# A Preliminary Report on The Syntheses of Oligonucleotide Primers in The National Research and Innovation Agency (NRIA)

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## Abstract

A PolyGen DNA Synthesizer is equipment that is used for synthesizing oligonucleotide primers for any amplification targets. Oligonucleotide primers are indispensable components for any Polymerase Chain Reaction (PCR)-based detections. In the present study a number of oligonucleotide primer sets were synthesized to target (1) the Human Insuline Glargin (HIG) and (2) the Human Erythropoietin (EPO), as well as (3) the RNA-dependent RNA Polymerase (RdRp) and (4) the Nucleocapsid (N) genes of the severe acute respiratory syndrome virus 2 (SARS-CoV-2). A solid-phase oligonucleotide synthesis method was used according to the default protocol of the Polygen's instrument to synthesize primers at a 40 nmol scale. The synthesized primers in this study were compared to commercially produced primers in their ability to amplify the gene target(s) in PCR and quantitative real-time PCR (qPCR) reactions. The first two sets of primers showed similar results in PCR compared to commercial primers; however, these primers were not tested for qPCR due to sample limitation. In contrast, the primer sets 3 and 4 were not able to produce amplicons in PCR reactions and only the primer set 4 successfully amplified the gene target in qPCR. These results indicate that the crude primers synthesized in this study are promising candidates for molecular detection and diagnostics, but these primers would benefit from further optimization for routine applications.

**Keywords:** oligonucleotides, DNA synthesizer, PCR, Indonesia

## Introduction

Polymerase Chain Reaction (PCR) is a molecular technique in which a target gene is amplified using a set of specific oligonucleotide primers. The technique is fundamental to molecular biology and is considered as the most important and practical in a laboratory for research in molecular biology (Kalendar *et al.,* 2017). PCR has also become a standard method that can be applied in many fields, especially for molecular detections and diagnostics (Ye *et al.,* 2012). However, factors such as methods to prepare the DNA template and conditions of PCR reaction can affect the outcome. Among all, the oligonucleotide primer is considered the most pivotal factor in the PCR amplification (Ye *et al.,* 2012).

The oligonucleotide primer is a chemically synthesized oligonucleotide, with a common length of 18-22 nucleotides. The primer sequence is a complementary sequence to the gene target (Pelt-Verkuil *et al.,* 2008). A set of oligonucleotide primers for a PCR contains forward and reverse primers. This primer set must bind to each end of the gene target

sequence for successful target amplification. Oligonucleotide primer sequences for PCR can be designed using publicly available software and are commonly available in primer databases. Depending on their sequence, the majority of commercial primers can be obtained at a relatively low cost. However, most commercial primers have not yet undergone experimental testing for specific applications, thus it cannot be assumed that the primers will provide the same outcomes under different experimental conditions. Therefore, an optimization of the PCR condition is crucial for molecular validation (Bustin & Hugget, 2017).

A solid-phase method is a common approach in the process of oligonucleotide synthesis. In principle, the method is generally applicable via synthetic chemistry and it has been applied to a variety of chemical reactions (Itakura *et al.,* 1984, Ellington & Pollard, 2000). The National Research and Innovation Agency (NRIA) has a PolyGen DNA Synthesizer PolyGen that uses a solid-phase method to synthesize oligonucleotides for molecular research and detection. The PolyGen DNA Synthesizer available in the NRIA is a 12-Column DNA Synthesizer that can be used to synthesize up to 12 oligonucleotides in a single run (Figure 1). This synthesizer is also suitable for creating oligonucleotide primers with various ranges, i.e., from 10 nmole – 1 µmole per column and from 1 µmole – 5 µmole with separate Slider (www.polygen.de).

This study aims to synthesize four sets of oligonucleotide primers, forward and reverse, namely: 1) AOX-F and AOX-R, (2) ARN-F and ARN-R, (3) RDX-F3 and RDX-B3, and (4) NCA-F3 and NCA-B3. These primers were synthesized using the PolyGen DNA Synthesizer that is available in the NRIA. The first set of primers is designed to target the Human Insuline Glargin (HIG) and the second set is designed to target the Human Erythropoietin (rhEPO) genes. The last two sets of primers are designed to target genes of the severe acute respiratory syndrome virus 2 (SARS-CoV-2), which are the RNA-dependent RNA Polymerase (RdRp) and the Nucleocapsid (N), respectively.

