



## A new RT-PCR assay for the revealing of Newcastle disease viruses by designing a pair of universal primers

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### Abstract

Newcastle disease viruses (NDVs) possess a single-stranded, non-segmented RNA and are classified into different strains based on their level of pathogenicity. Due to slight differences in the molecular makeup of the viral genome among these strains, employing a pair of primers for molecular diagnostics becomes essential. This study aims to establish a new approach to detect potential NDV infection by developing molecular methods. This was accomplished by performing a single RT-PCR reaction utilizing a newly designed universal primer set targeting a remarkably conserved area within the viral M gene. Various tools and resources were utilized to generate a set of primers, including the NCBI database and the Geneious Inspirational Software for Biologists. Ninety-four oropharyngeal swabs were collected from 66 chickens and 28 pigeons showing signs of ND. Viral RNA was extracted from samples, and M genes were amplified using conventional RT-PCR and real-time quantitative RT-PCR (qRT-PCR), followed by genomic sequencing and bioinformatics. The designed primers exhibited good quality, as indicated by a Delta G value of less than -5. This suggests that the primers are unlikely to cause any issues during the PCR process. Moreover, the amplification of the M gene was achieved successfully in both conventional RT-PCR and RT-PCR for approximately all collected samples from chickens and pigeons. This successful amplification was further verified through genomic sequencing and subsequent sequence analysis. These findings provide confirmation that the designed universal primers can effectively identify and quantify NDVs using PCR assay.

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### Introduction

Newcastle disease (ND) is a highly infectious disease of poultry presenting a global challenge due to its effect on acute respiratory conditions and its association with neurological symptoms, depression, and diarrhea. This disease is a major worldwide threat to the poultry industry (1,2). Disease severity is determined by the susceptibility of the host and the virulence of virus strains. These factors contribute significantly to the severity of the disease (3,4). ND is commonly transmitted through direct contact between infected birds or birds that act as carriers. The infected birds release the viruses through their feces, contaminating the

environment (5). The transmission of the disease can happen through direct contact with respiratory secretions and feces, contaminated water and food, and human clothing and equipment. NDVs can survive in the environment for several weeks, mainly in cold conditions (6). The disease is attributed to an avian paramyxovirus type 1 (APMV-1) belonging to the Paramyxoviridae family. NDV is an enveloped virus with an unsegmented RNA genome and a helical capsid (7,8). The genetic material of NDV consists of six genes responsible for encoding six different proteins. These proteins include the nucleoprotein, matrix protein, fusion protein, phosphoprotein, large polymerase protein, and hemagglutinin-neuraminidase (fusion) protein (9,10).

The stains of NDV can be classified into three groups based on their levels of pathogenicity: Lentogenic (non-virulent), Mesogenic (moderately virulent), and Velogenic (virulent). These groups are distinguished by variations in the amino acid numbers in the fusion protein, particularly at the hemagglutinin-neuraminidase cleavage site (11,12). In general, no or moderate clinical signs are associated with infection with a Lentogenic strain, while disease due to the Mesogenic strain may cause mortality to reach 25%. In comparison, infection with the Velogenic strain usually causes severe disease with a high mortality rate that may reach 100%. The high death rate after infection with this strain is explained that the virus can grow in several sites in the body, which usually causes infections in these sites and then the death of the animal (13-15). Since the NDV contains RNA, genetic mutations will likely occur in its genes (16-19). The reason for this is the occurrence and accumulation of mutations during replication cycles, caused by the deficiency of the correction machinery in RNA polymerases, which causes incorrect nucleotide insertion during the virus life cycle (20-22). Invariably, the three different virus strains are undoubtedly not identical in the RNA genome sequence of each strain (23,24). Besides observing the clinical signs of the disease, conducting laboratory tests is crucial to validate the presence of NDV infection and distinguish it from other diseases that display similar clinical signs, especially avian influenza and some types of bacterial diseases, particularly infection with *Mycoplasma* (25,26). Differential diagnostic techniques are necessary since multiple infections are likely to be detected. ELISA is widely used to detect viral antigens or antibodies (27-29). Molecular methods such as RT-PCR have become the best tool for NDV identification (30-32). However, gene-specific primers, besides genetic differences between virus strains, may be limited to diagnosing only a specific type of NDV (33). Therefore, the proposition of utilizing PCR-based protocols for virus detection holds numerous values in terms of rapid diagnosis, screening, and virus monitoring.

