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METHOD ARTICLE

REVISED Detection of herpes simplex virus 2: a SYBR-Green-based real-time PCR assay [version 2; peer review: 2 approved]

Previously titled: Rapid detection of herpes simplex virus 2: a SYBR-Green-based real-time PCR assay

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Abstract

The prevalence of Herpes simplex virus 2 (HSV2) is increasing at an alarming rate in the world. Most of the HSV2 cases are not diagnosed properly, although a range of molecular and serological diagnoses exist. Herein, we have reported a very rapid detection method specific for HSV2 using real-time PCR. The primers specific for HSV2 were designed using the Primer-BLAST tool and 120 base pairs of the polymerase gene were amplified using real-time PCR with SYBR Green dye. The designed primer pair was found highly efficient in detecting only HSV2 DNA, but not HSV1. The threshold cycle (Ct) value for HSV2 reactions by designed primers was found to be an average of 22.55 for a standard copy number of viral DNA that may denote the efficiency of the primers. The melting temperature (T_m) of the amplicon using designed primers (82.6⁰C) was also higher than that using reference primers (about 78⁰C), indicating the high GC content of the amplified template. The designed primer pair will help clinicians to detect the HSV2 DNA specifically and diagnose the associated disease rapidly.

Keywords

Herpes simplex virus 2, real-time PCR, rapid diagnosis, primer design

Open Peer Review

Reviewer Status  

Invited Reviewers

	1	2
version 2		
(revision)		
09 Nov 2021	report	report
		
version 1		
26 Jul 2021	report	report

1. **ASM Monjur Al Hossain**, University of Dhaka, Dhaka, Bangladesh
2. **Joyanta Modak**, Deakin University, Waurn Ponds, Australia

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Modhusudon Shaha (msshaha146@gmail.com)

Author roles: **Shaha M:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Writing – Original Draft Preparation; **Roy B:** Data Curation, Writing – Original Draft Preparation, Writing – Review & Editing; **Islam MA:** Resources, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: The author(s) declared that no grants were involved in supporting this work.

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First published: 26 Jul 2021, 10:655 <https://doi.org/10.12688/f1000research.53541.1>

REVISED Amendments from Version 1

The revised article is updated as per the reviewers' recommendation. Firstly, the title of the article is updated to 'Detection of herpes simplex virus 2: a SYBR-Green-based real-time PCR assay'. The introduction section is updated with more background information. We also included the limitations of this study in the conclusion section of this revised version.

Any further responses from the reviewers can be found at the end of the article

Introduction

Herpes simplex virus 2 (HSV-2), a highly infectious pathogen, is a member of the Herpesviridae family consisting of a double-stranded DNA material.^{1,2} Globally, about 417 million people are infected by HSV-2, with a prevalence of 11.3%.³ The most common age group with HSV-2 diseases is 15-49 years of age, with a slightly higher rate of infection in women.³ HSV-2 infection is characterized by painful genital ulcers with vesicular rash (watery blisters), which are highly contagious and spread viruses to other hosts upon direct contact.⁴

The diagnosis of HSV2 is complex and costly in developing countries like Bangladesh. Moreover, due to the high similarity of HSV2 with other human herpes viruses (HHV) such as HSV1, it is tough to confirm the type of the diagnosed HHV. Nowadays, molecular analysis exists to determine specific types of HHV, but identification of the virus type is solely dependent on nucleotide sequencing. Some primers for HSV1 and HSV2 detection are sufficiently available^{5,6} however, number of HSV2-specific primer is of scarce. In this case, developing a new real-time polymerase chain reaction (RT-PCR) method for HSV-2-specific diagnosis can facilitate HSV2 detection.

RT-PCR is the upgraded version of conventional PCR, invented by Kary Mullis in 1984, which aims to detect and quantify genes with dynamic expression profiles.⁷ Analysis by RT-PCR has several important steps including DNA extraction, designing primers and amplification.⁷ This study sheds light on the development of a RT-PCR assay to detect HSV2, which includes the design of compatible primers and amplification of HSV2 DNA using this primer set.