The human insuline glargine (HIG) is an analogue of human insuline that is produced synthetically using a non-pathogenic *Escherichia coli*. The insuline was developed due to its importance for the treatment of patients with diabetes mellitus type 1 (McKeage & Goa, 2011). The human Erythropoietin (EPO) is a theurapeutic agent protein that is important for the treatment of patient with anemia (Santoso *et al*., 2013). The primer AOX and ARN were designed to detect the genes HIG and EPO that were previously cloned in a plasmid, aimed for producing the recombinant theurapeutic proteins (Santoso *et al.,* 2013, Santoso *et al.,* 2014). Meanwhile, the SARS-CoV-2 is a new coronavirus causing a new disease called COVID-19 (Corman *et al.,* 2020). The primers RDX and NCA were designed to detect the RdRp and N genes of the SARS-CoV-2 that were previously cloned in a plasmid, aimed for development of rapid detection of COVID-19 in Indonesia (unpublished).

The demand for oligonucleotide primers in Indonesia for molecular research and detection is quite high and most of the time it relies on imported products. Considering the domestic production of oligonucleotide primers in Indonesia remains limited, the utilization of Polygen DNA Synthesizer in NRIA shows promise in its feasibility for further application and to supply the local demand of oligonucleotides for molecular research. To that extent, this study provides crucial data and important information on the potential use of the Polygen DNA Synthesizer in NRIA, to further support the potential production of oligonucleotides in Indonesia, especially for molecular research, detection, and diagnostics.

## Materials and Methods

**Samples Preparation.** The experiment used four plasmid DNA pJ603-EPO, PD902-HIG, pD454-RDRx, and PJ204-NCRx as a template DNA for the PCR validation (Table 1, Figure 1). Each plasmid DNA was isolated using a high-speed plasmid mini kit (Geneaid Biotech Ltd, Taiwan) according to the manufacturer's instructions. Briefly, *Escherichia coli* DH5α cells containing plasmids were grown on separate LB agar media plates with antibiotic ampicillin 100 µg/mL (Sigma–Aldrich GbmH, Switzerland) and grown overnight at 37°C. Colonies were inoculated into a 3 mL LB medium and grown overnight at 37°C. Cultured bacterial cells were lysed with lysis buffer, then plasmid DNA was banded in a spin column. Contaminants were removed with a wash buffer (containing ethanol) and the purified plasmid

DNA was eluted by a low salt elution buffer or TE (Tris-EDTA buffer pH 8).

**Oligonucleotide Synthesis.** The present study synthesized four sets of oligonucleotide primers by using a PolyGen DNA Synthesizer available in the NRIA (Table 1). Automated DMT-off oligonucleotide synthesis was conducted using a PolyGen DNA synthesizer according to the manufacturer's instructions (PolyGen, GmBH, Germany). All reagents and phosphoramidite monomer – a chemically modified DNA nucleotide for oligo synthesis were obtained from Carl Roth (Karlsruhe, Germany). Briefly, after each column of the column slider was filled with solid support containing the correct initial nucleotide (3'end of an oligo), the synthesis was initiated. The synthesis process was carried out at a scale of 40 nmol by adding a phosphoramidite monomer one by one using a repeated five-step cycle –deblocking, activating, coupling, capping, and oxidizing. The completed synthesis process was followed by material support cleavage using 32 % ammonia. Then, synthesized crude oligo was precipitated, eluted in ultra-pure water, and stored at -20 °C. (http://www.polygen.de).

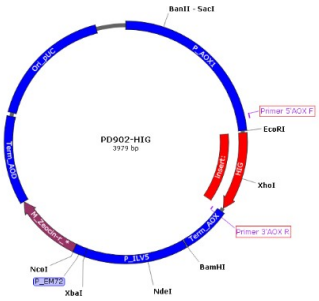


**Figure 1.a**. Map of plasmid pD902-HIG containing the Human Insuline Glargine (HIG) gene target (https://www.atum.bio).

