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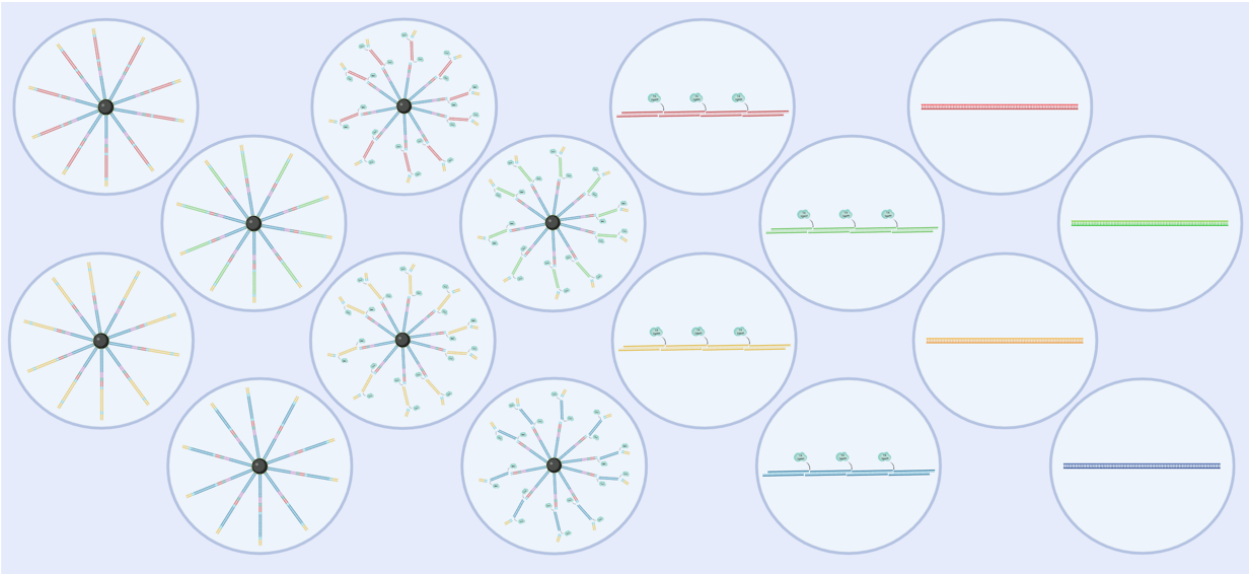

Multiplexed genes synthesis from oligos within microdroplets

Master's thesis in Biotechnology

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DEPARTMENT OF LIFE SCIENCES
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2025
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Acknowledgements, dedications, and similar personal statements in this thesis, reflect the author's own views.

Cover: Overview of multiplexed Golden Gate assembly within microdroplets utilizing barcoded beads. The different colors refers to different genes. Created in https://www.biorender.com/

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Abstract

This study investigates the adoption of DropSynth methodology for in-house gene synthesis and the development of a droplet-based Golden Gate (DropGG) assembly technique to address the inherent limitations of DropSynth, such as low accuracy (20-30%) and limited gene length (<1 kb). Motivated by the aim to lower cost and turn-around times associated with gene synthesis by transitioning to in-house production, the study involved assembling single genes using both DropSynth and Golden Gate methods within droplets, followed by scaling up to multiplexed gene assembly in a single reaction. Through a detailed evaluation of the DropSynth protocol, highlighting its weaker points, a novel workflow for DropGG was introduced, adapted for 96-gene assembly. The DropGG approach successfully demonstrated the assembly of genes up to 750 bp within droplets using barcoded beads, while also simplifying downstream processing steps. Collectively, these findings provide valuable insights into the strengths and limitations of droplet-based gene assembly technologies.

Keywords: Gene Synthesis, PCA, Golden Gate Assembly, Microdroplets

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Linnea Max, Gothenburg, January 2025

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1
Introduction

In the introduction chapter information about the project's background, aim, research questions and scope will be provided, as well as the disposition of the thesis.

1.1 Project Background

Zelezniak lab is a synbio lab that develops AI/ML modes to design functional DNA sequences, which are then tested and iterated upon through a cycle of HT build - HT test - AI/ML learn. This iterative process integrates both dry and wet lab techniques, aiming to expedite and economize the creation of synthetic genes. Currently, the synthetic genes are outsourced to gene synthesis vendors such as IDT and TWIST Bioscience. This outsourcing involves a turnaround time of approximately 4-6 weeks and a cost of around 1000 SEK for cloning a gene of roughly 1 kbp. The lab is actively seeking new technologies to synthesize genes in-house, aiming to cut down the turnaround time to 1-2 weeks and significantly reduce costs.

One promising technology is DropSynth (Fig. 2.1b), created by [Plesa et al., 2018], which uses polymerase cycling assembly (PCA) of oligonucleotides within droples, enabling multiplexed gene synthesis. This method can synthesize up to 10^4 genes at a cost as low as 2 $ per gene, though it is currently limited to gene lengths of around 1 kbp due to PCA constraints. Zelezniak lab aims to adopt and adapt this technology for our needs, specifically for synthesizing gene libraries designed by their AI models.

To overcome the gene length limitation, the lab proposes enhancing DropSynth by incorporating Golden Gate assembly within droplets, enabling the synthesis of genes longer than 1 kbp. Golden Gate technology has been shown to support assemblies in as high complexity as 52 fragments [Pryor et al., 2022]. [Lund et al., 2024] has shown in total 343 out of 458 genes have been successfully assembled from two oligo pools in a multiplexed and parallel way. We believed that by extending the Gold Gate assembly with droplets could further decrease the assembling bias rather than in an oligo pool mixture.

1.2 Aim

The project aimed to adapt DropSynth technology for gene synthesis, with the primary objective of synthesizing a gene library using this method. Additionally, the

study sought to develop a novel multiplexed Golden Gate-based droplet gene synthesis technology, termed DropGG. This included designing an experimental workflow for Golden Gate-based droplet gene synthesis and applying the new technology to synthesize a 2 kbp gene library.

1.3 Research Questions

The overarching goal of this project was to investigate time- and cost-effective methods for in-house gene synthesis. Based on this three research questions were formulated:

1. Can genes be synthesized on a laboratory bench without reliance on outsourced services?

2. Can genes of arbitrary size be synthesized effectively using in-house methods?

3. Is it possible to use Golden Gate assembly within droplets to synthesize genes in a multiplexed manner?

1.4 Project Scope

This project scoped to evaluate the DropSynth protocol for multiplexed gene synthesis within droplets, leveraging Polymerase Chain Assembly (PCA). Additionally, the scope included the development of a novel Golden Gate assembly-based method for multiplexed gene synthesis in droplets. Due to time constraints, the project was limited to synthesizing genes approximately 750 base pairs in length, assembled from up to four oligonucleotides for both methods.

1.5 Disposition of the Thesis

This thesis is structured into six chapters to provide a clear and logical progression of the research. The introduction outlines the background, objectives and significance of the study. The theory chapter presents the foundational concepts and previous research relevant to the methodologies and techniques employed. The method chapter details the experimental design, procedures, and protocols used to achieve the research objectives. The result chapter presents the key findings and observations, which are further analyzed and interpreted in the Discussion chapter. Finally, the conclusion summarizes the main outcomes, highlights their implications, and suggests directions for future research. Additional materials, including data, protocols, and supporting information, are provided in the supplementary material for reference and reproducibility.

