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Original Article

Development and performance evaluation of a multiplex fluorescent PCR method for the detection of fastidious pathogens causing respiratory infections in children

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Abstract

Introduction: This study was performed to develop a multiplex fluorescent PCR method for the concurrent detection of six fastidious bacteria associated with respiratory tract infections. These bacteria include *Streptococcus pneumoniae* (SPN), *Bordetella pertussis* (BP), *Neisseria meningitidis* (NM), *Legionella pneumophila* (LP), *Moraxella catarrhalis* (MC), and *Haemophilus influenzae* (HI).
Methodology: A multiplex fluorescent PCR test using SYBR Green as a DNA dye was developed and optimised. Clinical samples from 296 children with respiratory tract infections were then tested using the proposed method to assess its applicability for detecting the six pathogens.
Results: The SYBR Green-based multiplex fluorescent PCR method was successfully employed for the simultaneous identification of five pathogens through the analysis of melting curves and determination of the melting temperatures (*Tm*) values. However, the method exhibited limitations in distinguishing HI, necessitating separate detection using singleplex fluorescent PCR for this pathogen. In the methodological evaluation involving 296 clinical specimens, the multiplex fluorescent PCR successfully detected SPN, MC, NM, BP, and LP with sensitivities of 97.3%, 96.3%, 85.7%, 95.7%, and 80%, and areas under the ROC curve (AUC) of 0.977, 0.978, 0.927, 0.976 and 0.900, respectively.
Conclusions: Compared to conventional bacterial culture methods, the multiplex fluorescent PCR method with SYBR Green as the DNA dye is a sensitive and cost-effective approach for the simultaneous and rapid identification of five fastidious bacteria, making it a valuable alternative for clinical diagnostic laboratories.

Key words: Respiratory infections; child; fastidious bacterium; multiplex fluorescent PCR; SYBR green; melting curve.

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Introduction

Fastidious bacteria are a diverse group with strict growth environment and nutritional requirements [1]. They struggle to grow in typical environments, and *in vitro* cultivation usually requires the addition of special factors or nutritional components. Commonly found in the respiratory tract, these bacteria include *Streptococcus pneumoniae* (SPN), *Moraxella catarrhalis* (MC), *Legionella pneumophila* (LP), *Bordetella pertussis* (BP), *Neisseria meningitidis* (NM), and *Haemophilus influenzae* (HI). These bacteria are frequently pathogenic, particularly in children and immunocompromised individuals, causing severe invasive diseases, such as bacteraemia, meningitis, and pneumonia [2-4]. However, the cultivation of fastidious bacteria often yields false negative results because of their growth requirements, technical operations, transportation time, media, and culture plate selection. This drawback compromises the accurate diagnosis of respiratory tract infections, resulting in treatment delays for paediatric patients. Therefore, a rapid and accurate method for detecting fastidious bacteria in paediatric respiratory tract specimens should be developed [5].

In comparison with traditional bacterial culture methods, polymerase chain reaction (PCR) offers several advantages, including fast detection speed and high sensitivity [6-7]. Multiplex fluorescent PCR, using SYBR Green as a DNA dye, can be used as an efficient and cost-effective detection method. With melting curve analysis, this method can also be used to simultaneously detect multiple types of pathogens, such as bacteria [8], fungi [9], viruses [10], drug-resistant genes [11], and toxins genes [12]. Additionally, it eliminates the need for electrophoresis analysis of PCR products, which can be cumbersome and time-consuming. Therefore, in this study, we aimed to develop a multiplex fluorescent PCR method for the simultaneous detection of significant fastidious bacteria, including SPN, BP, NM, LP, HI, and MC, in paediatric respiratory tract specimens. We assessed the clinical diagnostic efficacy of this method and conducted its preliminary clinical applications.

Methodology

Strain and clinical samples sources

LP (ATCC33152) and BP (ATCC9340) were purchased from the Guangdong Institute of Microbiology (Guangdong Provincial Centre for Strain Preservation). HI (ATCC49247), MC (ATCC25238), NM (ATCC13090), and SPN (ATCC49619) were obtained from Kangtai Biological Technology Co., Ltd (Wenzhou, China). Clinical samples, including sputum, throat swabs, and bronchoalveolar lavage fluid (BALF), and clinical strains were obtained from paediatric patients with respiratory infections at Jinhua Central Hospital and Jinhua Maternal and Child Health Care Hospital between January 2022 and June 2023.

Main instruments

The main instruments used in this study were as follows: StepOne PLUS Real-Time PCR system and 2720 Thermal Cycler PCR instrument (Applied Biosystems Inc., USA); NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc., USA); DYY-6C electrophoresis system (Beijing Liuyi Instruments Factory, China); and Gel Doc XR + gel imaging system (Bio-Rad Laboratories, Inc., USA).

Main reagents

The main reagents used in this study were as follows: Genious 2× SYBR Green Fast qPCR Mix (low ROX premixed) purchased from ABclonal Biotech Co., Ltd. (USA) and Taq PCR Mix (2×, with blue dye), Ezup column-based bacterial genomic DNA extraction kit, SanPrep column-based PCR product purification kit, SanPrep column-based DNA gel recovery kit, and 4S Green nucleic acid dye procured from Shanghai Sangon Biotech Co., Ltd (China). LP commercial qPCR kit procured from Shanghai Zj Biotech Co., Ltd (China). BP commercial qPCR kit procured from Shengxiang Biotech Co., Ltd (China).

Primer design and synthesis

The 16S rRNA gene sequences (16S rDNA) encoding the target gene of each bacterial genus were searched in the NCBI gene database (https://www.ncbi.nlm.nih.gov). Primers were designed using the Primer Premier software to target the gene, and sequence specificity was confirmed via BLAST alignment (https://blast.ncbi.nlm.nih.gov). A total of six to seven pairs of primer sequences were designed for each pathogen (Supplementary Data 1), with synthesis conducted by Shanghai Sangon Biotech Co., Ltd. The primer sequences and relevant parameters for each bacterial genus are listed in Table 1.

Bacterial DNA extraction

Extraction of the DNA of standard strains: Bacterial suspensions of each standard strain were prepared with a final concentration of 1 × 10^5 CFU/mL. Bacterial DNA was extracted using an Ezup column-based bacterial genomic DNA extraction kit, which utilized an enhanced SDS lysis buffer and a high concentration of protein denaturing agent. This resulted in a significant improvement in lysis efficiency and a reduction in lysis time. The DNA extraction procedure was followed precisely according to the kit's instructions (Supplementary Data 2). The extracted DNA was aliquoted for storage at -20 °C.

Extraction of the DNA of strains from clinical specimens: a). Sputum samples: 0.5 mL of sputum sample was taken and mixed with an equal volume of a sputum liquefying agent (4% sodium hydroxide). After being incubated at room temperature (18–25 °C) for 15

Table 1. Characteristics of the primer sequences for the six fastidious bacteria.

Bacterial Species	Primer	Primer Sequence (5'-3')	GenBank Accession Number	Amplicon length	*Tm* value (°C)	References
SPN	SPN-F SPN-R	GGCAATGGACGGAAGT TGTAGCCCAGGTCATAAGG	NR_028665.1	858bp	86.22 ± 0.17	This study
BP	BP-F BP-R	GTTCGGAAAGAAAGATGTGA CACTCTAGCCCGGTAGTTAA	NR_025951.1	71bp	75.59 ± 0.10	This study
NM	NM-F NM-R	TCGGGAGCCGTAACAC CGGCTTGGCTACCCTCT	NR_104946.1	236bp	84.07 ± 0.15	This study
LP	LP-F LP-R	GGAGGAGGATTGATAGGTTA CGCTCGTTACGGGACT	NR_041742.1	664bp	85.14 ± 0.15	This study
HI	HI-F HI-R	TGGAGCGAATCTCATAAAG AATCATACCGTGGTAAACG	NR_044682.2	207bp	84.96 ± 0.12	This study
MC	MC-F MC-R	ACCAGTCGTTGGGTCTTT CAGGCGGTCTACTTATTGC	NR_028669.1	61bp	76.81 ± 0.14	This study

SPN: *Streptococcus pneumoniae*; BP: *Bordetella pertussis*; NM: *Neisseria meningitidis*; LP: *Legionella pneumophila*; HI: *Haemophilus influenzae*; MC: *Moraxella catarrhalis*; F: Forward primer; R: Reverse primer.

minutes and vortexed thrice, the mixture was centrifuged at 14,167 × g for 3 minutes to collect the precipitate for DNA extraction. b). BALF samples: BALF was centrifuged at 14,167 × g for 3 minutes to collect the precipitate for DNA extraction. c). Oropharyngeal swabs samples: The cotton swab head was cut off and placed in a 2 mL centrifuge tube. Then, 1 mL of sterile saline was added and vortexed thrice; the cotton swab was removed. The mixture was centrifuged at 14,167 × g for 3 minutes to collect the precipitate for DNA extraction.

Optimisation of the PCR amplification method

Preliminary experiments were conducted using singleplex PCR tests to verify the effectiveness of the primers for various bacteria. The primer concentration and annealing temperature for multiplex PCR were optimised. In the preliminary experiment, PCR amplification was performed using 10 μmol/L primers at volumes of 0.2 μL, 0.4 μL, 0.6 μL, and 0.8 μL each. Eventually, each pair of primers was optimized to a volume of 0.4 μL (0.2 μL for both forward and reverse primers at a concentration of 10 μmol/L). The annealing temperatures for PCR amplification were tested at 50 °C, 53 °C, 55 °C, 58 °C, 60 °C, 65 °C and finally preferred temperature of 55 °C. The amplification products were separated using a 1.5% agarose gel and observed under UV light. The PCR products were purified and subjected to sequencing in order to validate the presence and size of the amplicons. Additionally, the melting temperature (*Tm*) and melting curve of each amplicon were determined using StepOne Software v2.3 on the StepOnePlus Real Time PCR system.

Evaluation of the detection performance of multiplex fluorescent PCR

Sensitivity test: Gradient dilution (10^6–10^1 CFU/mL) of the six standard strains was performed to determine the lowest detection limit of multiplex fluorescent PCR.

Specificity test: The specificity of singleplex and multiple fluorescent PCR was evaluated using a variety of bacteria, including the standard strains HI (ATCC49247), MC (ATCC25238), NM (ATCC13090), and SPN (ATCC49619), LP (ATCC33152), BP (ATCC9340), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (ATCC 25923). Common pathogens isolated from clinical specimens were also included in this study, including *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Corynebacterium striatum*, *Staphylococcus haemolyticus*, *Enterococcus faecalis*, *Enterococcus faecium*, and *Staphylococcus epidermidis*. Additionally, the clinical strains closely related to the amplification target are included, such as *Haemophilus parainfluenzae*, *Haemophilus haemolyticus*, *Streptococcus pseudopneumoniae*, *Streptococcus salivarius*, *Streptococcus oris*, *Moraxella oslo*, and *Bordetella bronchiseptica*. The DNA of HI (ATCC49247), MC (ATCC25238), NM (ATCC13090), SPN (ATCC49619), LP (ATCC33152) and BP (ATCC9340) was used as positive controls, while ddH₂O was employed as a negative control, with the intention of evaluating the specificity of the newly established method.

Reproducibility test: The mean and standard deviation (SD) of *Tm* were calculated from five parallel experiments of three concentration gradients of each standard strain under identical PCR amplification conditions.