This study aims to establish a simple and reliable method for detecting NDVs by designing a novel pair of universal PCR primers. This was done by amplifying the viral M gene's highly conserved genetic fragment. The goal was accomplished by electronically designing and evaluating the quality of the primers. In addition, it was evaluated through laboratory work using conventional and quantitative RT-PCR, and the results were confirmed by genome sequencing and sequence analysis.

## Materials and methods

### Ethical approve

All work and analysis tests were conducted in strict accordance with the approved guidelines of the College of Veterinary Medicine, University of Basrah, Iraq. Approval issue number: 212 on December 10, 2018.

### Design of universal primers

The NCBI website was utilized to access the whole nucleotide sequence of the NDV M gene. Highly conserved nucleotide base sequences were extracted among several NDV isolates from diverse host species and then aligned to identify regions with extremely conserved sites utilizing Geneious Inspirational Software for Biologists ([www.geneious.com](http://www.geneious.com)). The set of universal primers was subsequently designed using the highly conserved region. Both forward and reverse primer sites were initially set by detecting nucleotide similarities of the selected virus strains (Figure 1).

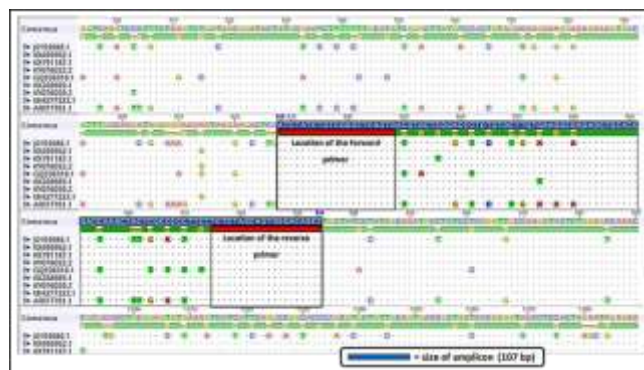


Figure 1: Identification of a highly conserved sequence of NDV M gene. The figure above shows the alignment of the nucleotide sequences between the different accession numbers of the partial M gene of multiple viral strains to designate conserved regions. This was subsequently used to design both primers. The primer's positions are marked in red, while the predicted size of the amplicon (107 bp) is indicated in blue.

To determine the designed primer quality, a software program (<https://eu.idtdna.com/calc/analyser>) was employed to check the capabilities of primer-dimer formation and cross-dimer formation. The primers were specifically designed to amplify a specific fragment of the M gene, resulting in a product size of 107 base pair (bp) (Table 1). They were prepared by Bioneer Company, Korea.

### Sample collection

Ninety-four oropharyngeal swabs were collected from local birds in different regions of Basrah. The samples were taken from 66 chickens and 28 pigeons displaying signs of ND. They were placed in a solution of phosphate-buffered saline (PBS) and glycerol, mixed in a 1:1 ratio. Afterward, they were carefully stored on ice and promptly sent to the College of Veterinary Medicine, University of Basrah laboratory. The samples underwent centrifugation at 1,000xg for 10 minutes. The resulting supernatant was carefully transferred to a newly labeled tube to prepare for RNA extraction.