2
Theory

In this chapter the theoretical background of the project is presented, including current state of gene synthesis and principles of Polymerase Chain Assembly, Golden Gate Assembly and droplet technology.

2.1 Current State of Gene Synthesis

The synthesis of synthetic DNA has become a cornerstone of modern biotechnology, enabling advancements in synthetic biology, therapeutic development, and data storage [Hoose et al., 2023]. Despite substantial progress, challenges persist in producing longer DNA sequences efficiently and cost-effectively. Traditional chemical synthesis methods, while effective for short nucleotides, face limitations when scaling up to sequences longer than 300 nucleotides. A key limitation lies in the elongation cycle efficiency, which refers to the effectiveness of incorporating each nucleotide into the sequence. With an elongation cycle efficiency of 99 %, the theoretical yield for an oligonucleotide of 200 nucleotides is 13 %, and the yield decreases further as the sequence length increases. This bottleneck constraints the development of complex synthetic genomes and large gene libraries essential for advanced research and industrial applications.

To address these challenges, several innovative approaches are under development. Examples include enzymatic DNA synthesis, microarray-based synthesis and rolling circle amplification. Several companies are leading efforts to advance gene synthesis technologies. Twist Bioscience, for instance, specializes in high-throughput DNA synthesis using a silicon microarray chip, enabling the production of large gene libraries with high accuracy. Meanwhile, DNA Script and Integrated DNA Technologies (IDT) develops enzymatic DNA synthesis technologies among other innovations. Though progress has been observed for several synthetic approaches, the challenge to produce DNA sequences of unlimited lengths and complexity remains.

2.2 Polymerase Chain Assembly

Polymerase Chain Assembly (PCA) is a gene synthesis technique that constructs long DNA sequences by assembling shorter oligonucleotides(oligos) [TerMaat et al., 2009]. Traditional PCA involves repeated cycles of hybridization and extension of single-stranded oligonucleotides by a polymerase enzyme, followed by a separate PCR

amplification to increase the quantity of the desired full-length sequence. An integrated approach combines the assembly and amplification steps into a single reaction using a high-speed thermocycler (Fig. 2.1a). However, challenges arise with longer sequences, such as 1-2 kbp, where competitive annealing and primer competition can limit the efficiency of the integrated PCA-PCR process.

The process typically starts with the desired gene sequence being split into shorter oligos, usually with a size ranging from 40 - 300 bp [Hoose et al., 2023]. Each oligo hybridizes with complementary overlaps of the neighboring oligonucleotides on the opposite strand. This process forms an annealed construct with alternating gaps in the sense and antisense strand. These gaps are subsequently filled in by a polymerase, creating a complete duplex DNA template ready for PCR amplification. External primers, complementary to the 5' ends of the duplex DNA template, amplify the target sequence and produce the final product.

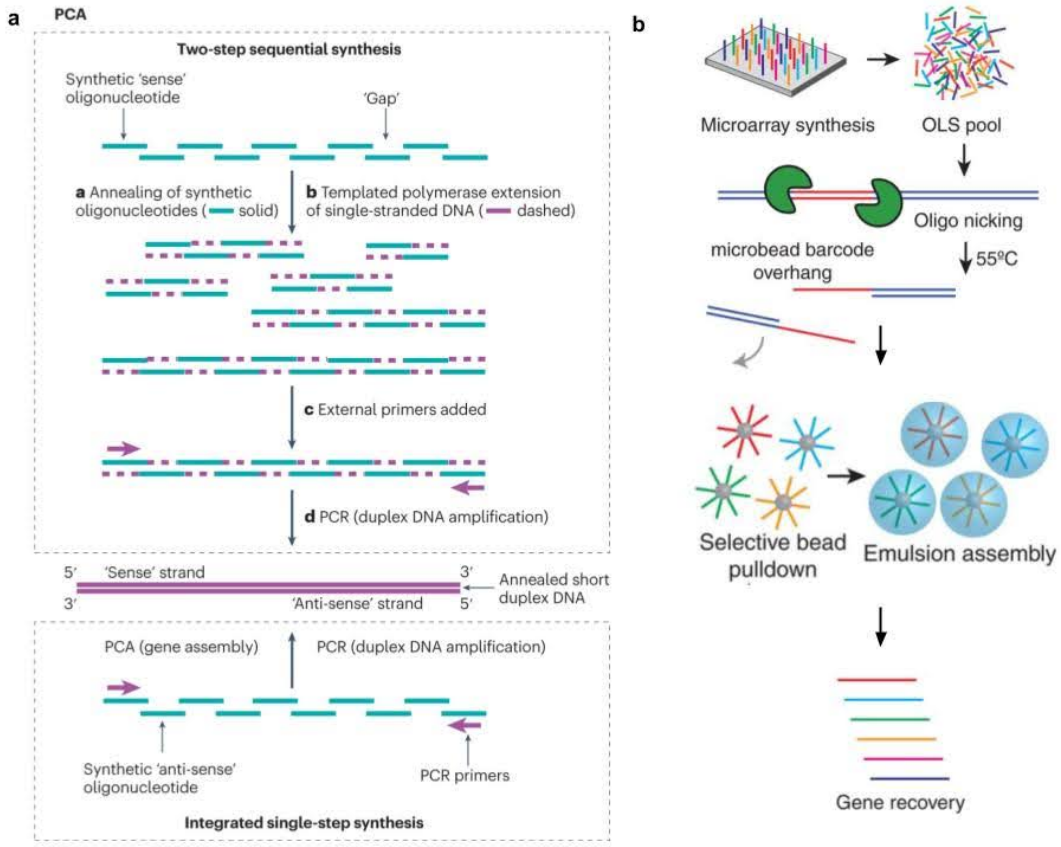

Figure 2.1: **a:** Overview of two step PCA-PCR (top) and integrated PCA-PCR (bottom). The target gene is divided into shorter oligonucleotides with overlapping sequences. The overlapping sequences anneal (step a) and a DNA polymerase fills in the gaps (step b) to create a template of the target gene. External primers are added (step c) and the target gene is amplified through PCR (step d). In the integrated PCA-PCR the external primers are added and the two reactions will take place simultaneously. Image adapted from [Hoose et al., 2023]. **b:** Overview of DropSynth technology. Microarray-derived oligonucleotides are amplified in a pooled format, and nicked to expose a gene-specific barcode, enabling binding to the corresponding barcoded beads. The beads are emulsified and oligos are assembled through PCA inside the droplets. The emulsion is broken and the assembled genes recovered. Image adapted from [Plesa et al., 2018].

DropSynth is technology developed by [Plesa et al., 2018] that utilizes the PCA principles for multiplexed gene synthesis in emulsions. Barcoded beads are used to compartmentalize oligos required for a specific gene assembly within emulsion droplets, enabling assembly of multiple genes in the same reaction (Fig. 2.1b). Using this technique they successfully constructed over 7000 synthetic genes at an estimated cost of $2 per gene employing a five-day workflow, demonstrating the scalability.