Methods**HSV2-specific primer design**

Appropriate primer design is a very important step to diagnose and quantify a specific microbial gene. An important, general step to design a primer set is to ensure the optimal melting temperature (T_m), balanced GC content, self-complementarity and self-3' complementarity.⁸ There are several online tools to develop and design primers, such as NCBI's **Primer-BLAST**.⁸ A known gene sequence of HSV2 virus collected from NCBI GenBank (MH697422.1, see *Underlying data*) was used for Primer-BLAST in the NCBI server keeping the parameters as default.

Using the online NCBI nucleotide Basic Local Alignment Search Tool (BLAST)⁸ several highly similar sequences of HSV1 and HSV2 were collected based on alignment score (quality of the alignment), query cover (nucleotide cover of searched sequence), Expected value (E, expected number of alignments by chance with a particular score; the lower the E value, the more significance the score) and identity with the reference sequence (**Figure 1a**), and then aligned using

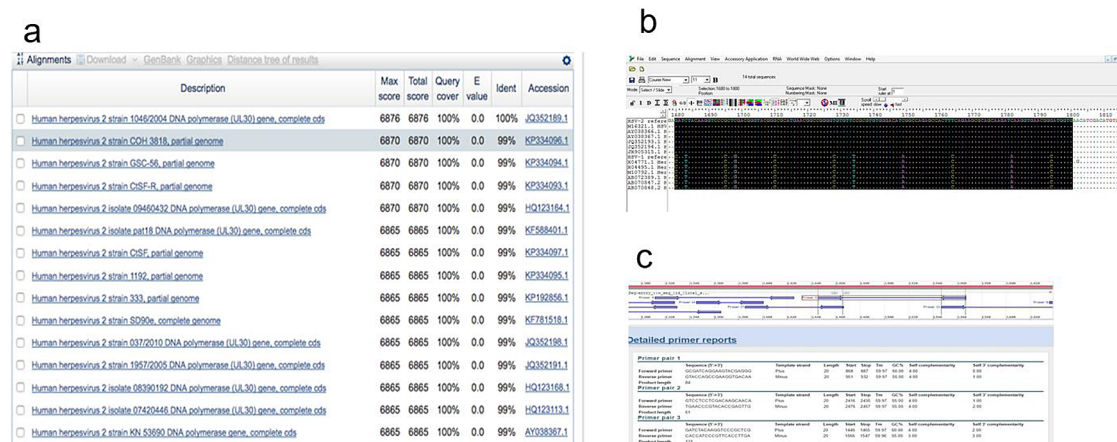


Figure 1. Primer design for real-time PCR specific to HSV2. (a) Collection of HSV sequences using NCBI-BLAST for designing a primer pair; (b) Alignment of HSV sequences and amplification region (selection region) using BioEdit software; (c) Different primer pair sets and associated properties; primer pair-3 was selected for further analysis.

BioEdit software version 7.2, keeping HSV2 sequences at the top (Figure 1b). The primers obtained were then checked to find the best region to anneal with HSV2 but not HSV1 (in order to develop the primer specific for HSV2). The region with maximum nucleotide matches to the reference HSV2 sequences and minimum nucleotide matches to the HSV1 sequences was selected. Among all sets of primers tested, primer-3 was found the best regarding properties like a greater number of nucleotide mismatches with HSV1 sequences, self-complementarity (a predictor secondary structures between a single primer or primer pairs), higher T_m compared to other not-selected primers) and GC content (in good range 55-60%), and most importantly, 3' self-complementarity (useful for predicting primer-dimers) (Figure 1c).