Methodological evaluation using clinical specimens

A total of 296 clinical specimens, including sputum, oropharyngeal swabs, and BALF, were tested to evaluate the diagnostic performance of both singleplex and multiplex fluorescent PCR detection methods. For the detection of HI, SPN, MC and NM, the traditional culture method and Targeted Next-Generation Sequencing (tNGS) method were employed as the ‘gold standard’ methods. If one of the two detection methods yielded a positive result, or if both methods were positive, the specimen was considered to be truly positive. Conversely, in the event that both detection methods were negative, the specimen was deemed to be truly negative. A similar approach was employed for the detection of BP and LP, with the commercial qPCR method and tNGS method serving as the ‘gold standard’ methods. The criteria for determining true positive and true negative results were consistent with those described above. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, and area under the receiver operating characteristic (ROC) curve of the singleplex and multiplex fluorescent PCR methods and the traditional culture method were calculated and compared using the test results of the ‘gold standard’ as a reference.

Commercial qPCR and tNGS

As the ‘gold standard’ methods for the detection of BP and LP, the commercial qPCR were performed according to the instructions, and tNGS was performed by Hangzhou Matrix Biotechnology Co., Ltd.

Commercial qPCR refers to the qPCR method in which the testing kit has obtained a registration certificate from the State Food and Drug Administration (SFDA) of China, thereby authorising its use in clinical practice. tNGS is a high-throughput sequencing technique that targets specific gene sequences using a large number of primers/probes [13]. It allows the use of a large number of primers/probes targeting specific gene sequences to amplify and capture a large number of target nucleic acid fragments extracted from the test sample by ultra multiplex PCR amplification/probe capture. The nucleic acid fragments of interest are subjected to high-throughput sequencing, followed by bioinformatics analysis of the resulting sequences [14]. This approach enables the highly sensitive and high-resolution identification of nucleic acids in the test sample [15].

Statistical analysis

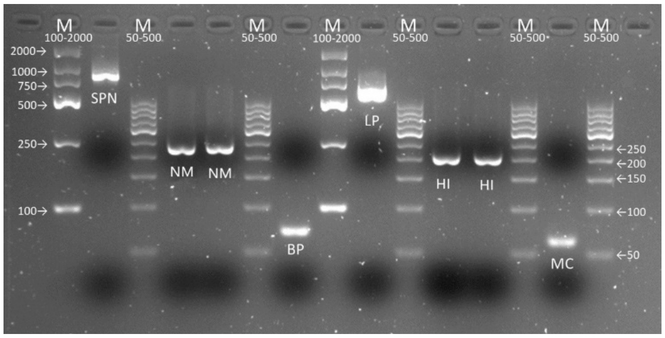
The diagnostic test indicators, including sensitivity, specificity, PPV, NPV, accuracy, and area under the ROC curve (AUC), were calculated using MedCalc 22.0 and SPSS 19.0 statistical software.

Results

Optimisation of the multiplex fluorescent PCR method and methodological establishment

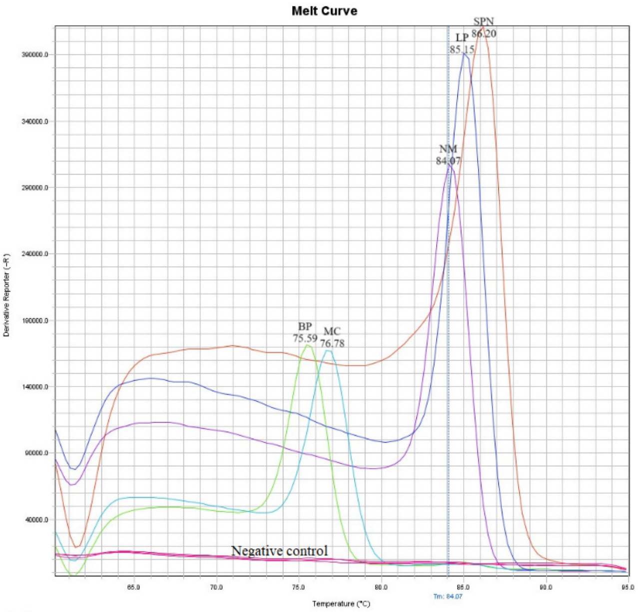
The optimised multiplex PCR method was set up with a total volume of 20 µL and composed of 10.0 µL of SYBR Green Fast qPCR Mix, 0.4 µL of each forward and reverse primer (10 µM), 2.0 µL (1ng for standard strain DNA) of the sample DNA template, and sterile double-distilled water to reach the final volume of 20.0 µL. The following amplification conditions were set for the multiplex PCR: initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 50 seconds, and fluorescent reading during extension at 72 °C.

Figure 1. Gel electrophoresis of the PCR amplification products for six fastidious bacteria.



SPN: *Streptococcus pneumoniae*; BP: *Bordetella pertussis*; NM: *Neisseria meningitidis*; LP: *Legionella pneumophila*; HI: *Haemophilus influenzae*; MC: *Moraxella catarrhalis*; M: Marker

Figure 2. The melting curve plots of the multiplex fluorescent PCR for the five bacteria.



SPN: *Streptococcus pneumoniae*; BP: *Bordetella pertussis*; NM: *Neisseria meningitidis*; LP: *Legionella pneumophila*; MC: *Moraxella catarrhalis*

Performance evaluation of multiplex and singleplex fluorescent PCR methods

Sensitivity Test: The multiplex fluorescent PCR and singleplex fluorescent PCR exhibited the capacity to detect bacterial concentrations as low as 10^2 CFU/mL. Consequently, the limit of detection (LoD) of the two methods was established at 10^2 CFU/mL.

Specificity Test: The multiplex fluorescent PCR method was successfully employed to identify the target genes of standard strains SPN, BP, NM, LP, and MC. In contrast, the remaining 20 strains and the double-distilled water control sample yielded negative results. Furthermore, the singleplex fluorescent PCR method successfully identified the target genes of the HI standard strains, while the results of the double distilled water control were negative. The electrophoresis result of the multiplex PCR for the five bacteria is shown in Figure 1. The melting curve plots of the multiplex fluorescent PCR for the five bacteria and the singleplex fluorescent PCR for HI, are shown in Figures 2 and 3, respectively.

Reproducibility Test: Each pathogen's *Tm* value demonstrates a high degree of stability, with fluctuations observed within a narrow range of 0.5 °C. The discrepancy in *Tm* values between different pathogens exceeds 1 °C, enabling unambiguous differentiation among the five pathogens (Table 1).

Evaluation of the clinical diagnosis performance of the '5+1' model

In 296 clinical samples, multiplex fluorescent PCR identified 72 cases of SPN, 26 cases of MC, 6 cases of NM, 22 cases of BP, and 4 cases of LP with sensitivities of 97.3%, 96.3%, 85.7%, 95.7%, and 80%, specificities of 98.2%, 99.3%, 99.7%, 99.6%, and 100%, and AUC of 0.977, 0.978, 0.927, 0.976, and 0.900, respectively (Figure 4). In comparison, bacterial culture identified 45 cases of SPN, 17 cases of MC, and 4 cases of NM, with sensitivities of 60.8%, 63.0%, and 57.1%, all showing 100% specificity. The AUC were 0.804, 0.815, and 0.786. Compared to the bacterial culture method, multiplex fluorescent PCR significantly increased the detection sensitivity of SPN, MC, and NM by 36.5%, 33.3%, and 28.6%, respectively. Additionally, the AUC were respectively increased by 0.173, 0.163, and 0.141.

Among the identical 296 clinical specimens, 101 instances of HI were identified using singleplex fluorescent PCR, demonstrating a sensitivity of 98.1% and specificity of 99.0%, with an area under the ROC curve of 0.983. In comparison, bacterial culture identified 83 cases of HI, with a sensitivity of 80.6% and specificity of 100%, resulting in an area under the ROC curve of 0.903. The sensitivity of singleplex fluorescent PCR improved by 17.5%, and the area

Figure 3. The melting curve plots of the singleplex fluorescent PCR for HI (*Haemophilus influenzae*).

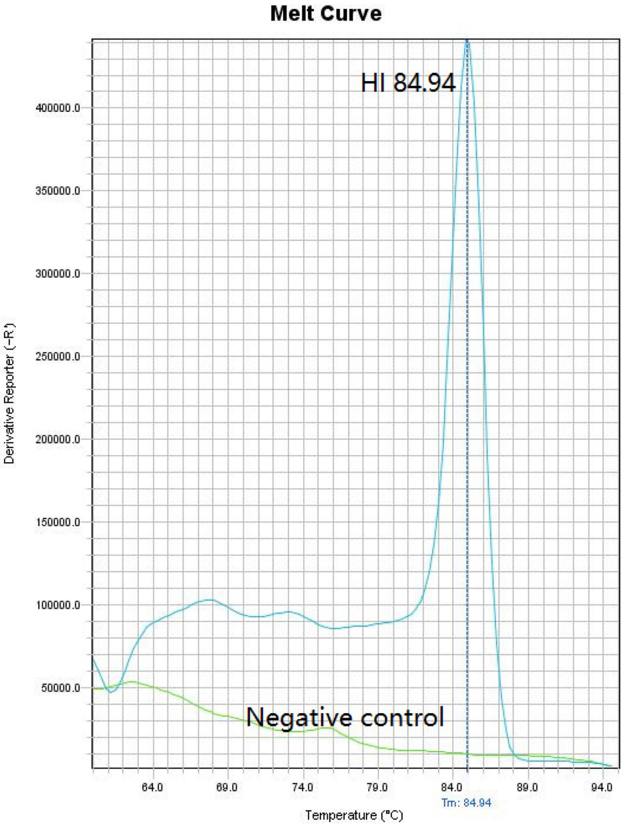
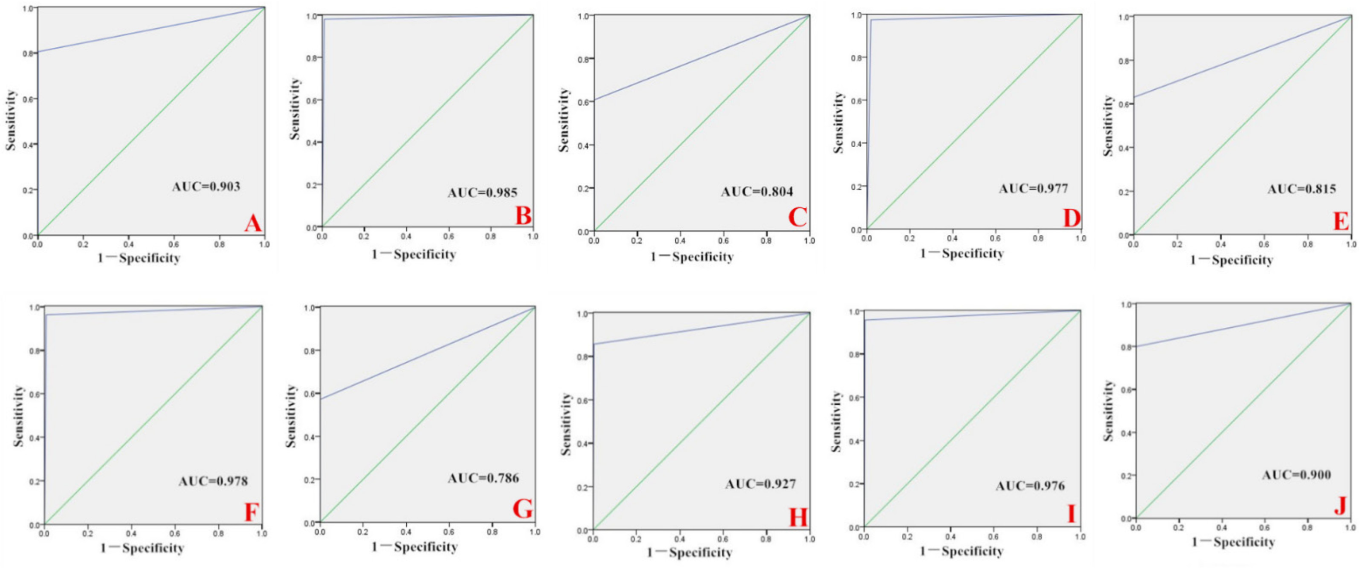


Figure 4. The AUC plots of different methods for the detection of fastidious respiratory bacteria: Figures A, C, E, and G show the AUC of HI, SPN, MC, and NM detected by culture method, respectively. Figure B shows the AUC of HI detected by the singleplex fluorescent method. Figures D, F, H, I, and J show the AUC of SPN, MC, NM, BP, and LP detected by the multiplex fluorescent PCR method.