The target genes are bioinformatically split into oligos with overlapping regions using a custom software (`https://github.com/PlesaLab/DropSynth_code_2023`). Prior to splitting, the gene sequence is analyzed to identify and remove internal

recognition sites for restriction enzymes used during processing. The sequence is then flanked by restriction sites for cloning and primer binding sites for assembly primers used in the PCA. After splitting, the oligos are flanked with BtsI-v2 sites, which facilitate the release of oligos from the barcoded beads. Additionally, gene-specific barcodes are incorporated at the 5' end of each oligo, flanked by BspQI nicking sites. Upon nicking, these sites will generate 12-bp overhangs that expose the barcodes. Common 15-nt primers are added to the 5' and 3' ends of all oligos, to enable pooled amplification. The oligos are synthesized and ordered as a pool. Preparatory steps, including amplification and nicking, are performed to process the oligos. The nicked oligos are subsequently bound to barcoded beads, each constructed with corresponding barcodes. These loaded beads are then encapsulated within a water-oil emulsion, where BtsI-v2 digestion releases the oligos into the droplets. The PCA process takes place inside the droplets and when the emulsion is broken the assembled genes are recovered. Downstream processing includes gel extraction, suppression PCR and purification to isolate and amplify target genes.

2.3 Golden Gate Assembly

Golden Gate Assembly is a molecular cloning method that enables the seamless and efficient assembly of multiple DNA fragments in a predefined order with a single reaction [HamediRad et al., 2019]. This technique leverages the unique properties of Type IIS restriction enzymes, such as BsaI, which cleave DNA at specific sites outside of their recognition sites, creating distinct 4-nucleotide overhangs. This allows for the design of overhangs that are independent of the enzyme's recognition site, enabling the removal of the recognition site to prevent further restriction activity. The complementary overhangs are joined together with a DNA ligase to produce a final scarless DNA sequence. There are several options of both Type IIS restriction enzymes and DNA ligases for Golden Gate assembly. [Pryor et al., 2020] suggests the choice of Type IIS restriction enzyme does not significantly impact the assembly fidelity. Rather it is the DNA ligase, overhang design and reaction conditions that affect the assembly fidelity. It has been observed that T7 DNA ligase exhibits a bias toward G/C-rich four-base overhangs, whereas T4 DNA ligase shows no significant bias.

Despite the efficiency and widespread use of Golden Gate assembly, the technique has some limitations. For instance, all DNA parts used in the assembly must be free of internal recognition sites for the Type IIS restriction enzyme employed. The presence of such sites can lead to undesired digestion, significantly reducing assembly efficiency. Given the relatively short recognition sequence of most Type IIS enzymes, these sites are quite common in biological DNA and often need to be removed through silent mutations, which adds complexity to the design process. Furthermore, the assembly relies on 4-bp overhangs, or sticky ends, generated during the digestion step to ensure proper alignment and ligation of DNA fragments. The choice of these overhangs is critical for the efficiency and success of the reaction.

The overhangs are usually designed using broad guidelines that aim to minimize

base pairing between non-complementary overhangs [Pryor et al., 2020]. This includes avoiding palindromic sequences and ensuring the same overhang pair is not used more than once in a single assembly reaction. Additionally, many modular cloning systems require at least two mismatched bases between non-complementary overhang sequences. Overhangs with 100 % A/T or G/C content are generally avoided, as they tend to ligate with low efficiency. While these principles help ensure efficient assembly, they can impose limitations when designing complex constructs. Ideal breakpoints within a target DNA sequence may conflict with these rules, complicating the assembly process.

There are tools to address this limitation, for example NEB:s Data-optimized Assembly Design (DAD), which generates customized high-fidelity overhang sets and divides the target sequence into high-fidelity assembly fragments. Compared to randomly or traditionally chosen overhangs the overhangs generated by DAD significantly improved the assembly fidelity. 100 % ligase fidelity can be predicted for up to approximately 20 overhang pairs, but as the number of overhang pairs is increased above this the fidelity decreases. It was also found that high-fidelity assemblies also can contain individual overhangs that violate the traditional overhang design rules. [Strzelecki et al., 2024] proposes an alternative approach where the binding energy of overhangs in combination with the reaction conditions may have a significant effect on assembly efficiency, which also contradicts the traditional design rules.

Several advancements have been achieved utilizing the Golden Gate Assembly method. For example, Lund et al. 2023 employed the DAD software and Golden Gate Assembly to successfully construct 343 out of 458 genes, ranging in lengths from 600 - 3500 bp. Additionally, [Pryor et al., 2022] demonstrated the potential of Golden Gate Assembly for synthesizing larger sequences by effectively assembling a 40 kb genome from 52 parts in a single reaction.

2.4 Droplet Technology

Droplet microfluidics has emerged as a pivotal technology in chemical and biological sciences, enabling high-throughput assays with the capacity to process thousands of samples simultaneously [Ding,]. This approach allows for precise compartmentalization of analytes and cells under controlled conditions, facilitating rapid and cost-effective analysis. With the increasing size of data sets associated with modern molecular science, the ability to perform large numbers of parallel experiments without raising experimental costs is crucial. The strategic use of droplets for sample segmentation serves as an effective approach to both minimize experimental costs and improve analytical efficiency. Droplet technology has been effectively utilized in drug screening applications, where droplets replace traditional microwell plates, reducing reagent consumption while increasing analytical throughput. Additionally, this technology has demonstrated considerable utility in single-cell RNA sequencing, enabling the high-throughput capture of individual cells. Within the droplets, RNA or DNA can be efficiently extracted and low copy targets amplified, facilitating detailed molecular analysis. These developments underscore the versatility and

potential of droplet technology in advancing both research and practical applications within life science.

Monodispersed emulsions are traditionally created using microfluidic devices, which ensure uniform droplet size critical for applications in biology. However, microfluidics requires specialized equipment and expertise, which can limit its accessibility. Hatori, Kim and Abate 2018 suggests an alternative method, particle-templated emulsification (PTE), where hydrogel particles and a water-oil solution are vortexed generating monodispersed droplets without the need for microfluidics. Although untemplated vortexed emulsions are not monodispersed, the addition of surfactant and particles in the solution drastically changes this. Using this method also ensures about a 99 % yield of single-particle droplets, which is especially important for our purposes.

3
Methodology

In this chapter the methodology used in the project is presented, including adoption of DropSynth technologies for generation of barcoded beads and emulsion Polymerase Chain Assembly (ePCA) and development of a Golden Gate Assembly in droplets (dropGG).

3.1 In-house Barcoded Beads Generation and Quality Control

3.1.1 Generation of Barcoded Beads 96X Format

The barcoded beads were manufactured according to the DropSynth protocol in a 96X format (supplementary material). To determine how many of the barcoded beads were manufactured correctly a primer with a fluorescent tag was designed to match with barcode 3. Some of the barcoded beads with barcode 3 was separated and mixed with the primer (5 μl beads, 2.5 μl Taq ligase buffer, 1 μl Taq ligase, 0.5 μl primer, 16 μl MQ-water) and then incubated in a thermomixer overnight to ensure binding of the primer to the beads (50 ℃ 3 h, 40 ℃ 3h, 30 ℃ 2h, 10 ℃ ∞), ramp rate 0.1℃/min, shaking 800 rpm). A control sample without primer was also included. Both samples were washed with B&W buffer 3X (2M NaCl, 1 mM EDTA, 10 mM Tris) on ice and then resuspended in 25 μl B&W buffer. The beads were then examined using flow cytometry. First the size and surface structure was examined and the beads were divided into size groups which were then analyzed separately. The fluorescence of the beads with primer and without primer was then measured and compared for the two groups. The fluorescence of the beads with primer was also examined separately to identify possible outliers.

