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Rapid Screening Methods for Universal Binding Peptide Aptamers Against SARS-CoV-2 Variant Spikes, Including Omicron Variants, and their Application to Diagnostic and Therapeutic Agents

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Abstract

The development of mRNA vaccines and oral drugs against SARS-CoV-2 has been useful in protecting against Covid-19 infection. Since then, however, many variants of delta and omicron strains with enhanced infectivity and immune escape capacity have emerged.

A 7-amino acid random peptide ribosome display library screening system was used to perform a rapid in vitro screening of peptide aptamers that universally bind to the SARS-CoV-2 wild-type, delta, and Omicron variant BA.1, BA.2, and BA.5 spike RBD (Receptor Binding Domain).

Screening resulted in four peptide aptamers that showed positive binding reactions in ELISA.

Interestingly, Amino Acid Sequence Determination of the four clones predicted that three of the four clones contain 2~3 Cys residues in their sequences, forming a complex higher-order structure with disulfide (S-S) bonds.

The 7-amino acid random peptide ribosome display library screening system allows for rapid in vitro screening of peptide aptamers that bind to other unknown emerging infectious disease pathogens that may be pandemic in the future.

The peptide aptamers are as small as 30 amino acids and can be easily synthesized and purified as peptides or proteins, or simply used as mRNA drugs.

Keywords: SARS-CoV-2; Variant; Omicron; Universal; Binding; Peptide aptamers; 7-amino acid random peptide ribosome display library screening system

Introduction

The Covid-19 pandemic is caused by SARS-COV-2. To date, more than 664 million people have been affected and 6.71 million have died [1-27].

An mRNA vaccine has been developed and has shown remarkable immunopreventive effects.

However, the emergence of SARS-COV-2 variants with increased infectivity, immune evasion, and altered virulence has continued since then in many parts of the world, and pandemics of these variants continue to occur [1-27].

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With continued mRNA vaccination, neutralizing antibody titers against the SARS-COV-2 variant could be maintained, but with the emergence of new variants appearing one after another, it becomes difficult to maintain neutralizing antibody titers.

The Omicron variant of the highly infectious SARS-COV-2 mutant, which emerged at the end of 2021, began as BA.1 and has mutated to BA.2 and BA.5. In January 2023, a BQ.1.1 strain derived from BA.5 and an XBB strain derived from BA.2 have emerged, although BA.5 is still the mainstream strain [1-31]. Furthermore, with the elimination of the zero-corona policy in China beginning in December 2022, it is estimated that there is an explosion of more than 900 million more infections at this point, and it is feared that new SARS-COV-2 variants, especially new Omicron lineages, may emerge in the future. Currently, the majority of cases are of the omicron type, which mainly infects the nasopharynx, but it is feared that the emergence of a new type, such as the delta type, with added lung invasiveness, would be a worldwide catastrophe.

Currently, the Omicron strain-compatible bivalent vaccine is used to address prevention of infection and severe disease. And in addition to the mRNA vaccine, the oral drugs Molnupiravir (Lagevrio, Merck) and nirmatrelvir+ritonavir (Paxlovid, Pfizer) are used for treatment [32-40].

As for antibody drugs, some of them have high therapeutic efficacy, but with the emergence of each new variant, the neutralizing antibody activity against the spike decreases, resulting in a short efficacy period, and it is necessary to constantly develop antibody drugs tailored to the new variant [4,41-54].

Antibody drugs are very effective in the treatment of immunocompromised patients with mild disease and risk factors such as severe cardiac disease, chronic respiratory disease, and obesity.

Antibody drugs are also expected to be effective in reducing the incidence of the disease in those who cannot be expected to acquire sufficient immunity through vaccination and in facilities with many elderly people with low basic physical ability.

Antibody drugs, by their nature as macromolecular biopharmaceuticals, take a long time to develop, and have problems such as difficulties in production and purification and their cost.

In addition, the constant use of antibody drugs for treatment is difficult because of the decreasing efficacy of antibody drugs with each new variant.

In recent years, attention has focused on the development of universal antibodies with neutralizing activity against several variants, but the emergence of new variants has weakened the neutralizing activity [55-70].

The omicron variant in particular is very strongly immuno evasive, and it is assumed that the spike mutant has an altered structure that makes it difficult for neutralizing antibodies to bind to it.

