

In an experiment conducted on pigs with *Chlamydia suis*, the results of a PCR and 2 different ELISA tests were compared. Culture was used as the gold standard in these tests. In the results obtained in that study conducted with 109 samples, culture and PCR results showed high agreement, while 2 different ELISA tests showed very poor performance. [51]

For the diagnosis of *Chlamydia trachomatis*, one of the common causes of infertility, a study was conducted by involving 100 women (81 of whom were infertile). In this study, PCR, ELISA, DIF (direct immune fluorescence) and ICT (immunochromatography test) methods were used to detect *Chlamydia trachomatis*. PCR was chosen as the gold standard. When PCR results were examined, it was seen that the *Chlamydia trachomatis* positive rate was approximately 35% in infertile women and 5% infertile women. PCR test results appear to be much more successful than other immunological tests. [52]

The experiment performed was used to compare the enterovirus results obtained with PCR and ELISA methods. ELISAs that can detect antibodies against IgM, IgA and IgG found in enteroviruses and PCR methods using primers specific to untranslated regions in the 5' region were used and the results were compared. In the results obtained, the sensitivity of ELISA tests was quite low and non-specific compared to PCR test results. [53]

In a more comprehensive study, the culture method and PCR method were compared for the diagnosis of *Chlamydia trachomatis*. In the study conducted with samples taken from 466 male and 290 female patients complaining of the disease; *Chlamydia trachomatis* prevalence was examined and the PCR method showed a higher detection rate in men and women than culture. The PCR method achieved more successful results than the culture method in samples taken from men and women, showing a sensitivity of over 90% in both groups. [54]

In the study conducted in which culture and PCR methods were compared for the diagnosis of *Chlamydia trachomatis* infection, samples were taken from 497 patients complaining of infection and 35 patients known to have infection. When the results of positive samples are compared, the sensitivity of the results obtained with the PCR method is 20% higher than that of culture. [55]

PCR is the amplification of the desired target nucleotide region. This process is carried out with the help of PCR reagents. The amplification process of the samples incubated at the targeted temperatures for the specified time intervals is achieved. If we give examples of PCR reagents; dNTPs, *Taq* DNA polymerase, PCR buffer and $MgCl_2$

Taq DNA polymerase ensures the extension of the region determined by the primers and is amplified with the help of dNTPs. Meanwhile, PCR buffer and $MgCl_2$ serve to create a suitable environment for *Taq*.

PCR reagents combined with nucleic acids are incubated at 95 °C primarily for DNA denaturation. For the annealing process, it is lowered to the specified temperature, generally between 55-65 °C. At this stage, target nucleotide sequences are matched with the primers. The last stage is an extension. At this stage, *Taq* polymerase takes part, the ideal working temperature is 72 °C.

In addition to the reagents listed in quantitative PCRs, probes are used. These probes, like primers, are specific to the target region. Probes, depending on their types, consist of a fluorophore probe that is excited by light at certain wavelengths and then emits radiation, and a quencher probe that prevents this radiation. There is a distance-dependent relationship between them; when they are side by side, the quencher probe prevents or reduces the fluorophore probe's radiation. However, when the gap between the two probes is opened with the endonuclease activity of *Taq* polymerase, there will be a difference between the initial and final luminescence states, as the quencher probe will not prevent or reduce the fluorescence probe's luminescence. This change is detected with sensitive sensors.

In the agarose gel method, an electrophoresis tank filled with electrically conductive buffer is used. PCR products move towards the anode in this electrical environment. In this method, discrimination is made according to molecule size. Since small molecules move faster than large molecules, the distinction between them is made accordingly. At the end of the process, analysis is performed with the marker.

Although the quantitative PCR method is fast and reliable, its biggest disadvantage is its price. Quantitative PCR device prices start from 30,000 € and go up to 100,000 €. The main reasons why it has this price are the components such as the camera and sensor it contains. In comparison, PCR is cheaper, but it has disadvantages due to factors such as gel electrophoresis taking a few hours, the use of chemicals, the risk of false results and being open to contamination. Therefore, in this study, an end-point feature PCR device will be used. This device, with appropriate components, will save both time and cost. We aim to

produce a kit that gives reliable results with the designed primer and probe. One of the biggest reasons why it is economical is that it reduces the cost significantly in terms of kits by using only fluorescent labels instead of using dual labels in probes.

Even though good results are obtained during the studies, these studies do not mean anything without validation. “Test validation is a very important process used in the laboratory to ensure that a new test performs as expected” (Robert et al., 2015).

The first of these validation tests is the cut-off score. “The cutoff value for a new diagnostic test for classifying cases as positive or negative may be determined utilizing some statistical techniques such as Mean ± 2SD and ROC curve” (Singh, 2006).

The mean value represents the center of a numerical cluster. This average value can be found by adding up all numerical values and dividing by the number of values in the data set.

“In statistics, the standard deviation is a measure of the amount of variation of a random variable expected about its mean. A low standard deviation indicates that the values tend to be close to the mean (also called the expected value) of the set, while a high standard deviation indicates that the values are spread out over a wider range.” (Bland et al., 1996)

“Mean ± 2SD method (Mean ± 2-Standard-Deviation method) is the application ofa 95% Confidence Interval of the mean for choosing a cutoff (Figure 2.1). This method may be carried out on a sample of adequate size of diagnosed cases (known negative cases). The upper limit of its 95% Confidence Interval (i.e. mean + 2SD) may be taken as a cutoff value. If a subject's test value comes greater than this cutoff value; then it may be considered positive” (Singh, 2006).

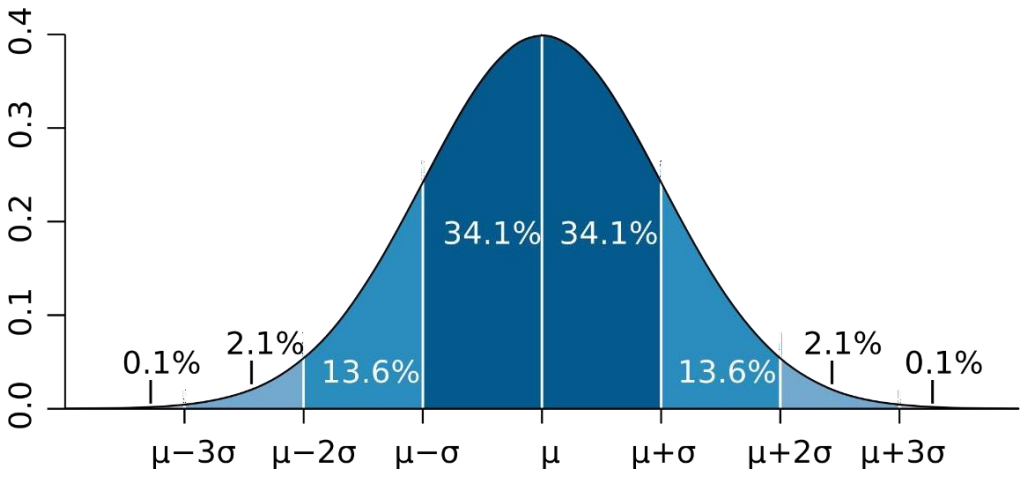

Figure 2.1. Normal distribution curve [56].

"Arguably among the most critical performance parameters for a diagnostic procedure are those related to the minimum amount of target that can be detected" (Bustin et al., 2009). "The limit of detection (LOD), also called analytical sensitivity, is a calculated value for the lowest concentration of analyte that can be detected by the assay" (Robert et al., 2015). The Clinical Laboratory Standards Institute (www.clsi.org), for example, defines LoD as "the lowest amount of analyte (measurand) in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value" (Nick et al., 2013).

"Conventionally, the LOD is reported as the estimate of the detection limit that can be achieved with 95% confidence. This determination requires Probit analysis involving testing of replicate" (Nick et al., 2013). "The LOD is determined by testing multiple specimens, typically 10 of each, across several dilutions. The results are then analyzed using probits (a unit of probability based on deviation from the mean of a standard deviation) and linear regression to calculate the LOD" (Robert et al., 2015).

"Analytical specificity is the ability of an analytical method to detect only the analyte that it was designed to measure" (Robert, et al. 2015).

"Analytical specificity is a test system parameter for laboratory-developed assays that verify that the test system does not cross-react with indigenous nucleic acid in the specimen or similar organisms. Analytical specificity is the ability of an assay to exclusively identify a target analyte or organism rather than a similar but different analyte in a specimen. Analytical specificity is determined by testing compounds with similar

genetic structures, organisms that represent the normal flora of the specimen, and organisms that cause similar disease states" (Lynne et al., 2007).

"Analytical specificity is the ability of an assay to exclusively identify the intended target substance or organism. For validation testing should include, a wide variety of samples and strains containing the target sequence (i.e. positive in the test) as well as samples and strains containing nucleic acids that should be negative in the assay" (Nick et al., 2013).