3.1.2 DropSynth Barcoded Beads Protocol Evaluation

To evaluate the DropSynth protocol for generation of barcoded beads all steps of the protocol were tested and confirmed to eliminate the barcoded beads being the reason for the failed PAZy assembly.

3.1.2.1 Hybridization

The first step of the barcoded beads protocol is to hybridize the anchor oligo, ligation oligo and barcode oligo, resulting in a fragment of 52 bp. To test if the hybridization

reaction could be improved a new protocol (44.5 μl TE buffer + 0.5 μl 5M NaCl, 3 min 100 ℃, cool down on bench) was tested and compared with the DropSynth protocol (5 μl T4 ligase buffer + 40 μl MQ-water, 3 min 70 ℃, ramp down to 60 ℃ for 1 min, 0.1 ℃/sec, ramp down to 50 ℃ for 1 min, 0.1 ℃/sec, ramp down to 40 ℃ for 1 min, 0.1 ℃/sec, ramp down to 30 ℃ for 1 min, 0.1 ℃/sec, place on ice). The amount of oligos in both reactions are 1.25 μl 100 μM Anchor oligo, 1.25 μl 100 μM ligation oligo and 2.5 ul 100 μM barcoded oligo. For both protocols two samples were made; one with all three oligos (52 bp) and one with only anchor oligo and ligation oligo (40 bp). All samples were loaded on a 5% TBE gel (100 V, 45 min) together with ligation oligo, barcoded oligo and anchor oligo for size comparison. For following steps 100 μl hybridize reactions were prepared for each of the four barcodes 1,2,4 and 9 according to the DropSynth protocol.

3.1.2.2 Ligation and Phosphorylation

The following steps of the protocol are ligation and phosphorylation of 5' end of the barcoded oligo. Four barcodes were tested for each step (barcodes 1, 2, 4 and 9). A mix of 1.25 μl T4 DNA ligase, 5 μl T4 DNA ligase buffer and 44 μl MQ was prepared and 9 μl of the mix was added to each of the four samples (containing 90 μl hybridize mixture each) and incubated in 16 ℃ for 1 h followed by 65 ℃ for 20 min. 12 μl of each sample was saved and the phosphorylation reaction was continued with the remaining 87 μl. A mix of 10 μl T4 PNK and 30 μl MQ was prepared, 8 μl of the mix was added to each sample and then they were incubated at 37 ℃ for 40 min followed by 65 ℃ for 20 min. The saved samples from all three steps (hybridization, ligation and phosphorylation) were loaded on a 5 % TBE gel (100 V, 45 min). The fragment produced by the phosphorylation reaction will be referred to as the 52-bp barcoded fragment.

3.1.2.3 Binding to Beads

The last step of the barcoded beads protocol is binding to the Streptavidin beads. As it was previously noted that the majority of barcoded fragments are not bound to the beads it was tested if a higher concentration of beads could increase the binding. 220 μl Streptavidin beads were washed two times with B&W buffer and resuspended in 440 μl B&W to a final concentration of 5 mg/ml. Two samples for each barcode were prepared; one with lower beads concentration (10 μl beads, 48 μl barcoded fragment) and one with higher concentration (100 μl beads, 48 μl barcoded fragment) and the binding reaction was performed in 37 ℃ for 30 min with shaking (2000 RPM). After binding, each sample was washed 8 times with B&W buffer (saving the supernatant from the binding reaction), 10 μl of beads was resuspended in 10 μ l of MQ water and heated at 70 ° C for 10 min. Heating will release the bond between the biotin and the Streptavidin beads. The supernatant from the heated beads was recovered and loaded on the gel, as well as the supernatant saved from the binding reaction.

3.2 Assembly of sfGFP with PCA

Two different designs, 4 and 5 oligos with overlapping sequences of 20 bp, were made for assembly of sfGFP. The length of the oligos were varying from 100 bp to 200 bp to achieve a Tm between 55 - 65 ℃ for all overlaps. The oligos were ordered as oligo pools, one for each design, with forward and reverse primers for each pool. The gene and oligo sequences as well as the primers are found in supplementary table 8 resp. 1 (No. 3 and No. 4).

The PCA was performed as a regular PCR protocol (Initial 98 ℃ 2 min, denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 20 s (Phusion) 40 s (PrimeStar), final extension 72 ℃ 5 min, 35 cycles), except that no primers were added, testing two different polymerases; PrimeStar and Phusion. After assembly gel electrophoresis was performed to check the size of the constructs. An additional round of PCR was performed with primers for all samples to amplify the correct assembly, and the size of the constructs were checked on a gel. Gel purification was performed for the samples from the second PCR and the purified samples were sent for sequencing.

The purified samples from the second PCR were inserted into a plasmid using Gibson assembly, transformed into E.coli and plated on LB plates. The colonies were then cultivated overnight in LB media and after plasmid extraction these samples were also sent for sequencing.

For the PCA assembly in droplets the same oligo pools as in the previous test was used and as Phusion showed slightly stronger bands in the previous PCA this polymerase was chosen. For each oligo pool two assembly reactions were made, one with primers and one without primers. The DropSynth protocol was followed for the emulsion PCA (ePCA) setup and the previously described PCA protocol was used. After ePCA an additional round of PCR was performed with primer for all samples. All samples were cloned into a vector using Gibson assembly and transformed into e.coli to be cultivated on LB plates overnight.

3.3 DropSynth Assembly of a PAZy Gene Library

The oligos for ePCA assembly of the PAZy gene library were designed using the code provided by Plesa lab (`https://github.com/PlesaLab/DropSynth_code_2023`). The PAZy library consists of 39 genes total where the genes were split into 4,5,6,7 or 8 oligos depending on the length of the gene (Supplementary table 2-7). The oligos were then ordered as an oligo pool.

The initial amplification and bulk amplification of the oligo pool was carried out as described in the DropSynth protocol, except for us using PrimeStar and extended primers. PCR samples after bulk amplification were gel extracted using the GeneJET Gel Extraction Kit. After purification the oligos were nicked using BspQI and the biotinylated fragment was removed using Streptavidin beads, preventing

this fragment from binding with the barcoded beads in the following steps.

The barcoded beads that we manufactured were pooled containing only the first 39 barcodes to facilitate full usage of all beads. Barcoded beads in 768X format provided by Hannah Eeckhaut from VIB-UGent Center for Medical Biotechnology, Belgium, were used as a positive control (will be referred to as control beads). The oligos were then bound to the barcoded beads using Taq ligase. To control if any oligos were bound to the beads a fraction of the beads were heated to 80 ℃ for 10 minutes to release the bond between the biotin and the Streptavidin beads. This was also done for the beads that were used to remove the biotinylated fragment and loaded on a gel together with the supernatant from the barcoded beads sample.