Setting up a RT-PCR reaction

The real-time PCR (based on SYBR Green fluorescent binding) with HSV1 and HSV2 template DNA were prepared using the designed primer pair (specific for HSV2) and control primer pair (common for both HSV1 and HSV2). In a 20ul reaction mix, 10ul of SYBR Green Master Mix (2X), 1ul of each reverse and forward primers at 6pM concentrations, 6ul of nuclease-free water and 2ul of template HSV2 DNA (extracted from an HSV2 lab strain) were added. The reagents were purchased from Qiagen (Germantown, MD, USA). The reaction conditions used to amplify the DNA were an initial 95°C for 20 seconds, 40 cycles of 3 seconds at 95°C and 30 seconds at 60°C, and a final cycle of 15 seconds at 95°C, 1 minute at 60°C and 15 seconds at 95°C.

Results and discussion

About 120 base pairs of HSV2 polymerase gene fragment were considered to be amplified using the designed primers, whereas in case of standard control primer pair both HSV1 and HSV2 were amplified (Figure 2a). The average threshold cycle (Ct) values for reaction by designed and control HSV2 primers were found to be 22.55 and 22.85 respectively, which indicates that the similar initial concentrations of DNA were amplified.^{9,10}

Observing the melting curve revealed that the designed primer was specific for HSV2. The peak of that amplification was stronger than that of reference primers, indicating the designed primers had a greater sensitivity to the HSV2 target gene than the reference primers (Figure 2b).^{11,12} The melting temperature (T_m) of the amplicon using the designed primers (82.6°C) was also higher than that of the reference primers (about 78°C). The explanation behind this may be a higher GC content of the product or larger fragments than the product amplified by reference primers.¹³ On the other hand, some unusual peaks for the negative controls were found in the melt curve, which have much higher Ct values (more than 35) and lower T_m of around 70-74°C, which indicates the possibilities of being either non-specific products or primer-dimers.¹⁴

In this assay, some secondary non-specific products or primer-dimers were observed in the melt curve, hence, the amplification reaction needs to be optimized. Increasing the annealing temperature of the reaction may reduce

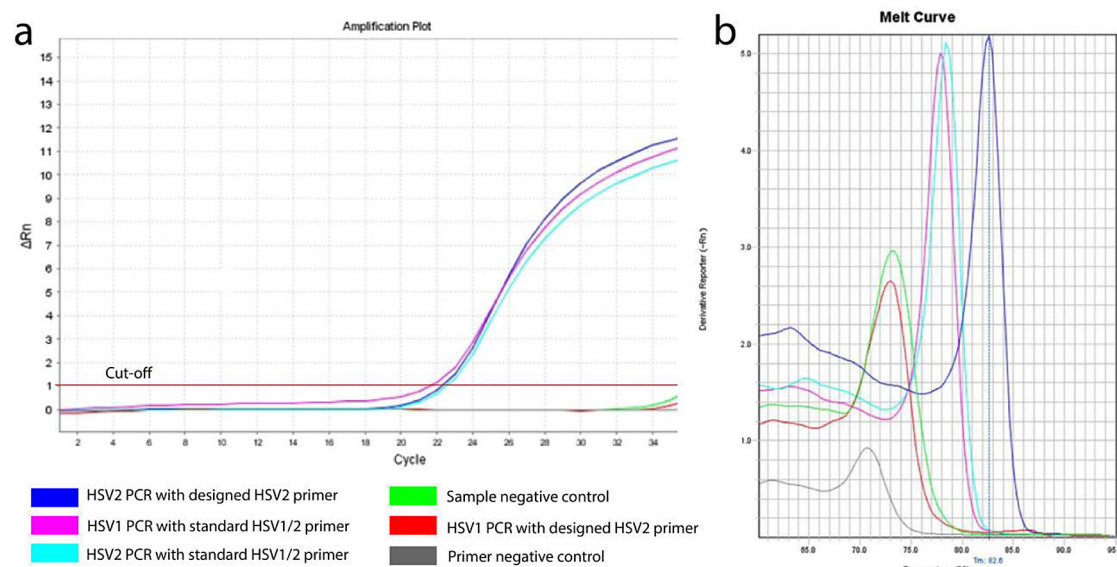


Figure 2. Amplification of HSV2 DNA by RT-PCR. (a) Amplification curves of HSV1 and HSV2 using designed (own) and standard (reference) primers; a lagging stage, logging cycle and plateau are clearly visible; (b) Melt curve of HSV1 and HSV2 using designed (own) and reference (reference) primers.