Table 1: Universal primers (forward and reverse) for the M gene of NDV

Gene	Forward primer	Reverse primer	Amplicon size
M	NDV-F-828-848 (21 bp) ATCTATCTGTCGGGCTCAGTG	NDV-R-934-915(20 bp) GGCTGTCCCACTGCTAGAGA	107 bp

### Viral RNA extraction

Viral RNA extraction was achieved on all samples using QIAamp viral RNA purification kit (Qiagen), following the manufacturer's guidelines. The extracted RNA was quantified by using a NanoDrop spectrophotometer. The RNA was then stored in a freezer ( $-20^{\circ}\text{C}$ ) until use.

### Conventional reverse transcriptase polymerase chain reaction (RT-PCR) and gel electrophoresis

The Bioneer's One Step RT-PCR kit was utilized to amplify a specific region of the viral M gene. This kit provides a streamlined and efficient process for conducting reverse transcription and PCR amplification in a single reaction. In this study, a forward primer (5'-ATCTATCTGTCGGGCTCAGTG-3') and a reverse primer (5'-GGCTGTCCCACTGCTAGAGA-3') were specifically designed for amplifying a 107 bp fragment of interest. The amplification process encompassed cDNA synthesis and subsequent PCR amplification, conveniently performed within a single tube using the same system.

For cDNA synthesis, the initial starting material of viral RNA used was at a concentration of 100 ng/ $\mu\text{l}$ . To ensure the accuracy of the PCR runs, two negative control samples were prepared for each run. One negative control lacked an RNA template, while the other lacked primers. Positive control samples were also included, representing viral RNA extracted from a live virus (LaSota) vaccine. These control samples were a reference for comparison and quality guarantee during the PCR procedure. The RT-PCR conditions employed in this study were as follows: cDNA synthesis was carried out at  $45^{\circ}\text{C}$  for 30 minutes. Afterward, an initial denaturation step was performed at  $95^{\circ}\text{C}$  for 2 minutes. Subsequently, 35 cycles were performed, which involved denaturation at  $95^{\circ}\text{C}$  for 10 seconds, followed by annealing at  $58^{\circ}\text{C}$  for 20 seconds, and extension at  $72^{\circ}\text{C}$  for 30 seconds in each cycle. After completing the cycling steps, the reaction was prolonged at  $72^{\circ}\text{C}$  for a final elongation period of 5 minutes. The reaction mixture was cooled down at a temperature of  $4^{\circ}\text{C}$  for 5 minutes to terminate the procedure. These thermal cycling conditions were optimized in the lab to obtain the best PCR results.

The amplified PCR products were detected on an agarose gel made of 1.5% agarose in TBE buffer and stained with ethidium bromide. For each sample, 10  $\mu\text{l}$  of the PCR product was added to the wells during loading. To estimate the size of the gene, a 100 bp DNA ladder was used, which provides reference bands of known sizes. Finally, the gel was exposed to a UV trans-illuminator to enable the visualization of the DNA bands.

### DNA sequencing and sequence analysis

For DNA sequencing, 10 microliters of the PCR product, along with 17 picomoles per microliter of both primers (the forward and reverse) that were used to amplify the viral M gene, was presented to Macrogen Company in South Korea. The concentrations and volumes of the samples and primers were certified according to Macrogen's protocol. The acquired sequences were subjected to editing, assembly, and alignment procedures using the Geneious Inspirational Software for Biologists, an approved tool for sequence analysis and genetic manipulation.

### Real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

The amplification process was employed to target and amplify the partial region of the M gene from NDV using the One Step Bright Green qRT-PCR Low Rox kit (ABM). The amplification process involved the use of the designed forward and reverse primers (5'-ATCTATCTGTCGGGCTCAGTG-3' and 5'-GGCTGTCCCACTGCTAGAGA-3', respectively) to target and amplify a 107 bp fragment of the gene. This kit provided a convenient and reliable method for conducting quantitative real-time PCR based on SYBR-Green, enabling efficient amplification and detection of the desired gene region. This integrated system facilitated the execution of both cDNA synthesis and PCR amplification within a single tube, simplifying the experimental process. During the RT-PCR, cDNA synthesis occurred at  $42^{\circ}\text{C}$  for 15 minutes. Subsequently, an initial denaturation step was performed at  $95^{\circ}\text{C}$  for 5 minutes. The amplification phase comprised 40 cycles, with denaturation occurring at  $95^{\circ}\text{C}$  for 15 seconds, followed by annealing at  $58^{\circ}\text{C}$  for 60 seconds. These optimized conditions ensured efficient and accurate amplification of the target gene region.