The ePCA was performed according to the DropSynth protocol, using PrimeStar and 60 bp assembly primers (containing an ITR sequence for suppression PCR). Additional ePCA:s was performed with shorter 20 bp assembly primers as well, excluding the ITR sequence. Amplification of final assemblies was performed with both single primer suppression PCR as well as regular PCR with the 20 bp assembly primers.

3.4 DropSynth ePCA protocol evaluation

3.4.1 Initial Amplification and Bulk Amplification

An annealing test for the initial amplification was performed with extended amplification primers (21 and 22 bp instead of 15 bp), where temperatures 52-62 ℃ were tested for the polymerases PrimeStar, Phusion and Sapphire over 15 cycles. The PAZy oligo pool was used as a template (diluted 1/10).

3.4.2 Nicking

The next step in the protocol is to nick the fragments with BspQI to expose the barcode. It was investigated whether the efficiency of this step could be improved by testing an alternative protocol. The test was performed with two different oligo pools; 20 bp and 25 bp overlap. A reaction mix was prepared by mixing 5 μl NEBuffer, 1 μl BspQI and 42 μ MQ-water. The mixture was divided into four samples, two for each oligo pool, containing 1 μg of DNA (from purified bulk amplification) resulting in a final volume of 10 μl. All four samples were incubated in 50 ℃ for 1 h, then two samples were left on the bench and the other two were incubated in 90 ℃ for 5 min and then placed on ice. All samples were then loaded on a 5 % TBE gel. For comparison to the original overnight protocol two previously nicked samples from the 20 bp and 25 bp overlap pools were also added to the gel.

To confirm correct nicking a checkpoint was added where a small part of the sample from each step(oligo pool before nicking, nicking reaction and after removing biotinylated fragments. The beads used to remove the biotinylated fragment were saved, washed and then resuspended in MQ-water before being heated in 80 ℃ for

10 min to release the fragments. The supernatant was recovered and loaded on the gel with the other samples.

3.4.3 Binding with Barcoded Beads

When examining the barcoded beads after the binding reaction by heating them in 80 ℃ for 10 min nothing could be seen on the gel and therefore it could not be confirmed that the ligation actually occurred. A ligation test was performed comparing the efficiency of Taq DNA ligase (used in the DropSynth protocol) and T4 DNA ligase using the 52-bp barcoded fragment (not bound to beads) and the nicked fragment from the 20 bp and 25 bp overlap pools. The reactions was prepared by mixing 1 μl phosphorylation mix (52-bp barcoded fragment, diluted ¼), 3 μl nicked pool (~200 ng/μl), 2 μl buffer (T4 DNA ligase buffer/Taq DNA ligase buffer), 1 μl enzyme (T4 DNA ligase/Taq DNA ligase) and 14 μl MQ-water. T4 ligase samples were incubated overnight at 16 ℃ ending with 10 min 65 ℃ and the DropSynth protocol was used for the Taq DNA ligase samples. The ligation mixtures were used as template in a PCR reaction with Sapphire (Initial 98 ℃ 2 min (denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 40 s)x30, final extension 72 ℃ 5 min) (primers No. 16 and No. 44 in Supplementary table 1) to amplify correct ligation. A PCR control was performed using the nicked pool as template.

An additional ligation test with increasing concentration of 52-bp barcoded fragments was performed with Taq DNA ligase, T4 DNA ligase and Hi-T4 DNA ligase. Firstly Taq DNA ligase and T4 DNA ligase was compared and the reactions were prepared by mixing 1 μl/ 2 μl/ 3 μl/ 5 μl phosphorylation mixture(diluted ¼), 2 μl buffer (T4 DNA ligase buffer/Taq DNA ligase buffer), 1 μl enzyme (T4 DNA ligase/ Taq DNA ligase), 3 μl nicked pool and MQ-water to 20 μl. The reactions were performed for 1 h, 45 ℃ for Taq DNA ligase samples and 16 ℃ +10 min 65 ℃ for T4 DNA ligase samples. Samples were loaded on 5 % TBE gel.

The test comparing Taq DNA ligase and Hi-T4 DNA ligase was performed as the previously described ligation test, apart from increasing the concentration of 52-bp barcoded fragment even further; low-concentration 4 μl phosphorylation mixture (not diluted) and high-concentration 8 μl phosphorylation mixture (not diluted). Reaction times were 1 h, 45 ℃ for all samples, with an additional 10 min 65 ℃ for Hi-T4 samples. Samples were loaded on 5 % TBE gel.

A test comparing reaction time for the binding reaction was performed with the barcoded beads and the nicked fragments using Taq DNA ligase. The samples were prepared by mixing 1,3 μg nicked DNA, 20 μl barcoded beads, 10 μl Taq ligase buffer, 4 μl Taq DNA ligase and MQ to 100 μl. One sample was incubated at 45 ℃ for 1 h and the other one at 45 ℃ overnight. 10 μl of each sample was washed with B&W buffer and then resuspended in 10 μl MQ-water, heated at 80 ℃ for 10 min before the supernatants were recovered. The supernatant was used as a template for PCR with Sapphire (Initial 98 ℃ 2 min (denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 40 s)x30, final extension 72 ℃ 5 min) (primers No. 16 and

No. 44 in Supplementary table 1). The amplified fragments were gel extracted using GeneJET Gel Extraction Kit and sent for sequencing.

3.4.4 ePCA Test

Firstly a PCA test was performed, without droplets, with the 20 bp and 25 bp overlap 4 gene pools. The reactions were prepared by mixing 20 μl loaded beads, 1 μl PrimeStar, 1 μl dNTP, 0.5 μl AsmF_40bpITR, 0.5 μl AsmR_40bpITR (No. 29 and 30 in Supplementary table 1), 10 μl PrimeStar buffer, 3.5 μl BtsI and MQ-water to 50 μl. Firstly the samples were incubated at 37 ℃ for 30 min in the thermomixer for BtsI to cut and release the fragments from the beads. Samples were then transferred to PCR tubes and the DropSynth protocol for ePCA was started (94 ℃ 2 min, (94 ℃ 15 s, 57 ℃ 20 s, 72 ℃ 45 s)x60, 72 ℃ 5 min, 4 ℃ ∞). Suppression PCR was performed using the assembly mixture as template (2 μl template, 4 μl 10 μM suppression primer(No. 31 in Supplementary table 1), 1 μl PrimeStar, 1 μl dNTP, 10 μl PrimeStar buffer, MQ-water to 50 μl)(Protocol: Initial 98 ℃ 2 min (denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 40 s)x30, final extension 72 ℃ 5 min). TA-cloning and colony PCR was performed with column purified suppression PCR samples as insert and colonies of around 1000 bp were sent for sequencing.

3.5 Extended Overlap PAZy Genes for ePCA

3.5.1 Extending Overlaps with PCR

To examine whether the length of overlaps were the reason for the previously failed assembly four genes out of the PAZy pool were selected for a change of design. Primers were designed to extend all overlaps to either 20 bp or 25 bp (No. 40-107 in Supplementary table 1). The primers were designed to be used after fragments had been cut with BtsI, meaning the necessary parts for nicking and the barcode would be removed. Therefore, primers were designed to add these parts after the overlaps had been extended (No. 45, 61, 78 and 95 in Supplementary table 1).

