BioChem

Article

New Method of Isothermal, Hairpin Assisted, Primer Independent Amplification of DNA

Denis Sergeevich Naberezhnov [1,2,*], Alexander Andreevich Alferov [1], Yuriy Borisovich Kuzmin [1] and Nikolay Evgenievich Kushlinskii [1]

[1] N.N. Blokhin National Medical Research Center of Oncology, 115522 Moscow, Russia; aleksandr.alferov@yahoo.com (A.A.A.); yriikuzmin@yandex.com (Y.B.K.); kne3108@gmail.com (N.E.K.)
[2] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 119991 Moscow, Russia
* Correspondence: denis@naberezhnov.com; Tel.: +7-906-175-3259

Abstract: The isothermal amplification of nucleic acids refers to processes that quickly increase the amount of DNA at a constant temperature. These methods are mainly developed as alternatives to PCR for cases in which the application of a thermal cycler is not possible or the assay method must be as rapid as possible. We have developed a new method of isothermal amplification based on the formation of hairpins at the ends of DNA fragments containing palindromic sequences and increased by the hydrolysis of one or both DNA strands by restriction endonuclease, known as hairpin-assisted isothermal reaction (HAIR). The key steps in HAIR are the formation of a self-complementary hairpin and the DNA breakage introduced by nickase. The end hairpins facilitate primer-free amplification, the amplicon strand cleavage by nickase produces additional 3′ ends that serve as new amplification points, and the amount of DNA can increase exponentially. The rate of amplification in HAIR is more than five times the rate of loop-mediated isothermal amplification (LAMP), and the total amount of DNA product of HAIR is more than double the amount of the LAMP product.

Keywords: nucleic acid amplification; isothermal amplification; primer-free amplification; hairpin-assisted isothermal reaction

Citation: Naberezhnov, D.S.; Alferov, A.A.; Kuzmin, Y.B.; Kushlinskii, N.E. New Method of Isothermal, Hairpin Assisted, Primer Independent Amplification of DNA. *BioChem* **2023**, *3*, 142–152. https://doi.org/10.3390/biochem3030010

Academic Editors: Simona Panni, Luana Licata and Raffaella Gallo

Received: 27 April 2023
Revised: 3 July 2023
Accepted: 9 August 2023
Published: 19 September 2023

1. Introduction

PCR is one of the most popular and functional methods for the analysis of nucleic acids. However, this method has a number of disadvantages that limit its greater application. The disadvantages of PCR are mainly manifested when it is used for molecular diagnosis. It is necessary to have the special, oversized equipment of a thermal cycler. Another disadvantage is the relatively long time needed for the analysis procedure. These disadvantages led to the development of isothermal amplification methods [1,2]. Isothermal amplification does not require stages of cooling and heating, unlike PCR. Amplification is performed at a constant temperature, which decreases the length of the procedure because time is not spent heating and cooling. In addition, using some methods of isothermal amplification, the amount of DNA increases by more than double at each stage of amplification [3]; this reaction is faster than a PCR. The replacement of thermocycling, in some cases, also allows the use of simpler and portable equipment such as a thermostat instead of a thermocycler.

Several isothermal amplification methods have been developed. The most famous method is loop-mediated isothermal amplification (LAMP) [4,5]. Among the other methods are strand displacement amplification (SDA) [6], nicking enzyme amplification reaction (NEAR) [5,7], whole-genome amplification (WGA) [8–10], helicase-dependent amplification (HDA) [11], recombinase polymerase amplification (RPA) [12], rolling circle amplification (RCA) [13], and others [14]. The different isothermal amplification methods have very different principles. In these methods, different sets of different primers, different enzymes, and even different methods of product detection are used. However, all methods are

performed at a constant temperature, from 37 °C for HDA to 72 °C for most others. Moreover, DNA polymerase with strong strand displacement activity but without exonuclease activity is used in all isothermal amplification methods. Bst DNA polymerase and its modifications—Bsm DNA polymerase, phi29 DNA polymerase [15], and modified Taq polymerase [16]—are used in the isothermal amplification of DNA.

Isothermal amplification is seldom used in research because of the difficult choice of primers and amplification conditions in comparison with PCR. Moreover, the product of the reaction in many cases is not a linear PCR fragment but a branched conglomerate, concatemer, or single-stranded DNA, which is inconvenient for research purposes. However, the reaction procedures are simple, the methods of amplification are specific and sensitive if optimal conditions are selected, and a reaction mixture of optimal composition is obtained because isothermal amplification methods have become widespread in clinical practice. In many ways, the spread of this method was associated with the COVID-19 epidemic [17] because dealing with the pandemic required the most rapid methods for diagnosing a pathogen. The short analysis time is the main, but not only, advantage of isothermal DNA amplification methods. Unlike PCR, isothermal amplification methods do not require a thermal cycler or thermal cycler with real-time detection, although real-time signal detection is still required in some modified methods. An ordinary thermostat is sufficient for isothermal methods, and the signal can be detected visually. Another interesting feature of these isothermal methods is their increased tolerance to chemical inhibitors often present in clinical samples [18–21].

Currently, isothermal amplification methods are used in a wide number of fields [22]: the detection of various pathogens in clinical samples, particularly COVID-19 [17]; food and samples taken from the environment [23–25]; diagnostics of infectious diseases [26–30]; and polymorphism detection [31]. Some isothermal methods endorsed for particular applications have already been approved by regulatory agencies such as the Food and Drug Administration (FDA), the European Medicines Agency (EMA), and even the World Health Organization (WHO) through the Foundation for Innovative New Diagnostics (FIND) for the molecular diagnostics of critical pathogens [32]; this has attracted further attention from diagnostics-oriented biotech companies.

Thus, although isothermal amplification methods are not the "gold standard" of diagnostics in the same way as PCR, they have found their application in molecular diagnostics in cases where speed is the main requirement of the test system and when the analysis is required in field conditions.

We developed a novel method termed hairpin-assisted isothermal reaction (HAIR). The main feature of this method, distinguishing it from other types of amplification methods, is that the amplification process is performed without the use of primers. The primers are required to initiate amplification only; after initiation, the reaction proceeds spontaneously.