AUC: Area under the curve; SPN: *Streptococcus pneumoniae*; BP: *Bordetella pertussis*; NM: *Neisseria meningitidis*; LP: *Legionella pneumophila*; HI: *Haemophilus influenzae*; MC: *Moraxella catarrhalis*

Table 2. Comparison of diagnostic efficacy of single or multiplex fluorescent PCR Vs culture for six fastidious pathogens.

Species	Reference test	Detection method	Diagnostic efficacy					
			Sensitivity	Specificity	AUC 95 CI%	PPV	NPV	Accuracy
HI	Culture and tNGS	Culture	80.6% (83/103)	100% (193/193)	0.903 (0.863-0.934)	100% (83/83)	90.61% (193/213)	93.24% (276/296)
		Single fluorescent PCR	98.1% (101/103)	99.0% (191/193)	0.985 (0.964-0.996)	98.1% (101/103)	99.0% (191/193)	98.6% (292/296)
SPN	Culture and tNGS	Culture	60.8% (45/74)	100% (222/222)	0.804 (0.754-0.848)	100% (45/45)	88.4% (222/251)	90.2% (267/296)
		Multiplex fluorescent PCR	97.3% (72/74)	98.2% (218/222)	0.977 (0.953-0.991)	94.7% (72/76)	99.1% (218/220)	98.0% (290/296)
MC	Culture and tNGS	Culture	63.0% (17/27)	100% (269/269)	0.815 (0.766-0.857)	100% (17/17)	96.4% (269/279)	96.6% (286/296)
		Multiplex fluorescent PCR	96.3% (26/27)	99.3% (267/269)	0.978 (0.954-0.991)	92.9% (26/28)	99.6% (267/268)	99.0% (293/296)
NM	Culture and tNGS	Culture	57.1% (4/7)	100% (289/289)	0.786 (0.735-0.831)	100% (4/4)	99.0% (289/292)	99.0% (293/296)
		Multiplex fluorescent PCR	85.7% (6/7)	99.7% (288/289)	0.927 (0.891-0.954)	85.7% (6/7)	99.7% (288/289)	99.3% (294/296)
BP	Commercial qPCR and tNGS	Multiplex fluorescent PCR	95.7% (22/23)	99.6% (272/273)	0.976 (0.952-0.990)	95.7% (22/23)	99.6% (272/273)	99.3% (294/296)
LP	Commercial qPCR and tNGS	Multiplex fluorescent PCR	80.0% (4/5)	100% (291/291)	0.900 (0.860-0.932)	100% (4/4)	99.7% (291/292)	99.7% (295/296)

SPN: *Streptococcus pneumoniae*; BP: *Bordetella pertussis*; NM: *Neisseria meningitidis*; LP: *Legionella pneumophila*; HI: *Haemophilus influenzae*; MC: *Moraxella catarrhalis*; AUC: Area Under Curve; 95% CI: 95% Confidence Interval; PPV: Positive Predictive Value; NPV: Negative Predictive Value.

under the ROC curve increased by 0.08 when compared to bacterial culture (Table 2).

Discussion

Fluorescent PCR detection methods can be divided into two main categories: fluorescent PCR based on fluorescent probes and fluorescent PCR based on double-stranded DNA-binding fluorescent dyes [16]. PCR dyes, such as SYBR Green, can be applied to any target sequence and are often cheaper than individually synthesised TaqMan probes. In a SYBR Green-based multiplex fluorescent PCR method, various pathogens can be identified by analysing the melting curves of the amplified PCR products [16].

In this study, a total of six to seven pairs of primer sequences were designed and testing for SPN, BP, NM, LP, HI, and MC. During the optimisation of the multiplex PCR method, only five pathogenic genes could be amplified, and HI could not be detected in the multiplex fluorescent PCR method. It may be related to the formation of primer dimers and mismatches between primers and templates when multiple primers are used in the same PCR system [17-18]. As such, separate detection using singleplex fluorescent PCR was required for HI. The LoD was determined to be 10^2 CFU/mL for both singleplex and multiplex fluorescent PCR assays. Specificity tests demonstrated that both multiplex and singleplex fluorescent PCR could amplify and detect the target genes of the standard strain, while other bacteria and double-distilled water controls produced negative results. Repeatability tests showed that the *Tm* value of each pathogen was stable, fluctuating within a narrow range of 0.5°C. The discrepancy in *Tm* values between the various pathogens exceeded 1°C, enabling the clear distinction of the five pathogens. A methodological assessment was conducted on 296 clinical specimens. The multiplex fluorescent PCR identified SPN, MC, NM, BP, and LP with sensitivities of 97.3%, 96.3%, 85.7%, 95.7%, and 80%, and AUC of 0.977, 0.978, 0.927, 0.976 and 0.900, respectively. In comparison to the bacterial culture method, the multiplex fluorescent PCR demonstrated a significant enhancement in the detection sensitivity of SPN, MC, and NM, with increases of 36.5%, 33.3%, and 28.6%, respectively. The singleplex fluorescent PCR revealed a sensitivity of 98.1% with an AUC of 0.985 for HI identification, representing a 17.5% increase over that of the bacterial culture method.

With this '5+1' model, a total of six fastidious bacteria could be detected. The clinical specimen testing results indicated that the positive rate of fastidious bacteria remarkably improved compared to traditional cultivation methods. However, the newly established multiplex fluorescent PCR method also has some limitations. Firstly, SYBR Green-based real-time PCR is its non-specific binding to any double-stranded DNA rather than solely targeting the desired DNA. Amplification curves alone cannot differentiate non-specific products such as primer dimers. Therefore, melting curve analysis is required to identify target genes [9,19,20]. The multiplex fluorescent PCR method developed in this study was effective in simultaneously detecting five of the six targeted fastidious pathogens. However, it could not reliably distinguish HI from the other pathogens in the multiplex format. Separate detection using singleplex fluorescent PCR was necessary for HI, indicating a limitation in the multiplex capability. Additionally, although the method demonstrated high sensitivity and specificity for most pathogens, the sensitivity for detecting NM (85.7%) and LP (80%) was lower compared to SPN, MC, and BP. The lower sensitivity for these two pathogens may be related to their low positivity rate in the local area and the insufficient number of positive clinical specimens. Therefore, more positive clinical specimens are needed to further evaluate the comprehensive detection capability of this method.

In conclusion, a multiplex fluorescent PCR method based on SYBR Green has been developed, capable of simultaneously amplifying the target genes of five respiratory pathogens (SPN, MC, NM, BP, and LP). Identification of these pathogens is achieved through the analysis of the *Tm* value and melting curve. Compared to conventional bacterial culture methods, this approach demonstrates improved efficiency and sensitivity in detecting fastidious bacteria, offering an attractive alternative for clinical diagnostic laboratories.

Ethical Approval

This research was approved by the Research Ethics Committee of Jinhua Municipal Central Hospital (No.2021-254).

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Authors' contributions

Xiaoyun Shan contributed to the study conception and design; Weixian Yang and Shihang Zhang performed the main part of data collection and identification of strains; Yijun Zhu and Jingchao Shi conducted the main experiments and drafted the manuscript, to which all authors provided critical comments.

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Conflict of interests

No conflict of interests is declared.

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Annex – Supplementary Items

EZ-10 Spin Column Bacterial Genomic DNA Mini-Preps Kit

CAT. NO.: B610423
Package: 50/100 Preps

Kit Contents

Components	50 Preps	100 Preps
Digestion Solution[A]	10 ml	20 ml
BD buffer	12 ml	24 ml
PW solution[E]	18 ml	36 ml
Wash Solution[B]	8 ml	16 ml
Elution Buffer [C]	5 ml	10 ml
Proteinase K[D]	10 mg	20 mg
UNIQ-10 Column	50	100
2.0-ml Collection Tube	50	100
Protocol	1	1

A. If Digestion Solution have precipitations, please heat to 55°C to dissolve precipitations

B. Before use, add 24 ml of 100% ethanol to 8ml Wash Solution for B610423(50 Preps), add 48 ml of 100% ethanol to 16ml Wash Solution for B610423 (100 Preps). If volume is not sufficient due to leaking during transportation, it is necessary to measure its volume and add ethanol accordingly.

C. Elution Buffer is 2.0 mmol/L Tris-HCl pH 8.5. Although Tris buffer pH 8.0 or water can be used, yield is 20% lower.

D. Before use, add 1 ml or 2 ml of sterilized water to the tube containing 10 mg or 20 mg of proteinase K, keep at -20 °C.

E. Before use, please add 12 ml isopropanol into PW solution for B610423 (50 Preps), add 24 ml isopropanol into PW solution for B610423(100 Preps)

Principle

This kit is designed for fast isolation of genomic DNA from bacterials. The kit contains a membrane embedded in a spin column for binding up to 10 µg of genomic DNA. According to G negative bactrials or G positive bactrial , after digestion solution and proteinase K treatment for G negative bactrials or lysozyme treatment and proteinase K digestion for G positive bactrial ,PW solution and wash buffer wash out Nucleotides, proteins, salts, and other impurities that do not bind to the Column, Purified genomic DNA can be applied in most molecular biology experiments including restriction digestion, PCR, Southern-blotting etc.

Applications

Genomic DNA extraction from different bacterials

Features

1. **Preparation of high quality genomic DNA from variable bactrials .**
2. **Rapid and economical. Entire procedure takes about 20 minutes (The time for preparation of samples is not included).**
3. **High yields**
4. **No phenol / chloroform extraction , no ethanol precipitation**

Procedure for Isolation of Genomic DNA from Variable bacterials

1. **G negative bacterials (Escherichia coli and so on)**

a. Transfer overnight culture (about $2x10^9$ cells) into centrifuge tube and centrifuge at 10000 *rpm* for 30 seconds, discard supernatant and keep pellet.

Note: Usually 1ml overnight culture have about $1\text{-}2x10^9$ bacterials.

b. Add 180 µl Digestion Solution into the pellets, resusspend them ,and then add 20 µl proteinase K (10mg/ml) , vortex , incubate at 56°C untill whole cells is lysed thoroughly. continue the process in step 3

Note: Usually incubation time for 30-60minutes, if you need RNA –free DNA, please add 20µl RNase (25mg/ml) and incubate at room temperature for 5 minutes .

2. **G positive bacterials (golden staphylococcal, orynebacteriadiphtheriae, streptococcal, and so on)**

a. Transfer overnight culture (about $2x10^9$ cells) into centrifuge tube and centrifuge at 10000 *rpm* for 30 seconds, discard supernatant and keep pellet.

b. Add 180 µl lysozyme solution（20mg/ml lysozyme，20mM Tris-HCl pH 8.0，2.5mM EDTA,1%Triton X-100）,and then incubate at 37°C for 30-60 minutes.

c. Add 20µl proteinase K ,vortex, incubate at 56°C until whole cells is lysed thoroughly. continue the process in step 3 **Note:** Usually incubation time for 30-60minutes, if you need RNA –free DNA , please add 20µl RNase A (25mg/ml) and incubate at room temperature for 5 minutes .