Firstly the original PAZy pool was amplified using the elongated version of the initial amplification primers (No. 35 and 36 in supplementary table 1) (2 μl template (diluted 1/10), 4 μl PrimeStar, 4 μl dNTP, 2 μl forward primer, 2 μl reverse primer, 40 μl PrimeStar buffer, MQ-water to 200 μl, divided over 4 PCR tubes á 50 μl) (Protocol: Initial 98 ℃ 2 min (denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 40 s)x15, final extension 72 ℃ 5 min). Amplified fragments were column purified using GeneJET PCR Purification Kit. Purified fragments were cut with BtsI by mixing 1 μg DNA with 1 μl BtsI, 5 μl NEBuffer and MQ-water to 50 μl and incubated at 37 ℃ for 15 min before column purification. The purified cut PAZy pool would be used as template for PCR reactions to extend overlaps.

All PCR reactions to extend overlaps was performed separately (32 in total, 0.5 μl template, 4 μl PrimeStar, 4 μl dNTP, 2 μl forward primer, 2 μl reverse primer, 40 μl

PrimeStar buffer, MQ-water to 200 μl, divided over 4 PCR tubes á 50 μl)(Protocol: Initial 98 ℃ 2 min (denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 40 s)x30, final extension 72 ℃ 5 min). All successfully amplified fragments were column purified. Samples failing the first amplification were amplified using Sapphire and samples showing a second band on the gel were gel extracted. The PCR to add the barcode and nicking sites was performed in the same way, but using each specific fragment from the previous PCR as template. Purified PCR samples were pooled according to the length of overlap, the volume of each sample depending on the measured concentration to contain an equal amount of each fragment.

3.5.2 ePCA and Downstream Processing

The following steps; nicking, removal of biotinylated fragments and binding with the barcoded beads was performed according to the DropSynth protocol.

After binding the nicked fragments to the barcoded beads the beads are washed with B&W buffer and resuspended in elution buffer. The oil solution was prepared by mixing 150 μl 2 % FluoSurf(Emulse) with 450 μl Fluorinated oil (Fluigent) in a 1.5 ml Eppendorf tube (per 100 μl reaction). The assembly reaction was prepared on ice. 40 μl loaded beads was mixed with 0.5 μl AsmF_40bpITR, 0.5 μl AsmR_40bpITR (No. 29 and 30 in Supplementary table 1), 50 μl KAPA HiFi 2X Mastermix (KAPA Biosystems), 1 μl BSA (20 mg/ml), 1 μl MQ-water. 7 μl BtsI-v2 was added right before adding the reaction mixture to the bottom of the oil phase and creating the emulsion by vortexing on max speed for 3 min. The emulsions were allowed to settle for a few minutes to let all droplets gather at the top of the oil phase. The emulsion was picked up with a pipette and placed in PCR tubes with 50 μl each. Two different ePCA protocols were tested, with either 37 ℃ or 55 ℃ for the initial BtsI cutting (37/55 ℃ 1.5 h, 94 ℃ 2 min, (94 ℃ 15 s, 57 ℃ 20 s, 72 ℃ 45 s)x60, 72 ℃ 5 min, 4 ℃ ∞).

The emulsion was broken by adding 100 μl 1H,1H,2H,2H-Perfluoro-1-octanol per 100 μl ePCA mixture in a 1.5 ml Eppendorf tube, vortexing at maximum speed for 1 min and centrifuging at 14,700 x g for 10 min. The aqueous solution that settles on top of the oil phase is recovered with a pipette and transferred to a clean 1.5 ml tube.

Some of the assembly mixture was used as template (2 μl template/ 50 μl PCR reaction) for PCR with the 20 bp assembly primers (No. 38 and 39 Supplementary table 1), while the rest of the assembly mixture was gel extracted (cut band 500-1000 bp) with GeneJET Gel Extraction Kit. The gel extracted DNA was used as a template for suppression PCR. The reaction was prepared by mixing 1 μl template, 4 μl suppression primer (No. 31 in supplementary table), 0.5 μl Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 10 μl Q5® Reaction buffer, 1 μl dNTP and MQ-water to 50 μl(protocol: 95 ℃ 3 min, (98 ℃ 15 s, 58 ℃ 30 s, 72 ℃ 15 s)x25, 72 ℃ 1 min). The PCR mixture was column purified using DNA Clean & Concentrator -25 (Zymo Research) and the size was checked on 1 % agarose gel. The purified DNA was used as a template(0.5 μl template/50 μl) for

gene specific PCR with PrimeStar (Protocol: Initial 98 ℃ 2 min (denaturation 98 ℃ 15 s, annealing 56 ℃ 15 s, extension 72 ℃ 40 s)x15, final extension 72 ℃ 5 min) using primers No. 175-180 in supplementary table 1.

3.6 DropGG Assembly of a Gene Library

3.6.1 Initial mRFP Assembly

To test the potential of Golden Gate assembly of oligos mRFP was split, using the NEB SplitSet tool, into 4 and 5 oligos (Supplementary table 9). Common primer sequences and cutting sites for BsaI were added to each oligo and were then ordered as oligo pools as described by [Lund et al., 2023]. To prepare the oligo pools for cloning after assembly overhangs complementary to the pGGAselect vector was added to the first and last oligo of each pool using PCR. Then a first test of Golden Gate assembly was performed for both pools using the pGGAselect vector provided by NEB. As the first assembly was unsuccessful two different programs were tested for 4 oligos, one short (37 ℃ 1 h, 60 ℃ 5 min) and one long ((37 ℃ 5 min, 16 ℃ 5 min)X65 cycles, 60 ℃ 10 min). Assembly samples were transformed into e.coli and plated on LB plates. Colony PCR was then performed to confirm insert size.

When continuing with Golden Gate assembly in droplets only the 4 oligo pool was tested, with the addition of testing NEB Golden Gate mix compared with making our own mix of reagents(2 μl T4 DNA ligase buffer, 1.5 μl T4 DNA ligase, 0.9 μl BsaI, total volume 20 μl) . Reaction set-up was performed as the previous test apart from not adding the vector and the DropSynth protocol for making emulsions were followed. As the shorter program proved more effective in the previous test this was also used for the droplet assembly. The assembly mixture was used as the template for PCR to amplify the correctly assembled genes.

3.6.2 DropGG

Primers were constructed to change the design of the oligos of the 4 genes used in the previous ePCA test to suit Golden Gate assembly, i. e. overlaps were shortened to 4-bp and flanking cutting sites for BsaI was added (primers No. 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136-147 in Supplementary table 1). Additional primers were used to add barcodes and nicking sites to enable binding to the barcoded beads (primers No. 151-162, 45, 62, 78 and 95 in Supplementary table 1). The design change was then performed as described in section 3.3.3.1 Extending overlaps with PCR.

The following steps; nicking and binding to the barcoded beads was performed as described in the DropSynth protocol except that the reaction time for binding was shortened from overnight to 1 h. The oil solution was prepared by mixing 150 μl 2 % FluoSurf(Emulse) with 450 μl Fluorinated oil (Fluigent) in a 1.5 ml Eppendorf tube (per 100 μl reaction). The dropGG reaction was prepared on ice by mixing 40 μl loaded beads with 10 μl T4 DNA ligase buffer, 6 μl T4 ligase, 3.6 μl BsaI-v2 and

MQ-water to 100 μl. A control sample with only beads loaded with oligos for mRFP was created. BsaI-V2 was added right before creating the emulsion by adding the reaction solution to the bottom of the oil phase and vortexing on maximum speed for 3 min. The emulsion was transferred to PCR tubes, 50 μl in each, and placed in the thermocycler at 37 ℃ overnight, with a finishing step of 60 ℃ for 10 min.

