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Analytical sensitivity and specificity of the HNF1A gene primers using the qPCR method in the suspected patient with MODY3

Sensitivitas dan Spesifisitas Analitik Primer Gen HNF1A Menggunakan Metode qPCR Pada Pasien Terduga MODY3

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ABSTRACT

Background: *Maturity onset diabetes of the young (MODY) is a rare monogenic type of diabetes, but it accounts for 1–5% of diabetes cases and typically presents before the age of 25. Among the 14 subtypes, MODY type 3 (MODY3) is the most common, particularly in Asian populations, and is associated with mutations in the hepatocyte nuclear factor 1 alpha (HNF1A) gene. Diagnosing MODY often presents challenges due to its varied clinical symptoms. Molecular testing based on qPCR offers a more accurate, relatively time-efficient, and cost-effective method for detecting HNF1A gene mutations.*
Objective: *This study aimed to validate the sensitivity and specificity of the primer for exon 4 of HNF1A in patients suspected of having MODY3 using qPCR with SYBR Green.*
Methods: *The samples included patients under 25 years old with a family history of diabetes mellitus, without obesity or ketoacidosis. Sensitivity was analyzed using probit regression based on amplification results from 10 DNA template dilutions with six replications. Analytical specificity was tested using a paired t-test against* Plasmodium vivax *spike DNA with three replications.*
Results: *The analytical sensitivity of the HNF1A exon 4 primer was 3.16 × 10 $^{-7}$ ng/µL. The specificity test showed a p-value < 0.05, indicating no significant difference between target DNA and spike DNA variations.*
Conclusion: *The HNF1A exon 4 primer demonstrated high sensitivity and good specificity, supporting its potential for qPCR-based detection of MODY as a preliminary validation.*

Keywords: *HNF1A, MODY, qPCR, sensitivity, specificity, validation method*

ABSTRAK

Latar Belakang: Maturity onset diabetes of the young (MODY) merupakan diabetes monogenik yang jarang, namun mencakup 1–5% kasus diabetes dan umumnya terjadi sebelum usia 25 tahun. Dari 14 subtipe, MODY3 merupakan yang paling umum, terutama pada populasi Asia, dan berkaitan dengan mutasi gen *Hepatocyte Nuclear Factor 1 Alpha* (HNF1A). Diagnosis MODY sering sulit karena variasi gejala klinis. Pemeriksaan molekuler berbasis qPCR menjadi metode yang lebih akurat, cepat, dan relatif efisien untuk mendeteksi mutasi gen HNF1A.
Tujuan: Penelitian ini bertujuan untuk memvalidasi sensitivitas dan spesifisitas primer ekson 4 gen HNF1A pada pasien yang dicurigai MODY3 menggunakan qPCR dengan SYBR Green.
Metode: Sampel penelitian adalah pasien berusia < 25 tahun dengan riwayat keluarga diabetes melitus, tanpa obesitas dan ketoasidosis. Sensitivitas dianalisis menggunakan regresi probit berdasarkan hasil amplifikasi dari 10 variasi pengenceran DNA dengan 6 replikasi. Spesifisitas dianalisis menggunakan uji t berpasangan terhadap DNA spike *Plasmodium vivax* dengan 3 replikasi.

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Hasil: Sensitivitas analitik primer ekson 4 HNF1A sebesar 3,16 × 10^{-7} ng/µL. Uji spesifisitas menunjukkan nilai $p < 0,05$, yang menandakan tidak terdapat perbedaan signifikan antara DNA target dan DNA spike.
Kesimpulan: Primer ekson 4 HNF1A menunjukkan sensitivitas tinggi dan spesifisitas yang baik, sehingga berpotensi digunakan sebagai metode deteksi MODY berbasis qPCR pada tahap validasi awal.

Kata kunci: HNF1A, MODY, qPCR, sensitivitas, spesifisitas, validasi metode

INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disease caused by reduced insulin production or insulin resistance or both, which is characterized by high levels of glucose in the blood (hyperglycemia). According to IDF data, Indonesia ranked fifth with 19.47 million DM patients in 2021, and is expected to increase to 28.57 million by 2045.[1] Therefore, appropriate steps are needed to manage this disease so that the number of patients does not increase every year.