## Results

### Universal primers efficiency

To confirm the efficiency of the designed universal primers, it is important to investigate the possibility of forming secondary structures, including primer dimer and cross dimer. These secondary structures can limit the performance of the primers. To assess these possibilities, a program available at <https://eu.idtdna.com/calc/analyzer> was utilized. This allowed the possible secondary structures to be evaluated, providing specific information on the suitability of the primers for successful amplification. By addressing

these concerns, the reliability and efficiency of the primers for subsequent experiments were optimized.

The analysis of the designed primers using the Delta G values indicated that the ratio of secondary structure formation was minimal. This suggests that the presence of secondary structures, such as primer dimer and cross dimer, would not significantly affect or delay the amplification of the target gene. The low ratio of secondary structure formation further supports the suitability and reliability of the primers for efficient gene amplification (Figure 2).

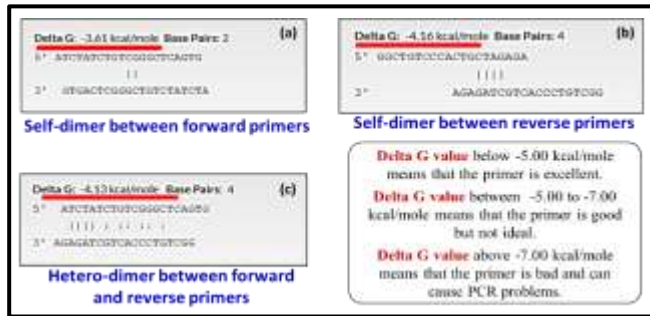


Figure 2: Assessment of the efficiency of the universal primers. The Delta G values for self-dimer and hetero-dimer formation of the primers were less than -5, indicating their excellent quality. This means the primers are unlikely to cause any issues during PCR, ensuring reliable and efficient amplification.

**Detection of NDV by conventional RT-PCR**

The PCR-amplified products were analyzed by separating them on an agarose gel stained with ethidium bromide. This allowed easy visualization of the DNA bands. Among the 94 samples examined, 92, specifically 64/66 from chickens and 28/28 from pigeons, exhibited visible bands on the agarose gel. Figure 3 illustrates the separation of the specific PCR products in the study.

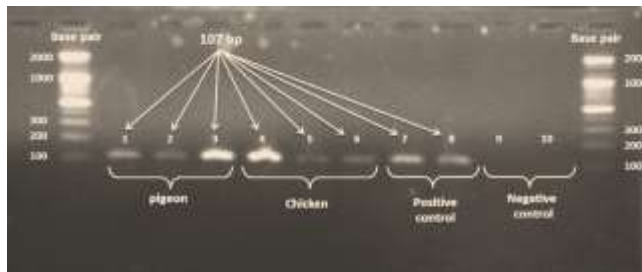


Figure 3: The visualized PCR product of the partial M gene of NDV on an agarose gel. The results revealed successful amplification of a 107 bp fragment from oropharyngeal specimens of pigeons (lanes 1-3) and chickens (lanes 4-6). The positive control samples (lanes 7 and 8) also showed the expected amplification. In contrast, the negative control samples (lanes 9 and 10) exhibited no amplification signal.

**Sequencing and sequence analysis**

The PCR products underwent DNA sequencing to determine the nucleotide sequence, and the resulting sequences were analyzed using Geneious Inspirational Software for Biologists. The edited sequences displayed clear and readable DNA traces without evidence of secondary structures in both the forward and reverse directions (Figure 4). The software combined two DNA sequence sides (forward and reverse) to create a continuous sequence of 107 nucleotides. This sequence corresponds to the region between the forward and reverse primers (Figure 5). The obtained DNA sequence was compared to sequences in the GenBank database through the alignment process. The alignment results provided confirmation of infection with the NDV (Figure 6).