The emulsion was broken and aqueous phase recovered as done in 3.3.3.2 ePCA and downstream processing. The assembly mixture was used as a template for gene specific PCR using primers No. 17, 18 and 177-180 in Supplementary table 1.

4

Results

In this chapter, the findings of the project are presented. This includes adoption and evaluation of DropSynth technologies and development of dropGG.

4.1 Adoption of DropSynth Technology

The adoption of the DropSynth technology was performed in three steps; PCA (without emulsion) and ePCA (with emulsion) of sfGFP, ePCA of the PAZy library and the redesign of 4 PAZy genes for ePCA with extended overlaps. This was done to test parts of the methodology before generating the small library to identify issues and adjusting the protocol thereafter.

4.1.1 Assembly of sfGFP with PCA

For the assembly of sfGFP with PCA from oligos two designs were made; one by splitting the sfGFP into 4 oligos and the other by splitting the sfGFP into 5 oligos. The assembly was performed with and without primers and an additional round of PCR where the assembly mixture is used as template was used to amplify the correct assemblies.

After the PCA of sfGFP no clear bands could be identified on the gel (Fig. 4.1a) but after the second PCR the bands around the correct size (783 bp) are clearly visible (Fig. 4.1b). We purified the correct size band, ligate into a vector, and sequencing results proved that the sfGFP was assembled correctly.

We further tested if the PCA for sfGFP gene could be done within droplets in the ePCA way with Phusion polymerase. The same sfGFP oligo pools as in the previous experiment was used. For each oligo pool, two different PCR strategies were tested, one by adding primers (No. 14 and No. 15 in the supplementary table 1) in the emulsion mixture, and the other without adding primers. As seen in Fig. 4.1c all samples show the correct assembly size after amplification (lane 5-8), though 5 oligos without primer (lane 8) seems to be slightly weaker than the other samples. Green colonies could be seen on all plates except for the group of 5 oligo with primer, confirming correct assembly of sfGFP. There seem to be no significant difference between the two strategies of with and without primers.

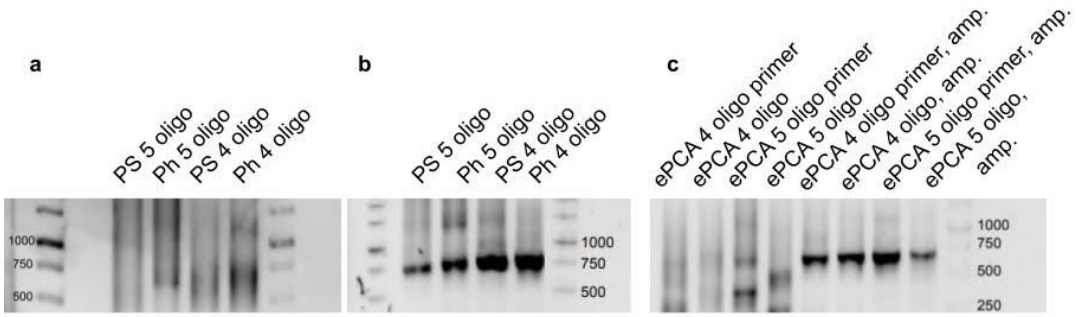

Figure 4.1: a&b: Result from PCA assembly of sfGFP from 4 and 5 oligos, comparing polymerases PrimeStar(PS) and Phusion(Ph), correct assembly size 783 bp. **a:** PCA with no primers and **b:** after one round of PCR with primers. **c:** Result from ePCA of sfGFP with and without primers using Phusion, correct assembly size 783 bp.

4.1.2 ePCA of PAZy Library with DropSynth Protocol

The emulsion PCR (ePCA) was performed according to the DropSynth protocol which can be found in the supplementary material. No bands around the correct sizes (500-1500 bp) were observed after ePCA or after amplification of ePCA. An additional test where PCA without droplets was performed to investigate if the problem was the droplets, but it seems that this was not the case. When designing the oligo, it was found that most overlaps were lower than the expected length of 20 bp and the Tm of the overlaps were also lower than expected, around 51-53 ℃ when they should be 58-62 ℃.

4.1.3 Design Change of PAZy Genes for ePCA

To investigate whether the lengths of overlaps was the reason for the previous ePCA not working, four genes out of the PAZy pool were chosen for a change of design. As the previous overlaps were around 15-20 bp two new designs were made; one where all overlaps are 20 bp and one where all overlaps are 25 bp. Moreover, a new pool of barcoded beads was made containing only the four barcodes of the genes with changed design. ePCA with the 20 bp- and 25 bp- overlap pools were performed with the same protocol as previously. When checking the assembly mixture on agarose gel, nothing could be visualized around the correct size (750 bp) indicating that the assembly did not work.

Based on the findings from the evaluation in 4.2.3.3 ePCA test, it was determined that 25 bp overlaps performed better than 20 bp overlaps in PCA. Furthermore, both 55 ℃ (suggested by NEB manual) and 37 ℃ (in dropSynth protocol) were effective for BstI digestion. Consequently, 25 bp overlaps were adopted for subsequent ePCA designs. Both ligation with a pMD18-T vector and gene-specific PCR was performed with the processed ePCA samples and the resulting products were sent for sequencing. Correct assembly could be confirmed for two out of the four genes in

the 25 bp overlap pool (Fig. 4.2) but no genes were assembled in the 20 bp overlap pool. This result indicates that it was in fact the length of the overlaps in addition to the choice of polymerase in the ePCA reaction that was the issue for the first PAZy assembly. The reason for no assembly confirmation on the remaining two genes is that the design of the fragments did not allow for different enough primers and as a result SAY37583.1 was amplified instead of SAY37584.1 and SAY37587.1. Due to time limitations this could not be adjusted.

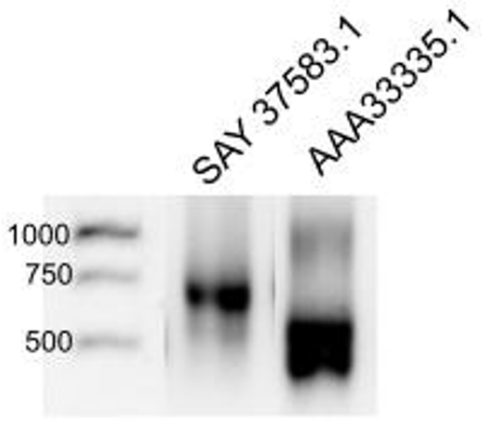

Figure 4.2: Gene specific PCR using processed ePCA 25 bp- overlap 55 ℃ as template.

4.2 Evaluation of DropSynth Protocol

While performing the extended overlap test for ePCA, we simultaneously tested and evaluated the steps of the DropSynth protocol to identify the potential cause for the previous PAZy ePCA not being successful. During this process, we also introduced several checkpoints throughout the protocol to ensure accuracy at each stage.