"Linear range is the linearity of an analytical procedure is its ability to give test results which are directly proportional to the concentration of analyte. Ideally, a linear relationship should be maintained across the entire range of the analytical procedure. Linearity may be assessed by testing dilutions of a quantified standard. Results should be evaluated by calculation of a regression line" (Nick et al., 2013).

"Results should not be extrapolated beyond the established linear dynamic range of the assay. Consequently, the quantitative range of the assay should ideally encompass the range expected from clinical samples. The standard curve should include a minimum of four points and the upper and lower values of the standards should be within 1 log of the top and bottom, respectively, of the reported quantitative range. Positive results above or below the quantitative range should be reported appropriately (e.g. "Positive, greater than xx copies/mL" or "Positive, less than xx copies/mL" respectively)" (Nick et al., 2013).

"Precision, or reproducibility, is a measure of the agreement between replicate analyses (using identical procedures) of a homogeneous analyte; they include inter-run, intra-run, and inter-operator variability. While the two terms are used interchangeably, historically, the term precision is generally applied to quantitative assays, while reproducibility is used with qualitative analyses" (Robert et al., 2015).

"An assay may be precise but inaccurate. The precision of an analytical procedure can be expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision can be determined by repeat testing of samples. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under

prescribed conditions" (Nick et al., 2013).

"Robustness is a measure of the capacity of the method to remain unaffected by small variations in method parameters. The verification of the robustness allows the determination of the total failure rate of the assay. To verify the robustness, negative samples are generally spiked with quantitation standards; then, inhibitions observed and robustness of the assay is calculated" (Liesbet et al., 2016).

"The validation of clinical accuracy requires comparison of the assay to an appropriate "gold standard" method using sequential clinical samples obtained in real clinical situations. The number of samples tested will vary depending on the availability of suitable clinical material. They should, wherever possible, include a wider range of concentrations of positive samples as well as negative samples" (Nick et al., 2013).

"There are a number of ways to express accuracy, but the most common is diagnostic sensitivity and specificity (formerly referred to as clinical sensitivity and specificity). The accuracy data can be displayed in a 2 x 2 table (Figure 2.2)" (Lynne, 2007).

"Clinical sensitivity can be expressed mathematically as a percentage of the number of true-positive results divided by the number of true positive results plus the number of false-negative results. Clinical specificity is mathematically expressed as a percentage of the number of true-negative results divided by the number of true-negative results plus the number of false-positive results" (Robert et al., 2015).

New Test	Subjects with the disease	Subjects without the disease	Total
Positive	a (TP)	b (FP)	a +b (TP + FP)
Negative	c (FN)	d (TN)	c +d(FN + TN)
Total	a +c (TP + FN)	b +d(FP + TN)	a+ b+ c+ d (TP+TN+FP+FN)

Figure 2.2. Diagnostic accuracy [57].

3. MATERIALS & METHODS

3.1. Materials

3.1.1. Chemicals

The chemicals used during the experiment are listed. (Table 3.1)

Table 3.1. Chemicals.

dNTP Mix	Sigma Aldrich
PCR Buffer	Thermo Fisher Scientific
Primers	Thermo Fisher Scientific
Probe	Sigma Aldrich
$MgCl_2$	Sigma Aldrich
Taq Polymerase	Thermo Fisher Scientific
PCR Grade Water	Thermo Fisher Scientific

3.2. Methods

For the purpose of the study, it will be used with the end-point feature PCR device, which has a very affordable price and is available at Denovo Biotechnology. In this way, it is planned to save both cost and time during the work and in the future. Instead of using both quencher and fluorophore probe labels, which are compatible with the working principle of the device to be used, experiments will be carried out with only the fluorophore probe label. If this study is effective/successful without a quencher probe; It is anticipated that the cost will decrease since one of the high-cost probes will not be used among the diagnostic kit components.

3.2.1. Primer and probe desing

Tools from NCBI were used for primer design. The points to be taken into consideration while making this design are determined as follows; Conservative sequence regions will be targeted for *Chlamydia trachomatis*. Nucleotide sequences of the regions of interest will be entered into the program in FASTA format. The melting temperature of

the primers is important for the study. While designing primers, the following features are included in the tool: The lengths will be chosen to be between 18-23 nucleotides and the G/C ratio will be between 40-60%. When selecting the nucleotide sequence for the study, the desired temperature will be selected as 57-62 ^{o}C. Additionally, care will be taken to ensure that the difference between the melting temperatures of the two primers is not more than 3 ^{o}C. It is not desirable for the annealing temperature to be too low or too high for the progress of the study. Because in these two cases, non-specific bindings or low numbers of PCR products may be encountered.

To avoid false positivity through cross-contamination, the comparison will be made with the nucleotide sequences of human and other sexually transmitted infections from the database. Primers with a mispriming/false-priming score of more than 12 or a self-complementarity score of 8 and above will not be selected. In addition, primer sequences with a self 3' complementarity score of 3 and above will not be selected. When selecting the appropriate primer pair, pairs with long repeat sequences and dinucleotide repeats will be avoided. Having base sequences with high Gibbs Free energy values at the 3' end of the primers prevents non-specific binding, so primers containing GC sequences will be preferred.

For probe design, the tools on the website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) will be used. Care will be taken to ensure that the probe length is 18-27 nucleotides. As with the primers, care should be taken to ensure that the melting temperature is between 57-62 ^{o}C and that the nucleotide sequence does not contain long-run or dinucleotide repetitive sequences. After checking the self complementarity, self 3' complementarity and mispriming/false-priming scores, nucleotide sequences with high values will not be used.

When determining PCR protocols, the total number of cycles and those involved in this cycle; denaturation, annealing and elongation temperatures will be determined. While determining these temperatures, the primer and probe operating range for the annealing temperature will be worked within the most appropriate range of 57-62 ^{o}C. In the extension range, 95-98 ^{o}C will be used. In addition, the amounts of chemicals used will be determined and the most optimum working conditions will be tried to be achieved.

3.2.2. Statistical analysis

Excel software functions were used for the analysis records and numerical operations related to these records (addition, subtraction, multiplication, division, finding the average value, standard deviation calculation). The graph created regarding the results, the curve line of the graph, and the equation of the curve line were created in the same waywith the help of Excel. The following methods were used when analyzing the results recorded in Excel.

3.2.3. Cut-off score

The cut-off score represents the lowest limit required for positive, successful or valid results to occur in the study or research. The general opinion in PCR studies is that it is called the gold standard and the success rate is at least 95%. Threshold or Cutoff represents the probability of the prediction being correct in binary classification. It represents the variation between false positives and false negatives. In this study, when determining the cut-off score, 22 negative samples with known results will be studied and then the fluorescence intensity reading values obtained will be recorded. The mean and standard deviation values of the recorded fluorescence intensity readings will be found. Afterward, the Mean ± 2SD method (Mean ± 2-Standard-Deviation method) will be used to determine the cut-off score. The value found will then be compared with the results of negative and known positive samples. Negative samples are expected to be measured as true-negative, and positive samples are expected to be measured as true-positive. The expected success rate to achieve a successful result in these measurements is at least 95%.

3.2.4. Analytical sensitivity

The ability of the test to detect very low concentrations of a particular substance ina biological sample. Analytical sensitivity is often referred to as the limit of detection (LoD). LoD is the lowest concentration at which an analyte in a sample can be consistently detected ≥95% of the time. LoD is the number of genome copies of the analyte that can be detected, infectious dose, colony-forming units, plaque-forming units, etc. can be represented as. To determine analytical sensitivity, an end-point dilution is used until the assay no longer detects the target of interest in more than 95% of replicates.

To determine the limit of detection value, bacterial load copies/ml will be studied repeatedly with samples known and response rates will be determined. Afterwards, a graph will be created according to bacterial load and response percentages and the trend line of this graph will be calculated. “Traditionally, the LOD is reported as an estimate of the limit of detection that can be achieved with 95% confidence. “This determination requires Probit analysis involving testing of replicates” (Nick et al., 2013). determined by testing the sample. The results are then analyzed using probits (a unit of probability based on the deviation from the mean of the standard deviation) and linear regression to calculate the LOD” (Robert et al., 2015). If the value corresponding to 95% in the probit chart is put into the equation obtained from the trend line, the minimum bacterial load range that can be measured with a success rate of at least 95% is found.

3.2.5. Analytical specificity

“Analytical specificity is the ability of an analytical method to detect only the analyte that it was designed to measure” (Robert, et al. 2015).

Analytical specificity refers to the ability of an assay to measure on particular organism or substance, rather than others, in a sample. It is critical to verify that the assay’s primers are specific to the target. Cross-reactivity may occur when genetically related organisms are present in a patient specimen. These organisms mimic the intended target, which results in the assay’s primers cross-reacting or annealing to these genetically related organisms which will generate a false positive result. The primary interaction with endogenous or exogenous substances may not bind to the intended target. After the target-oriented amplification, the target cannot be detected and a potential false negative result is produced.