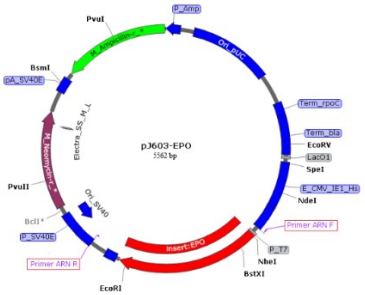


**Figure 1.b**. Map of plasmid pJ603-EPO containing the Human Erythropoietin (EPO) gene target (https://www.atum.bio)

**Table 1**. The oligonucleotide primers synthesized in this study

| Plasmid/template | Genes Target | Primer code | Size (bp) | Reference |
|---|---|---|---|---|
| PD902-HIG | Human Insulin Glargine (HIG) | AOX-Forward (AOX-F)<br>AOX-Reverse (AOX-R) | 500 | Santoso *et al.,* 2014 |
| pJ603-EPO | Human Erythropoietin (EPO) | ARN-Forward (ARN-F)<br>ARN-Reverse (ARN-R) | 600 | Rubiyana *et al.,* 2020 |
| pD454-RDRX | RNA-dependent RNA Polymerase (RdRp) | RDX-Forward (RDX-F3)<br>RDX- Reverse (RDX-B3) | 208 | This study |
| pJ204-NCRx | Nucleocapside (N) | NCA-Forward (NCA-F3)<br>NCA-Reverse (NCA-B3) | 244 | This study |

**Molecular Validation using PCR.** The PCR amplification for HIG and rhEPO genes was conducted using Gotaq® Green Master Mix (Promega, USA) on an Axygen MaxyGene II Thermal Cycler (Corning, USA) according to the manufacturer's protocols. Each 25 µl reaction mixture contained 12.5 µl of 2× Master Mix, 1 µl of each 5× diluted forward and reverse synthesized primers, 8.5 µl of RNA-free water, and 2 µl of plasmid DNA as a template. The thermal cycling condition of HIG detection was 2 min denaturation at 94 °C, followed by 25 cycles of each 1 min denaturation at 94 °C, annealing at 55 °C, and extension at 72 °C, respectively, and then elongation at 7 min at 72 °C. The PCR condition for the rhEPO detection was 2 min denaturation at 94 °C, followed by 25 cycles of each 1 min denaturation at 94 °C, annealing at 58 °C and extension at 72 °C, respectively, and then 7 min elongation at 72 °C. The PCR amplification of RdRp and N genes was conducted using the NZY PCR master mix (NZYtech) on an Axygen MaxyGene II Thermal Cycler (Corning, USA) according to the manufacturer's protocols. Each 25 µl reaction mixture contained 12.5 µl of 2x Master Mix, 1.5 µl of 10x diluted forward and reverse primers, 8.5 µl of RNA-free water, and 1 µl of plasmid DNA as a template. The thermal cycling condition of N and RdRp detection was 2 min

denaturation at 95 °C, followed by 40 cycles of each 5 sec denaturation at 95 °C, 20 sec annealing at 57 °C, and 5 min extension at 72 °C, respectively and then 5 min elongation at 72 °C. Finally, amplified products of each primer pair were examined and visualized by 1 % agarose gel electrophoresis.

**Molecular Validation using qPCR.** The qPCR amplification was only performed to detect the RdRp and N genes of SARS-CoV-2. The qPCR amplification was conducted using SensiFAST SYBR one-step mix with No-Rox (Bioline) in a BioRad CFX96 qPCR machine (BioRad). Each 20 µl reaction mixture contained 10 µl of Master Mix, 0.8 µl of 10× diluted forward and reverse primers, 7 µl of RNA-free water, 0.4 µl RiboSafe rNase inhibitor, and 1 µl of plasmid DNA as a template. The thermal cycling condition for RdRp and N detection was 2 min denaturation at 95 °C, followed by 40 cycles of each 5 sec denaturation at 95 °C, 20 sec annealing at 57 °C, and 5 sec extensions at 72 °C, respectively, and then 5 min elongation at 72 °C. Melting curve optimization was done at 55-59 °C and an increment for 5 sec. Finally, amplified products of each primer pair were examined using the CFX Maestro analysis software for the BioRad CFX real-time PCR system (BioRad).