The sequence of the main (internal) pair of primers consists of the complementary target sequence, Nt.BstNBI nickase recognition sequence, and a palindrome sequence. The second pair of primers (outer primers) are analogous to the outer primers of LAMP and NEAR. A pair of outer primers anneals upstream of the binding site of the internal primers to displace the initial product strand from the DNA matrix. All primers have no further function once the amplification process has been initiated. A schematic of the main stages of amplification initiation is shown in Figure 1.

Internal primers anneal to the complementary part on the target sequence at the first stage (Figure 1a) followed by DNA polymerase synthesis of a complementary strand displacing the template strand (Figure 1b–d). A product with a terminal AT repeat at the 5′ end is formed (Figure 1d). The outer primer anneals on the DNA template (Figure 1d) and DNA polymerase synthesizes a new chain, displacing the product with the AT terminal repeat at the 3′ end (Figure 1e,f). The first internal primer anneals on single-stranded DNA containing the AT repeat at the 3′ end (Figure 1f). The complementary strand is displaced by DNA polymerase and a product containing repeats at both ends of DNA is formed (Figure 1g). The outer primer is annealed again (Figure 1g) and the strand containing

terminal repeats is displaced (Figure 1h,i), forming a product capable of self-amplification (Figure 1i,j). During the initiation of amplification, different products are formed, which can also act to initiate amplification. It should be noted that the described method is not the only possible means to initiate replication. Theoretically, any method for obtaining a DNA duplex with terminal repeats can be used to initiate replication. Such methods include PCR, HDA, and RPA.

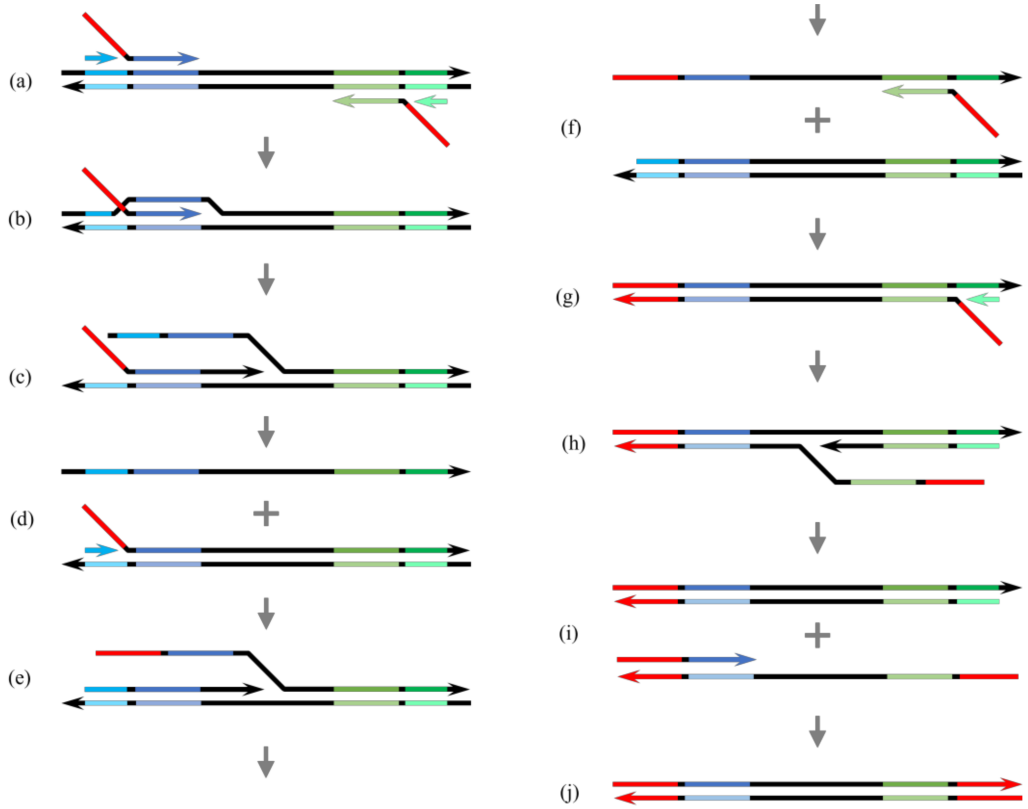


Figure 1. Initiation of HAIR amplification. Formation of a self-amplifying product. (**a**) Primer annealing sites; (**b**) local denaturation of the DNA template and annealing of the first internal primer; (**c**) displacement of the complementary strand by DNA polymerase; (**d**) completion of the synthesis of the strand with AT repeat synthesis and annealing of the first internal primer on the DNA template; (**e**) displacement of the strand with the AT repeat by DNA polymerase; (**f**) complete displacement of the strand with the AT repeat and annealing of the second internal primer; (**g**) formation of a DNA duplex with the AT repeat and annealing of the second outer primer; (**h**) displacement of the strand with two AT repeats; (**i**) complete displacement of the chain with two AT repeats and annealing of the first internal primer; (**j**) formation of a DNA duplex with terminal AT repeats capable of self-amplification.

The product containing AT repeats is capable of self-amplification (Figure 2). The form of the product containing AT terminal repeats is in equilibrium with the formed product in which the terminal repeats are turned inward to form hairpins (Figure 2b). Synthesis initiates in the hairpin form of DNA and the complementary strand is displaced by DNA polymerase (Figure 2c). DNA hairpins with a repeat at one end are formed (Figure 2d), which is also in equilibrium with the DNA duplex with end hairpins (Figure 2e) that are capable of self-amplification, after which the double-length product is formed (Figure 2f). The double-length product is cleaved by nickase (Figure 2g) and the DNA polymerase displaces the strand (Figure 2h), producing a single self-amplifying product (Figure 2i,j) similar to the initial product, and the cycle is repeated. The result of several cycles of self-amplification of the product that was not cut with nickase is a long concatemer, which is the main product of the reaction (Figure 2k). Many other products are also formed during

amplification, such as oligonucleotides consisting of AT repeats, but the concatemer is the main product of HAIR.