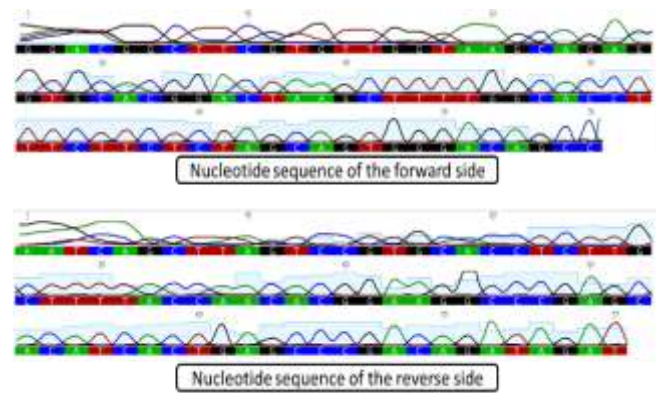


Figure 4: Forward and reverse nucleotide sequencing of partial M gene of NDV.

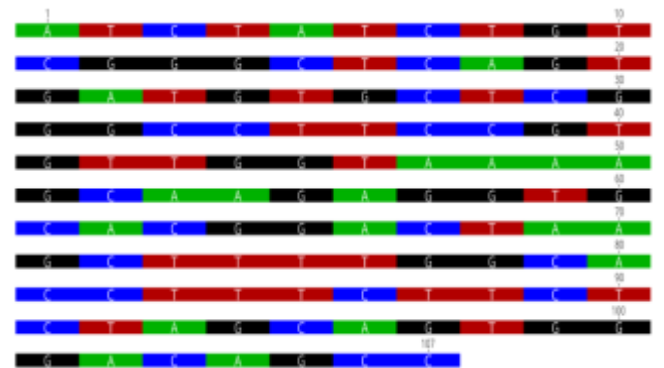


Figure 5: Final DNA sequence of partial matrix (M) gene (107 bp) of NDV. The forward priming site, indicated by the sequence "ATCTATCTGTGTCGGGCTCAGTG," is located from nucleotide position 1 to 21 in the assembled sequence. Similarly, the reverse priming site, "GGCTGTCCCACTGCTAGAGA," is found between nucleotide positions 107 to 88. The assembled sequence has a length of 107 base pairs, matching the size of the PCR product visualized on the agarose gel.

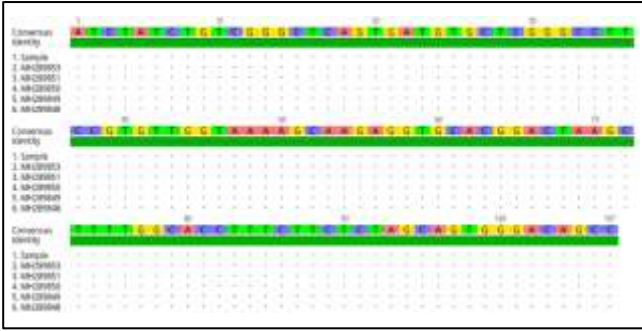


Figure 6: Displaying the alignment of the amplified viral gene fragment with other fragments available in the GenBank database. The analysis of the tested sample (1) and the available sequences of the NDV from online databases (2-6) showed complete sequence matching. This finding provides strong evidence confirming the presence of NDV infection in the birds.