Antibody drugs bind easily to the convex surface of a molecule, but lose their binding ability when a conformational change occurs due to amino acid mutation on the surface of the molecule.

Molecular concavities, grooves, and clefts are well conserved and sometimes have enzyme active centers. However, molecular concavities, grooves, and clefts are places where amino acid mutations are less likely to occur, but also where macromolecular antibodies cannot penetrate and bind.

Therefore, in this study, we attempted in vitro screening of "7-amino acid peptide aptamers" that can bind not only to convex surfaces of molecules but also to concave surfaces, grooves, and clefts.

For screening, the ribosomal display method [71-81], which links genes and protein molecules on a one-to-one basis, was employed, allowing for larger library sizes.

In this ribosome display method [71-81], the stop codon of the gene DNA is omitted and in vitro transcription and translation are performed in a cell-free protein synthesis system to create a protein-mRNA-ribosome complex (PRM complex, ternary complex), followed by affinity selection against the target antigen, and clones that bind to the target antigen are purified.

Affinity selection (panning) (71, 72, 81–84) can be done once a day, and even if panning is repeated six times, rapid screening can be done in about a week's process.

A 7-amino acid random peptide ribosome display library [71-81] was constructed and screened for peptide aptamers that bind to the SARS-CoV-2 wild-type spike RBDs. Among them, we selected peptide aptamers that can bind not only wild-type spike RBDs but also delta variant, omicron variant, BA.1, BA.2, and BA.5 spike RBDs.

The peptide aptamers selected in this screening bind to the common epitope of the spike RBD of SARS-CoV-2 variants, including the omicron variant, and are potential universal binding peptide aptamers for all SARS-CoV-2 variants.

These peptide aptamers are approximately 30 amino acids in length, including a 7 amino acid binding site and a surrounding scaffold sequence. They can be easily obtained by peptide synthesis and purification. In addition, peptide aptamer proteins can be easily synthesized and purified

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in bacteria such as E. coli. These peptide aptamers can be diagnostic and therapeutic agents.

Recently, in the very early stages of an emerging infectious disease outbreak with pathogenic potential, the identification of the pathogen by Next-generation sequencing is used to identify the pathogen and its component proteins.

While the development of diagnostics can be done quickly with PCR and other genetic diagnostics, the development of diagnostics and therapeutics for pathogen proteins requires the complicated, expensive, and time-consuming process of antibody production.

In such cases, antibody production becomes even more difficult for pathogens that repeatedly mutate, such as the SARS-CoV-2 variant in this case.

In this study, we screened universal binding peptide aptamers against SARS-CoV-2 variants using a 7-amino acid random peptide ribosome display library screening system, demonstrating the utility of this method for screening peptide aptamers as antibody alternatives.

Materials and Methods

The structure of 7-amino acid random peptide ribosome display library (GW019PAL); (Figure 1)

The 7-amino acid random peptide ribosome display library (GW019PAL) shown in Figure 1 was provided by GeneWorld Inc. The structure is T7 promoter, SD sequence, GS linker, scafold1 sequence (SF1), 7 amino acid random peptide, scafold2 sequence (SF2), GS linker, followed by human fibronectin (2177-2250) lacking stop codon [71-81.

Immobilization of SARS-CoV-2 spike RBD antigens on Magnetic Beads

SARS-CoV-2 spike RBD; wild type (TAIYO NIPPON SANSO Corporation) was immobilized on NHS-Activated Magnetic Beads (Pierce™ NHS-Activated Magnetic Beads, 88826 [85]). (according to the product manual).

Affinity selection(Panning) [71,72,81-84]; (Figure 2)

Protein synthesis was performed by in vitro transcription and translation reactions using an E. coli cell-free protein synthesis system (TAIYO NIPPON SANSO Corporation, Cell-free Musaibou-Kun; N Mini SS, A238-0303) (according to the product manual).

The 7-amino acid random peptide ribosome display library (GW019PAL) is lacking a stop codon, resulting in the formation of a protein (peptide)-ribosome-mRNA complex (PRM complex, ternary complex).

The PRM complex is mixed with the above SARS-CoV-2 wild-type spike RBD (Taiyo Nippon Sanso) immobilized on Magnetic Beads and reacted at 4°C for 1 hour. After the reaction, the Magnetic Beads are washed 10 times with ice-cold PBS, the mRNA is dissociated and purified, and RT-PCR (30 cycles) is performed to produce a purified peptide ribosome display library construct. (This process is called the affinity selection cycle, or Panning).