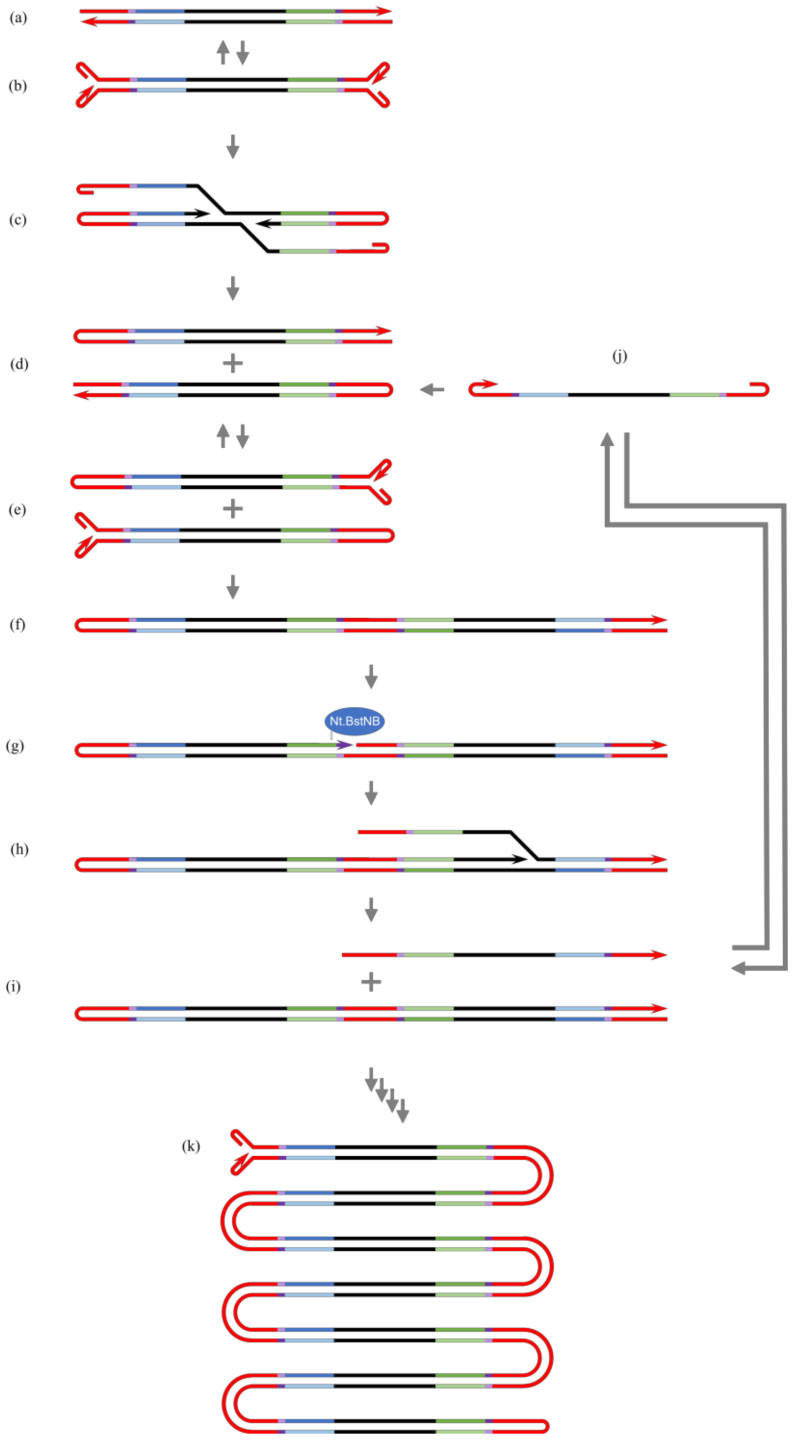


Figure 2. Self-amplification of a DNA duplex with terminal AT repeats. (**a**) Linear form of self-amplifying DNA duplex; (**b**) hairpin form of self-amplifying DNA duplex; (**c**) self-amplification of the DNA duplex and displacement of DNA chains; (**d**) formation of single hairpin-form products with one terminal AT repeat; (**e**) formation of hairpins from AT repeats and synthesis of the chain; (**f**) formation of a double-length product; (**g**) cleavage of the DNA duplex by nickase and formation of a new 3′ end; (**h**) displacement of the strand by DNA polymerase; (**i**) formation of a single-stranded monomeric form of the product; (**j**) annealing of the AT 3′ end repeats to themselves; (**k**) after several cycles of self-amplification without the participation of nickase, a long concatemer is formed.

In fact, amplification initiation and amplification itself are two different reactions, in which initiation is responsible for specificity and amplification itself for visualization. This duality may have potential advantages as it allows HAIR to be combined with NEAR, PCR, HDA, and RPA. In this case, the selection of amplification conditions (amplification initiation) seems to be more flexible because the researcher can choose the initiation method that is the most suitable option for their purposes. In this paper, we will use NEAR and PCR to obtain self-amplification of a DNA duplex with terminal repeats; then, we will show the characteristics of HAIR as an independent type of amplification and for amplification of the target sequence in the plasmid.

2. Materials and Methods

2.1. Materials

Sequences of DNA amplicon and primers are presented in the supplementary data in Table S1. Primers were synthesized by Lumiprobe (Moscow, Russia). Buffer mixes and reagents were purchased from the following manufacturers: Bst 2.0 DNA polymerase, Nt.BstNBI nickase from NEB (MA, USA), EvaGreen, and dNTP mix from Syntol (Moscow, Russia); FaiI restriction endonuclease, B buffer, ROSE buffer, and gel loading buffer from SibEnzyme (Novosibirsk, Russia); and agarose and TAE buffer from PanEco (Moscow, Russia).

2.2. HAIR Reaction Mix

HAIR amplification was carried out in a 20 μL reaction mixture containing 0.1 μM of each primer; 500 μM of each dNTP; 50 mM Tris-HCl, pH 8.5, 100 mM KCl; 2 mM $MgCl_2$; 0.1 u/ml Nt.BstNBI; and 0.1 u/ml Bst 2.0 polymerase. For real-time fluorescence-based monitoring of amplification, EvaGreen was added to the mastermix to a final concentration of 1×. The mixture was chilled on ice, Bst 2.0 DNA polymerase and Nt.BstNBI nickase were added directly to each PCR well, and amplification was immediately started at 56 °C.