At this stage, for the specific use of *Chlamydia trachomatis*, the kit to be studied will be tested with different versions and the fluorescence intensity readings obtained from the prototype system will be recorded. These values will be compared to the recorded cutoff score and a check will be made to see if any false positive results will be obtained.

3.2.6. Linear range

"Linear range is the linearity of an analytical procedure is its ability to give test results which are directly proportional to the concentration of analyte. Ideally, a linear relationship should be maintained across the entire range of the analytical procedure. Linearity may be assessed by testing dilutions of a quantified standard. Results should be evaluated by calculation of a regression line" (Nick et al., 2013).

For this study, standard samples with known bacterial load will be used. Fluorescence intensity values obtained from the studied samples (via the fluorescence probe) will be recorded. A graph will be created to show the relationship between recorded fluorescence signal intensities and bacterial load. The range where this relationship is linear is the desired and interesting part. The fluorescence intensity value obtained in the optimum working region of the study is expected to increase or decrease in direct proportion to the viral load in the sample studied.

3.2.7. Precision

"Precision, or reproducibility, is a measure of the agreement between replicate analyses (using identical procedures) of a homogeneous analyte; they include inter-run, intra-run, and inter-operator variability. While the two terms are used interchangeably, historically, the term precision is generally applied to quantitative assays, while reproducibility is used with qualitative analyses" (Robert et al., 2015).

Precision is a measure of dispersion of results for a repeatedly tested sample. Precision is the variability in the data from replicate determinations of the same homogeneous sample under normal assay conditions. Precision includes within assay variability, repeatability (within-day variability), and reproducibility (day-to-day variability). Precision may be established without the availability of a "gold" standard as it represents the scatter of the data rather than the exactness (accuracy) of the reported result.

To perform this test, standard samples with known bacterial loads will be used. Each Fluorescence Intensity Reading result obtained from the samples studied in the prototype

system will be recorded and the bacterial load calculation will be made for each sample using the found in the linear range study. Then, the mean and standard deviations of these results will be calculated. Since the standard deviation increases as the numerical values increase, the coefficient of variation value, which is the percentage expression of the ratio of the standard deviation to the average, was examined. Then, the coefficient of variation value will be calculated using the mean value and standard deviation. "The coefficient of variation measures the variability of a series of numbers independently of the unit of measurement used for these numbers. In order to do so, the coefficient of variation eliminates the unit of measurement of the standard deviation of a series of numbers by dividing it by the mean of these numbers. The coefficient of variation can be used to compare distributions obtained with different units, such as, for example, the variability of the weights of newborns (measured in grams) with the size of adults (measured in centimeters). The coefficient of variation is meaningful only for measurements with a real zero (i.e., "ratio scales") because the mean is meaningful (i.e., unique) only for these scales" (Abdi et al., 2010).

3.2.8. Diagnostic sensitivity and Diagnostic specificity

"The validation of clinical accuracy requires comparison of the assay to an appropriate "gold standard" method using sequential clinical samples obtained in real clinical situations. The number of samples tested will vary depending on the availability of suitable clinical material. They should, wherever possible, include a wider range of concentrations of positive samples as well as negative samples" (Nick et al., 2013).

Diagnostic sensitivity is the ratio of the samples that have positive results to those that also turn out to be positive as a result of the test. It shows how well one can select and distinguish samples in a positive population. Giving false results causes the positive sample to show false-negative results.

Diagnostic specificity is the rate at which the samples in the population withnegative results are also negative as a result of the test. It shows the rate at which negative samples in the population can be selected. A specific test should not show a false-positive result due to another pathogen.

The prototype Fluorescence Intensity Reading values of the samples to be used in the study and whose results are known will be recorded, and then the bacterial loads of the samples will be found using the equation obtained from the linear range chart. A graph and equation will be created to see the similarity of the bacterial loads found with the diagnostic kit and the measurements in the reference system.

4. RESULTS & DISCUSSION

4.1. Primers and Probe

For primer design, primer samples were created with nucleotide sequences from different regions of *Chlamydia trachomatis*. The most suitable primer pair was selected among these samples. This primer pair was designed according to the nucleotide sequence taken from the 16S rRNA region, which is a conservative region of *Chlamydia trachomatis*. After the primer design, the probe design was also made. (Table 4.1) FAM fluorophore was chosen for the fluorophore probe. FAM fluorophore was selected according to the end-point PCR feature to be used.

Table 4.1. Primers and probe.

Forward Primer	(5'-GCATCCAATCAATCAGATTTCT-3')
Reverse Primer	5'-GTGTATTTTTGCAACTCCTCCT-3')
FAM Probe	(5'-ACAATATTCGCTGTATCAGAGCCAG-3')

The results of the optimization studies carried out when determining PCR protocols are as follows; The total number of cycles is 40. Before starting these cycles, the initial denaturation phase was waited at 95 ^{o}C for 6 minutes. During each cycle, it was waited at 95 ^{o}C for 15 seconds during the denaturation phase, at 60 ^{o}C for 30 seconds during the annealing phase, and at 72 ^{o}C for 15 seconds during the extension phase.

As a result of the optimization studies, the values of the chemicals used that provide the most optimum working conditions were found as follows; forward primer (0.625 µl), reverse primer (0.625 µl), dNTP mix (0.5 µl), PCR buffer (2.5 µl), $MgCl_2$ (1.5 µl), *Taq* Polymerase (0.5 µl), PCR grade H_2O (17.75 µl), *Chlamydia trachomatis* sample (1 µl).

4.2. Cut-off Score

In the study, 51 samples with known positivity and negativity were studied. The results of 22 of these samples are negative and the results of 29 are positive. (Table 4.2) After working with these samples, Fluorescence Intensity Reading values were recorded. The cutoff score was found by taking the average and standard deviation of the results

obtained from negative samples. The mean value of negative samples was found to be (29,87) and the standard deviation value was (0,593). The formula (mean value + 2 Standard Deviation) was used to determine the cut-off and the cut-off value was found to be (31,055). (Table 4.3) With these values, the results of negative and positive samples were compared. In 22 negative samples, 21 successful results and 1 false positive result (95,5%) were obtained. When positive results were checked according to the determined cut-off value, out of 29 positive samples, 28 successful results were obtained and 1 false negative result (96,6%) was obtained. Considering the success of both negative and positive results, a generally accepted success rate of 95% was achieved.

Table 4.2. List of negative and positive samples with fluorescence intensity reading values.

Sample	Reference System QualitativeDiagnosis	Prototype System Fluorescence IntensityReading
N01	-	30,48
N02	-	30,50
N03	-	30,45
N04	-	29,42
N05	-	29,45
N06	-	29,47
N07	-	30,53
N08	-	29,09
N09	-	30,42
N10	-	29,12
N11	-	29,16
N12	-	31,55 (False positive)
N13	-	29,25
N14	-	30,40
N15	-	29,27
N16	-	30,57
N17	-	29,30
N18	-	30,38
N19	-	29,32
N20	-	30,60
N21	-	30,02
N22	-	30,03
P01	+	30,23 (False negative)
P02	+	31,56
P03	+	31,69
P04	+	31,42
P05	+	31,81
P06	+	35,93
P07	+	38,84
P08	+	33,02
P09	+	35,35
P10	+	35,65
P11	+	42,05
P12	+	42,64
P13	+	42,05
P14	+	42,92
P15	+	39,43

P16	+	42,64
P17	+	42,92
P18	+	48,76
P19	+	47,88
P20	+	48,46
P21	+	47,58
P22	+	51,95
P23	+	57,20
P24	+	62,15
P25	+	54,00
P26	+	42,68
P27	+	42,81
P28	+	48,69
P29	+	47,79

Table 4.3. Cut-off score calculation.

Mean	29,87
SD	0,593
Mean+2SD	31,055

The high detection rate of positive results can be attributed to the high sample size studied. On the contrary, the approximately 1% lower success rate of the negative sample may be due to working with fewer samples. Higher rates can be reached by increasing the number of samples. It should also be noted that inaccurate results could be due to errors in the current study. Since the number of samples in the negative group is small, any error affects and changes the result much more. In line with all these opinions, this study may yield more positive results when carried out more comprehensively. Because when a study is conducted with a larger sample, changes may occur in the mean values and standard deviation values, and these results/changes may change the course of the study.