#### Detection of NDV by Real-Time RT-PCR

Using Real-Time RT-PCR, the universal primers designed for the NDV successfully amplified the partial matrix (M) gene. The amplified DNA interacted with fluorescent molecules, producing a fluorescent signal, indicating the presence of the virus (Figure 7). The number of positive samples made with this assay was equivalent to that obtained in the conventional RT-PCR. These results provide confirmation that the designed primers are suitable for detecting suspected cases of NDV. This can be illustrated that the primer binding site is compatible with all subtypes of the viral genome, which was confirmed during the design phase. In addition, they can be utilized to determine the viral load in samples, indicating the disease's severity. These findings demonstrate the potential future applications of these primers in diagnosing and monitoring NDV infections.

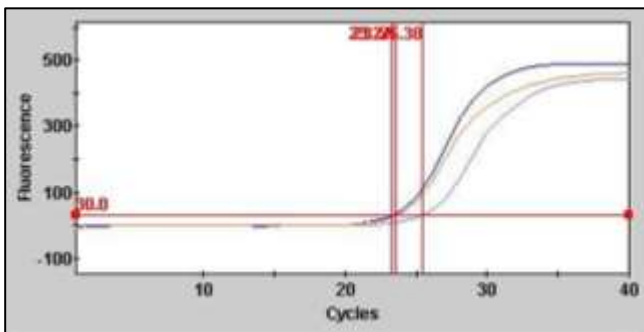


Figure 7: Illustrating the successful detection of the partial matrix (M) gene of NDV using Real-Time RT-PCR. Positive results were observed in the samples collected from chickens, pigeons, and the positive control (live virus vaccine). This confirms the presence of the NDV in these samples.

#### Discussion

NDV is categorized into different pathogenic strains based on fusion (F) protein variations. These strains exhibit different levels of virulence and can infect a wide diversity of avian species (34). The accuracy and reliability of PCR-based methods in detecting virus strains primarily depend on the selection of primer sequences (35). The efficiency of the assay regarding sensitivity and specificity is greatly influenced by the careful design and specificity of the primers chosen. RT-PCR assays that amplify the viral genome are considered a specific diagnostic method for confirming NDV infection (36). We have placed our focus on these techniques, as they have been established to be highly dependable when detecting and confirming the virus within a sample. Our importance lies in utilizing primers that guarantee positive results and yield specific outcomes within a specific timeframe.

The primer sets commonly used for PCR-based detection of NDV are generally suitable for identifying specific virus strains predominant in bird populations. The design of these primers has been accurately performed. It effectively targets conserved regions within the virus's genome, allowing for the detection and identification of various strains circulating in avian species (37). Using the same primer sets repeatedly can obstruct virus detection and increase the probability of obtaining false-negative results. Moreover, Undetected or unexpected viral strains can lead to false-negative consequences, where infection in the host will be unrecognized. It is crucial to periodically reassess and modify the primer sets to ensure accurate and reliable virus detection.

The results from the experiments conducted in this study confirmed that the PCR amplification was performed using this specific pair of primers from almost all the tested samples. The samples that did not yield a positive result in PCR analysis could potentially belong to birds infected with a disease resembling ND, such as influenza, infectious bronchitis, or some bacterial infections, such as infection with *Mycoplasma* (38,39). The positive results were further validated through sample sequencing and subsequent sequence analysis, which yielded highly accurate nucleotide sequence data, ensuring the reliability and efficacy of the designed primers. Genomic sequencing approaches have been extensively validated to confirm PCR results for any detection, regardless of the causative agent involved (40). The success in achieving high-quality sequencing results, devoid of secondary structure or tracer interference, can be attributed to the appropriate PCR conditions employed and the utilization of high-quality primers during the gene amplification.

The high efficiency of the primers used in this study, characterized by their minimal probability of forming secondary structures such as self-dimers and hetero-dimers, played a significant role in accomplishing optimal results.

Additionally, it should be noted that this pair of primers also showed excellent performance when used in quantitative (Real-Time) RT-PCR assay, which further confirms their efficacy and dependability. The optimization of any PCR primers is valuable for quantifying the virus and obtaining a better understanding of the disease's severity and viral load. It enables more accurate measurements, creating a clearer picture of the disease's impact regardless of the causative agent (41,42). Based on the positive results obtained in this study, employing the designed pair of primers for virus detection and quantification is strongly advised. These primers have demonstrated their effectiveness in conventional and Real-Time RT-PCR methodologies, making them highly reliable and suitable for accurate analysis.