2.3. Amplification of BRAF Gene Cloned in pUC19

The BRAF gene was amplified with the primers 24-F3c-P and 20-B3c-P, inserted into the multiple cloning site (MCS) of pUC19, digested with SmaI, and then used as a template. A total 1 ng of the resulting plasmid pUC19-BRAF_15ex was added to the HAIR reaction mix. pUC19-BRAF_15ex and controls were added in equimolar quantities. Fluorescence intensities were measured with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

2.4. Gel Electrophoresis

A 10 μL aliquot of reaction mix was mixed with 10× agarose gel loading buffer and subjected to electrophoretic analysis on a 0.5% agarose gel containing ethidium bromide. The amplification products were visualized using ChemiDoc Touch Gel Imaging System (Bio-Rad, USA).

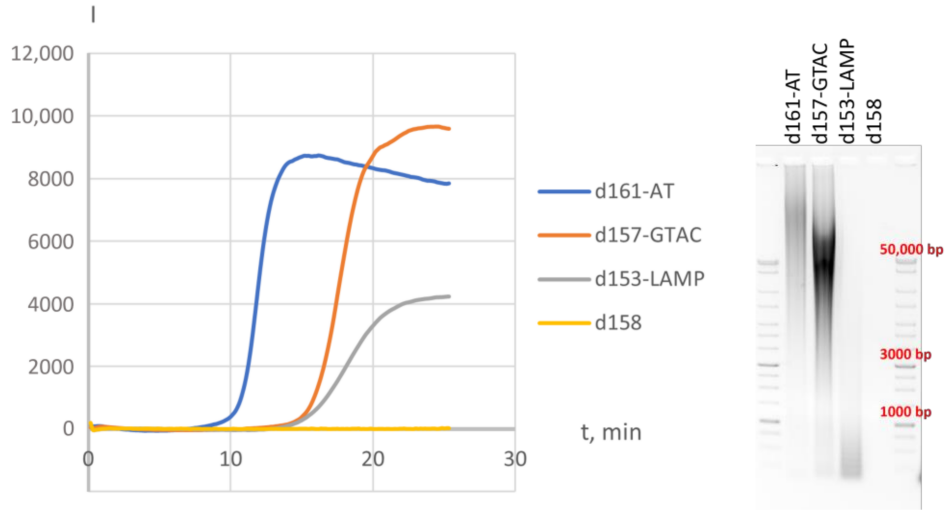
3. Results

3.1. Variations in HAIR

The key steps in HAIR are the formation of a self-complementary hairpin and the DNA breakage introduced by nickase. The end hairpins make primer-free amplification possible. However, in this case, the number of 3′ ends is reduced and the amount of DNA can only increase linearly. Strand digestion by nickase produces additional 3′ ends that serve as new amplification points, and the amount of DNA can increase exponentially.

Different sequences of the end hairpin of self-amplifying products of HAIR can be used. We tested AT and GTAC repeats as terminal repeats. DNA duplexes containing fifteen AT repeats (d161-AT) and seven GTAC repeats (d157-GTAC) were obtained using standard PCR and added at equimolar concentrations to the mixture containing buffer components, nucleoside triphosphates, Bst polymerase, and nickase but not containing primers.

The amplification reaction mixture was incubated at 56 °C, which is optimal for Nt.BstNBI. The DNA duplex without repeats (d158) and the LAMP amplicon (d153-LAMP) were used as controls. Both DNA duplexes (d161-AT and d157-GTAC) are efficient at self-amplification (Figure 3) but d161-AT amplifies faster than d157-GTAC. It should be noted that the rate of HAIR exceeds the rate of LAMP. This may be because the rate of folding of the end hairpin exceeds the rate of primer annealing. It is impossible to accurately compare the rate of these processes as they use different principles of amplification. The rate of self-amplification of d157-GTAC is more than four times that of d153-LAMP, and the rate of self-amplification of d161-AT is more than five times that of d153-LAMP. Moreover, the total amount of DNA produced by HAIR is more than double the amount of that produced by LAMP because the amount of product is limited by the number of nucleosides rather than triphosphates. The product of HAIR is a concatemer over 50 kb (Figure 3b) for both cases of repeats, which corresponds to a concatemer of more than 500 monomers. In the case of the AT repeat, amplification is faster than for the GTAC repeat, which is likely due to the lower melting temperature of the sequence and large number of variants of hairpin formations.



(**a**) (**b**)

Figure 3. Comparison of the amplification of DNA duplexes containing AT and GTAC terminal repeats and LAMP amplicon using (**a**) real-time amplification and (**b**) agarose gel electrophoresis.

We studied the kinetics of the accumulation of the product of HAIR and the formation of the reaction product (Figure 4). The kinetics were first studied in the absence of nickase and then after its addition using agarose gel electrophoresis. When DNA polymerase was added, the band of the DNA duplex became blurry. This means that the length of the reaction products is heterogeneous. This, in turn, means the rate of turning of DNA ends to hairpins is heterogenous or that inactive DNA duplexes are formed. Full-length concatemers are formed after 20 min of incubation. The length of concatemers is very different, from a few monomers to several thousand monomers. After the addition of nickase, the amount of product increases exponentially and reaches a maximum after 8 min of incubation. It should also be noted that, in this case, the concatemers are shorter than concatemers obtained through the simultaneous addition of polymerase and nickase. The length of concatemers is no more than 20 residues versus more than 500 monomers in the standard mix.