4.3. Limit of Detection

To determine the Limit of Detection value (LoD), 10 samples with bacterial loads ranging from 213 copies/ml to 259 copies/ml were studied 16 times with each bacterial load. (Table 4.4) 100% response was achieved in samples with bacterial loads of 259, 247 and 244 copies/ml. Variable response percentages were observed in samples with 242, 239, 236, 232, and 230 copies/ml bacterial loads. A 0% response was detected in samples with bacterial loads of 224 and 213 copies/ml. For probit analysis, a graph was created using these values with bacterial load and response percentages. (Figure 4.1) When the graph was created with all values, a secondary graph was created because the 0% and 100% response values in the graph prevented the graph from being in a linear order. (Figure 4.2) In this graph, 0% and 100% response values, which make the graph horizontal, are not used. The trend line value results of the chart are $y=0.2251x-47.842$, $R^2 = 0.9832$. It is desired that the R^2 value found be close to 1 because this is an indicator of a standard measurement. The R^2 value we obtained is very close to 1, which shows that the graph is standard and quite successful. An equation with 2 unknowns at the probit table and trend line value will be used to find the lowest viral load for which the Limit of Detection value gives a 95% response rate. In the probit chart (Figure 4.3), the value of 6.64 corresponds to the value of 95%. When the probit value was written instead of y in the formula $y=0.2251x-47.842$ on the trend line, the lowest viral load that could be measured with a 95% success rate was found to be 242 copies/ml. (Table 4.5)

Table 4.4. Responding results of the limit of detection.

Bacterial Load (copies/ml)	Samples Responded	Total Samples	Responding (%)	Probits
259	16	16	100,0	
247	16	16	100,0	
244	16	16	100,0	
242	15	16	93,8	6,54
239	14	16	87,5	6,15
236	9	16	56,3	5,16
232	5	16	31,3	4,51
230	2	16	12,5	3,85
224	0	16	0,0	
213	0	16	0,0	

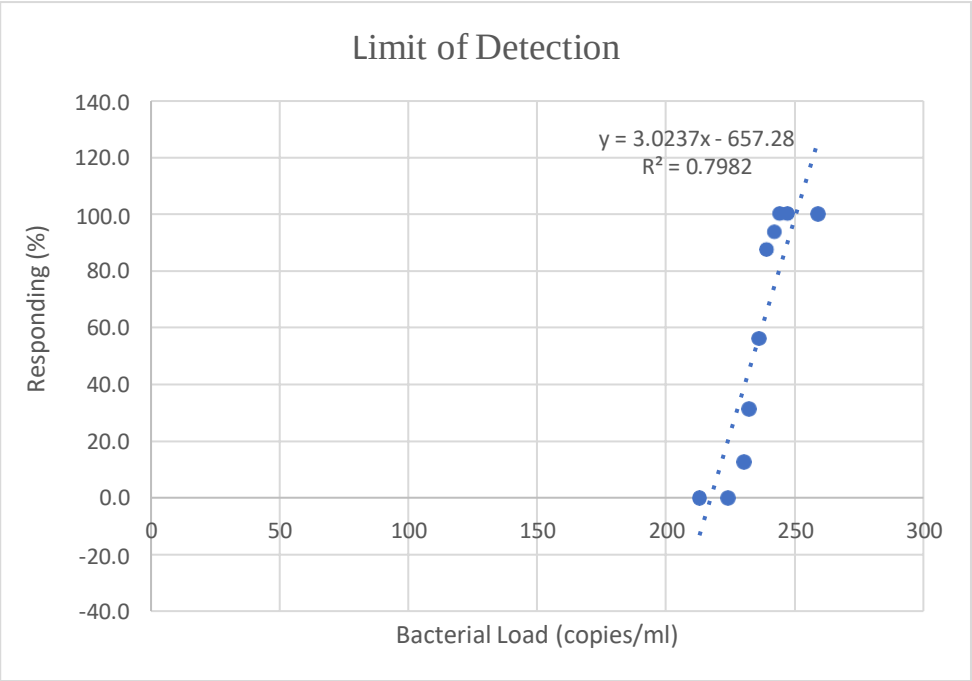

Figure 4.1. First limit of detection graph.

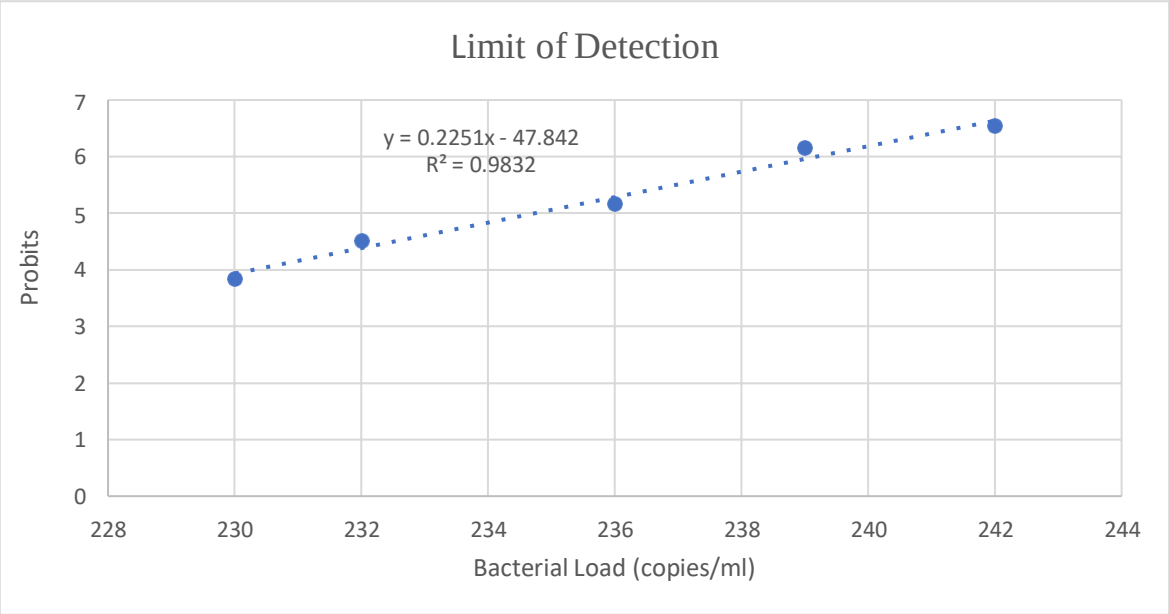

Figure 4.2. Second limit of detection graph.

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.29	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Figure 4.3. Transformation of percentage to probits.

Table 4.5. Prohibit and LoD values.

Prohibit for 95%	6,64
LoD	242

4.4. Analytical Specificity

"Analytical specificity is the ability of an assay to exclusively identify the intended target substance or organism. For validation testing should include, a wide variety of samples and strains containing the target sequence (i.e. positive in the test) as well as samples and strains containing nucleic acids that should be negative in the assay" (Nick et al., 2013).

To test the results of the study against cross-activities, it was tested with 18 reference infection samples. (Table 4.6) The Fluorescence Intensity Reading values of these studied samples were recorded one by one and evaluated according to the determined cut- off score. Looking at the results, negative results were obtained in all 18 samples. These results show that the study yielded successful results despite the risk of cross-activity. In the future, a more comprehensive test can be conducted by expanding the sample group. Although negative results were obtained, infection samples were encountered with fluorescence intensity reading values close to the cut-off score. The reason for this may be that there may be sequence similarities with the samples.

Table 4.6. Fluorescence intensity reading results of different infections.

Sample	Reference System Qualitative Diagnosis	Prototype System Fluorescence Intensity Reading	Prototype System Qualitative Diagnosis
HSV1	-	28,65	-
HSV2	-	29,76	-
EBV	-	29,81	-
CMV	-	28,76	-
HIV1	-	29,94	-
HAV	-	28,99	-
HCV	-	30,32	-
E. coli	-	30,11	-
MT	-	29,34	-
HPV16	-	30,18	-
TTV	-	29,39	-
HHV8	-	30,29	-
HHV6	-	29,45	-
H. pylori	-	30,31	-
Brucella	-	29,52	-
MRSA	-	30,37	-
HBV	-	29,56	-
TOXO	-	29,65	-

4.5. Linear Range

"Results should not be extrapolated beyond the established linear dynamic range of the assay. Consequently, the quantitative range of the assay should ideally encompass the range expected from clinical samples. The standard curve should include a minimum of four points and the upper and lower values of the standards should be within 1 log of the top and bottom, respectively, of the reported quantitative range. Positive results above or below the quantitative range should be reported appropriately (e.g. "Positive, greater than xx copies/mL" or "Positive, less than xx copies/mL" respectively)" (Nick et al., 2013).

To show that the study gave consistent and accurate results in variable viral loads, 6 standard samples with known bacterial load and increasing without changing at a certain rate were studied. (Table 4.7) After the measurements, the fluorescence intensity reading values corresponding to each viral load were recorded and a graph was created using the fluorescence intensity reading values obtained with these viral loads. (Figure 4.4) When the trend line of this graph was created, the values y= 0,2933x – 66.143, R^2 = 0,993 were

reached. The R^2 value obtained is very close to 1, which indicates how standard the study and the graph created are in variable measurement values and how successful the measurement is.

Table 4.7. Fluorescence intensity reading values of standard samples.