3. **Add 200µl BD buffer, and vortex thoroughly, then incubate at 70°C for ten minutes.**

Note: Precipitation maybe happen, but disappear after incubate at 70°C for ten minutes. if the solution still turbid, means bacterial still not be fully lysed and the number of genomics is decreased and contaminated.

4. **Add 200** µl **anhydrous alcohol, and vortex thoroughly.**

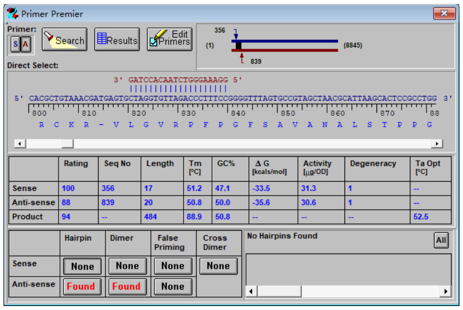
Note: Maybe some fibriform suspends after add anhydrous alcohol, but this can not affect the extraction of bacterial genomics .

5. **Put UNIQ-10 column into the collection tube, and then transfer the solution and whole fibriform suspends into UNIQ-10 column, keep at room temperature for 2 minutes ,centrifuge at 12000 *rpm* for 3 minutes, and discard the flow-through in the tube.**
6. **Put UNIQ-10 column into the collection tube again, add 500µl PW solution, and then centrifuge at 10000 *rpm* for 1 minutes, and discard the flow-through in the tube.**
7. **Put UNIQ-10 column into the collection tube again, add 500µl Wash solution, and then centrifuge at 10000 *rpm* for 1 minutes, and discard the flow-through in the tube.**
8. **Put UNIQ-10 column into the collection tube again, and then centrifuge at 10000 *rpm* for 2 minutes, thus residual alcohol is dispelled.**
9. **Transfer the column to a clean 1.5 ml microfuge tube. Add 50µl of Elution Buffer into the center part of the membrane in the column and incubate at 60°C for 5 minutes. Spin at 10000 rpm for 1 minutes. Store DNA in freezer at –20°C.**

Note: It is important to add the Elution Buffer into the center part. Pre-warm Elution Buffer at 65-80°C could increase elution efficiency.

The initial designed primer sequences

SPN-F1-GAATCTTCGGCAATGGA
SPN-R1-GGAAAGGGTCTAACACCTAG



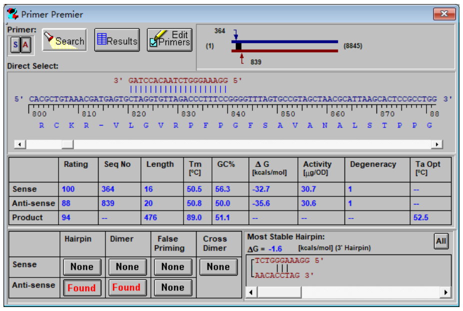
```
      3' GATCCACAATCTGGGAAAGG 5'
         ||||||||||||||||||||
5' CACGCTGTAAACGATGAGTGCTAGGTGTTAGACCCTTTCCGGGGTTTAGTGCCGTAGCTAACGCATTAAGCACTCCGCCTGG 3'
   800       810       820       830       840       850       860       870       88
    R  C  K  R  -  V  L  G  V  R  P  F  P  G  F  S  A  V  A  N  A  L  S  T  P  P  G
```

	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	356	17	51.2	47.1	-33.5	31.3	1	--
Anti-sense	88	839	20	50.8	50.0	-35.6	30.6	1	--
Product	94	--	484	88.9	50.8	--	--	--	52.5

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	Found	Found	None	

No Hairpins Found

SPN-F2-GGCAATGGACGGAAGT
SPN-R2-GGAAAGGGTCTAACACCTAG



```
      3' GATCCACAATCTGGGAAAGG 5'
         ||||||||||||||||||||
5' CACGCTGTAAACGATGAGTGCTAGGTGTTAGACCCTTTCCGGGGTTTAGTGCCGTAGCTAACGCATTAAGCACTCCGCCTGG 3'
   800       810       820       830       840       850       860       870       88
    R  C  K  R  -  V  L  G  V  R  P  F  P  G  F  S  A  V  A  N  A  L  S  T  P  P  G
```

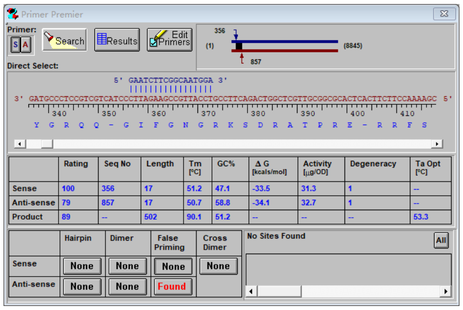
	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	364	16	50.5	56.3	-32.7	30.7	1	--
Anti-sense	88	839	20	50.8	50.0	-35.6	30.6	1	--
Product	94	--	476	89.0	51.1	--	--	--	52.5

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	Found	Found	None	

Most Stable Hairpin:
ΔG = -1.6 [kcals/mol] (3' Hairpin)

```
TCTGGGAAAGG 5'
     |||
AACACCTAG 3'
```

SPN-F3-GAATCTTCGGCAATGGA
SPN-R3-GCTACGGCACTAAACCC



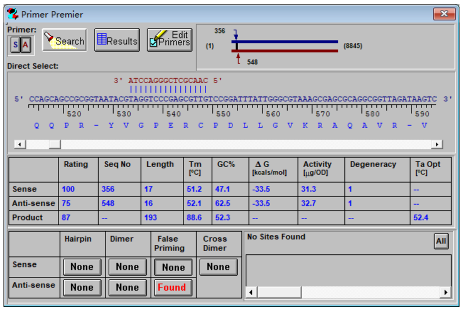
```
      5' GAATCTTCGGCAATGGA 3'
         |||||||||||||||||
3' GATGCCCTCCGTCGTCATCCCTTAGAAGCCGTTACCTGCCTTCAGACTGGCTCGTTGCGGCGCACTCACTTCTTCCAAAAGC 5'
       340       350       360       370       380       390       400       410
   Y  G  R  Q  Q  -  G  I  F  G  N  G  R  K  S  D  R  A  T  P  R  E  -  R  R  F  S
```

	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	356	17	51.2	47.1	-33.5	31.3	1	--
Anti-sense	79	857	17	50.7	58.8	-34.1	32.7	1	--
Product	89	--	502	90.1	51.2	--	--	--	53.3

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	None	None	Found	

No Sites Found

SPN-F4-GAATCTTCGGCAATGGA
SPN-R4-CAACGCTCGGGACCTA



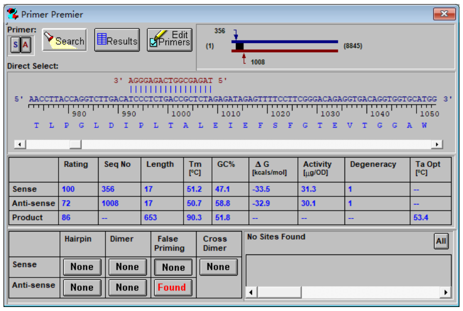
```
      3' ATCCAGGGCTCGCAAC 5'
         ||||||||||||||||
5' CCAGCAGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTAGATAAGTC 3'
          520       530       540       550       560       570       580       590
   Q  Q  P  R  -  Y  V  G  P  E  R  C  P  D  L  L  G  V  K  R  A  Q  A  V  R  -  V
```

	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	356	17	51.2	47.1	-33.5	31.3	1	--
Anti-sense	75	548	16	52.1	62.5	-33.5	32.7	1	--
Product	87	--	193	88.6	52.3	--	--	--	52.4

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	None	None	Found	

No Sites Found

SPN-F5-GAATCTTCGGCAATGGA
SPN-R5-TAGAGCGGTCAGAGGGA



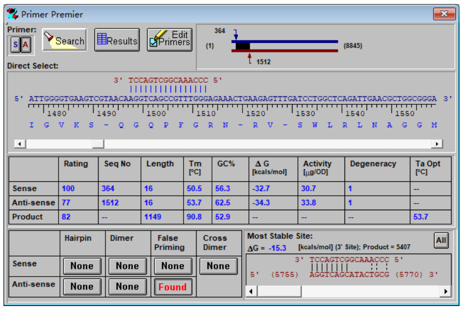
```
      3' AGGGAGACTGGCGAGAT 5'
         |||||||||||||||||
5' AACCTTACCAGGTCTTGACATCCCTCTGACCGCTCTAGAGATAGAGTTTTCCTTCGGGACAGAGGTGACAGGTGGTGCATGG 3'
          980       990       1000      1010      1020      1030      1040      1050
   T  L  P  G  L  D  I  P  L  T  A  L  E  I  E  F  S  F  G  T  E  V  T  G  G  A  W
```

	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	356	17	51.2	47.1	-33.5	31.3	1	--
Anti-sense	72	1008	17	50.7	58.8	-32.9	30.1	1	--
Product	86	--	653	90.3	51.8	--	--	--	53.4

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	None	None	Found	

No Sites Found

SPN-F6-GGCAATGGACGGAAGT
SPN-R6-CCCAAACGGCTGACCT



```
      3' TCCAGTCGGCAAACCC 5'
         ||||||||||||||||
5' ATTGGGGTGAAGTCGTAACAAGGTCAGCCGTTTGGGAGAAACTGAAGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGGA 3'
      1480      1490      1500      1510      1520      1530      1540      1550
   I  G  V  K  S  -  Q  G  Q  P  F  G  R  N  -  R  V  -  S  W  L  R  L  N  A  G  G  M
```

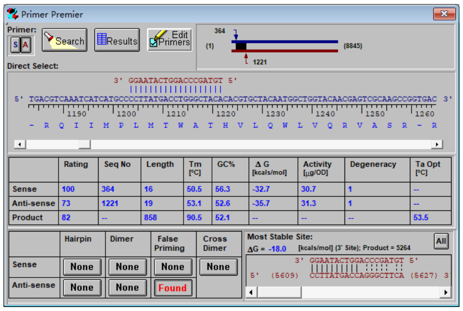
	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	364	16	50.5	56.3	-32.7	30.7	1	--
Anti-sense	77	1512	16	53.7	62.5	-34.3	33.8	1	--
Product	82	--	1149	90.8	52.9	--	--	--	53.7

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -15.3 [kcals/mol] (3' Site); Product = 5407

```
             3' TCCAGTCGGCAAACCC 5'
                ||||||||    :: :
5'  (5755)      AGGTCAGCATACTGCG (5770) 3'
```

SPN-F7-GGCAATGGACGGAAGT
SPN-R7-TGTAGCCCAGGTCATAAGG



```
      3' GGAATACTGGACCCGATGT 5'
         |||||||||||||||||||
5' TGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGCTGGTACAACGAGTCGCAAGCCGGTGAC 3'
          1190      1200      1210      1220      1230      1240      1250      1260
   -  R  Q  I  I  M  P  L  M  T  W  A  T  H  V  L  Q  W  L  V  Q  R  V  A  S  R  -  R
```