non-specific amplification of the product.¹⁵ Here, the annealing temperature of the reaction was 60°C; it would be better to use a slightly higher annealing temperature to get only the specific product. However, the annealing temperature should not be too high, as it would reduce the yield of the desired product.¹⁶ The optimal annealing temperature can easily be obtained using gradient real-time PCR.¹⁶ On the other hand, the concentration of primers can slightly be decreased to prevent the formation of primer-dimer if formed.^{15,17}

The optimization of Magnesium ion (Mg^{2+}) concentrations is also an important factor to achieve specific and desired product amplification.¹⁸ It has been documented that the higher the Mg^{2+} concentration, the higher the affinity of primers towards hybridization, leading to the formation of primer-dimers and non-specific priming.^{15,18} Furthermore, to verify the SYBR Green reaction specificity, an additional agarose gel electrophoresis can be performed using the product and non-specific bands, to confirm the presence of a single PCR product.^{16,19} As the SYBR Green fluorescent dye binds with double-stranded DNA and provides fluorescence at the same time as the amplification is running (hence being a real-time assay), all the steps of the protocol should be handled carefully and aseptically. That is why the lack of aseptic conditions may lead to the contamination of the reaction, giving rise to secondary non-specific products.²⁰

Conclusions

According to the results, the annealing temperature of the reaction should be slightly increased to get specific amplification of HSV2. Furthermore, the primer concentration needs to be decreased to prevent primer-dimer formation. On the other hand, the concentration of Mg^{2+} in the buffer system can also be reduced to promote the specificity and efficiency of the reaction. Observing the real-time PCR assay, I believe that my designed primers do not need to be altered as the specificity of determining HSV2 is good enough compared with other primers used.

One limitation of this study is that we were not able to include clinical samples to validate the results of HSV2-specific PCR. However, some modifications to this assay such as proper optimization of the reaction and the use of a probe, specific to that gene, can render it appropriate to be used in a diagnostic laboratory.⁷ As SYBR Green assays detects all dsDNA, the introduction of a probe (consisting of fluorescent-labeled target-specific oligonucleotides that increase sensitivity and specificity) may establish this assay as a diagnostic test to detect HSV2. Furthermore, to establish this assay as a diagnostic test, positive control with a housekeeping gene (constitutive genes expressed in all cells) is necessary.²¹ This may promote the efficiency and reliability of this test.

Data availability

Underlying data

Figshare: HSV-2 primer and qPCR Ct values, <https://doi.org/10.6084/m9.figshare.14603934.v4>.²²

This project contains the designed HSV2 primer pair and the cycle threshold (Ct) values of HSV2- and HSV1-positive samples generated using qPCR.

NCBI GenBank: Human alphaherpesvirus 2 isolate HSV2_1 DNA polymerase (UL30) gene, complete cds. Accession number: MH697422.1; <https://www.ncbi.nlm.nih.gov/protein/MH697422.1>.

NCBI GenBank: Human herpesvirus 2 strain RR 60279 DNA polymerase gene, complete cds. Accession number: AY038366.1; <https://www.ncbi.nlm.nih.gov/protein/AY038366.1>.

NCBI GenBank: HIV-1 clone MNT3-2-12 from USA gag protein (gag) gene, partial cds. Accession number: AY038267.1; <https://www.ncbi.nlm.nih.gov/protein/AY038267.1>.