Maturity-onset diabetes of the young (MODY) is a monogenic form of diabetes caused by a genetic mutation in a single gene. MODY accounts for 1-5% of all diabetes cases and typically appears in young people, typically during adolescence and early adulthood. Clinical criteria for differentiating MODY from other types of diabetes include disease onset before 25 years of age, the absence of pancreatic beta-cell autoantibodies, sustained insulin secretion with serum C-peptide levels >200 pmol/L, and a history of hyperglycemia in at least two consecutive generations.[2,3]

There are 14 distinct subtypes of MODY. Among these, MODY type 3 is the most common subtype found in Asian populations. MODY3 is associated with mutations in the HNF1A (hepatocyte nuclear factor 1 alpha) gene, accounting for the majority of MODY cases, with a frequency between 30–50%.[4,5] Establishing a diagnosis of MODY requires various laboratory tests, including serum and urine glucose, glycated hemoglobin (HbA1c), lipid profile, liver enzymes, kidney function markers, and high-sensitivity C-reactive protein (hs-CRP) measurement.[3] However, the clinical criteria vary widely, and a definitive diagnosis can only be made after these criteria are present. Therefore, faster and more accurate methods are needed for the early detection of MODY. Early detection is crucial for preventing long-term complications associated with diabetes, such as microvascular and macrovascular disease.[2] Molecular-based genetic testing, such as qPCR, can be a relatively faster, more precise, and more cost-effective option. The qPCR method is capable of detecting genetic mutations with high accuracy because it uses specially designed primers to specifically target specific genes.[6]

To ensure that the qPCR method used produces accurate and reliable data before routine use, method validation is a necessary first step.[7,8] The main components of validation include accuracy, precision, linearity, robustness, ruggedness, limit of quantification (LOQ), limit of detection (LOD), also known as sensitivity, and specificity. Analytical sensitivity is defined as the ability of a method to detect very low concentrations of an analyte in a sample, while analytical specificity is defined as the ability of a method to distinguish between the target and other substances that may be present in the sample.[9] This study aimed to evaluate the analytical sensitivity and specificity of HNF1A gene primers using qPCR as a preliminary step in method validation for MODY3 detection. The results demonstrate the capability of qPCR to detect the HNF1A gene and confirm its specificity for diagnosing MODY3. Assessing the method's sensitivity and specificity is essential to ensure accurate and rapid diagnosis, supporting early detection, appropriate treatment, and prevention of misdiagnosis. This preliminary

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study specifically validated HNF1A exon 4 primers using the SYBR Green qPCR method in patients suspected of MODY3.

METHODS

Study design

This research was conducted in December 2024 at the Molecular Biology Laboratory, Department of Medical Laboratory Technology, Poltekkes Kemenkes Bandung. The research flow diagram is shown in Figure 1.