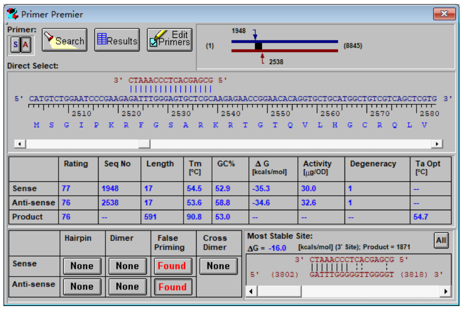
	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	100	364	16	50.5	56.3	-32.7	30.7	1	--
Anti-sense	73	1221	19	53.1	52.6	-35.7	31.3	1	--
Product	82	--	858	90.5	52.1	--	--	--	53.5

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -18.0 [kcals/mol] (3' Site); Product = 5264

```
             3' GGAATACTGGACCCGATGT 5'
                ||||||||||| ::::: ::
5'  (5609)      CCTTATGACCAGGGCTTCA (5627) 3
```

BP-F16- TGGCAGGAAAGAAACGG
BP-R16- GCGAGCACTCCCAAATC



```
      3' CTAAACCCTCACGAGCG 5'
         |||||||||||||||||
5' CATGTCTGGAATCCCGAAGAGATTTGGGAGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTG 3'
          2510      2520      2530      2540      2550      2560      2570      2580
   M  S  G  I  P  K  R  F  G  S  A  R  K  R  T  G  T  Q  V  L  H  G  C  R  Q  L  V
```

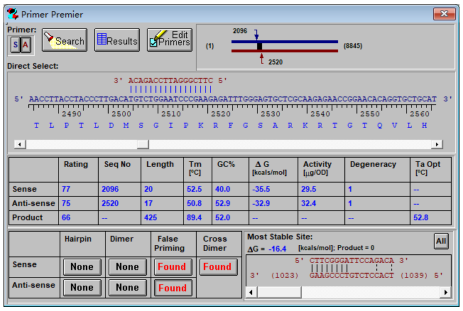
	Rating	Seq No	Length	Tm [ºC]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [ºC]
Sense	77	1948	17	54.5	52.9	-35.3	30.0	1	--
Anti-sense	76	2538	17	53.6	58.8	-34.6	32.6	1	--
Product	76	--	591	90.8	53.0	--	--	--	54.7

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -16.0 [kcals/mol] (3' Site); Product = 1871

```
             3' CTAAACCCTCACGAGCG 5'
                |||||||| ::     :
5'  (3802)      GATTTGGGGTTGGGGT (3818) 3'
```

BP-F17- GTTCGGAAAGAAAGATGTGA
BP-R17- CTTCGGGATTCCAGACA



```
                     3' ACAGACCTTAGGGCTTC 5'
                        |||||||||||||||||
5' AACCTTACCTACCCTTGACATGTCTGGAATCCCGAAGAGATTTGGGAGTGCTCGCAAGAGAACCGGAACACAGGTGCTGCAT 3'
        2490      2500      2510      2520      2530      2540      2550      2560
   T  L  P  T  L  D  M  S  G  I  P  K  R  F  G  S  A  R  K  R  T  G  T  Q  V  L  H
```

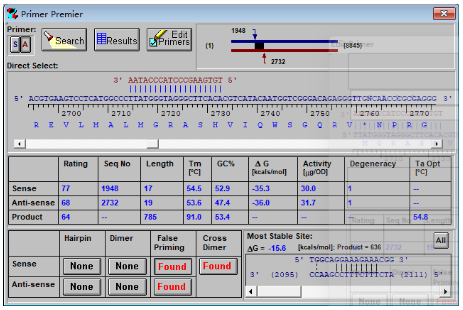
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	77	2096	20	52.5	40.0	-35.5	29.5	1	--
Anti-sense	75	2520	17	50.8	52.9	-32.9	32.4	1	--
Product	66	--	425	89.4	52.0	--	--	--	52.8

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -16.4 [kcals/mol]; Product = 0

```
           5' CTTCGGGATTCCAGACA 3'
              |||||||     :  :
3'  (1023)    GAAGCCCTGTCTCCACT (1039) 5'
```

BP-F18- TGGCAGGAAAGAAACGG
BP-R18- TGTGAAGCCCTACCCATAA



```
                     3' AATACCCATCCCGAAGTGT 5'
                        |||||||||||||||||||
5' ACGTGAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTCATACAATGGTCGGGACAGAGGGTTGNCAACCCGCGAGGG 3'
        2700      2710      2720      2730      2740      2750      2760      2770
   R  E  V  L  M  A  L  M  G  R  A  S  H  V  I  Q  W  S  G  Q  R  V  *  N  P  R  G
```

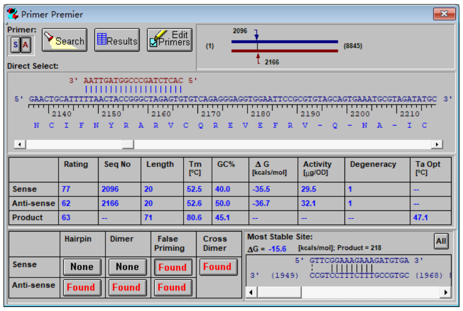
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	77	1948	17	54.5	52.9	-35.3	30.0	1	--
Anti-sense	68	2732	19	53.6	47.4	-36.0	31.7	1	--
Product	64	--	785	91.0	53.4	--	--	--	54.8

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -15.6 [kcals/mol]; Product = 636

```
           5' TGGCAGGAAAGAAACGG 3'
              :    ||||||||
3'  (2095)    CCAAGCCTTTCTTTCTA (2111) 5'
```

BP-F19- GTTCGGAAAGAAAGATGTGA
BP-R19- CACTCTAGCCCGGTAGTTAA



```
               3' AATTGATGGCCCGATCTCAC 5'
                  ||||||||||||||||||||
5' GAACTGCATTTTTAACTACCGGGCTAGAGTGTGTCAGAGGGAGGTGGAATTCCGCGTGTAGCAGTGAAATGCGTAGATATGC 3'
      2140      2150      2160      2170      2180      2190      2200      2210
   N  C  I  F  N  Y  R  A  R  V  C  Q  R  E  V  E  F  R  V  -  Q  -  N  A  -  I  C
```

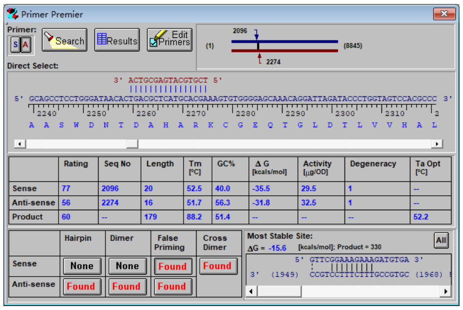
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	77	2096	20	52.5	40.0	-35.5	29.5	1	--
Anti-sense	62	2166	20	52.6	50.0	-36.7	32.1	1	--
Product	63	--	71	80.6	45.1	--	--	--	47.1

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	Found	Found	Found	

Most Stable Site:
ΔG = -15.6 [kcals/mol]; Product = 218

```
           5' GTTCGGAAAGAAAGATGTGA 3'
              :    ||||||||
3'  (1949)    CCGTCCTTTCTTTGCCGTGC (1968)
```

BP-F20- GTTCGGAAAGAAAGATGTGA
BP-R20- TCGTGCATGAGCGTCA



```
                     3' ACTGCGAGTACGTGCT 5'
                        ||||||||||||||||
5' GCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGTGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCC 3'
     2240      2250      2260      2270      2280      2290      2300      2310      2
   A  A  S  W  D  N  T  D  A  H  A  R  K  C  G  E  Q  T  G  L  D  T  L  V  V  H  A  L
```

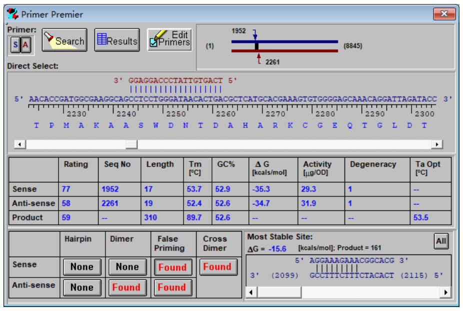
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	77	2096	20	52.5	40.0	-35.5	29.5	1	--
Anti-sense	56	2274	16	51.7	56.3	-31.8	32.5	1	--
Product	60	--	179	88.2	51.4	--	--	--	52.2

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	Found	Found	Found	

Most Stable Site:
ΔG = -15.6 [kcals/mol]; Product = 330

```
           5' GTTCGGAAAGAAAGATGTGA 3'
              :    ||||||||
3'  (1949)    CCGTCCTTTCTTTGCCGTGC (1968)
```

BP-F21-AGGAAAGAAACGGCACG
BP-R21-TCAGTGTTATCCCAGGAGG



```
                     3' GGAGGACCCTATTGTGACT 5'
                        |||||||||||||||||||
5' AACACCGATGGCGAAGGCAGCCTCCTGGGATAACACTGACGCTCATGCACGAAAGTGTGGGGAGCAAACAGGATTAGATACC 3'
         2230      2240      2250      2260      2270      2280      2290      2300
   T  P  M  A  K  A  A  S  W  D  N  T  D  A  H  A  R  K  C  G  E  Q  T  G  L  D  T
```

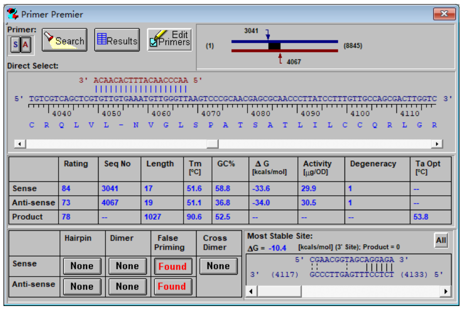
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	77	1952	17	53.7	52.9	-35.3	29.3	1	--
Anti-sense	58	2261	19	52.4	52.6	-34.7	31.9	1	--
Product	59	--	310	89.7	52.6	--	--	--	53.5

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	None	Found	Found	

Most Stable Site:
ΔG = -15.6 [kcals/mol]; Product = 161

```
           5' AGGAAAGAAACGGCACG 3'
                ||||||||
3'  (2099)    GCCTTTCTTTCTACACT (2115) 5'
```

Hi-F1-CGAACGGTAGCAGGAGA
Hi-R1-AACCCAACATTTCACAACA



```
            3' ACAACACTTTACAACCCAA 5'
               |||||||||||||||||||
5' TGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGACTTGGTC 3'
     4040      4050      4060      4070      4080      4090      4100      4110
   C  R  Q  L  V  L  -  N  V  G  L  S  P  A  T  S  A  T  L  I  L  C  C  Q  R  L  G  R
```

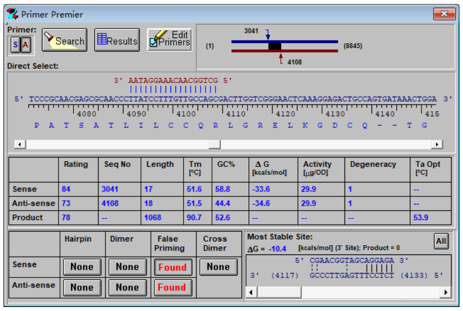
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	84	3041	17	51.6	58.8	-33.6	29.9	1	--
Anti-sense	73	4067	19	51.1	36.8	-34.0	30.5	1	--
Product	78	--	1027	90.6	52.5	--	--	--	53.8

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -10.4 [kcals/mol] (3' Site); Product = 0

```
           5' CGAACGGTAGCAGGAGA 3'
              ::    :     ||||||
3'  (4117)    GCCCTTGAGTTTCCTCT (4133) 5'
```