NCBI GenBank: Human herpesvirus 2 strain 1605/2007 DNA polymerase (UL30) gene, complete cds. Accession number: JQ352193.1; <https://www.ncbi.nlm.nih.gov/protein/JQ352193.1>.

NCBI GenBank: Human herpesvirus 2 strain 1541/2008 DNA polymerase (UL30) gene, complete cds. Accession number: JQ352194.1; <https://www.ncbi.nlm.nih.gov/protein/JQ352194.1>.

NCBI GenBank: Human herpesvirus 2 isolate HSV-2v_pat1 DNA polymerase (UL30) gene, complete cds. Accession number: JX905315.1; <https://www.ncbi.nlm.nih.gov/protein/JX905315.1> NCBI GenBank: Human herpesvirus 1 UL30 gene for DNA polymerase, complete cds. Accession number: AB072389.1; <https://www.ncbi.nlm.nih.gov/protein/AB072389.1>.

NCBI GenBank: Human herpesvirus 1 gene for DNA polymerase, complete cds, clone: HSV-1 R98/0. Accession number: AB070847.2; <https://www.ncbi.nlm.nih.gov/protein/AB070847.2>.

NCBI GenBank: Herpes simplex virus type 1 DNA polymerase gene. Accession number: X04771.1; <https://www.ncbi.nlm.nih.gov/protein/X04771.1>.

NCBI GenBank: Herpes simplex virus type 1 strain ANG gene for DNA polymerase. Accession number: X04495.1; <https://www.ncbi.nlm.nih.gov/protein/X04495.1>.

NCBI GenBank: Herpes simplex virus type 1 (KOS) ori-L region and polymerase (pol) gene, complete cds. Accession number: M10792.1; <https://www.ncbi.nlm.nih.gov/protein/M10792.1>.

NCBI GenBank: Human herpesvirus 1 gene for DNA polymerase, complete cds, clone: HSV-1 R98/3. Accession number: AB070848.2; <https://www.ncbi.nlm.nih.gov/protein/AB070848.2>.

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Version 2

Reviewer Report 30 November 2021

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Joyanta Modak

School of Medicine, Deakin University, Waurin Ponds, VIC, Australia

The authors have addressed all the comments. I don't have any further comments on this manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of expertise is microbiology and molecular biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 22 November 2021

<https://doi.org/10.5256/f1000research.78555.r99493>

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ASM Monjur Al Hossain

Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka, Dhaka, Bangladesh

Shaha *et al.* addressed all the issues scientifically. Therefore, the manuscript can be indexed.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug delivery

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 05 October 2021

<https://doi.org/10.5256/f1000research.56937.r93395>

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**Joyanta Modak**

School of Medicine, Deakin University, Waurin Ponds, VIC, Australia

Shaha *et al.*, aimed to develop a new primer for the detection of herpes simplex virus 2 using real-time PCR. They used the Primer-BLAST tool to design a primer that can detect only HSV2, not HSV1. However, there are a few issues about the manuscript that need to be addressed.

1. Title of the manuscript – this is not a rapid antigen test using a kit or immunochromatographic test that can be done in 5 minutes. So, the word “Rapid” should be removed from the title.
2. There are quite a few published articles that developed a real-time PCR method for the detection of HSV2. In the introduction, authors should acknowledge previously published articles and should describe what is lacking in the previous method and why they are developing new methods.
3. What type of samples were used, e.g. clinical sample, extracted DNA, lab strains etc.
4. How many samples were used to validate the method?
5. What are the sensitivity and specificity of the primer?
6. Please provide a table for the new and reference primer.
7. Authors should write results and discussions together instead of results alone.
8. The authors should highlight the limitation of the method such as the number of samples used.
9. Please delete the word “my” in the conclusion.
10. Authors should consider improving the flow of writing.
11. Please update the references with the most recent articles.