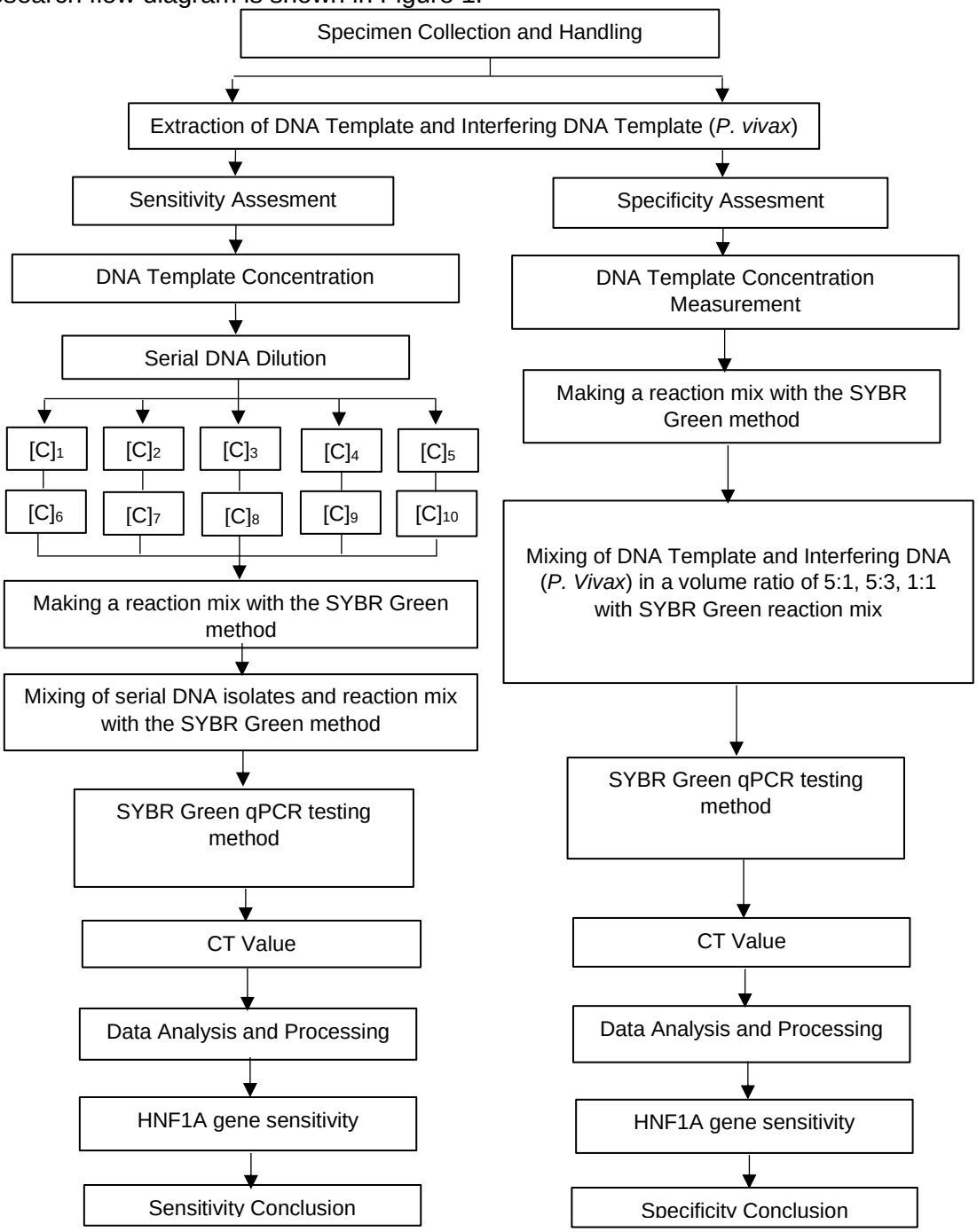

Figure 1. Research Flow Scheme

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This research was quantitative descriptive because it describes, examines, and explains what is being studied as it is, and draws conclusions from the observed phenomena using numbers. Furthermore, no treatment was performed on samples and/or clinical specimens.

Data Source and Sampling Procedure

The research unit consisted of the remaining EDTA blood specimen from one suspected MODY3 patient from Majalaya Regional Hospital. Inclusion criteria were DM patients aged less than 25 years with a family history of DM. Exclusion criteria included DM patients with obesity and ketoacidosis. Sampling was based on available specimens that met the criteria.

Variables of the Study

The variables in this study consisted of DNA template concentration and the presence of interfering DNA (*Plasmodium vivax*) as independent variables, while the dependent variable was the cycle threshold (Ct) value obtained from qPCR amplification, which reflects the analytical sensitivity and specificity of the HNF1A gene primer.

Measurement and Instrument

1 tube of EDTA blood specimen from 1 suspected MODY3 patient as template DNA, 1 tube of EDTA blood specimen from 1 confirmed positive *Plasmodium vivax* patient as interfering DNA, Wizard ® Genomic DNA purification Kit (Promega), GoTaq Master Mix Kit (Promega), HNF1A exon 4 Target Gene Primer 200 nM in 20 µL (Macrogen). Primer sequences can be seen in Table 1.