Hi-F2-CGAACGGTAGCAGGAGA
Hi-R2-GCTGGCAACAAAGGATAA



```
                     3' AATAGGAAACAACGGTCG 5'
                        ||||||||||||||||||
5' TCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGACTTGGTCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGA 3'
          4080      4090      4100      4110      4120      4130      4140      415
   P  A  T  S  A  T  L  I  L  C  C  Q  R  L  G  R  E  L  K  G  D  C  Q  -  -  T  G
```

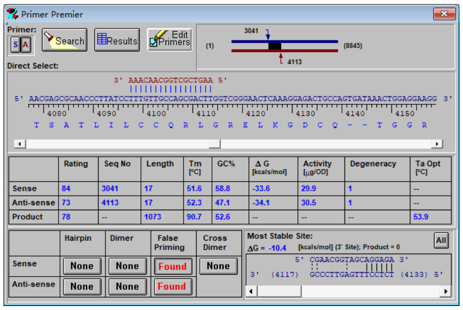
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	84	3041	17	51.6	58.8	-33.6	29.9	1	--
Anti-sense	73	4108	18	51.5	44.4	-34.6	29.9	1	--
Product	78	--	1068	90.7	52.6	--	--	--	53.9

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -10.4 [kcals/mol] (3' Site); Product = 0

```
           5' CGAACGGTAGCAGGAGA 3'
              ::    :     ||||||
3'  (4117)    GCCCTTGAGTTTCCTCT (4133) 5'
```

Hi-F3-CGAACGGTAGCAGGAGA
Hi-R3-AAGTCGCTGGCAACAAA



```
                     3' AAACAACGGTCGCTGAA 5'
                        |||||||||||||||||
5' AACGAGCGCAACCCTTATCCTTTGTTGCCAGCGACTTGGTCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGG 3'
      4080      4090      4100      4110      4120      4130      4140      4150
   T  S  A  T  L  I  L  C  C  Q  R  L  G  R  E  L  K  G  D  C  Q  -  -  T  G  G  R
```

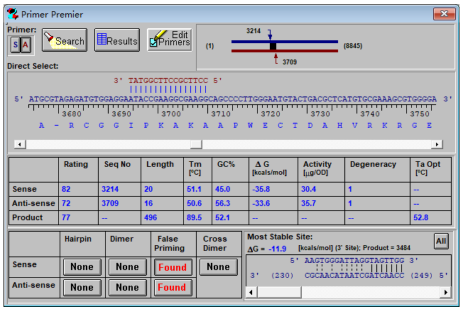
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [μg/OD]	Degeneracy	Ta Opt [°C]
Sense	84	3041	17	51.6	58.8	-33.6	29.9	1	--
Anti-sense	73	4113	17	52.3	47.1	-34.1	30.5	1	--
Product	78	--	1073	90.7	52.6	--	--	--	53.9

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -10.4 [kcals/mol] (3' Site); Product = 0

```
           5' CGAACGGTAGCAGGAGA 3'
              ::    :     ||||||
3'  (4117)    GCCCTTGAGTTTCCTCT (4133) 5'
```

Hi-F4-AAGTGGGATTAGGTAGTTGG
Hi-R4-CCTTCGCCTTCGGTAT



```
                3' TATGGCTTCCGCTTCC 5'
                   ||||||||||||||||
5' ATGCGTAGAGATGTGGAGGAATACCGAAGGCGAAGGCAGCCCCTTGGGAATGTACTGACGCTCATGTGCGAAAGCGTGGGGA 3'
         3680      3690      3700      3710      3720      3730      3740      3750
    A  -  R  C  G  G  I  P  K  A  K  A  A  P  W  E  C  T  D  A  H  V  R  K  R  G  E
```

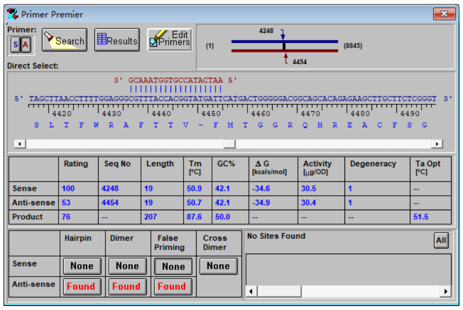
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	82	3214	20	51.1	45.0	-35.8	30.4	1	--
Anti-sense	72	3709	16	50.6	56.3	-33.6	35.7	1	--
Product	77	--	496	89.5	52.1	--	--	--	52.8

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -11.9 [kcals/mol] (3' Site); Product = 3484

```
           5' AAGTGGGATTAGGTAGTTGG 3'
              :: : :::::  |||||||
3'  (230)  CGCAACATAATCGATCAACC  (249)  5'
```

Hi-F5-TGGAGCGAATCTCATAAAG
Hi-R5-AATCATACCGTGGTAAACG



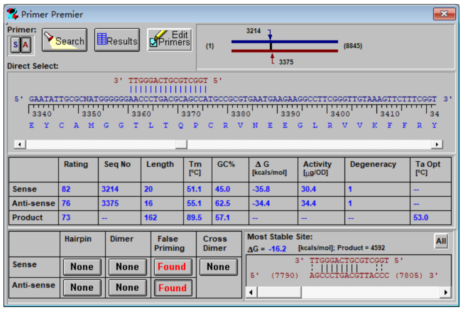
```
                3' GCAAATGGTGCCATACTAA 5'
                   |||||||||||||||||||
5' TAGCTTAACCTTTTGGAGGGCGTTTACCACGGTATGATTCATGACTGGGGGACGGCAGCACAGAGAAGCTTGCTTCTCGGGT 3'
        4420      4430      4440      4450      4460      4470      4480      4490
    S  L  T  F  W  R  A  F  T  T  V  -  F  M  T  G  G  R  Q  H  R  E  A  C  F  S  G
```

	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	100	4248	19	50.9	42.1	-34.6	30.5	1	--
Anti-sense	53	4454	19	50.7	42.1	-34.9	30.4	1	--
Product	76	--	207	87.6	50.0	--	--	--	51.5

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	None	None
Anti-sense	Found	Found	Found	

No Sites Found

Hi-F6-AAGTGGGATTAGGTAGTTGG
Hi-R6-TGGCTGCGTCAGGGTT



```
                3' TTGGGACTGCGTCGGT 5'
                   ||||||||||||||||
5' GAATATTGCGCNATGGGGGGAACCCTGACGCAGCCATGCCGCGTGAATGAAGAAGGCCTTCGGGTTGTAAAGTTCTTTCGGT 3'
   3340      3350      3360      3370      3380      3390      3400      3410      34
   E  Y  C  A  M  G  G  T  L  T  Q  P  C  R  V  N  E  E  G  L  R  V  V  K  F  F  R  Y
```

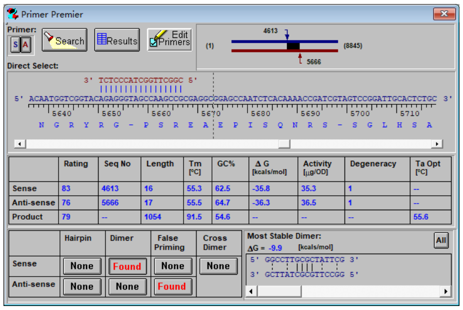
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	82	3214	20	51.1	45.0	-35.8	30.4	1	--
Anti-sense	76	3375	16	55.1	62.5	-34.4	34.4	1	--
Product	73	--	162	89.5	57.1	--	--	--	53.0

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -16.2 [kcals/mol]; Product = 4592

```
             3' TTGGGACTGCGTCGGT 5'
                :  ||||||||   ::
5'  (7790)  AGCCCTGACGTTACCC  (7805)  3'
```

Nm-F1-GGCCTTGCGCTATTCG
Nm-R1-CGGCTTGGCTACCCTCT



```
            3' TCTCCCATCGGTTCGGC 5'
               |||||||||||||||||
5' ACAATGGTCGGTACAGAGGGTAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGATTGCACTCTGC 3'
        5640      5650      5660      5670      5680      5690      5700      5710
    N  G  R  Y  R  G  -  P  S  R  E  A  E  P  I  S  Q  N  R  S  -  S  G  L  H  S  A
```

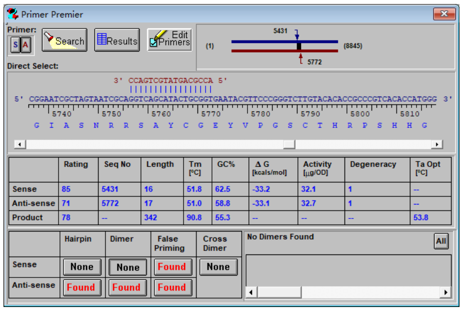
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	83	4613	16	55.3	62.5	-35.8	35.3	1	--
Anti-sense	76	5666	17	55.5	64.7	-36.3	36.5	1	--
Product	79	--	1054	91.5	54.6	--	--	--	55.6

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	None
Anti-sense	None	None	Found	

Most Stable Dimer:
ΔG = -9.9 [kcals/mol]

```
5' GGCCTTGCGCTATTCG 3'
     :  :  ||||  :  :
3' GCTTATCGCGTTCCGG 5'
```

Nm-F2-TCGGGAGCCGTAACAC
Nm-R2-ACCGCAGTATGCTGACC



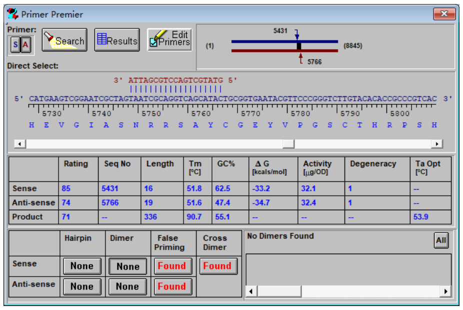
```
                3' CCAGTCGTATGACGCCA 5'
                   |||||||||||||||||
5' CGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGG 3'
       5740      5750      5760      5770      5780      5790      5800      5810
    G  I  A  S  N  R  R  S  A  Y  C  G  E  Y  V  P  G  S  C  T  H  R  P  S  H  H  G
```

	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	85	5431	16	51.8	62.5	-33.2	32.1	1	--
Anti-sense	71	5772	17	51.0	58.8	-33.1	32.7	1	--
Product	78	--	342	90.8	55.3	--	--	--	53.8

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	Found	Found	Found	

No Dimers Found

Nm-F3-TCGGGAGCCGTAACAC
Nm-R3-GTATGCTGACCTGCGATTA



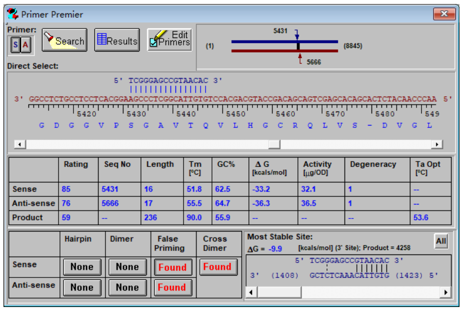
```
                3' ATTAGCGTCCAGTCGTATG 5'
                   |||||||||||||||||||
5' CATGAAGTCGGAATCGCTAGTAATCGCAGGTCAGCATACTGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCAC 3'
     5730      5740      5750      5760      5770      5780      5790      5800
   H  E  V  G  I  A  S  N  R  R  S  A  Y  C  G  E  Y  V  P  G  S  C  T  H  R  P  S  H
```

	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	85	5431	16	51.8	62.5	-33.2	32.1	1	--
Anti-sense	74	5766	19	51.6	47.4	-34.7	32.4	1	--
Product	71	--	336	90.7	55.1	--	--	--	53.9