This affinity selection cycle (Panning) was repeated for 6 cycles to purify peptide aptamers that bind to SARS-CoV-2 spike RBD; wild type (TAIYO NIPPON SANSO Corporation).

Subcloning into the E. coli expression vector pT7-FLAG-1 (Sigma-Aldrich;P1118) [86,87] and peptide aptamer protein synthesis and purification

The region flanking the peptide aptamer of the 6th panning RT-PCR product peptide ribosome display library construct is PCR amplified with primers containing NotI, XmaI restriction enzyme sites and subcloned into NotI, XmaI restriction site of the E. coli expression vector pT7-FLAG-1.

After sonication and centrifugation of the bacterial resuspension, the supernatant was purified for peptide aptamers (proteins) using an anti-FLAG M2 antibody affinity gel (Sigma-Aldrich; A2220) [88]. (Refer to the product manual.)

ELISA [89-102]

SARS-CoV-2 spike RBD protein at a concentration of 1ug/ml; wild type, delta (TAIYO NIPPON SANSO Corporation), BA.1 (Sino Biological,40592-V08H121), BA.2 (AcroBioSystems, SPD-C522g), BA.5 (Sino Biological,40592-V08H131) are immobilized on an ELISA separator plate (Sumitomo Bakelite, MS-8508M).

ELISA was performed by direct method. Plates are immobilized with each of SARS-CoV-2 wild type, delta, BA.1, BA.2, and BA.5 spike RBD proteins, and peptide aptamers after flag-tag purification (cloned proteins purified by panning and protein synthesized) are reacted at 37°C for 30 minutes and then washed.

Further, Anti-DDDDK-tag pAb-HRP-DirecT (MBL,PM020-7) is reacted at 37°C for 30 minutes and washed. After that, SureBlue TMB 1-Component Microwell Peroxidase Substrate (KPL, 5120-0075) is added and the reaction is chromogenized. Then, add a reaction stopper (1N H_2SO_4) to stop the color development, and determine the color by visual inspection.

It is important to select peptide aptamer clones that are only chromogenic with the spike RBD protein, but not almost or completely chromogenic with the control BSA-containing blocking buffer, as a positive ELISA reaction. This will eliminate peptide clones that bind non-specifically to the plate as much as possible.

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Nucleotide Sequence and Amino Acid Sequence Determination of Peptide Aptamers [103-107]

Sequencing primers (for pT7-FLAG-1) and Applied Biosystems BigDye Terminator v3.1 were added to the pT7-FLAG-1 plasmid subcloned with peptide aptamers, and cycle sequencing reactions were performed and analyzed with SeqStudio Genetic Analyzer.

The peptide aptamer sequence was analyzed with the genetic analysis software Genetyx and translated into an amino acid sequence.

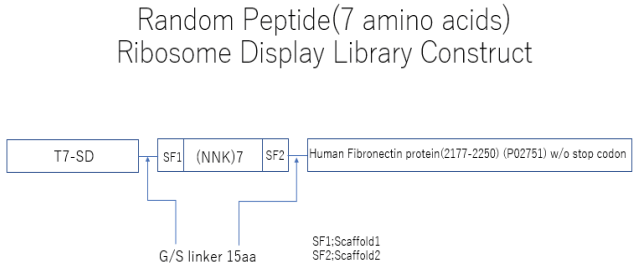

Figure 1: The structure of 7-amino acid random peptide ribosome display library (GW019PAL) [71-81].

The structure of the 7-amino acid random peptide ribosome display library (GW019PAL) consists of a 7 promoter, SD sequence, GS linker, scafold1 sequence (SF1),7 amino acid random peptide, scafold2 sequence (SF2), GS linker, human fibronectin lacking stop (2177-2250).