Table 1. Sequence Primary Forward and Reverse primers for the HNF1A gene

HNF1A gene	Primary Forward (5'"3')	Primary Reverse (5'"3')	PCR Product Length (BP)	Annealing Temperature (˚C)
Exon 1	TGCAAGGAGTTTGGTTTGTG	GAAGGTCATGGGGACTCAAC	536	58
Exon 2	CCTCAGGGTTGACAAGGTTC	TGTGTAATGGGGATGGTGAA	395	57
Exon 3	GCCATGGCAATGAGAAAGAA	GGCAACTGGACAGCCTTTTA	387	55
Exon 4	GGCAGAGCTCAGCTTCTCAG	AAGGAGTGGCATGAATGGAA	464	55
Exon 5	GCCTAAGCAAACCAATGGAG	CAGCTGCTGAGACCTACGAG	391	58
Exon 6	CCAACCTCATCTTTCCTTGG	AATGAATGAATGAGTCCCAGTG	400	61
Exon 7	CTCTGGGAAGGAGAGGTGGT	GTCCCAGAGACACATGCAGA	397	63
Exon 8	TTTTGAAAATCAGCCCTGGA	CTGGAGGCCTCAGTGTCTG	389	59
Exon 9	ACCAAGCAGGTAAGGTCCAG	CTTCCTCACAGCAGCCCTA	375	61
Exon 10	TGAGTACCCCTAGGGACAGG	CCTGCCTTCCCTGTTAGCTT	640	62

Data Collection

DNA extraction

EDTA blood specimens from suspected patients and EDTA blood specimens confirmed positive for *Plasmodium vivax* were extracted using the Wizard ® Genomic DNA purification Kit. Then, the concentration and purity of template and interfering DNA were measured using a NanoDrop NP80 Spectrophotometer at wavelengths of 260 and 280 nm.

Exon selection

qPCR was performed targeting the HNF1A gene from exons 1–10. Then, the HNF1A gene in exon 4 was selected for use in the analytical sensitivity test because it is the most commonly found mutation.[5,10]

DNA template dilution

The DNA template was serially diluted in 10 dilution variations in a final volume of 50 µL each to be used in 6 qPCR replications.

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qPCR for measuring analytical sensitivity

The reaction mix was made with a final volume of 25 µL containing 1X Go Taq qPCR master mix, reverse primer, forward primer, CXR reference dye, nuclease-free water, and DNA template. The qPCR process on the Tianlong Gentier 96 was conditioned as follow: a pre-denaturation phase of 1 cycle, a temperature of 95°C, for 2 minutes; a denaturation phase of 40 cycles, a temperature of 95°C, for 15 seconds; an annealing phase of 40 cycles, a temperature of 55°C, for 1 minute; and an extension phase of 40 cycles, a temperature of 60°C, for 1 minute. The annealing temperature of 55°C was taken from Mohammadi's research (2019).[11] This procedure was performed on 10 concentration variations with 6 replications. Each replication was accompanied by a negative control.

qPCR to measure analytical specificity

The target gene was amplified in 3 replicate experiments, and the target gene that had been spiked with interfering DNA using 3 varying concentrations of interfering DNA was also amplified in 3 replicate experiments. The volume and conditions of the qPCR used were the same as those described in the qPCR steps to measure analytical sensitivity. Each replicate was accompanied by a negative control.

Ethical Considerations

This study received ethical approval from the Health Research Ethics Commission (KEPK) of the Directorate of Health Polytechnic, Ministry of Health, Bandung (No. 51/KEPK/EC/XII/2024).

Data analysis

Data Obtained

Data for analytical sensitivity testing consisted of 60 primary data points in the form of Ct values obtained from 6 replications at 10 levels of serial dilution of DNA template. The data for the analytical specificity test consisted of 12 primary data points in the form of Ct values obtained from 3 replications of the baseline specimen and 3 replications of 3 variations in the concentration of interfering DNA, accompanied by negative controls.

Data Processing

Analytical sensitivity tests were conducted using probit analysis. Meanwhile, analytical specificity tests were conducted using the SPSS paired t-test.