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	None	None	Found	

No Dimers Found

Nm-F4-TCGGGAGCCGTAACAC
Nm-R4- CGGCTTGGCTACCCTCT



```
                5' TCGGGAGCCGTAACAC 3'
                   ||||||||||||||||
3' GGCCTCTGCCTCCTCACGGAAGCCCTCGGCATTGTGTCCACGACGTACCGACAGCAGTCGAGCACAGCACTCTACAACCCAA 5'
             5420      5430      5440      5450      5460      5470      5480      549
    G  D  G  G  V  P  S  G  A  V  T  Q  V  L  H  G  C  R  Q  L  V  S  -  D  V  G  L
```

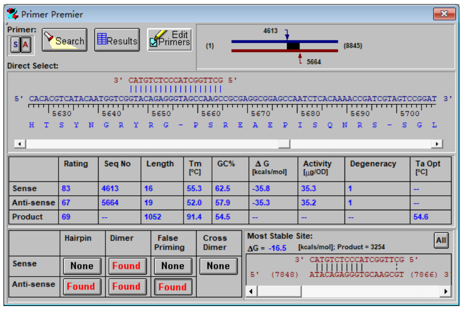
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	85	5431	16	51.8	62.5	-33.2	32.1	1	--
Anti-sense	76	5666	17	55.5	64.7	-36.3	36.5	1	--
Product	59	--	236	90.0	55.9	--	--	--	53.6

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -9.9 [kcals/mol] (3' Site); Product = 4258

```
             5' TCGGGAGCCGTAACAC 3'
                  :       |||||||
3'  (1408)  GCTCTCAAACATTGTG  (1423)  5'
```

Nm-F5-GGCCTTGCGCTATTCG
Nm-R5-GCTTGGCTACCCTCTGTAC



```
                3' CATGTCTCCCATCGGTTCG 5'
                   |||||||||||||||||||
5' CACACGTCATACAATGGTCGGTACAGAGGGTAGCCAAGCCGCGAGGCGGAGCCAATCTCACAAAACCGATCGTAGTCCGGAT 3'
       5630      5640      5650      5660      5670      5680      5690      5700
   H  T  S  Y  N  G  R  Y  R  G  -  P  S  R  E  A  E  P  I  S  Q  N  R  S  -  S  G  L
```

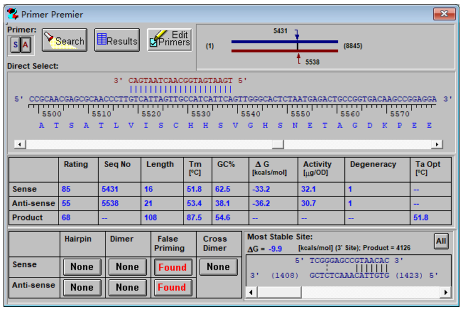
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	83	4613	16	55.3	62.5	-35.8	35.3	1	--
Anti-sense	67	5664	19	52.0	57.9	-35.3	35.2	1	--
Product	69	--	1052	91.4	54.5	--	--	--	54.6

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	None
Anti-sense	Found	Found	Found	

Most Stable Site:
ΔG = -16.5 [kcals/mol]; Product = 3254

```
             3' CATGTCTCCCATCGGTTCG 5'
                 ||||||||||     :
5'  (7848)  ATACAGAGGGTGCAAGCGT  (7866)  3
```

Nm-F6- TCGGGAGCCGTAACAC
Nm-R6- TGAATGATGGCAACTAATGAC



```
                 3' CAGTAATCAACGGTAGTAAGT 5'
                    |||||||||||||||||||||
5' CCGCAACGAGCGCAACCCTTGTCATTAGTTGCCATCATTCAGTTGGGCACTCTAATGAGACTGCCGGTGACAAGCCGGAGGA 3'
     5500      5510      5520      5530      5540      5550      5560      5570
    A  T  S  A  T  L  V  I  S  C  H  H  S  V  G  H  S  N  E  T  A  G  D  K  P  E  E
```

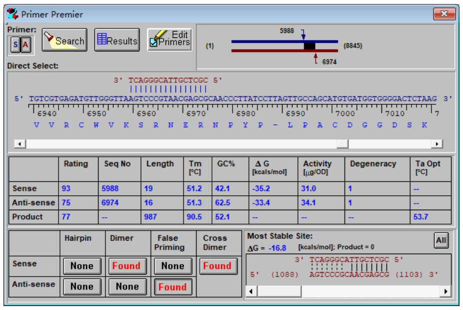
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	85	5431	16	51.8	62.5	-33.2	32.1	1	--
Anti-sense	55	5538	21	53.4	38.1	-36.2	30.7	1	--
Product	68	--	108	87.5	54.6	--	--	--	51.8

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -9.9 [kcals/mol] (3' Site); Product = 4126

```
          5' TCGGGAGCCGTAACAC 3'
               :       |||||||
3'  (1408)  GCTCTCAAACATTGTG (1423) 5'
```

LP-F1- CGTAGGAATATGCCTTGAA
LP-R1- CGCTCGTTACGGGACT



```
                 3' TCAGGGCATTGCTCGC 5'
                    ||||||||||||||||
5' TGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTATCCTTAGTTGCCAGCATGTGATGGTGGGGACTCTAAG 3'
   6940      6950      6960      6970      6980      6990      7000      7010      7
  V  V  R  C  W  V  K  S  R  N  E  R  N  P  Y  P  -  L  P  A  C  D  G  G  D  S  K
```

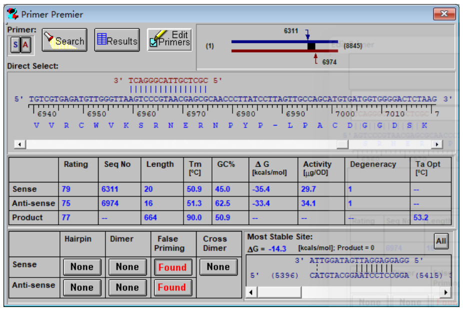
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	93	5988	19	51.2	42.1	-35.2	31.0	1	--
Anti-sense	75	6974	16	51.3	62.5	-33.4	34.1	1	--
Product	77	--	987	90.5	52.1	--	--	--	53.7

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -16.8 [kcals/mol]; Product = 0

```
          3' TCAGGGCATTGCTCGC 5'
             :::::::  ||||||||
5'  (1088)  AGTCCCGCAACGAGCG (1103) 3'
```

LP-F2- GGAGGAGGATTGATAGGTTA
LP-R2- CGCTCGTTACGGGACT



```
                 3' TCAGGGCATTGCTCGC 5'
                    ||||||||||||||||
5' TGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTATCCTTAGTTGCCAGCATGTGATGGTGGGGACTCTAAG 3'
   6940      6950      6960      6970      6980      6990      7000      7010      7
  V  V  R  C  W  V  K  S  R  N  E  R  N  P  Y  P  -  L  P  A  C  D  G  G  D  S  K
```

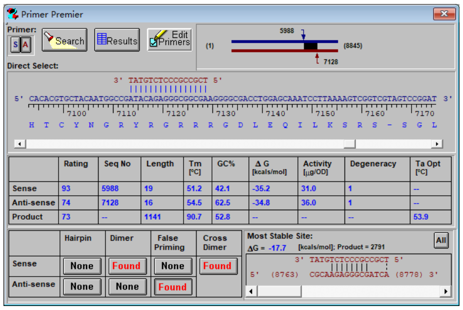
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	79	6311	20	50.9	45.0	-35.4	29.7	1	--
Anti-sense	75	6974	16	51.3	62.5	-33.4	34.1	1	--
Product	77	--	664	90.0	50.9	--	--	--	53.2

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -14.3 [kcals/mol]; Product = 0

```
          3' ATTGGATAGTTAGGAGGAGG 5'
               :      |||||||
5'  (5396)  CATGTACGGAATCCTCCGGA (5415) 3'
```

LP-F3- CGTAGGAATATGCCTTGAA
LP-R3- TCGCCGCCCTCTGTAT



```
                 3' TATGTCTCCCGCCGCT 5'
                    ||||||||||||||||
5' CACACGTGCTACAATGGCCGATACAGAGGGCGGCGAAGGGGCGACCTGGAGCAAATCCTTAAAAGTCGGTCGTAGTCCGGAT 3'
        7100      7110      7120      7130      7140      7150      7160      7170
  H  T  C  Y  N  G  R  Y  R  G  R  R  R  G  D  L  E  Q  I  L  K  S  R  S  -  S  G  L
```

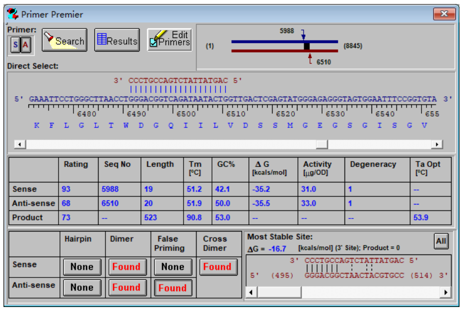
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	93	5988	19	51.2	42.1	-35.2	31.0	1	--
Anti-sense	74	7128	16	54.5	62.5	-34.8	36.0	1	--
Product	73	--	1141	90.7	52.8	--	--	--	53.9

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -17.7 [kcals/mol]; Product = 2791

```
          3' TATGTCTCCCGCCGCT 5'
                 ||||||||    :
5'  (8763)  CGCAAGAGGGCGATCA (8778) 3'
```

LP-F4- CGTAGGAATATGCCTTGAA
LP-R4- CAGTATTATCTGACCGTCCC



```
                 3' CCCTGCCAGTCTATTATGAC 5'
                    ||||||||||||||||||||
5' GAAATTCCTGGGCTTAACCTGGGACGGTCAGATAATACTGGTTGACTCGAGTATGGGAGAGGGTAGTGGAATTTCCGGTGTA 3'
        6480      6490      6500      6510      6520      6530      6540      655
  K  F  L  G  L  T  W  D  G  Q  I  I  L  V  D  S  S  M  G  E  G  S  G  I  S  G  V
```

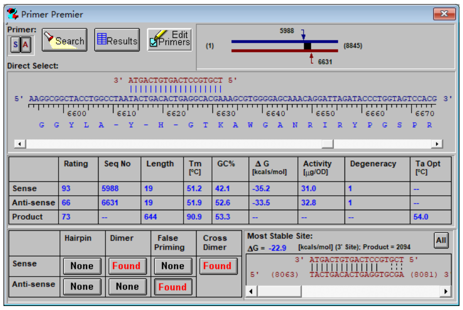
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	93	5988	19	51.2	42.1	-35.2	31.0	1	--
Anti-sense	68	6510	20	51.9	50.0	-35.5	33.0	1	--
Product	73	--	523	90.8	53.0	--	--	--	53.9

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	Found
Anti-sense	None	Found	Found	

Most Stable Site:
ΔG = -16.7 [kcals/mol] (3' Site); Product = 0

```
         3' CCCTGCCAGTCTATTATGAC 5'
            |||||||    :   ::
5'  (495)  GGGACGGCTAACTACGTGCC (514) 3'
```

LP-F5- CGTAGGAATATGCCTTGAA
LP-R5- TCGTGCCTCAGTGTCAGTA



```
                 3' ATGACTGTGACTCCGTGCT 5'
                    |||||||||||||||||||
5' AAGGCGGCTACCTGGCCTAATACTGACACTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG 3'
        6600      6610      6620      6630      6640      6650      6660      6670
    G  G  Y  L  A  -  Y  -  H  -  G  T  K  A  W  G  A  N  R  I  R  Y  P  G  S  P  R
```