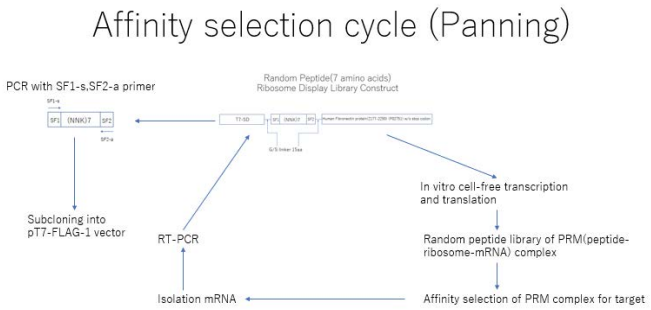

Figure 2: Principle for screening peptide aptamers that bind to target antigens from a 7-amino acid random peptide ribosome display library [71,72,81-84].

The Affinity Selection (panning) step consists of the following five steps.

Step 1: The 7-amino acid random peptide ribosome display library (Figure 1) was T7 promoter, SD sequence, GS linker, scafold1 sequence (SF1), 7 amino acid random peptide, scafold2 sequence (SF2), GS linker, followed by human fibronectin (2177-2250) lacking the stop codon.

Step 2: 7-amino acid random peptide ribosome display library constructs are transcribed and translated in an E. coli S-30 cell-free protein synthesis system, then stopped by cooling on ice, and the ribosomal complex is stabilized by increasing magnesium concentration.

Step 3: Perform affinity selection on immobilized target antigens with a random peptide library. Peptide-ribosome-mRNA complexes that bind to the target antigen are purified and selected. In this process, nonspecific peptide-ribosome-mRNA complexes are removed by washing.

Step 4: The bound peptide-ribosome-mRNA complex (PRM complex) is dissociated and the mRNA is extracted.

Step 5: Isolated mRNA is reverse transcribed to cDNA and cDNA is amplified by PCR for 30 cycles. (RT-PCR). The amplified DNA is subjected to the next affinity selection cycle (panning) to further purify the peptide aptamer. This affinity selection cycle (panning) is performed for 6 cycles.

Results

A 7-amino acid random peptide ribosome display library was panned against SARS-CoV-2 wild-type spiked RBD protein and 30 clones were picked up.

Insert-check PCR was performed on these peptide aptamer clones, and 25 clones had a peptide aptamer insert of 7 amino acid length.

To introduce the flag tag at the N-terminus into these 25 peptide aptamer clones, PCR was performed using primers with restriction enzymes and These clones were subcloned into the E. coli expression vector pT7-FLAG-1.

The peptide aptamer was then protein synthesized in E. coli, and the E. coli bacteria were crushed and Flag-peptide aptamer was purified on an anti-FLAG M2 antibody affinity gel column.

25 peptide aptamer clones were examined by ELISA for binding to SARS-CoV-2 wild type as well as delta, BA.1, BA.2, and BA.5 spike RBD.

The results showed that 4 peptide aptamer clones showed coloration and binding in ELISA to all of the SARS-CoV-2 wild-type, delta-type, BA.1, BA.2, and BA.5 spike RBDs.

The start time and degree of coloration in ELISA were similar for all four clones.

In this experiment, the ELISA was determined strictly by visual inspection for the blocking protein containing BSA, which was the control. This means that only clones that were very weakly chromogenic or negative for the blocking protein containing BSA were selected.

Table 1 shows the amino acid sequences of the 7-amino acid peptide aptamer clones of these four clones.

These 4 clones are expected to bind to the common

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epitope of the spike RBD of SARS-CoV-2 wild type, Delta, and Omicron strains BA.1, BA.2, and BA.5.

3 of the 4 clones have two or more Cys residues within the peptide aptamer. The N-terminal side scaffold sequence SF1 of the peptide aptamer contains a Cys residue, which is expected to be a complex higher-order structure between this Cys residue and two or more Cys residues within the peptide aptamer [108-112]. The remaining one of the 4 clones is expected to have a linear structure. However, there is a Tyr residue within this peptide aptamer that is expected to contribute to binding to the target [113-118].

Table 1: Amino acid sequence of the SARS-CoV-2 variant universal binding peptide aptamer clone.

Clone Name	Amino acid sequence
S-WT6-3	SSSGCCI
S-WT8-24	PSSCCRL
S-WT8-4	VCGCGAC
S-WT3	SSYSSNS

Three of the four clones have two or more Cys residues within the peptide aptamer; the Cys residues are located in the N-terminal scaffold sequence SF1 of the peptide aptamer, which is expected to be a complex higher order structure [108-112]. The remaining one clone is a linear structure. However, Tyr residues are present within this peptide aptamer and are expected to contribute to binding to the target [113-118].