Analytical Sensitivity (Probit Analysis)

A linear regression curve is created against the Ct value of each dilution series to obtain the R^2 value, and the dilution limit is determined until the curve produces an R^2 value ≥ 0.950, which is expected to produce a result inaccuracy of less than 5%. Then, the proportion of replications that still provide a positive value is calculated compared to the total number of replications. In this study, the positive value referred to is the one that provides a positive R^2 value ≥ 0.950. Next, the obtained proportion value is calculated again using the formula 5+NORMSINV(P) to obtain the probit value. The probit value is the result of converting the proportion to probability units on a standard deviation scale. In determining analytical sensitivity, the probit point used is C95, which is 6.64 (based on the Finney table), as can be seen in Figure 2.

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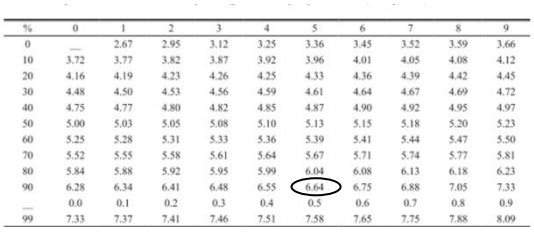

%	0	1	2	3	4	5	6	7	8	9
0	—	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.25	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
—	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Figure 2. Finney Table for Percentage Proportion Transformation to Probit Value

The C95 point is defined as the point at which 95% of the samples containing that concentration of analyte are declared positive. The value of 6.64 was then plotted onto a linear regression curve to obtain the log10 concentration value and converted to a concentration. The resulting concentration value represents the lowest DNA template concentration that still yields a positive value.

Analytical Specificity (Statistical Test)

The data obtained is first tested using the Shapiro-Wilk normality test to determine whether the distribution of the data is normal or not. The data distribution is determined to be normal if the significance value is P > 0.05.[12.] Then, the data was tested using the SPSS paired t-test.

RESULTS

In this study, EDTA blood specimens from suspected MODY patients that have been extracted are hereinafter referred to as target DNA, while blood specimens that are positive for *Plasmodium vivax* that have been extracted are hereinafter referred to as interfering DNA. After obtaining the target DNA and interfering DNA, the concentration of both DNA specimens was measured, and the target DNA concentration was obtained at 19,050 ng/µL, and the Plasmodium vivax interfering DNA at 35,850 ng/µL. The results of the target DNA extraction from 1 specimen of a suspected MODY patient were then diluted in stages to a dilution of 10^9.

Table 2. Ct Value of qPCR SYBR Green Method

Dilution	Ct Value of qPCR SYBR Green Method					
	Replication 1	Replication 2	Replication 3	Replication 4	Replication 5	Replication 6
10^0	20.03	20.15	20.33	20.35	19.95	19.76
10^1	21.41	21.47	22.81	21.68	22.42	20.82
10^2	24.79	25.79	26.11	25.07	25.29	24.89
10^3	28.80	28.90	29.13	28.95	28.50	28.72
10^4	31.50	31.26	32.72	32.24	32.20	32.38
10^5	34.39	32.43	34.60	33.38	33.02	35.63
10^6	34.11	34.33	35.47	34.06	34.09	32.65
10^7	32.43	33.95	34.18	33.74	33.93	34.55
10^8	32.68	32.71	34.49	35.27	35.15	34.65
10^9	32.01	33.90	34.77	35.23	33.26	34.86

In determining analytical sensitivity, 60 Ct values were obtained from 6 replications of the qPCR process using the SYBR Green method against 10 variations in target DNA dilution concentrations to detect the HNF1A gene (Table 2). The Ct values obtained tended to increase along with increasing levels of target DNA dilution. Then, a linear regression curve of the Ct value data was created for each replication (Table 3).

In Table 3, it can be observed that at dilution 10^1 (concentration 1.905 x 10^0 ng/µL), all replications (1–6) gave an R2 value ≥ 0.950. Meanwhile, at dilution 10^2 (concentration 1.905 x 10^{-1} ng/µL), only 3 replications showed an R2 value ≥ 0.950. Then, all replications

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(1–6) again gave an R2 value ≥ 0.950 at dilution 10^3 (concentration 1.905 x 10^{-2} ng/µL); 10^4 (concentration 1.905 x 10^{-3} ng/µL); and 10^5 (concentration 1.905 x 10^{-4} ng/µL). However, at dilution 10^6 (concentration 1.905 x 10^{-5} ng/µL), only 5 replicates gave R2 values ≥ 0.950. Starting from replicates 7 to 9, no replicates gave R2 values ≥ 0.950.