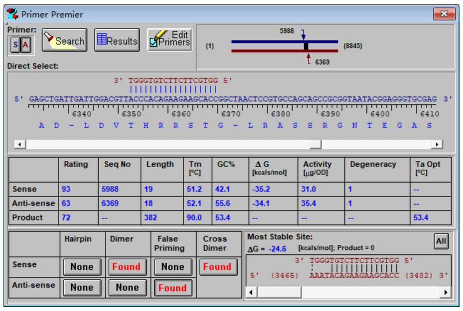
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	93	5988	19	51.2	42.1	-35.2	31.0	1	--
Anti-sense	66	6631	19	51.9	52.6	-33.5	32.8	1	--
Product	73	--	644	90.9	53.3	--	--	--	54.0

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -22.9 [kcals/mol] (3' Site); Product = 2094

```
          3' ATGACTGTGACTCCGTGCT 5'
             |||||||||||||   :::
5'  (8063)  TACTGACACTGAGGTGCGA (8081) 3
```

LP-F6- CGTAGGAATATGCCTTGAA
LP-R6- GGTGCTTCTTCTGTGGGT



```
                 3' TGGGTGTCTTCTTCGTGG 5'
                    ||||||||||||||||||
5' GAGCTGATTGATTGGACGTTACCCACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAG 3'
        6340      6350      6360      6370      6380      6390      6400      6410
    A  D  -  L  D  V  T  H  R  R  S  T  G  -  L  R  A  S  S  R  G  N  T  E  G  A  S
```

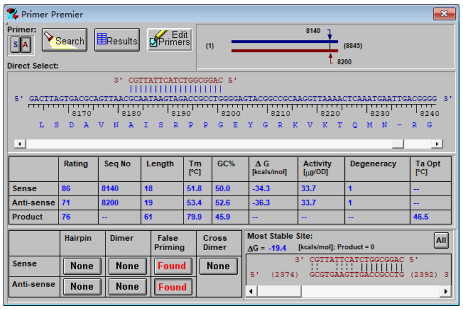
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	93	5988	19	51.2	42.1	-35.2	31.0	1	--
Anti-sense	63	6369	18	52.1	55.6	-34.1	35.4	1	--
Product	72	--	382	90.0	53.4	--	--	--	53.4

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	Found	None	Found
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -24.6 [kcals/mol]; Product = 0

```
          3' TGGGTGTCTTCTTCGTGG 5'
             :    |||||||||||||
5'  (3465)  AAATACAGAAGAAGCACC (3482) 3'
```

MC-F1- ACCAGTCGTTGGGTCTTT
MC-R1- CAGGCGGTCTACTTATTGC



```
                 3' CGTTATTCATCTGGCGGAC 5'
                    |||||||||||||||||||
5' GACTTAGTGACGCAGTTAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG 3'
        8170      8180      8190      8200      8210      8220      8230      8240
    L  S  D  A  V  N  A  I  S  R  P  P  G  E  Y  G  R  K  V  K  T  Q  M  N  -  R  G
```

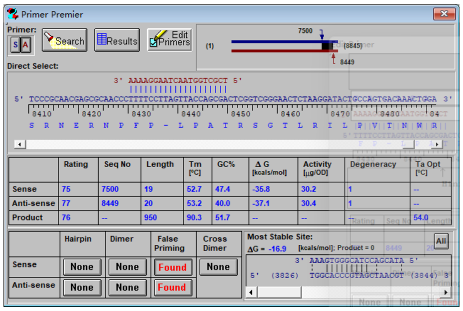
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	86	8140	18	51.8	50.0	-34.3	33.7	1	--
Anti-sense	71	8200	19	53.4	52.6	-36.3	33.7	1	--
Product	76	--	61	79.9	45.9	--	--	--	46.5

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -19.4 [kcals/mol]; Product = 0

```
          3' CGTTATTCATCTGGCGGAC 5'
             ::    ::::  |||||||||
5'  (2374)  GCGTGAAGTTGACCGCCTG (2392) 3
```

MC-F2- ATACGACCTACGGGTGAAA
MC-R2- TCGCTGGTAACTAAGGAAAA



```
        3' AAAAGGAATCAATGGTCGCT 5'
           ||||||||||||||||||||
5' TCCCGCAACGAGCGCAACCCTTTTCCTTAGTTACCAGCGACTCGGTCGGGAACTCTAAGGATACTGCCAGTGACAAACTGGA 3'
    8410     8420     8430     8440     8450     8460     8470     8480     84
 S  R  N  E  R  N  P  F  P  -  L  P  A  T  R  S  G  T  L  R  I  L  P  V  T  N  W  R
```

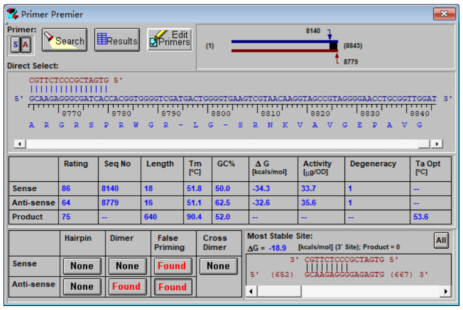
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	75	7500	19	52.7	47.4	-35.8	30.2	1	--
Anti-sense	77	8449	20	53.2	40.0	-37.1	30.4	1	--
Product	76	--	950	90.3	51.7	--	--	--	54.0

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -16.9 [kcals/mol]; Product = 0

```
             3' AAAGTGGGCATCCAGCATA 5'
                :  ||||||||| :   :
5'  (3826)      TGGCACCCGTAGCTAACGT (3844) 3'
```

MC-F3- ACCAGTCGTTGGGTCTTT
MC-R3- GTGATCGCCCTCTTGC



```
   CGTTCTCCCGCTAGTG 5'
   ||||||||||||||||
5' GCAAGAGGGCGATCACCACGGTGGGGTCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGTTGGAT 3'
         8770     8780     8790     8800     8810     8820     8830     8840
   A  R  G  R  S  P  R  W  G  R  -  L  G  -  S  R  N  K  V  A  V  G  E  P  A  V  G
```

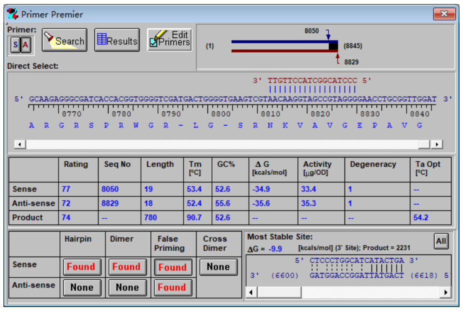
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	86	8140	18	51.8	50.0	-34.3	33.7	1	--
Anti-sense	64	8779	16	51.1	62.5	-32.6	35.6	1	--
Product	75	--	640	90.4	52.0	--	--	--	53.6

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	Found	Found	

Most Stable Site:
ΔG = -18.9 [kcals/mol] (3' Site); Product = 0

```
           3' CGTTCTCCCGCTAGTG 5'
                |||||||||
5'  (652)     GCAAGAGGGGAGAGTG (667) 3'
```

MC-F4- CTCCCTGGCATCATACTGA
MC-R4- CCCTACGGCTACCTTGTT



```
                                                    3' TTGTTCCATCGGCATCCC 5'
                                                       ||||||||||||||||||
5' GCAAGAGGGCGATCACCACGGTGGGGTCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGTTGGAT 3'
         8770     8780     8790     8800     8810     8820     8830     8840
   A  R  G  R  S  P  R  W  G  R  -  L  G  -  S  R  N  K  V  A  V  G  E  P  A  V  G
```

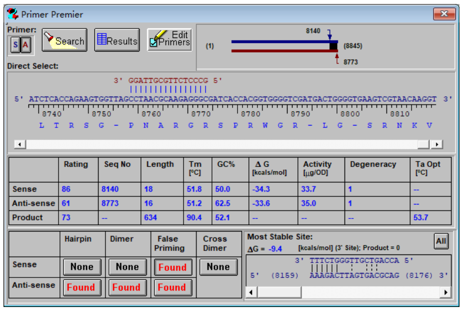
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	77	8050	19	53.4	52.6	-34.9	33.4	1	--
Anti-sense	72	8829	18	52.4	55.6	-35.6	35.3	1	--
Product	74	--	780	90.7	52.6	--	--	--	54.2

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	Found	Found	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -9.9 [kcals/mol] (3' Site); Product = 2231

```
             5' CTCCCTGGCATCATACTGA 3'
                :: :::::: :  |||||||
3'  (6600)      GATGGACCGGATTATGACT (6618) 5
```

MC-F5- ACCAGTCGTTGGGTCTTT
MC-R5- GCCCTCTTGCGTTAGG



```
        3' GGATTGCGTTCTCCCG 5'
           ||||||||||||||||
5' ATCTCACCAGAAGTGGTTAGCCTAACGCAAGAGGGCGATCACCACGGTGGGGTCGATGACTGGGGTGAAGTCGTAACAAGGT 3'
      8740     8750     8760     8770     8780     8790     8800     8810
    L  T  R  S  G  -  P  N  A  R  G  R  S  P  R  W  G  R  -  L  G  -  S  R  N  K  V
```

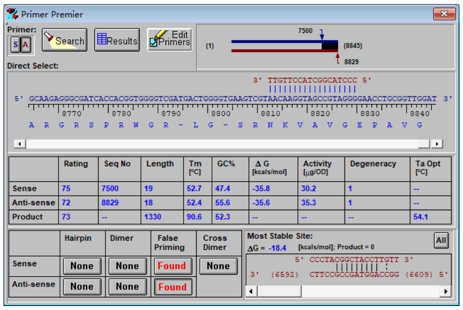
	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	86	8140	18	51.8	50.0	-34.3	33.7	1	--
Anti-sense	61	8773	16	51.2	62.5	-33.6	35.0	1	--
Product	73	--	634	90.4	52.1	--	--	--	53.7

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	Found	Found	Found	

Most Stable Site:
ΔG = -9.4 [kcals/mol] (3' Site); Product = 0

```
             3' TTTCTGGGTTGCTGACCA 5'
                ||||||    :   :::
5'  (8159)      AAAGACTTAGTGACGCAG (8176) 3'
```

MC-F6- ATACGACCTACGGGTGAAA
MC-R6- CCCTACGGCTACCTTGTT



```
                                                    3' TTGTTCCATCGGCATCCC 5'
                                                       ||||||||||||||||||
5' GCAAGAGGGCGATCACCACGGTGGGGTCGATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAGGGGAACCTGCGGTTGGAT 3'
         8770     8780     8790     8800     8810     8820     8830     8840
   A  R  G  R  S  P  R  W  G  R  -  L  G  -  S  R  N  K  V  A  V  G  E  P  A  V  G
```

	Rating	Seq No	Length	Tm [°C]	GC%	Δ G [kcals/mol]	Activity [µg/OD]	Degeneracy	Ta Opt [°C]
Sense	75	7500	19	52.7	47.4	-35.8	30.2	1	--
Anti-sense	72	8829	18	52.4	55.6	-35.6	35.3	1	--
Product	73	--	1330	90.6	52.3	--	--	--	54.1

	Hairpin	Dimer	False Priming	Cross Dimer
Sense	None	None	Found	None
Anti-sense	None	None	Found	

Most Stable Site:
ΔG = -18.4 [kcals/mol]; Product = 0

```
             5' CCCTACGGCTACCTTGTT 3'
                     |||||||||  :
3'  (6592)      CTTCCGCCGATGGACCGG (6609) 5'
```