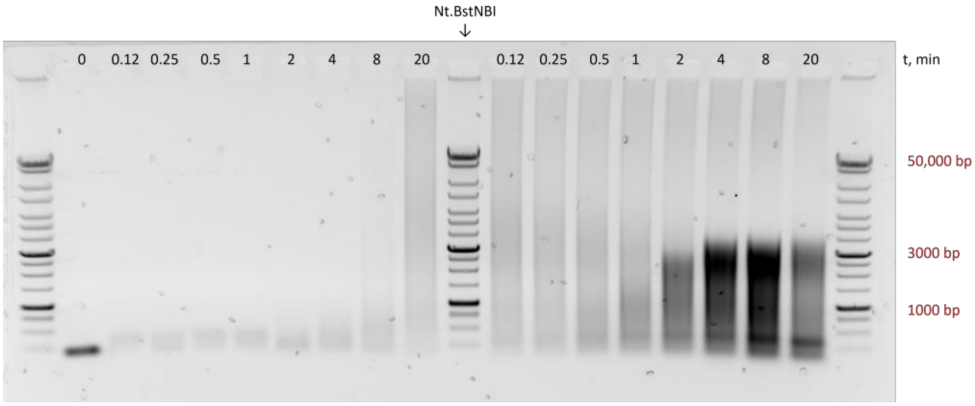
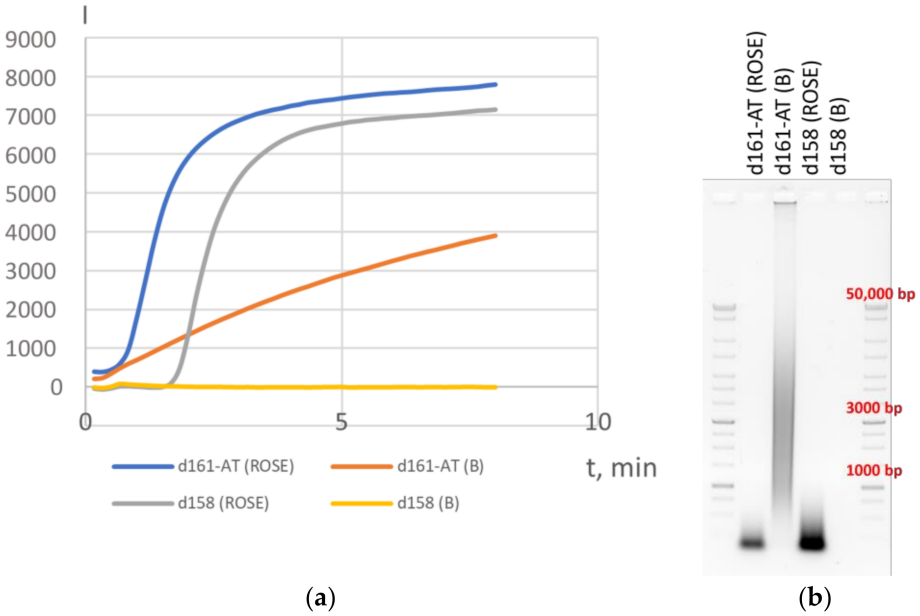


Figure 4. Time dependence of the amount and type of product of amplification.

Additional 3′ ends can be added by DNA double-strand breaks separately from DNA single-strand breaks. Concatemers can be digested by restriction endonuclease in the AT sequence. The AT repeat can be hydrolyzed by the thermostable restriction endonuclease FaiI. This restriction endonuclease can be used in the reaction. Cleavage by restriction endonuclease leads to the shortening of the terminal repeats after every amplification cycle; however, AT repeats can be annealed with each other and lengthen the AT sequence. In this case, the product of amplification is a short rather than a long concatemer. The disadvantage of FaiI is the need for different optimal buffers for FaiI and Bst polymerases: the FaiI restriction endonuclease is not active in the Bst polymerase buffer (isothermal amplification buffer), and the Bst polymerase has low activity in the FaiI restriction endonuclease buffer (B). We tested the possibility of self-amplification of d161-AT in buffer B and ROSE buffer, in which FaiI is 100% active. The DNA duplex d158 without repeats was used as a control. The optimal working temperature of FaiI is 50 °C, but it is also highly active at higher temperatures (up to 68 °C). The results of amplification and gel electrophoresis are shown in Figure 5.



(**a**) (**b**)

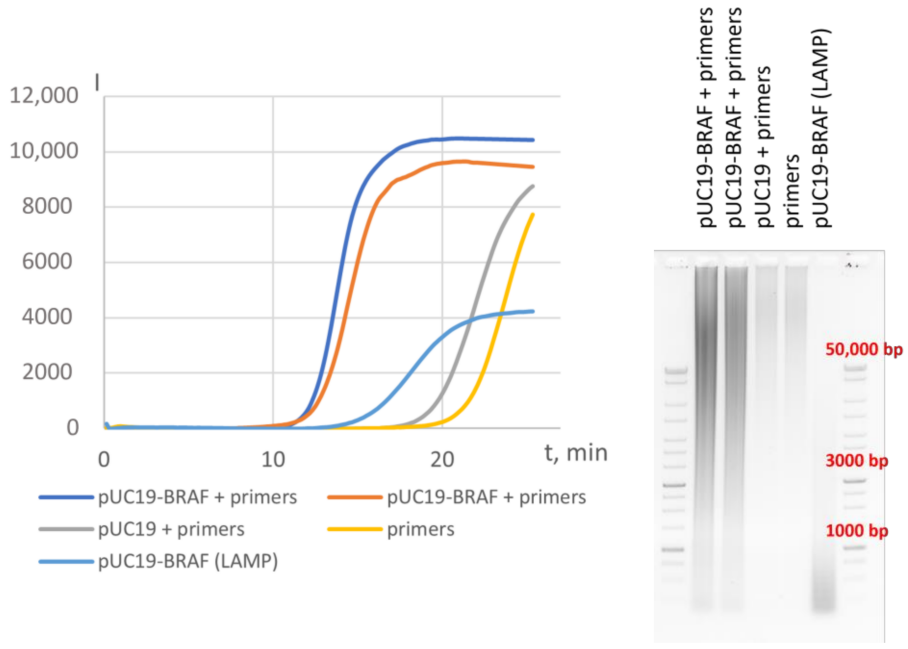
Figure 5. Comparison of amplification of DNA duplexes in the presence of restriction endonuclease FaiI using (**a**) real-time amplification and (**b**) agarose gel electrophoresis.