## Results

Four sets of oligonucleotide primers were successfully synthesized in this study using a PolyGen DNA Synthesizer, PolyGen, GmBh, Germany (Table 1). These four sets of primers were compared to the corresponding four sets of commercial primers, aiming to evaluate their performances. The performance was tested using molecular techniques PCR (for all primers) and qPCR (for SARS-CoV-2 primers).

**Molecular Validation using PCR.**

The PCR amplification of primers for detection of HIG and EPO showed no differences in the PCR amplification performance between the commercial primers and the primers synthesized in this study (Figure 2). However, for SARS-CoV-2 detection (NCA and RDX), only the commercial primers were able to successfully amplify the target. No amplicons were obtained using the crude primers synthesized in this study (Figure 3).

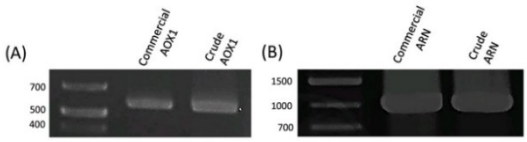


**Figure 2**. Molecular validation by PCR to detect genes (A) HIG using AOX1 primer sets and (b) EPO using ARN primer sets. The commercial primers AOX and ARN were compared with the primers that were synthesized in this study. Amplicons of the correct sizes were produced by each primer pair (target gene 500 bp for HIG and 600bp for EPO). 1Kbp ladder was used as a marker.

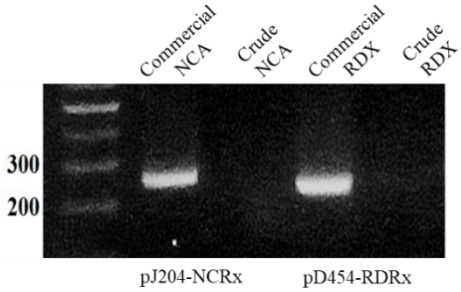


**Figure 3**. Molecular validation by PCR to detect RdRp and N genes of SARS-CoV-2, using primers RDX and NCA, respectively. Only the commercial primers were able to produce the 208 bp and 244 bp amplicons corresponding to RdRp and N genes, respectively. 1 Kbp ladder was used as a marker.

**Molecular Validation using qPCR.**

The qPCR amplification by NCA primers showed different performances between the commercial primers and the primers synthesized in this study (Figure 4). In qPCR, the commercial primers NCA showed better performance than the primers synthesized in this study, indicated by a better Cq of these primers. However, for RDX primers, only the commercial primers RDX showed amplification in qPCR, while the primers synthesized in this study showed no amplification signals (Figure 5).

## Discussion

The aim of this study is to synthesized oligonucleotide primers using the PolyGen DNA Synthesizer that is available in the NRIA. Four sets of primers have been successfully synthesized during the study. However, each set of primers showed different PCR and qPCR amplification performances.

It is possible that some primers were unsuccessful in amplifying their target gene(s) as the synthesized primers were in their crude state and not yet purified, thus affecting their

performances. Crude primers may contain truncated oligonucleotides fragments that will affect the downstream performance such as PCR and or qPCR amplification (Hecker & Riil, 2018). Therefore, the purification step is important for any synthesized primers prior to their use in a further downstream application. Purification can be done by various methods, for example using silica gel column purification, HPLC desalting, polyacrylamide electrophoresis, and MALDI-TOF (Dellinger *et al.,* 2008).

| Set Primers NCA, plasmid pJ204-NCRx | No | Sampel code | Cq |
|---|---|---|---|
| Commercial | 1 | pJ204-3 | 7.73 |
| | 2 | pJ204-4 | 3.36 |
| | 3 | pJ204-6 | 5.81 |
| synthesized in NRIA | 4 | pJ204-3 | 24.87 |
| | 5 | pJ204-4 | 22.77 |
| | 6 | pJ204-6 | 24.39 |