The C95 probit value of 6.64 (Figure 2) was entered into the linear equation to obtain the Log10 Concentration value of -7.4998 and converted to a concentration of 3.16 x 10-7. This value is the lowest DNA concentration that still provides an R2 value ≥ 0.950.

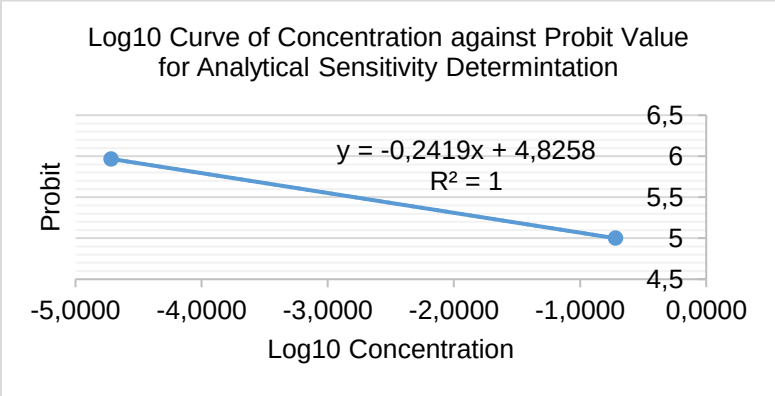

Figure 2. Log10 Curve of Concentration against Probit Value for SYBR Green Analytical Sensitivity Determination

Table 3. R^2 Value of qPCR SYBR Green Method

Dilution	Ct Value of qPCR SYBR Green Method					
	Replication 1	Replication 2	Replication 3	Replication 4	Replication 5	Replication 6
10^0	N/A	N/A	N/A	N/A	N/A	N/A
10^1	1.000	1.000	1.000	1.000	1.000	1.000
10^2	0.945	0.915	0.999	0.941	0.998	0.897
10^3	0.960	0.965	0.997	0.961	0.997	0.951
10^4	0.980	0.980	0.997	0.979	0.994	0.974
10^5	0.989	0.972	0.995	0.978	0.985	0.985
10^6	0.963	0.971	0.980	0.958	0.969	0.898
10^7	0.869	0.936	0.910	0.911	0.928	0.876
10^8	0.800	0.854	0.856	0.899	0.911	0.844
10^9	0.717	0.822	0.813	0.873	0.830	0.812

Table 4. Probit Values in Determining Analytical Sensitivity of the SYBR Green Method

Concentration (ng/µL)	Log10 Concentration	N Total	N Post	Proportion	Probit
1.905 x 10^1	1.2799	6	6	1.000	N/A
1.905 x 10^0	0.2799	6	6	1.000	N/A
1.905 x 10^{-1}	-0.7201	6	3	0.500	5,000
1.905 x 10^{-2}	-1.7201	6	6	1.000	N/A
1.905 x 10^{-3}	-2.7201	6	6	1.000	N/A
1.905 x 10^{-4}	-3.7201	6	6	1.000	N/A
1.905 x 10^{-5}	-4.7201	6	5	0.833	5.96
1.905 x 10^{-6}	-5.7201	6	0	0.000	N/A
1.905 x 10^{-7}	-6.7201	6	0	0.000	N/A
1.905 x 10^{-8}	-7.7201	6	0	0.000	N/A

Next, the proportion of the number of replications that provide an R2 value ≥ 0.950 is calculated against the total replications, so that the probit value can be obtained, which can be observed in Table 4. The Log10 Concentration values (-0.7201 and -4.7201) are

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plotted against their probit values (5.000 and 5.96) so that a linear equation is obtained: y = -0.2419x + 4.8258. The linear regression curve in Figure 3 produces an R2 value = 1, which provides high confidence.