The d161-AT is amplified linearly in buffer B and exponentially in ROSE buffer. However, the control DNA duplex d158, which does not contain repeats, is amplified in the ROSE buffer, but the result of the amplification is a high-molecular-weight product. The

composition of the ROSE buffer is unknown. This buffer may contain nucleic acids. Therefore, components of the buffer may be amplified rather than the control DNA duplex. This is supported by the fact that the amplification of d158 and blank reaction mix is a simultaneous amplification (data not shown). Thus, despite the fact that the use of the restriction endonuclease FaiI may be preferable to the use of the nicking endonuclease Nt.BstNBI due to the thermal stability of FaiI and the possibility of varying the amplification temperature, the use of FaiI in HAIR requires the selection of the buffer composition.

3.2. Application of HAIR for Target Determination

To test the possibility of the application of HAIR, we used a plasmid with a cloned BRAF gene sequence. The plasmid without the BRAF gene sequence was used as a control. Controls without the DNA template and LAMP method were also used. Results of HAIR are shown in Figure 6.



(**a**) (**b**)

Figure 6. Amplification of the cloned BRAF gene using (**a**) real-time amplification and (**b**) agarose gel electrophoresis.

The product of HAIR amplified after 10 min of incubation (Figure 6a) and the concatemer (Figure 6b) was similar to the d161-AT self-amplification concatemer. A non-specific product was amplified after 20 min of incubation and was probably the result of self-hybridization of primers because it appeared simultaneously in the controls without DNA template. However, the non-specific amplicon has a different molecular weight from the proper products (Figure 6b). Tiny time differences between the amplification of specific and non-specific amplicons were expected because they are typical for all types of amplification that use a nicking enzyme [33].

4. Discussion

HAIR, similar to parvovirus replication, is a self-priming rotating hairpin replication (RHR) [34]. The HAIR amplicon consists of terminal repeats, a nick site, and a target sequence, analogous to the genome of the parvovirus. AT repeats are similar to inverted terminal repeats in the parvovirus genome, and the results of Nt.BstNBI nickase are similar to NS1 protein-induced single-stranded DNA breaks. The amplification mechanism also resembles parvovirus replication. Attempts to use a process similar to parvovirus replication for DNA amplification have been made in the past [35,36]; however, the resulting

amplification methods were extremely inefficient due to the use of non-repeating sequences as terminal repeats.

Hairpin-assisted isothermal reactions mainly rely on several molecular biological processes. The first is the formation of hairpins at the ends of the DNA in the form of a fork, in which the 3′ and 5′ ends of the DNA are turned inward. Two main mechanisms for the formation of such forms have been proposed (type S and type C) [37,38]: the first (type S) implies the small-scale melting of the double helix; the other mechanism (type C) involves the melting of a large region, which is favored by nearby AT-rich sequences. This large region of melting would allow hairpins to fold on both strands, leading to cruciform formation. Presumably, hairpin formation in HAIR proceeds according to the type C mechanism and is related to dsDNA fluctuations (or "breathing")—that is, the spontaneous denaturation–renaturation of DNA. It is specifically manifested at the ends in AT-rich DNA regions and with an increase in temperature [39]. If DNA segments contain palindromic sequences, the formation of a secondary structure in the form of hairpins is possible. One of the hairpins has a 3′ end that can act as a primer for DNA polymerase. Another process used by HAIR is to digest one of the DNA's strands by nicking endonuclease exposing the 3′ end. DNA polymerase starts from this nick, uses another uncleaved strand as a template to synthesize a new double-stranded nucleic acid, and displaces the old strand to serve as a template for the next round of nucleic acid synthesis. The single-strand breaking of DNA produces new, additional active 3′ ends containing the AT repeat and the reaction of amplification is accelerated. This mechanism is important in SDA and NEAR, from which it follows that HAIR is a variety of this amplification.

Unfortunately, the similarity of the mechanism of amplification of HAIR with NEAR makes it prone to the disadvantages of NEAR. This can be observed when applying HAIR for BRAF amplification (Figure 6a). A non-specific amplification appears 10 min after target amplification, although the relative time of the non-specific amplification is more than twice the time of specific amplification. This result is not a surprise and is well-described in the literature [33]. However, since the specificity of HAIR mainly depends on the stage of initiation, and NEAR, PCR, HDA, or RPA can potentially elicit the initiation of HAIR, each of these can be used to increase the specificity of the reaction. NEAR, HDA, and RPA are more suitable for initiation because they use similar amplification conditions to HAIR, i.e., a strand-displacement DNA polymerase and a compatible buffer. In addition, NEAR, HDA, and RPA can be more specific in this case than when they are used alone. This is because of the lower primer concentration required to initiate amplification, which is known to increase the specificity of amplification [40]. Thus, HAIR should be considered primarily as a DNA amplification method for those cases in which primers prevent the reaction of DNA amplification for any reason. For example, primers can be fixed to a solid state because they are only needed to initiate amplification. The advantage of immobilized primers is that their interaction with each other is physically excluded and the formation of primer dimers (a problem for all types of amplification) is prevented. At the same time, the immobilization of primers should in no way affect the speed and efficiency of amplification reaction since primers do not participate in the reaction itself. Therefore, the immobilization of primers can increase the specificity of the amplification.

5. Conclusions

HAIR is a new and interesting type of amplification for science and for clinical application. The scientific value of HAIR lies in the fact that this is the first effective type of amplification of DNA that is independent of primers. The amplification requires only enzymes and nucleoside triphosphates. An interesting feature of HAIR is the high rate of amplification, which is more than five times that of LAMP. HAIR sheds light on the mechanism of the formation of terminal hairpins of DNA. The process of hairpin formation has not yet been studied, so we can assume that this process is slow and inefficient; however, the high rate of HAIR amplification suggests otherwise, which was not known before. The remarkably fast rate of HAIR also allows us to consider it as a new molecular

diagnostic method. This reaction is compatible with a nickase-cleavable fluorescent beacon [41]; therefore, the amplified product could be unambiguously identified by real-time amplification. At the same time, HAIR, as a type of nicking enzyme amplification, has many disadvantages, such as difficulties in primer selection, the amplification of non-specific products, and the inability to vary the amplification temperature [33,42]. However, these difficulties can be overcome by careful selection of amplification conditions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biochem3030010/s1. Table S1: Sequences of DNA amplicon and primers.