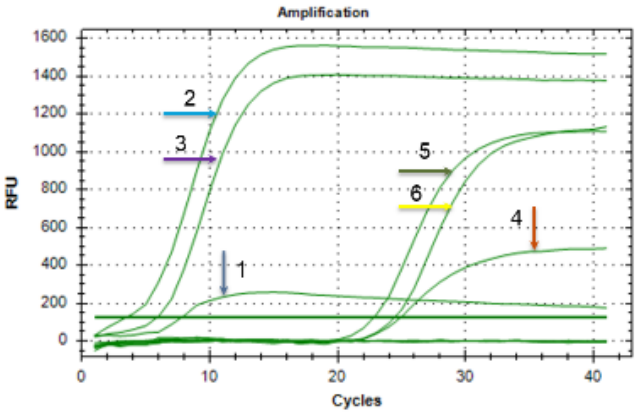


**Figure 4**. Molecular validation by qPCR to detect the N gene of SARS-CoV-2 using the NCA primers. The commercial NCA primer resulted in a better Cq compared to the NCA primer set synthesized in this study. The numbered arrow corresponds to the Cq tables of each template sample.

| Set Primers RDX, plasmid pD454-RDRx | No | Sampel | Cq |
|---|---|---|---|
| commercials | 1 | pD454-3 | 7.88 |
| | 2 | pD454-4 | 6.73 |
| | 3 | pD454-6 | 19.90 |
| synthesized in NRIA | 4 | pD454-3 | n/a* |
| | 5 | pD454-4 | n/a |
| | 6 | pD454-6 | n/a |

*n/a = no amplification signal

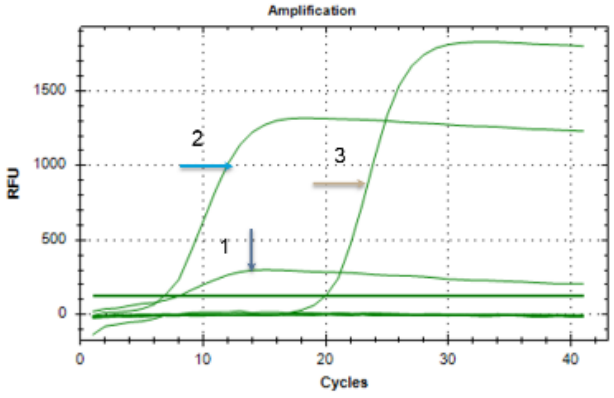


**Figure 5.** Molecular validation by qPCR to detect the RdRp gene of SARS-CoV-2, using the RDX primers. The commercial RDX primers were able to amplify the target gene, whereas no amplification was obtained from the RDX primers synthesized in this study. The numbered arrow corresponds to the Cq tables of each template sample.

This study used a solid-phase synthesis approach to obtain the four sets of oligonucleotide primers, namely (1) AOX-F and AOX-R, (2) ARN-F and ARN-R, (3) RDX-F3 and RDX-B3, and (4) NCA-F3 and NCA-B3 (Table 1). The first two sets of the primers were designed to detect the human insuline glargine (HIG) and human erythropoietin (EPO) genes, while the last two sets of primers were designed to detect the viral RNA SARS-CoV-2 that caused the COVID-19. The goal of this study is the application of PolyGen DNA Synthesizer that is available in the NRIA to obtain nucleotide primers for routine experiment in the NRIA, for example for detection of RdRp and N genes of the SARS-CoV-2. The molecular PCR and qPCR were used to validate the performances of these primers However, the primer optimization to detect the genes targeted was outside the scope of this study.