Discussion

In this study, we show that a 7-amino acid random peptide ribosome display library screening system can be used to rapidly screen in vitro for peptide aptamers that exhibit universal binding to any of multiple SARS-CoV-2 variants [1-31].

The 7-amino acid random peptide ribosome display library [71-81] is a premade library, so it does not require immunization of animals, as is the case with antibody production.

Furthermore, screening for peptide aptamers can be easily performed in a very short process, just one week.

Specifically, affinity selection (panning) [71,72,81-84] is repeated six times against the target antigen, and binding to the target antigen can be easily determined by visual ELISA.

The spike RBD of SARS-CoV-2 is easily mutated, and if antibody drugs are developed for each spike variant of the spike RBD, further mutations will reduce the neutralizing activity of the developed antibody drugs.

Therefore, it is difficult to use antibody drugs as therapeutic agents.

Developing antibody drugs for each variant would be very complicated and costly, and would not make much sense.

Therefore, the development of a universal antibody is desired. Such antibodies have already been developed [4,41-70], but it is not known whether they have sufficient neutralizing activity against the continuously emerging variants of omicron strains [4,41-70].

While BA.5 has been the dominant strain since the end of 2022, variants such as BQ.1.1, strain derived from BA.5 and XBB strain derived from BA.2 have been emerging [119-125].

In particular, XBB.1.5 has immune escape ability against neutralizing antibodies produced by omicron-type bivalent vaccines [(conventional strain (strain of origin)/Omicron strain BA.1) or (conventional strain (strain of origin)/ Omicron strain BA.4-5)]. There are currently no antibody drugs that exhibit effective neutralizing antibody activity against XBB.1.5 [99,121,125-144].

This is because mutations in the Spike molecule change the conformation of the Spike, and the neutralizing activity of the antibody is easily lost. Because antibodies are macromolecules, they are good at recognizing the convex surfaces of molecules, but have difficulty penetrating and binding to the concavities, grooves, and clefts of molecules.

Therefore, we wondered if peptide aptamers could be utilized as antibody-like drugs to replace antibody drugs.

To this purpose, we attempted an in vitro screening of "7-amino acid middle-molecule peptide aptamers" that can bind to molecular convexities as well as concavities, grooves, and clefts.

The 7-amino acid random peptide ribosome display library screening system was used to examine whether universal peptide aptamers that bind to a common epitope of the SARS-CoV-2 variant spike RBD, including variants of the Omicron lineage, could be screened.

Normally, when the target antigen for screening is the SARS-CoV-2 wild strain spiked RBD, the ELISA chromogenic binding reaction is performed with the SARS-CoV-2 wild strain spiked RBD, but at this time, ELISA screening of peptide aptamer clones that can bind to the Omicron lineage delta, BA.1, BA.2, and BA.5 spike RBDs as well as the SARS-CoV-2 wild strain spike RBD will be performed. In this way, we thought it would be possible to screen for universal peptide aptamers that can bind to multiple epitopes commonly present in the Omicron lineage spike RBDs, including the SARS-CoV-2 wild strain spike RBD.

The results of the experiment allowed screening of four peptide aptamers that showed universal binding to SARS-

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CoV-2 wild type, delta type, Omicron lineage BA.1, BA.2, and BA.5 spike RBD.

It will be necessary in the future to measure the neutralizing activity and binding affinity of these four peptide aptamer clones.

If these peptide aptamers have neutralizing activity, they could be used as a therapeutic alternative to antibody drugs.

In addition, the peptide aptamer is a medium-sized molecule about 30 amino acid length even with the surrounding scaffold sequence. Peptide synthesis and purification and protein synthesis and purification by bacteria are considered easy.

Moreover, multiple peptide aptamers are easily obtained through screening, and peptide aptamers are easily multivalent and multiplexed due to their small molecular weight of about 30 amino acids. It is also easy to further increase the specificity and affinity of the peptide aptamer to the target antigen by making it multivalent and multiplexed.

Since each peptide aptamer can bind to a different epitope, multivalency and multiplexing may be effective against immune evasion.

In addition, recombination of monovalent, multivalent, or multiplexed peptide aptamers with the Fc region of IgG can easily add effector functions such as ADCC and CDC, and this higher-molecular weight can also extend the half-life in the blood.