Author Contributions: D.S.N., investigation, resources, and writing—review and editing; Y.B.K., methodology; A.A.A., methodology; N.E.K., supervision, data curation, resources, methodology, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Asiello, P.J.; Baeumner, A.J. Miniaturized Isothermal Nucleic Acid Amplification, a Review. *Lab Chip* **2011**, *11*, 1420–1430. [CrossRef]
2. Kim, J.; Easley, C.J. Isothermal DNA Amplification in Bioanalysis: Strategies and Applications. *Bioanalysis* **2011**, *3*, 227–239. [CrossRef] [PubMed]
3. Savonnet, M.; Aubret, M.; Laurent, P.; Roupioz, Y.; Cubizolles, M.; Buhot, A. Kinetics of Isothermal Dumbbell Exponential Amplification: Effects of Mix Composition on LAMP and Its Derivatives. *Biosensors* **2022**, *12*, 346. [CrossRef] [PubMed]
4. Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28*, e63. [CrossRef] [PubMed]
5. Van Ness, J.; Van Ness, L.K.; Galas, D.J. Isothermal Reactions for the Amplification of Oligonucleotides. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 4504–4509. [CrossRef] [PubMed]
6. Walker, G.T.; Fraiser, M.S.; Schram, J.L.; Little, M.C.; Nadeau, J.G.; Malinowski, D.P. Strand Displacement Amplification—An Isothermal, in Vitro DNA Amplification Technique. *Nucleic Acids Res.* **1992**, *20*, 1691–1696. [CrossRef] [PubMed]
7. Qian, C.; Wang, R.; Wu, H.; Ji, F.; Wu, J. Nicking Enzyme-Assisted Amplification (NEAA) Technology and Its Applications: A Review. *Anal. Chim. Acta* **2019**, *1050*, 1–15. [CrossRef]
8. Li, Y.; Kim, H.-J.; Zheng, C.; Chow, W.H.A.; Lim, J.; Keenan, B.; Pan, X.; Lemieux, B.; Kong, H. Primase-Based Whole Genome Amplification. *Nucleic Acids Res.* **2008**, *36*, e79. [CrossRef]
9. Dean, F.B.; Nelson, J.R.; Giesler, T.L.; Lasken, R.S. Rapid Amplification of Plasmid and Phage DNA Using Phi29 DNA Polymerase and Multiply-Primed Rolling Circle Amplification. *Genome Res.* **2001**, *11*, 1095–1099. [CrossRef]
10. Deleye, L.; Tilleman, L.; Vander Plaetsen, A.-S.; Cornelis, S.; Deforce, D.; Van Nieuwerburgh, F. Performance of Four Modern Whole Genome Amplification Methods for Copy Number Variant Detection in Single Cells. *Sci. Rep.* **2017**, *7*, 3422. [CrossRef]
11. Vincent, M.; Xu, Y.; Kong, H. Helicase-Dependent Isothermal DNA Amplification. *EMBO Rep.* **2004**, *5*, 795–800. [CrossRef]
12. Piepenburg, O.; Williams, C.H.; Stemple, D.L.; Armes, N.A. DNA Detection Using Recombination Proteins. *PLoS Biol.* **2006**, *4*, e204. [CrossRef]
13. Goo, N.-I.; Kim, D.-E. Rolling Circle Amplification as Isothermal Gene Amplification in Molecular Diagnostics. *BioChip J.* **2016**, *10*, 262–271. [CrossRef]
14. Бодулев, О.Л.; Сахаров, И.Ю. Изотермические Методы Амплификации Нуклеиновых Кислот И Их Применение В Биоанализе. Биохимия **2020**, *85*, 174–196. [CrossRef]
15. Which DNA Polymerase Is Best for My Isothermal Amplification Reaction? | NEB. Available online: https://international.neb.com/faqs/2013/10/24/which-dna-polymerase-is-best-for-my-isothermal-amplification-reaction (accessed on 14 October 2022).
16. Ignatov, K.B.; Barsova, E.V.; Fradkov, A.F.; Blagodatskikh, K.A.; Kramarova, T.V.; Kramarov, V.M. A Strong Strand Displacement Activity of Thermostable DNA Polymerase Markedly Improves the Results of DNA Amplification. *Biotechniques* **2014**, *57*, 81–87. [CrossRef]
17. De Felice, M.; De Falco, M.; Zappi, D.; Antonacci, A.; Scognamiglio, V. Isothermal Amplification-Assisted Diagnostics for COVID-19. *Biosens. Bioelectron.* **2022**, *205*, 114101. [CrossRef] [PubMed]