The process of oligonucleotide synthesis using the solid-phase method includes repeated detritylation/deprotection, coupling, capping, and oxidation cycle steps in which one nucleotide is added at each synthesis cycle (Temsamani *et al.,* 1995). The synthesis begins with the removal of 4,4'-dimethoxytrityl (DMT), a protection group, from a 5'-hydroxyl group of protected monomer nucleotide (phosphoramidite) using trichloroacetic acid (TCA) in

dichloroethane. This process allows the first phosphoramidite to be coupled with the upcoming one (Ellington & Pollard, 2000). The following step in this synthesis process is an activation/coupling in which the incoming protected phosphoramidite is being protonated using weakly acidic activator tetrazole and immediately coupled with the deprotected phosphoramidites. Noted that even with the most efficient chemistry and reagents of the highest purity, it is impossible to achieve 100 % coupling efficiency at each cycle of synthesis. Capping is the step to inactivate the uncoupled first phosphoramidite, preventing deletion product during the synthesis process. A combination of incomplete coupling, capping, and detritylation causes deletion of mutations, resulting in a shorter oligonucleotide, especially the n-1 product (Temsamani *et al.,* 1995). In the final cycle step, the internucleotide linkage is oxidized to a phosphotriester for a more stable oligonucleotide structure. A mixture of polynucleotides and a series of truncated shorter polynucleotides most likely are present in the final product (Itakura *et al.,* 1984). These steps have been shown in this study to be effective in generating primers that are of good quality but need further purification process, nonetheless.

Polymerase Chain Reaction (PCR) is a common technique to amplify specific gene targets. The method uses a set of oligonucleotide primers that are specific for the gene target to ensure the target amplification. Any impurities may affect the downstream amplification, and therefore these primers need further optimization to get a better amplification result (Dellinger *et al.,* 2008). However, these preliminary results using crude primers, particularly those used to amplify the HIG and EPO genes, showed promising performances albeit in their current crude states. At the very minimum, this study successfully showed the feasibility of generating working primers that are comparable to commercially obtained primers. It is also likely that different oligonucleotides will have different properties depending on their sequences. Additionally, we were only testing these sequences under a particular condition and reagents, thus there are opportunities to explore more on optimizing these primers' ability to amplify their gene target(s). Moreover, this study only reported the molecular validation of the primers using PCR and qPCR to the extent of whether or not amplicons were obtained. Further analysis of the PCR product, such as cloning the amplicon to a plasmid and sequencing it, is important to confirm whether the correct genes target was amplified.

Studies reported the utilization of DNA PolyGen Synthesizer (PolyGen, GmBh, Germany) to synthesize a hairpin-free DNA synthetic for nucleic acid detection (Lu *et al.,* 2015), a synthetic DNA for a cyclic enzymatic amplification method (Cui *et al.,* 2010), and a DNA synthetic for a microfluidic paper-based analytic device (Wei *et al.,* 2015). The present study reported the synthesis of oligonucleotide primers using the PolyGen DNA Synthesizer (GmBh, Germany) available in the National Research and Innovation Agency. Some promise was shown by the primers synthesized in this study. However, to obtain satisfactory performances, this study recommends performing purification of the crude primers and sequencing the amplification product. Further optimization of these primers under different PCR conditions would also be beneficial.

Aiming to supply the local demand for oligonucleotide primers in Indonesia, it is expected that oligonucleotides can be produced and synthesized domestically. This would benefit the development of molecular research in Indonesia, especially for the purpose of detection and diagnostics. Also, it can be applied for many biotechnological applications, for example, to design synthetic genes, to study the regulation of gene expression, to investigate specific protein production (Itakura *et al.,* 1984), and also for cancer treatment (Abnous *et al.,* 2017).

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contributed to providing supervision. All authors have read and agreed with the content of this manuscript.

## References


Abnous, K., Danesh, N. M., Ramezani, M., Lavaee, P., Jalalian, S. H., Yazdian-Robati, R., ... & Taghdisi, S. M. (2017). A novel aptamer-based DNA diamond nanostructure for in vivo targeted delivery of epirubicin to cancer cells. *RSC Advances, 7*(25), 15181-15188. https://doi.org/10.1039/C6RA28234B.

Bustin, S., & Huggett, J. (2017). qPCR primer design revisited. *Biomolecular Detection and Quantification, 14*, 19-28. https://doi.org/10.1016/j.bdq.2017.11.001.

Corman, V. M., Landt, O., Kaiser, M., Molenkamp, R., Meijer, A., Chu, D. K., ... & Drosten, C. (2020). Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Eurosurveillance, 25*(3), 2000045.