In determining analytical specificity, 12 Ct values were obtained, consisting of 3 Ct values on the target DNA and 9 Ct values on 3 replications of the target DNA spiked with 3 variations in the concentration of interfering DNA. The ratio of target DNA and interfering DNA used was 5:1 (15 µL for target DNA and 3 µL for interfering DNA), 5:3 (10 µL for target DNA and 6 µL for interfering DNA), and 1:1 (9 µL for target DNA and 9 µL for interfering DNA). Table 5 shows that the lowest Ct value obtained was 17.78, and the highest Ct value was 22.34.

Table 5. Ct value for determining the analytical specificity of the SYBR Green method

Template Composition	Ct value			Average
	R1	R2	R3	
Baseline Specimen	20.512	19.824	19.754	20.030
HNF1A : *P.vivax* (5:1)	17.777	19.605	19.762	19.048
HNF1A : *P.vivax* (5:3)	22.340	19.324	19.301	20.322
HNF1A : *P.vivax* (1:1)	19.410	19.340	19.480	19.410

Note: The baseline specimen contains only target DNA, while HNF1A: *P. vivax* contains target DNA spiked with *P.vivax* interfering DNA; R is replication.

Before proceeding to the paired t-test, a Shapiro-Wilk normality test was conducted to determine whether the distribution of the obtained Ct values was normal. Table 6 shows that all data obtained were normally distributed, as indicated by a significance value of P > 0.05. Therefore, the statistical test can be continued with a paired t-test.

Table 6. Shapiro Wilk Normality Test for SYBR Green Ct Data

Class	Sig.
Baseline Specimen	0.159
HNF1A : *P.vivax* (5:1)	0.130
HNF1A : *P.vivax* (5:3)	0.166
HNF1A : *P.vivax* (1:1)	1.00

Analytical specificity testing was continued with a paired t-test to compare the average Ct value in the baseline specimen with the average Ct value at each concentration variation of interfering DNA. The results of the paired t-test for each pair of concentration variations yielded a significance value of P > 0.05 and a 95% confidence level (Table 7). This indicates that there is no significant difference between the average Ct value of the baseline specimen and the average Ct value at each concentration variation of interfering DNA. Therefore, it is concluded that the primers used can still detect the HNF1A gene specifically, even though there is interfering DNA in the specimen.

Table 7. SYBR Green paired t-test

Pair	Sig
Baseline–HNF1A : *P.vivax* (5:1)	0.382
Baseline–HNF1A : *P.vivax* (5:3)	0.739
Baseline–HNF1A : *P.vivax* (1:1)	0.132

DISCUSSION

This study aimed to determine the analytical sensitivity and specificity of HNF1A gene primers in suspected MODY3 patients using qPCR. The obtained data were processed and analyzed using a statistical approach using probit regression to determine the limit of detection (C95), while the determination of analytical specificity was carried out using a paired t-test.

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To determine the detection limit, the target DNA was serially diluted in 10 variations (dilution 10^0–10^9) with 6 replications per concentration point, along with a negative control. Then, it was observed at which dilution the curve still gave a positive value, namely, the R2 value ≥ 0.950 (hereinafter referred to as N Pos). Analytical sensitivity was calculated based on the relationship between the target DNA concentration and the positive detection level.

Based on the observation results, there was instability in the results at dilution 10^2 because there were only 3 positive values (N Pos = 3). Meanwhile, at higher dilution levels, starting from 10^3 to 10^5, the number of N pos data obtained was greater than the data at dilution 10^2. All replications at dilution levels 10^3 to 10^5 produced positive values (N Pos = 6). However, at dilution 10^6, the results showed instability again, indicated by the N pos value decreasing as the dilution increased. Based on these observations, there was instability in the results at several dilutions, indicated by N Pos < 6, namely at dilutions 10^2, 10^6, 10^7, 10^8, and 10^9.