Sample	Reference System Quantitative DiagnosisBacterial Load (copies/ml)	Prototype System Fluorescence Intensity Reading
Standard 1	1000	223,49
Standard 2	900	204,38
Standard 3	800	164,97
Standard 4	700	137,86
Standard 5	600	114,86
Standard 6	500	77,33

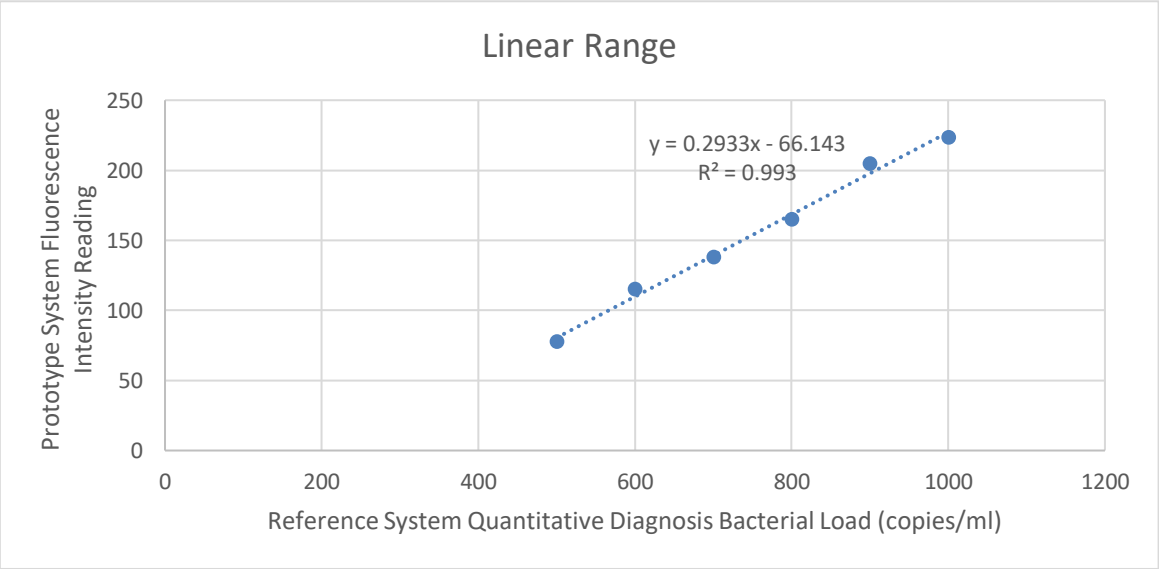

Figure 4.4. Linear range graph.

4.6. Precision

In order to demonstrate that the study was reproducible and that viral load measurement was stable and sensitive, 16 repeated tests were performed using a standard with a known viral load of 600 copies/ml and the results were recorded. (Table 4.8) Fluorescence intensity reading values obtained from the measurement of each sample were recorded. In order to find the viral loads corresponding to the recorded values, the equation

y = 0,2933x – 66,143 obtained from the trend line created in the linear range graph was used. Viral load values were calculated. Considering the results obtained, the average viral load of the 16 samples studied was found to be 599,38. Our average value is 99,89% similar to the reference standard sample value. The average value obtained is almost exactly the same as the standard example and shows how successful the study was. The standard deviation of the fluorescent intensity reading results obtained from 16 samples is 13,6. Since the standard deviation increases as the numerical values increase, the coefficient of variation value, which is the percentage expression of the ratio of the standard deviation to the average, was examined. This value was found to be 2,27%. (Table 4.9)

Table 4.8. Bacterial load measurement comparison of reference system and prototype system for standard samples.

Sample	Reference System Quantitative Diagnosis Bacterial Load (copies/ml)	Prototype System Fluorescence Intensity Reading	Prototype System Quantitative Diagnosis BacterialLoad (copies/ml)
Standard 5 R01	600	103,14	577
Standard 5 R02	600	117,15	625
Standard 5 R03	600	106,67	589
Standard 5 R04	600	112,83	610
Standard 5 R05	600	108,61	596
Standard 5 R06	600	110,91	604
Standard 5 R07	600	108,87	597
Standard 5 R08	600	109,51	599
Standard 5 R09	600	116,41	622
Standard 5 R10	600	102,75	576
Standard 5 R11	600	113,32	612
Standard 5 R12	600	106,82	590
Standard 5 R13	600	110,66	603
Standard 5 R14	600	108,43	595
Standard 5 R15	600	109,51	599
Standard 5 R16	600	108,87	597

Table 4.9. Coefficient of variation value.

	Replicates	Standard Deviation	Mean	Coefficient of variation (%)
Total variance	16	13,60	599,38	2,27

4.7. Diagnostic Sensitivity and Diagnostic Specificity

In the study, we caught negative samples with a success rate of 95,5% and positive samples with a success rate of 96,6%. (Table 4.10) In addition, the viral loads of the samples that were above the fluorescence intensity reading value we took when determining the cut-off score. (Table 4.11) That is, the samples we described as positive, were found with the fluorescence intensity reading value obtained from the samples and the trend line formula we obtained from the linear range graph. The measurements of our reference system and prototype were recorded and a chart was created for relational dimensioning. (Figure 4.5) After the chart was created, the trend line was also created.

The result from the trend line is $y=0,9305x+106,54$ $R^2=0,8973$. When the results are compared, this R^2 value is an expected result. The reason is the difference between the viral load measurements of the reference system and the prototype measurement. In prototype measurements, the results were higher than in reference systems. But these results should not mislead us into thinking that all results are like this, because; When the viral load was calculated with fluorescent intensity reading values in the tests performed with 16 samples with 600 copies/ml viral load, the average value was 599,38. This result tells us; It indicates that the test is successful in detecting positive samples regardless of viral load. If the viral load change is wanted to be monitored in studies to monitor and evaluate the stages of the disease, the average value of our study is 99,89% successful when working with 600 copies/ml samples. However, below these values, our study was conducted with samples with a viral load of 231-334 copies/ml.

Table 4.10. Accuracy

Diagnostic Specificity	95,5
Diagnostic Sensitivity	96,6

Table 4.11. Bacterial load measurement comparison of reference system and prototype system for positive samples

Sample	Reference SystemQualitative Diagnosis	Prototype System FluorescenceIntensity Reading	Prototype System Quantitative DiagnosisBacterial Load (copies/ml)
N01	-	30,48	-
N02	-	30,50	-
N03	-	30,45	-
N04	-	29,42	-
N05	-	29,45	-
N06	-	29,47	-
N07	-	30,53	-
N08	-	29,09	-
N09	-	30,42	-
N10	-	29,12	-
N11	-	29,16	-
N12	-	31,55	333 (False positive)
N13	-	29,25	-
N14	-	30,40	-
N15	-	29,27	-
N16	-	30,57	-
N17	-	29,30	-
N18	-	30,38	-
N19	-	29,32	-
N20	-	30,60	-
N21	-	30,02	-
N22	-	30,03	-
P01	231	30,23	(False negative)
P02	243	31,56	333
P03	244	31,69	334
P04	245	31,42	333
P05	245	31,81	334
P06	246	35,93	348
P07	255	38,84	358
P08	257	33,02	338
P09	263	35,35	346
P10	266	35,65	347
P11	273	42,05	369
P12	276	42,64	371
P13	277	42,05	369
P14	285	42,92	372
P15	289	39,43	360
P16	294	42,64	371
P17	295	42,92	372
P18	300	48,76	392
P19	304	47,88	389
P20	305	48,46	391
P21	312	47,58	388
P22	320	51,95	403
P23	324	57,20	421
P24	332	62,15	437
P25	334	54,00	410
P26	294	42,68	371
P27	295	42,81	371
P28	300	48,69	392
P29	304	47,79	388

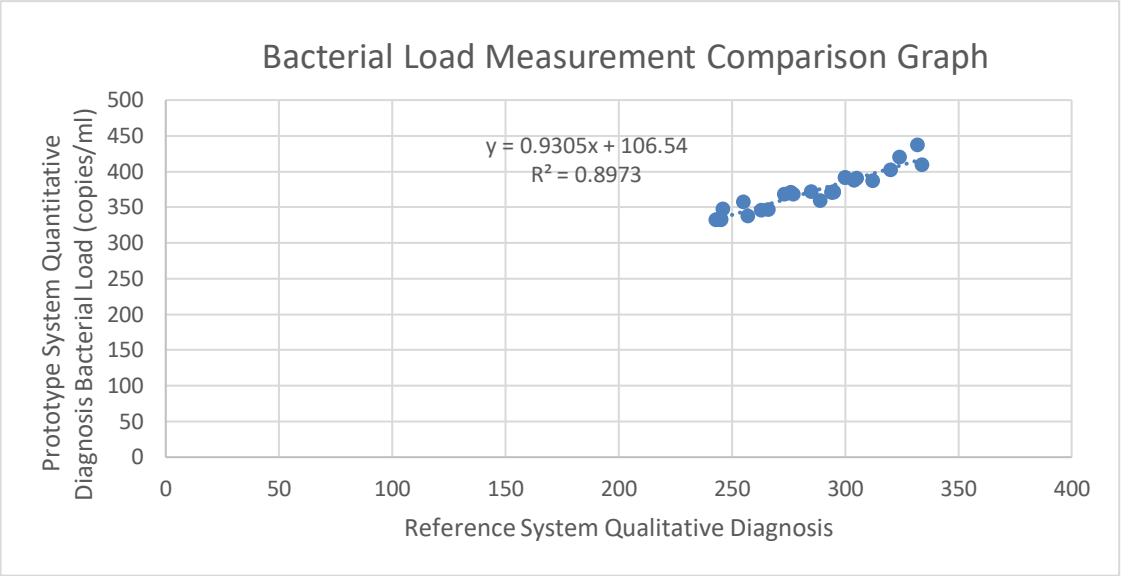

Figure 4.5. Reference system and prototype system bacterial load measurement comparison graph.