Cui, L., Ke, G., Wang, C., & Yang, C. J. (2010). A cyclic enzymatic amplification method for sensitive and selective detection of nucleic acids. *Analyst, 135*(8), 2069-2073. https://doi.org/10.1039/c0an00215a.

Dellinger, D. J., Betley, J. R., Wyrzykiewicz, T. K., & Caruthers, M. H. (2005). Synthesis of DNA using a new two-step cycle. In *Oligonucleotide Synthesis* (pp. 1-16). Humana Press. https://doi.org/10.1385/1-59259-823-4:001.

Ellington, A., & Pollard Jr, J. D. (2000). Introduction to the synthesis and purification of oligonucleotides. *Current Protocols in Nucleic Acid Chemistry,* (1), A-3C.

Hecker, K. H., & Rill, R. L. (1998). Error analysis of chemically synthesized polynucleotides. *Biotechniques, 24*(2), 256-260. https://doi.org/10.2144/98242st01.

Itakura, K., Rossi, J. J., & Wallace, R. B. (1984). Synthesis and use of synthetic oligonucleotides. *Annual Review of Biochemistry, 53*(1), 323-356. https://doi.org/10.1146/annurev.bi.53.070184.001543.

Kalendar, R., Muterko, A., Shamekova, M., & Zhambakin, K. (2017). In silico PCR tools for a fast primer, probe, and advanced searching. In *PCR* (pp. 1-31). Springer, New York, NY.

Lv, Y., Cui, L., Peng, R., Zhao, Z., Qiu, L., Chen, H., ... & Tan, W. (2015). Entropy beacon: a hairpin-free DNA amplification strategy for efficient detection of nucleic acids. *Analytical Chemistry, 87*(23), 11714-11720. https://doi.org/10.1021/acs.analchem.5b02654.

McKeage, K., & Goa, K. L. (2001). Insulin glargine. *Drugs, 61*(11), 1599-1624. https://doi.org/10.2165/00003495-200161110-00007.

van Pelt-Verkuil, E., Van Belkum, A., & Hays, J. P. (2008). *Principles and technical aspects of PCR amplification.* Springer Science & Business Media. https://doi.org/10.1007/978-1-4020-6241-4.

PolyGen (2022). 12-Column DNA Synthesizer. 12 Oligonucleotide syntheses per run. Retrieved from http://www.polygen.de/12_saeulen_synthesizer_e.html.

Rubiyana, Y., Soejoedono, R. D., & Santoso, A. (2020). Enhancement of transient erythropoietin protein expression by valproic acid in CHO-K1 suspension adapted cells. *Indonesian Journal of Biotechnology, 25*(1), 28-32. https://doi.org/10.22146/ijbiotech.52621.

Santoso, A., Rubiyana, Y., Herawati, N., Wijaya, S. K., Wardiana, A., & Ningrum, R. A. (2013). Heterologous expression and characterization of human erythropoietin in P*ichia pastoris*. *International Journal of Pharma and Bio Sciences. 4*(4), 187-196.

Santoso, A., Septisetyani, E. P., Meiyanto, E., Putri, D. D. P., & Ningrum, R. A. (2014). Expression of modified recombinant human erythropoietin in CHOK1 cells and its in vitro proliferation assay in TF1 cells. *Indonesian Journal of Pharmacy, 25*(1), 9.(2014). https://doi.org/10.14499/indonesianjpharm25iss1pp9.

Temsamani, J., Kubert, M., & Agrawal, S. (1995). Sequence identity of the n-1 product of a synthetic oligonucleotide. *Nucleic acids research, 23*(11), 1841-1844. https://doi.org/10.1093/nar/23.11.1841.

Wei, X., Tian, T., Jia, S., Zhu, Z., Ma, Y., Sun, J., ... & Yang, C. J. (2015). Target-responsive DNA hydrogel mediated "stop-flow" microfluidic paper-based analytic device for rapid, portable and visual detection of multiple targets. *Analytical Chemistry, 87*(8), 4275-4282. https://doi.org/10.1021/acs.analchem.5b00532.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*(1), 1-11. https://doi.org/10.1186/1471-2105-13-134.