Is the rationale for developing the new method (or application) clearly explained?

Partly

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

No

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: My area of expertise is microbiology and molecular biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Oct 2021

Modhusudon Shaha, National Institute of Biotechnology, Dhaka, Bangladesh

Thank you so much for your thoughtful and constructive comments on the manuscript.

- *Title of the manuscript – this is not a rapid antigen test using a kit or immunochromatographic test that can be done in 5 minutes. So, the word “Rapid” should remove from the title.*
- **Response:** The title is revised as ‘Detection of herpes simplex virus 2: a SYBR-Green-based real-time PCR assay’.
- *There are quite a few published articles that developed a real-time PCR method for the detection of HSV2. In the introduction, authors should acknowledge previously published articles and should describe what is lacking the previous method and why they are developing new methods.*
- **Response:** The required section is updated as per the reviewer’s recommendation.
- *What type of samples were used, e.g. clinical sample, extracted DNA, lab strains etc.*
- **Response:** We used an HSV2 lab strain and extracted DNA to perform the PCR assay. It has been updated in the Methods section of the manuscript.

- *How many samples were used to validate the method?*
- **Response:** We performed three experiments to confirm the specificity of the used primers to HSV2 strains. We also cross-checked the results with an HSV1 lab strain. The detailed results were described in the Results section of the manuscript.
- *What are the sensitivity and specificity of the primer?*
- **Response:** The primers were of improved sensitivity and specificity compared to the standard primer set that was used to amplify both HSV1 and HSV2 DNAs (described in the Results section). As we tried to declare a new HSV2-specific primer set, we did not determine the sensitivity and specificity of the primers.
- *Please provide a table for the new and reference primer.*
- **Response:** The designed primers used for this study were given in the data availability section.
- *Authors should write results and discussions together instead of results alone.*
- **Response:** The Results section is updated as Results and Discussion.
- *The authors should highlight the limitation of the method such as the number of samples were used.*
- **Response:** The manuscript was updated with the inclusion of the limitation statement.
- *Please delete the word "my" in the conclusion.*
- **Response:** Required section is updated.
- *Authors should consider improving the flow of writing.*
- **Response:** The manuscript was checked again and revised accordingly.

Competing Interests: No competing interests

Reviewer Report 07 September 2021

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ASM Monjur Al Hossain

Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Dhaka, Dhaka,

Bangladesh

First, I would like to thank all the authors for their nice write up. The authors explained the methods clearly. In introduction, the authors clearly describe the background of the project. They sum up the conclusion nicely. They also included necessary citations. However, I have some queries for the researchers:

1. In the methods section, what are the default parameters used in the Primer-BLAST tool for designing the primer?
2. What is the rationale behind selecting MH697422 as the reference one?
3. What are the sequences of primer-3?
4. In the results section, have you tried using different Mg++ concentrations, higher melting temp. to check whether the non-specific melting curve was still there or not?

Addressing these points, the manuscript can be indexed.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Drug delivery

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 18 Oct 2021

Modhusudon Shaha, National Institute of Biotechnology, Dhaka, Bangladesh

Thank you so much for your constructive comments on this. To design the appropriate primers, we used the default parameters of Primer-BLAST tool of NCBI database. More specifically, max Tm difference of 3°C (min Tm- 67°C and max Tm 63°C), PCR product size of min 70 bp, and max 200 bp. With regards to the use of MH697422 as a reference sequence, this accession contains the complete sequence of DNA polymerase gene of HSV2, on which we were interested.

The sequences of primer-3 are given in the data availability section which was deposited to Figshare. Additionally, the primer sequences are given below-

Forward

GATCTACAAGGTCCCGCTCG

Reverse

CACCATCCCGTTCACCTTGA

Regarding question 4, we used a commercially available PCR master mix (described in the Methods section) for PCR amplification. Therefore, we did not use different Mg++ concentrations.

Competing Interests: No competing interest

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