5. CONCLUSION

In this research, using an end-point PCR device; The aim was to develop an economical *Chlamydia trachomatis* diagnostic kit that could provide rapid results and be accurate to certain standards. For this disease, which affects hundreds of millions of people every year, it is vital that the kit is easily accessible, economical, provides fast and accurate results. Although antigen kits promise economical and fast results, they are not as successful as PCR in terms of providing reliable results. The culture method, on the other hand, has a disadvantage in terms of speed and accurate results. Since PCR devices are very expensive today, an economical and end-point PCR device from Denovo Biotechnology was used in this study. Since the probe used in this PCR device only has a fluorescent label, it is much cheaper than commercial kits that use dual label probes.

In the study, 51 samples with known positivity and negativity were studied. The results of 22 of these samples are negative and the results of 29 are positive. To determine a cut-off score from the obtained values, the mean and standard deviation values of the negative values were found. The mean value of negative samples was found to be (29,87) and the standard deviation value was (0,593). The formula (mean value + 2 Standard Deviation) was used to determine the cut-off and the cut-off score was found to be (31,055). (Table 4.3) With these values, the results of negative and positive samples were compared. In 22 negative samples, 21 successful results and 1 false positive result (95,5%) were obtained. When positive results were checked according to the determined cut-off score, out of 29 positive samples, 28 successful results were obtained and 1 false negative result (96,6%) was obtained. Considering the success of both negative and positive results, a generally accepted success rate of 95% was achieved.

The high detection rate of positive results can be attributed to the high sample size studied. On the contrary, the approximately 1% lower success rate of the negative sample may be due to working with fewer samples. Higher rates can be reached by increasing the number of samples. It should also be noted that inaccurate results could be due to errors in the current study. Since the number of samples in the negative group is small, any error affects and changes the result much more. In line with all these opinions, this study may yield more positive results when carried out more comprehensively. Because when a study is conducted with a larger sample, changes may occur in the mean values and standard

deviation values, and these results/changes may change the course of the study.

As an additional validation test to the study, the youden index can also be used when determining the cut-off score. “The Youden index, is defined as the maximum of the sum of sensitivity and specificity minus one. The cut-off score, where the maximum is achieved, provides an optimal threshold for the clinicians to use the diagnostic test for classification if equal weight is placed on sensitivity and specificity” (Youden, 1950).

LoD is the lowest concentration at which an analyte in a sample can be consistently detected ≥95% of the time. To determine analytical sensitivity, an end-point dilution is used until the assay no longer detects the target of interest in more than 95% of replicates. To determine the Limit of Detection value (LoD), 10 samples with bacterial loads ranging from 213 copies/ml to 259 copies/ml were studied 16 times with each bacterial load. (Table 4.4) 100% response was achieved in samples with bacterial loads of 259, 247 and 244 copies/ml. Variable response percentages were observed in samples with 242, 239, 236, 232, and 230 copies/ml bacterial loads. A 0% response was detected in samples with bacterial loads of 224 and 213 copies/ml.

For probit analysis, a graph was created using these values with bacterial load and response percentages. (Figure 4.1) When the graph was created with all values, a secondary graph was created because the 0% and 100% response values in the graph prevented the graph from being in a linear order. (Figure 4.2) In this graph, 0% and 100% response values, which make the graph horizontal, are not used. The trend line value results of the chart are y=0.2251x-47.842, $R^2 = 0.9832$. It is desired that the R^2 value found be close to 1 because this is an indicator of a standard measurement. The R^2 value we obtained is very close to 1,which shows that the graph is standard and quite successful. An equation with 2 unknowns at the probit table and trend line value will be used to find the lowest viral load for which the Limit of Detection value gives a 95% response rate. In the probit chart (Figure 4.3), the value of 6.64 corresponds to the value of 95%. When the probit value was written instead of y in the formula y=0.2251x-47.842 on the trend line, the lowest viral load that could be measured with a 95% success rate was found to be 242 copies/ml. (Table 4.5)

The range in which the LoD value is studied seems to be a reliable and accurate range, so instead of working with a larger bacterial load group, a larger repeat group can be studied if desired.

"Analytical specificity is a test system parameter for laboratory-developed assays that verify that the test system does not cross-react with indigenous nucleic acid in the specimen or similar organisms. Analytical specificity is the ability of an assay to exclusively identify a target analyte or organism rather than a similar but different analyte in a specimen. Analytical specificity is determined by testing compounds with similar genetic structures, organisms that represent the normal flora of the specimen, and organisms that cause similar disease states" (Lynne et al., 2007).

To test the results of the study against cross-activities, it was tested with 18 reference infection samples. (Table 4.6) The Fluorescence Intensity Reading values of these studied samples were recorded one by one and evaluated according to the determined cut- off score. Looking at the results, negative results were obtained in all 18 samples. Although negative results were obtained, infection samples were encountered with fluorescence intensity reading values close to the cut-off score. The reason for this may be that there may be sequence similarities with the samples.

The study evaluated the 18 different infections it was compared with as negative and showed a 100% success rate. However, fluorescence rates close to the cut-off score were observed in some infections. The reasons for this may have arisen during the operation. In order to prevent this, working repeatedly with these infections or with more and different disease groups can provide clearer and more reliable results when taking a commercial step.

"Results should not be extrapolated beyond the established linear dynamic range of the assay. Consequently, the quantitative range of the assay should ideally encompass the range expected from clinical samples. The standard curve should include a minimum of four points and the upper and lower values of the standards should be within 1 log of the top and bottom, respectively, of the reported quantitative range. Positive results above or below the quantitative range should be reported appropriately (e.g. "Positive, greater than

xx copies/mL" or "Positive, less than xx copies/mL" respectively)" (Nick et al., 2013).

To show that the study gave consistent and accurate results in variable viral loads, 6 standard samples with known bacterial load and increasing without changing at a certain rate were studied. (Table 4.7) After the measurements, the fluorescence intensity reading values corresponding to each viral load were recorded and a graph was created using the fluorescence intensity reading values obtained with these viral loads. (Figure 4.4) When the trend line of this graph was created, the values y= 0,2933x – 66.143, R^2 = 0,993 were reached. The R^2 value obtained is very close to 1, which indicates how standard the study and the graph created are in variable measurement values and how successful the measurement is.

As an improvement for the linear range study, it can be considered to bring the bacterial loads of standard samples closer to the minimum and maximum values. A cut-off score of 242 load/ml or similar bacterial load values can be taken for the minimum value. As a result of the tests performed with gradually increased bacterial loads, the maximum value can be found after reaching the plateau point in the graph. When a broad table is made, it becomes clearer at what ranges the kit works more stably.

"An assay may be precise but inaccurate. The precision of an analytical procedure can be expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision can be determined by repeat testing of samples. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions" (Nick et al., 2013).

In order to demonstrate that the study was reproducible and that viral load measurement was stable and sensitive, 16 repeated tests were performed using a standard with a known viral load of 600 copies/ml and the results were recorded. (Table 4.8) Fluorescence intensity reading values obtained from the measurement of each sample were recorded. In order to find the viral loads corresponding to the recorded values, the equation y = 0,2933x – 66,143 obtained from the trend line created in the linear range graph was used. Viral load values were calculated.

Considering the results obtained, the average viral load of the 16 samples studied was found to be 599,38. Our average value is 99,89% similar to the reference standard sample value. The average value obtained is almost exactly the same as the standard example and shows how successful the study was. The standard deviation of the fluorescent intensity reading results obtained from 16 samples is 13,6. Since the standard deviation increases as the numerical values increase, the coefficient of variation value, which is the percentage expression of the ratio of the standard deviation to the average, was examined. This value was found to be 2,27%. (Table 4.9) Of course, the success of thisstudy also depends on the number of viral loads studied. Before the development of the kit and a possible commercialization step, the results can be compared and the consistencyrate can be checked by working with a larger standard sample group.