The instability of the results at dilution 10^2 is likely due to a technical error during the process. This technical error can occur due to a reduction in target DNA particles during the dilution process, due to some target DNA particles not being removed from the pipette tip. This technical error can lead to biased concentration estimates. Schmidt et al. (2023) in their study stated that random errors can cause data to be nonlinear.[13]

The presence of less N Pos data at dilution 10^2 compared to N Pos data at dilution 10^3 causes the data to be non-linear. This is not in line with the basic law of dilution proposed by Sari & Kuntari (2019) in their research that dilution of a solution causes a decrease in the concentration of substances in the solution, so that, along with the increase in the dilution value, the concentration level will be lower, which means that N pos will be less.[14]

Then, further instability of the results occurred at dilution 10^6 with 5 positive values (N Pos = 5), while at dilutions 10^7-10^9, all data were negative (N Pos = 0). This indicates that dilution 10^6 was the last concentration that still produced a positive value. Meanwhile, instability of the results at dilutions 10^6–10^9 likely occurred due to too high a dilution, so that the DNA concentration in the sample was too low to react with the qPCR reagent, affecting the efficiency of amplification and detection in qPCR analysis accurately.[15]

Next, the sensitivity data processing was continued by finding the probit value of the replication proportion obtained using the formula 5+NORMSINV (P). Then, from the probit value, a regression equation formula was obtained with x as the log10 value of the concentration to be sought and y as the C95 probit point of 6.64. The log10 value of the concentration obtained was then converted to a concentration, which was 3.16×10^{-7} ng/µL.

Setyawati and Zubaidah (2021) stated that a DNA concentration of 0.01 - 0.1 µg per µL of template solution is sufficient for PCR, so the analytical sensitivity of the primers used in this study can detect DNA concentrations lower than the recommended DNA concentration.[16] In addition, the HNF1A primers used have a lower detection limit than other gene primers in the literature. In a study of the invA gene primer in *Salmonella enterica*, Sturza et al. (2021) obtained an LoD of 2×10^{-5} ng/µL.[17] Kim et al. (2025) obtained a primer LoD for the detection of *Listeria monocytogenes* and *Listeria innocua* of 5×10^{-5} ng/µL.[18] However, specific literature on the analytical sensitivity of qPCR for the HNF1A gene is very limited, so the development of other research methods is needed to obtain more complete data in selecting a qPCR method for confirming the diagnosis of MODY.

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Analytical specificity was determined by mixing target DNA with interfering DNA (*Plasmodium vivax*) in ratios of 5:1, 5:3, and 1:1. The paired t-test result showed a P value > 0.05. This indicates that the qPCR method used can specifically detect the HNF1A gene and is not significantly affected up to a concentration of interfering DNA of 35.85 ng/µL.

According to Burd (2010), referring to CLSI guidelines, analytical specificity test results are not graded as "good" or "poor," but rather are based on the presence or absence of interference from organisms or interfering substances at a given concentration. [7] Significant interference is likely to occur if the concentration of interfering substances is very high. Therefore, CLSI (2005) recommends that interference studies use the highest concentration of organisms or interfering substances likely to be found in the actual specimen. [8]

qPCR-based molecular testing offers several advantages, such as offering a relatively more accurate method, saving time and costs, and being more sensitive and specific in detecting HNF1A gene mutations compared to conventional methods that require various complex clinical criteria that are prone to misdiagnosis.[19,20] qPCR can help determine the type of DM caused by genetic mutations, thus having implications for the treatment and management of DM patients with MODY3. However, this method is not a standardized method for detecting the HNF1A gene.

A method needs to be validated before use to ensure the reliability, accuracy, and reproducibility of results, particularly in research, clinical diagnostics, or laboratory testing. Analytical sensitivity and specificity are components of this validation test. The preliminary results of the primary analytical sensitivity and specificity validation of the HNF1A qPCR method are promising for clinical laboratory application, but further development is needed to obtain accurate and reliable results for diagnosis.

However, this study also has several limitations. One is the inability to determine the point of mutation in suspected MODY type 3 specimens because sequencing was not performed. Therefore, further sequencing is necessary for further research. Furthermore, due to time constraints, the validation parameters of the method studied focused solely on sensitivity and susceptibility.

CONCLUSION

The HNF1A exon 4 primers showed an analytical sensitivity of 3.16 x 10^{-7} ng/µL and good specificity. The results of this study serve as a preliminary validation test for the qPCR method for HNF1A gene detection in MODY patients. However, further testing of other validation test components and the use of healthy human DNA spikes are needed to ensure the accuracy of the qPCR method as a diagnostic tool for MODY3.

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