18. Bachmann, L.H.; Johnson, R.E.; Cheng, H.; Markowitz, L.E.; Papp, J.R.; Hook, E.W. Nucleic Acid Amplification Tests for Diagnosis of Neisseria Gonorrhoeae Oropharyngeal Infections. *J. Clin. Microbiol.* **2009**, *47*, 902–907. [CrossRef] [PubMed]
19. Craw, P.; Mackay, R.E.; Naveenathayalan, A.; Hudson, C.; Branavan, M.; Sadiq, S.T.; Balachandran, W. A Simple, Low-Cost Platform for Real-Time Isothermal Nucleic Acid Amplification. *Sensors* **2015**, *15*, 23418–23430. [CrossRef]
20. Kaneko, H.; Kawana, T.; Fukushima, E.; Suzutani, T. Tolerance of Loop-Mediated Isothermal Amplification to a Culture Medium and Biological Substances. *J. Biochem. Biophys. Methods* **2007**, *70*, 499–501. [CrossRef]
21. Mori, Y.; Notomi, T. Loop-Mediated Isothermal Amplification (LAMP): A Rapid, Accurate, and Cost-Effective Diagnostic Method for Infectious Diseases. *J. Infect. Chemother.* **2009**, *15*, 62–69. [CrossRef]
22. Oliveira, B.B.; Veigas, B.; Baptista, P.V. Isothermal Amplification of Nucleic Acids: The Race for the Next "Gold Standard". *Front. Sens.* **2021**, *2*, 752600. [CrossRef]
23. Mahmoudian, L.; Melin, J.; Mohamadi, M.R.; Yamada, K.; Ohta, M.; Kaji, N.; Tokeshi, M.; Nilsson, M.; Baba, Y. Microchip Electrophoresis for Specific Gene Detection of the Pathogenic Bacteria V. Cholerae by Circle-to-Circle Amplification. *Anal. Sci.* **2008**, *24*, 327–332. [CrossRef]
24. Zhao, Y.; Chen, F.; Li, Q.; Wang, L.; Fan, C. Isothermal Amplification of Nucleic Acids. *Chem. Rev.* **2015**, *115*, 12491–12545. [CrossRef]
25. Chow, W.H.A.; McCloskey, C.; Tong, Y.; Hu, L.; You, Q.; Kelly, C.P.; Kong, H.; Tang, Y.-W.; Tang, W. Application of Isothermal Helicase-Dependent Amplification with a Disposable Detection Device in a Simple Sensitive Stool Test for Toxigenic Clostridium Difficile. *J. Mol. Diagn.* **2008**, *10*, 452–458. [CrossRef] [PubMed]
26. Lee, S.Y.; Lee, C.N.; Mark, H.; Meldrum, D.R.; Lin, C.W. Efficient, Specific, Compact Hepatitis B Diagnostic Device: Optical Detection of the Hepatitis B Virus by Isothermal Amplification. *Sens. Actuators B Chem.* **2007**, *127*, 598–605. [CrossRef]
27. Torres-Chavolla, E.; Alocilja, E.C. Nanoparticle Based DNA Biosensor for Tuberculosis Detection Using Thermophilic Helicase-Dependent Isothermal Amplification. *Biosens. Bioelectron.* **2011**, *26*, 4614–4618. [CrossRef] [PubMed]
28. Rohrman, B.; Richards-Kortum, R. Inhibition of Recombinase Polymerase Amplification by Background DNA: A Lateral Flow-Based Method for Enriching Target DNA. *Anal. Chem.* **2015**, *87*, 1963–1967. [CrossRef] [PubMed]
29. Hellyer, T.J.; Nadeau, J.G. Strand Displacement Amplification: A Versatile Tool for Molecular Diagnostics. *Expert Rev. Mol. Diagn.* **2004**, *4*, 251–261. [CrossRef]
30. Mycobacterium Tuberculosis Detection via Rolling Circle Amplification—Analytical Methods (RSC Publishing). Available online: https://pubs.rsc.org/en/content/articlelanding/2011/ay/c0ay00529k (accessed on 20 July 2023).
31. Li, J.; Deng, T.; Chu, X.; Yang, R.; Jiang, J.; Shen, G.; Yu, R. Rolling Circle Amplification Combined with Gold Nanoparticle Aggregates for Highly Sensitive Identification of Single-Nucleotide Polymorphisms. *Anal. Chem.* **2010**, *82*, 2811–2816. [CrossRef]
32. Guichón, A.; Chiparelli, H.; Martínez, A.; Rodríguez, C.; Trento, A.; Russi, J.C.; Carballal, G. Evaluation of a New NASBA Assay for the Qualitative Detection of Hepatitis C Virus Based on the NucliSens Basic Kit Reagents. *J. Clin. Virol.* **2004**, *29*, 84–91. [CrossRef]
33. Tan, E.; Erwin, B.; Dames, S.; Ferguson, T.; Buechel, M.; Irvine, B.; Voelkerding, K.; Niemz, A. Specific versus Nonspecific Isothermal DNA Amplification through Thermophilic Polymerase and Nicking Enzyme Activities. *Biochemistry* **2008**, *47*, 9987–9999. [CrossRef]
34. Tattersall, P.; Ward, D.C. Rolling Hairpin Model for Replication of Parvovirus and Linear Chromosomal DNA. *Nature* **1976**, *263*, 106–109. [CrossRef] [PubMed]
35. Park, D.; Ellington, A.D.; Jung, C. Selection of Self-Priming Molecular Replicators. *Nucleic Acids Res.* **2019**, *47*, 2169–2176. [CrossRef] [PubMed]
36. Jung, C.; Ellington, A.D. A Primerless Molecular Diagnostic: Phosphorothioated-Terminal Hairpin Formation and Self-Priming Extension (PS-THSP). *Anal. Bioanal. Chem.* **2016**, *408*, 8583–8591. [CrossRef] [PubMed]
37. Bikard, D.; Loot, C.; Baharoglu, Z.; Mazel, D. Folded DNA in Action: Hairpin Formation and Biological Functions in Prokaryotes. *Microbiol. Mol. Biol. Rev.* **2010**, *74*, 570–588. [CrossRef] [PubMed]
38. Lilley, D.M. The Kinetic Properties of Cruciform Extrusion Are Determined by DNA Base-Sequence. *Nucleic Acids Res.* **1985**, *13*, 1443–1465. [CrossRef]
39. von Hippel, P.H.; Johnson, N.P.; Marcus, A.H. 50 Years of DNA 'Breathing': Reflections on Old and New Approaches. *Biopolymers* **2013**, *99*, 923–954. [CrossRef]
40. Wang, D.-G.; Brewster, J.D.; Paul, M.; Tomasula, P.M. Two Methods for Increased Specificity and Sensitivity in Loop-Mediated Isothermal Amplification. *Molecules* **2015**, *20*, 6048–6059. [CrossRef]
41. Zheleznaya, L.A.; Perevyazova, T.A.; Zheleznyakova, E.N.; Matvienko, N.I. Some Properties of Site-Specific Nickase BspD6I and the Possibility of Its Use in Hybridization Analysis of DNA. *Biochemistry* **2002**, *67*, 498–502. [CrossRef]
42. Wang, L.; Qian, C.; Wu, H.; Qian, W.; Wang, R.; Wu, J. Technical Aspects of Nicking Enzyme Assisted Amplification. *Analyst* **2018**, *143*, 1444–1453. [CrossRef]