This result shows us that there is a 99.89% similarity between the reference system and the prototype system. At this stage, it is important whether the diagnostic kit will only look for negative positivity in commercial planning or whether it will be used during viral load measurement due to changes in the progression of the disease. The test captures positive samples according to the gold standard, regardless of viral load, which is also seen in the precision test and is successful in this regard.

However, if the disease course of the infected person is to be monitored on a periodic basis and measurement of the viral load number is important; Based on the data available, bacterial load measurement is quite successful in samples with 600 copies/ml, but as the bacterial load decreases, the measurement also decreases.

In order to see to what viral load range we can make standard measurements in these viral load calculations, viral loads should be studied with decreasing viral loads up to 242 copies/ml viral load, as in the future works in the linear range test, and then viral loads should be calculated using the linear graph equation and compared with the reference system, and a 95% success rate is achieved. The lowest viral load region measured with should be determined.

In order to measure these viral load measurements more precisely, studies should be conducted with a larger sample size and more repetitions. Small deviations due to the small

number of test groups lead to major changes due to the person or material/chemical used. A study may be developed in the future to further refine viral load measurement and to what range the golden ratio is measured.

Following these successful results, the steps that can be taken during the commercialization phase are; Repeated studies can be conducted with larger sample groups and different viral loads. In the short term, improvements can be made to the current diagnostic kit. *Chlamydia trachomatis* was chosen for the experiment because it infects millions of people every year. However, what is important here is not the infection being studied, but the development of a diagnostic kit that gives satisfactory results together with the end-point PCR device. Looking at the results, this has been achieved. Following this successful result, the next step could be to study the current method not only with this infection but also for other suitable diseases. Even diagnostic kits for more than one disease can be prepared depending on the status and progress of the studies. Studies on other diseases can be planned in the medium and long term.

REFERENCES

[1] Sexually transmitted disease surveillance 2015. Atlanta: *Department of Health and Human Services*, 2016.

[2] 2014 Sexually Transmitted Diseases Surveillance. Available online: (accessed on 15 June 2016).

[3] World Health Organization, *Global incidence and prevalence of selected curable sexually transmitted infections - 2008*. Geneva: World Health Organization, 2012. Accessed: Jan. 21, 2024.

[4] L. Z. A. Lugo *et al.*, "Cytokine profiling of samples positive for Chlamydia trachomatisand Human papillomavirus," *PLoS One*, vol. 18, no. 3, p. e0279390, Mar. 2023, doi: 10.1371/journal.pone.0279390.

[5] R. J. Bastidas, C. A. Elwell, J. N. Engel, and R. H. Valdivia, "Chlamydial intracellular survival strategies," *Cold Spring Harb Perspect Med*, vol. 3, no. 5, p. a010256, May 2013, doi: 10.1101/cshperspect.a010256.

[6] W. R. Gransden, "Topley and Wilson's Microbiology and Microbial Infections, 9th edition (CD-ROM)," *J Clin Pathol*, vol. 52, no. 3, pp. 237–238, Mar. 1999.

[7] C. M. Black, "Current methods of laboratory diagnosis of Chlamydia trachomatis infections," *Clin Microbiol Rev*, vol. 10, no. 1, pp. 160–184, Jan. 1997, doi: 10.1128/CMR.10.1.160.

[8] "Sexually transmitted infections (STIs): annual data tables," GOV.UK. Accessed: Jan. 21, 2024.

[9] W. M. Geisler, C. Wang, S. G. Morrison, C. M. Black, C. I. Bandea, and E. W. Hook, "The natural history of untreated Chlamydia trachomatis infection in the interval between screening and returning for treatment," *Sex Transm Dis*, vol. 35, no. 2, pp. 119–123, Feb. 2008, doi: 10.1097/OLQ.0b013e318151497d.

[10] K. Manavi, "A review on infection with Chlamydia trachomatis," *Best Pract Res Clin Obstet Gynaecol*, vol. 20, no. 6, pp. 941–951, Dec. 2006, doi:

10.1016/j.bpobgyn.2006.06.003.

[11] W. E. Stamm, “Chlamydia trachomatis infections: progress and problems,” *J Infect Dis*, vol. 179 Suppl 2, pp. S380-383, Mar. 1999, doi: 10.1086/513844.

[12] M. J. W. van de Laar and S. A. Morré, “Chlamydia: a major challenge for public health,” *Euro Surveill*, vol. 12, no. 10, pp. E1-2, Oct. 2007, doi: 10.2807/esm.12.10.00735- en.

[13] P. M. Mitchell, L. F. White, L. M. Rahimi, D. Jenkins, and E. M. Schechter-Perkins, “335 Predictors of Gonorrhea and Chlamydia in Emergency Department Patients,” *Annals of Emergency Medicine*, vol. 60, no. 4, p. S119, Oct. 2012, doi: 10.1016/j.annemergmed.2012.06.313.

[14] S. M. Rogers *et al.*, “Concordance of chlamydia trachomatis infections within sexual partnerships,” *Sex Transm Infect*, vol. 84, no. 1, pp. 23–28, Feb. 2008, doi: 10.1136/sti.2007.027029.

[15] A. R. Markos, “The concordance of Chlamydia trachomatis genital infection between sexual partners, in the era of nucleic acid testing,” *Sex Health*, vol. 2, no. 1, pp. 23–24, 2005, doi: 10.1071/sh04030.

[16] J. F. Peipert, “Clinical practice. Genital chlamydial infections,” *N Engl J Med*, vol. 349, no. 25, pp. 2424–2430, Dec. 2003, doi: 10.1056/NEJMcp030542.

[17] D. Scott Lamontagne *et al.*, “Incidence and reinfection rates of genital chlamydial infection among women aged 16-24 years attending general practice, family planning and genitourinary medicine clinics in England: a prospective cohort study by the Chlamydia Recall Study Advisory Group,” *Sex Transm Infect*, vol. 83, no. 4, pp. 292–303, Jul. 2007, doi: 10.1136/sti.2006.022053.

[18] J. C. M. Heijne, S. A. Herzog, C. L. Althaus, G. Tao, C. K. Kent, and N. Low, “Insights into the timing of repeated testing after treatment for Chlamydia trachomatis: data and modelling study,” *Sex Transm Infect*, vol. 89, no. 1, pp. 57–62, Feb. 2013, doi: 10.1136/sextrans-2011-050468.

[19] M. Malhotra, S. Sood, A. Mukherjee, S. Muralidhar, and M. Bala, “Genital

Chlamydia trachomatis: An update," *Indian J Med Res*, vol. 138, no. 3, pp. 303–316, Sep. 2013.

[20] J. Schachter, "Infection and Disease Epidemiology," In: R. S. Stephens, Ed., Chlamydia: *Intracellular Biology, Pathogenesis and Immunity, ASM Press*, Washington DC, 1999, pp. 139-169.

[21] H. R. Taylor, M. J. Burton, D. Haddad, S. West, and H. Wright, "Trachoma," *Lancet*, vol. 384, no. 9960, pp. 2142–2152, Dec. 2014, doi: 10.1016/S0140-6736(13)62182-0.

[22] Global Network for Neglected Tropical Diseases. Trachoma interactive fact sheet. Accessed February 6, 2011.

[23] N. C. Nwokolo, B. Dragovic, S. Patel, C. Y. W. Tong, G. Barker, and K. Radcliffe, "2015 UK national guideline for the management of infection with Chlamydia trachomatis," *Int J STD AIDS*, vol. 27, no. 4, pp. 251–267, Mar. 2016, doi: 10.1177/0956462415615443.

[24] E. Lanjouw, S. Ouburg, H. J. de Vries, A. Stary, K. Radcliffe, and M. Unemo, "2015 European guideline on the management of Chlamydia trachomatis infections," *Int J STD AIDS*, vol. 27, no. 5, pp. 333–348, Apr. 2016, doi: 10.1177/0956462415618837.

[25] J. A. White, "Manifestations and management of lymphogranuloma venereum," *Curr Opin Infect Dis*, vol. 22, no. 1, pp. 57–66, Feb. 2009, doi: 10.1097/QCO.0b013e328320a8ae.

[26] R. J. Bastidas, C. A. Elwell, J. N. Engel, and R. H. Valdivia, "Chlamydial intracellular survival strategies," *Cold Spring Harb Perspect Med*, vol. 3, no. 5, p. a010256, May 2013, doi: 10.1101/cshperspect.a010256.

[27] T. Darville and T. J. Hiltke, "Pathogenesis of genital tract disease due to Chlamydia trachomatis," *J Infect Dis*, vol. 201 Suppl 2, no. Suppl 2, pp. S114-125, Jun. 2010, doi: 10.1086/652397.

[28] A. Aghaizu *et al.*, "What is the cost of pelvic inflammatory disease and how much could be prevented by screening for chlamydia trachomatis? Cost analysis of the Prevention of Pelvic Infection (POPI) trial," *Sex Transm Infect*, vol. 87, no. 4, pp. 312– 317, Jun. 2011, doi: 10.1136/sti.2010.048694.