The Nucleocapsid protein of SARS-CoV-2 is highly conserved and has been used as a diagnostic marker for antigen testing.

Although detailed data are not presented here, a number of anti-nucleocapsid peptide aptamer clones were also successfully screened using the 7 amino acid random peptide ribosome display library screening method.

In addition, multivalency and multiplexing of these multiple anti-nucleocapsid peptide aptamers will enable the development of more sensitive antigen test reagents that are comparable to the sensitivity of PCR test reagents.

Conclusions and Perspectives

In this study, we demonstrated that our 7-amino acid random peptide ribosome display library screening system can be used to rapidly screen for middle-molecule peptide aptamers that can bind to target antigens in a very short period of time (approximately one week).

In addition, multiple peptide aptamers obtained in screening can be multivalent, multiplexed, and recombined with molecules having effector functions at will.

In the present study, taking the SARS-CoV-2 variant as an example, we were able to obtain multiple peptide aptamers that can bind to several common epitopes present in the variant.

This 7-amino acid random peptide ribosome display library screening system is expected to be an effective tool for screening peptide aptamers for research, diagnosis, and therapy targeting various proteins of pathogens through rapid analysis of pathogen structures using next-generation sequencers in the event of new emerging infectious disease outbreaks.

As shown in this study, when an emerging infectious disease pathogen repeatedly makes mutations, such as SARS-CoV-2 variant, the peptide aptamer screening system is useful as a rapid screening method for universal binders to epitopes commonly present in variant strains.

The SARS-CoV-2 Omicron lineage has repeatedly made mutations and Omicron sub-lineages have emerged one after another, resulting in increased infectivity and immune evasion ability, and now the XBB.1.5 sub-lineage is increasing. The 7-amino acid random peptide ribosome display library screening system is a method for screening peptide aptamers as early as within a week, so it is easy to pan directly against the XBB.1.5 spike RBD to obtain peptide aptamers and a rapid way to combat the Omicron lineage.

The 7-amino acid random peptide ribosome display library screening system is a potential alternative to polyclonal and monoclonal antibody production and may be useful in antibody-based research, diagnostics, and therapy.

In particular, the 7-amino acid random peptide ribosome display library screening system we have developed can be applied to the field of cancer research, enabling rapid screening of peptide aptamers that target cancer antigens.

Furthermore, by making peptide aptamers multivalent and multiplexed, or by creating recombinant peptide aptamers with effector molecules that have cell-killing effects, they may be applied as diagnostic and therapeutic tools for various cancer cells.

Peptide aptamers are small, with a molecular weight of about 30 amino acids, and if peptide aptamer recombinants of monomeric, multimeric, and multiplexed forms with an added effector function are not utilized in the form of proteins, but are applied in medicine as mRNA drugs like the SARS-CoV-2 mRNA vaccine by Pfizer and Moderna, Inc., it would eliminate the need for protein synthesis and purification and open the way for the early development of therapeutic drugs.

Recently, Felicity Liew et al. reported that although IgG and IgA antibodies in the blood are maintained after mRNA

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vaccination, IgA antibodies in the nasal mucosa, which are necessary for protection against viral infection, rapidly decrease is one reason for the difficulty in infection protection [145]. Therefore, multivalent and multiplexed peptide aptamers that universally bind to SARS-CoV-2 variant spikes, including omicron variants, are expected to be used as mRNA agents for nasal delivery to protect against (prevent) infection and for post-infection treatment by neutralizing the virus [146-150].

Author Contributions

Nakanobu Hayashi designed the experiments. Nakanobu Hayashi, Chikako Abe, and Jiro Kikuchi were in charge of the experiments; Masahiro Ueda assisted in the selection and acquisition of reagents necessary for the research; Momoko Hayashi and Sakura Hayashi collected and organized the references for the papers; Koyu Suzuki, Masahiko Sugitani, Hiroaki Taniguchi, Toru Wake discussed and reviewed the experiments and results. Yusuke Furukawa reviewed and supervised the final contents.

Nakanobu Hayashi, Chikako Abe, Jiro Kikuchi and Yusuke Furukawa, these authors contributed equally. All agreed on the content of the paper.

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Conflict of interest

Nakanobu Hayashi is the founder and shareholder of GeneTry, Inc. The other authors declare that no competing interests exist.

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