Studies have extensively reviewed in vitro diagnostic methods for NDV, including serological tests, hemagglutination and hemagglutination inhibition (HI), ELISA, immunofluorescence, and neutralization tests. In addition, molecular methods such as RT-PCR play a big role in diagnosing the disease. Although serological diagnostics are widely used, molecular tests are gold standards because of their high sensitivity and specificity (37,43,44). In support of these scientific views, applying molecular approaches for diagnosis, virus screening, and virus monitoring is very important. Applying our developed method will have an effective role in this investigation by reducing potential errors. Therefore, the importance and benefits of our study are obtaining satisfactory results in a limited time away from repeating experiments with inappropriate outcomes.

## Conclusion

In conclusion, this innovative PCR-based assay allows for the diagnosis of any virus strain of NDV across various host species, all within single PCR tubes and within a short period. This represents a substantial speed improvement compared to previous PCR protocols used for disease diagnosis.

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## Conflict of interest

The authors affirm that they have no conflicts of interest to disclose.

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## اختبار جديد لتفاعل البلمرة المتسلسل العكسي للكشف عن فيروسات مرض نيوكاسل من خلال تصميم زوج من البادئات الشاملة

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### الخلاصة

تحتوي فيروسات مرض نيوكاسل على جينوم الحمض النووي الريبي غير المجزأ ويتم تصنيفها إلى سلالات مختلفة بناءً على مستوى قدرتها المرضية. بسبب الاختلافات الطفيفة في التركيب الجزيئي للجينوم الفيروسي بين هذه السلالات، يصبح من الضروري استخدام زوج من البادئات للتشخيص الجزيئي. الهدف من هذه الدراسة هو إنشاء نهج جديد للكشف عن عدوى فيروس مرض نيوكاسل المحتملة عن طريق تطوير الطرق الجزيئية. تم تحقيق ذلك عن طريق إجراء تفاعل البلمرة التقليدي

المصممة جودة جيدة، كما يتضح من قيمة دلتا ج أقل من ٥. يشير هذا إلى أنه من غير المحتمل أن تسبب البادئات أي مشاكل أثناء عملية تفاعل البوليميراز المتسلسل. علاوة على ذلك، تم تحقيق تضخيم الجين M بنجاح في كل من اختبار تفاعل البلمرة التقليدي للنسخ العكسي والكمي التقليدي والكمي لغالبية العينات التي تم جمعها من الدجاج والحمام. تم التحقق من هذا التضخيم الناجح من خلال إجراء التسلسل الجيني وتحليل التسلسل اللاحق. توفر هذه النتائج تأكيداً على أن البادئات العالمية المصممة يمكنها الكشف عن فيروس مرض نيوكاسل بشكل فعال باستخدام اختبار البلمرة المتسلسل.

للنسخ العكسي والكمي باستخدام مجموعة مصممة حديثاً من البادئات الشاملة التي تستهدف منطقة ثابتة للغاية من الجين الفيروسي م. لإنشاء مجموعة من البادئات، تم استخدام عدد من المواقع الإلكترونية بما في ذلك قاعدة بيانات المركز الوطني لمعلومات التكنولوجيا الحيوية والبرنامج الجيني الملهم لعلم الأحياء. تم جمع ٩٤ مسحة فموية بلعومية من ٦٦ دجاجة و ٢٨ حمامة ظهرت عليها علامات واضحة لمرض نيوكاسل. تم استخراج الحمض النووي الريبي الفيروسي من العينات وتم تضخيم الجين م باستخدام اختبار تفاعل البلمرة التقليدي للنسخ العكسي والكمي، متبوعاً بالتسلسل الجيني والمعلوماتية الحيوية. أظهرت البادئات