[29] A. Malik, S. Jain, S. Hakim, I. Shukla, and M. Rizvi, "Chlamydia trachomatis infection & female infertility," *Indian J Med Res*, vol. 123, no. 6, pp. 770–775, Jun. 2006.

[30] J. D. Carter and A. P. Hudson, "The evolving story of Chlamydia-induced reactive arthritis," *Curr Opin Rheumatol*, vol. 22, no. 4, pp. 424–430, Jul. 2010, doi: 10.1097/BOR.0b013e32833a43a2.

[31] D. Taylor-Robinson and A. Keat, "Observations on Chlamydia trachomatis and other microbes in reactive arthritis," *Int J STD AIDS*, vol. 26, no. 3, pp. 139–144, Mar. 2015, doi: 10.1177/0956462414533319.

[32] B. de Barbeyrac *et al.*, "Chlamydia trachomatis in subfertile couples undergoing an in vitro fertilization program: a prospective study," *Eur J Obstet Gynecol Reprod Biol*, vol. 129, no. 1, pp. 46–53, Nov. 2006, doi: 10.1016/j.ejogrb.2006.02.014.

[33] K. A. Cunningham and K. W. Beagley, "Male genital tract chlamydial infection: implications for pathology and infertility," *Biol Reprod*, vol. 79, no. 2, pp. 180–189, Aug. 2008, doi: 10.1095/biolreprod.108.067835.

[34] G. A. Greendale, S. T. Haas, K. Holbrook, B. Walsh, J. Schachter, and R. S. Phillips, "The relationship of Chlamydia trachomatis infection and male infertility," *Am J Public Health*, vol. 83, no. 7, pp. 996–1001, Jul. 1993, doi: 10.2105/ajph.83.7.996.

[35] P. Joki-Korpela, N. Sahrakorpi, M. Halttunen, H.-M. Surcel, J. Paavonen, and A. Tiitinen, "The role of Chlamydia trachomatis infection in male infertility," *Fertil Steril*, vol. 91, no. 4 Suppl, pp. 1448–1450, Apr. 2009, doi: 10.1016/j.fertnstert.2008.06.051.

[36] A. Nasr El-Din, H. Sorour, M. Fattouh, and M. Abu El-Hamd, “Evaluation of the role of Chlamydia trachomatis in primary male infertility,” *Int J Clin Pract*, vol. 75, no. 10, p. e14702, Oct. 2021, doi: 10.1111/ijcp.14702.

[37] “Tubal factor infertility, with special regard to chlamydial salpingitis - PubMed.” Accessed: Jan. 22, 2024. doi: 10.1097/00001432-200402000-00010

[38] R. B. Ness *et al.*, “Chlamydia antibodies, chlamydia heat shock protein, and adverse sequelae after pelvic inflammatory disease: the PID Evaluation and Clinical Health (PEACH) Study,” *Sex Transm Dis*, vol. 35, no. 2, pp. 129–135, Feb. 2008, doi: 10.1097/olq.0b013e3181557c25.

[38] R. B. Ness *et al.*, “Chlamydia antibodies, chlamydia heat shock protein, and adverse sequelae after pelvic inflammatory disease: the PID Evaluation and Clinical Health (PEACH) Study,” *Sex Transm Dis*, vol. 35, no. 2, pp. 129–135, Feb. 2008, doi: 10.1097/olq.0b013e3181557c25.

[39] D. T. Fleming and J. N. Wasserheit, “From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection,” *Sex Transm Infect*, vol. 75, no. 1, pp. 3–17, Feb. 1999, doi:10.1136/sti.75.1.3.

[40] M. Laga *et al.*, “Non-ulcerative sexually transmitted diseases as risk factors for HIV-1transmission in women: results from a cohort study,” *AIDS*, vol. 7, no. 1, pp. 95–102, Jan. 1993, doi: 10.1097/00002030-199301000-00015.

[41] R. Mishori, E. L. McClaskey, and V. J. WinklerPrins, “Chlamydia trachomatis infections: screening, diagnosis, and management,” *Am Fam Physician*, vol. 86, no. 12, pp.1127–1132, Dec. 2012.

[42] D. Hu, E. W. Hook, and S. J. Goldie, “Screening for Chlamydia trachomatis in women 15 to 29 years of age: a cost-effectiveness analysis,” *Ann Intern Med*, vol. 141, no. 7, pp. 501–513, Oct. 2004, doi: 10.7326/0003-4819-141-7-200410050-00006.

[43] C. A. Wisniewski *et al.*, “Optimal Method of Collection of First-Void Urine for Diagnosis of Chlamydia trachomatis Infection in Men,” *J Clin Microbiol*, vol. 46,

no. 4, pp. 1466–1469, Apr. 2008, doi: 10.1128/JCM.02241-07.

[44] J. Fang, C. Husman, L. DeSilva, R. Chang, and L. Peralta, "Evaluation of self-collected vaginal swab, first void urine, and endocervical swab specimens for the detection of Chlamydia trachomatis and Neisseria gonorrhoeae in adolescent females," *J Pediatr Adolesc Gynecol*, vol. 21, no. 6, pp. 355–360, Dec. 2008, doi: 10.1016/j.jpag.2008.03.010.

[45] H. C. Wiesenfeld, "Screening for Chlamydia trachomatis Infections in Women," *N Engl J Med*, vol. 376, no. 8, pp. 765–773, Feb. 2017, doi: 10.1056/NEJMcp1412935.

[46] L. H. Bachmann *et al.*, "Nucleic acid amplification tests for diagnosis of Neisseria gonorrhoeae and Chlamydia trachomatis rectal infections," *J Clin Microbiol*, vol. 48, no. 5,pp. 1827–1832, May 2010, doi: 10.1128/JCM.02398-09.

[47] R. L. Cook, S. L. Hutchison, L. Østergaard, R. S. Braithwaite, and R. B. Ness, "Systematic review: noninvasive testing for Chlamydia trachomatis and Neisseria gonorrhoeae," *Ann Intern Med*, vol. 142, no. 11, pp. 914–925, Jun. 2005, doi: 10.7326/0003-4819-142-11-200506070-00010.

[48] P. Horner *et al.*, "Enhanced Enzyme Immunoassay with Negative-Gray-Zone Testing Compared to a Single Nucleic Acid Amplification Technique for Community-Based Chlamydial Screening of Men," *J Clin Microbiol*, vol. 43, no. 5, pp. 2065–2069, May 2005, doi: 10.1128/JCM.43.5.2065-2069.2005.

[49] Jennifer B et al., "Chlamydia trachomatis: discovery of a new strain "*J New Zeal Med Assoc*. 2007;120:86–7.

[50] A. ZO, A. A.Z, T. H. Tang, and rm Hassan, "Comparison between RT-PCR and ELISA for the detection of HBV in blood donors.," *Biohealth Science Bulletin*, Jan. 2010.

[51] K. Sachse, E. Grossmann, C. Jäger, R. Diller, and H. Hotzel, "Detection of Chlamydia suis from clinical specimens: comparison of PCR, antigen ELISA, and culture," *Journal of Microbiological Methods*, vol. 54, no. 2, pp. 233–238, Aug. 2003, doi: 10.1016/S0167- 7012(03)00040-X.

[52] A. A. H. S. and K. A. S. Mohammed, “Comparison of immunological and molecular methods for the detection of Chlamydia trachomatis among infertile women in Basra, Iraq,” *Annals of Tropical Medicine and Public Health*, vol. 23, pp. 232–237, 2020, doi: 10.36295/ASRO.2020.232237.

[53] M. E. Craig, P. Robertson, N. J. Howard, M. Silink, and W. D. Rawlinson, “Diagnosis of Enterovirus Infection by Genus-Specific PCR and Enzyme-Linked Immunosorbent Assays,” *Journal of Clinical Microbiology*, vol. 41, no. 2, pp. 841–844, Feb. 2003, doi: 10.1128/jcm.41.2.841-844.2003.

[54] A. Bianchi *et al.*, “An evaluation of the polymerase chain reaction amplicor Chlamydia trachomatis in male urine and female urogenital specimens,” *Sex Transm Dis*, vol. 21, no. 4, pp. 196–200, 1994, doi: 10.1097/00007435-199407000-00003.

[55] W. H. Vogels, P. C. van Voorst Vader, and F. P. Schröder, “Chlamydia trachomatis infection in a high-risk population: comparison of polymerase chain reaction and cell culture for diagnosis and follow-up,” *J Clin Microbiol*, vol. 31, no. 5, pp. 1103–1107, May 1993, doi: 10.1128/jcm.31.5.1103-1107.1993.

[56] “Expected value,” *Wikipedia*. Jan. 23, 2024. Accessed: Feb. 04, 2024.

[57] S. A. Shaikh, “Measures derived from a 2 x 2 table for an accuracy of a diagnostic test,” *Journal of Biometrics & Biostatistics*, vol. 02, no. 05, 2011. doi:10.4172/2155-6180.1000128