4.2.1 Barcoded Beads Manufacturing

The barcoded beads were manufactured based on Pierce Streptavidin Magnetic Beads (ThermoFisher) and contain a covalently attached library of barcoded oligonucleotides, which could be able to bind with reverse complementary oligonucleotides on the 5' end of the gene fragments. Every bead-attached oligo contains a 40 base, double-strand anchoring sequence, 12 base, bead-specific barcode. The split fragments from the same gene share the same reverse complementary barcode sequence which could bind with the beads with the same barcode specifically. These barcoded beads were manufactured in 96x format in-house according to the Dropsynth protocol [Plesa et al., 2018], since the barcode oligos were also ordered in 96x format.

The barcoded fragment to be bound with the beads is constructed from three oligos; anchor oligo, ligation oligo and barcode oligo, which are hybridized, ligated and

phosphorylated (Fig. 4.3a). To test the hybridization reaction two samples were prepared, one with all three oligos and one with only anchor and ligation oligo. This was done to examine if a difference in size could be observed between the two samples, which would indicate the barcode oligo being assembled correctly. Furthermore an alternative protocol for hybridization was tested for possible improvement of efficiency. As a size difference could be visualized between the samples with two and three oligos it was assumed that the fragment was hybridized correctly (Fig. 4.3b). No significant improvement could be identified between the DropSynth protocol and the new protocol.

The following two steps of the protocol, ligation and phosphorylation, were examined by comparing the size of the fragment in each step as the size should remain the same. This was performed for four barcodes separately, barcodes 1,2,4 and 9. No size difference could be identified between the steps for any of the four barcodes, indicating that the barcoded fragment is correctly constructed (Fig. 4.3c).

Finally the binding of the barcoded fragment to the Streptavidin beads was examined by testing two different concentrations of beads, as it was previously noted that a large portion of the barcoded fragment remained in the supernatant after the binding reaction. To analyze what is bound to the beads they were heated to release the biotin-streptavidin bond. As seen in Fig. 4.3d the binding was not improved by a higher concentration of beads, rather the opposite. For the low concentration heated beads samples a fragment of correct size could be visualized but not for the high concentration beads samples. From these results it was concluded that the barcoded beads are constructed correctly and no changes should be made to the protocol, though a check can be performed by saving some samples from each step and finally heating a part of barcoded beads and load all samples on a 5 % TBE gel to confirm that all samples has the same size, 52 bp.

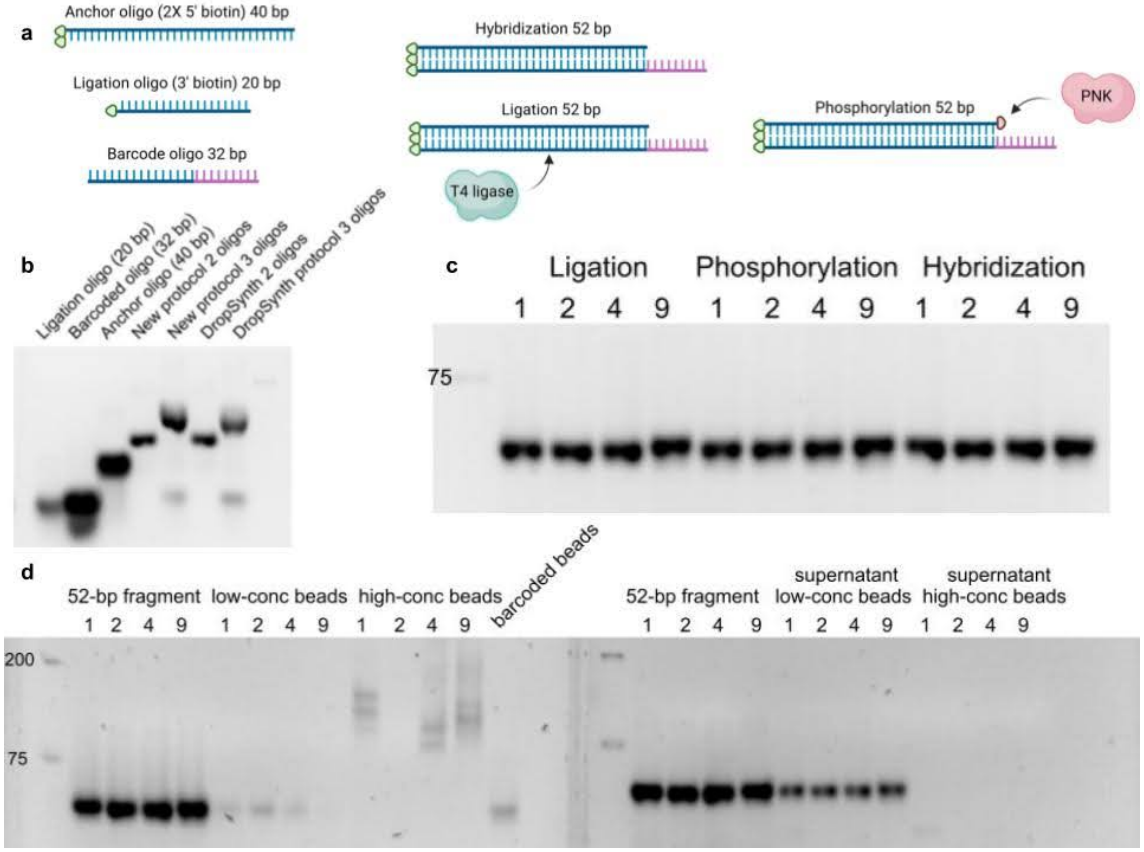

Figure 4.3: **a:** Construction of barcoded fragments (52 bp) to bind with Streptavidin beads from anchor oligo (40 bp), ligation oligo (20 bp) and barcode oligo (32 bp). **b:** Hybridization test of two protocols with anchor and ligation oligos (2 oligos) or with anchor, ligation and barcode oligos (3 oligos). **c:** Comparison of the three first steps in the barcoded beads protocol; hybridization, ligation and phosphorylation. **d:** Binding of barcoded fragments to Streptavidin beads with low concentration of beads compared with high concentration of beads. To the right: supernatant from heating the beads, to the left: supernatant from binding reaction.

4.2.2 Quality Control of Barcoded Beads

After constructing the beads a quality control was performed. We further incubate a fluorescence QC primer (No. 34 in Supplementary table 1) with our manufactured beads from well A3, following the same steps for incubating the beads with split fragments. The beads were washed three times before flow cytometry analysis. FSC and BSC results reveal that the size of the beads seem not to be uniform. We gated the beads into two groups according to their sizes, i.e., group A was gated for a more clustered population (58.9%), and group B was gated for a more dispersed population (45.95%) (Fig. 4.4a). We analyzed beads with and without QC primers incubation, and they both exhibited the same pattern of one clustered population (group A) and one dispersed population (group B).

The beads with QC primer incubation exhibited a distinct fluorescence shift compared with the beads without QC primer incubation in both group A (Fig. 4.4b) and

group B (Fig. 4.4c). This shift suggests that the fluorescent primers were successfully bound to the beads, which means the beads could have been successfully barcoded, so that the QC primer could bind with the barcode nucleotides. Only 1.49% and 9.24% were observed for group A (Fig. 4.4d) and group B (Fig. 4.4e) respectively to be outliers, indicating that the majority of the beads harbor barcodes and could bind with QC primer well.

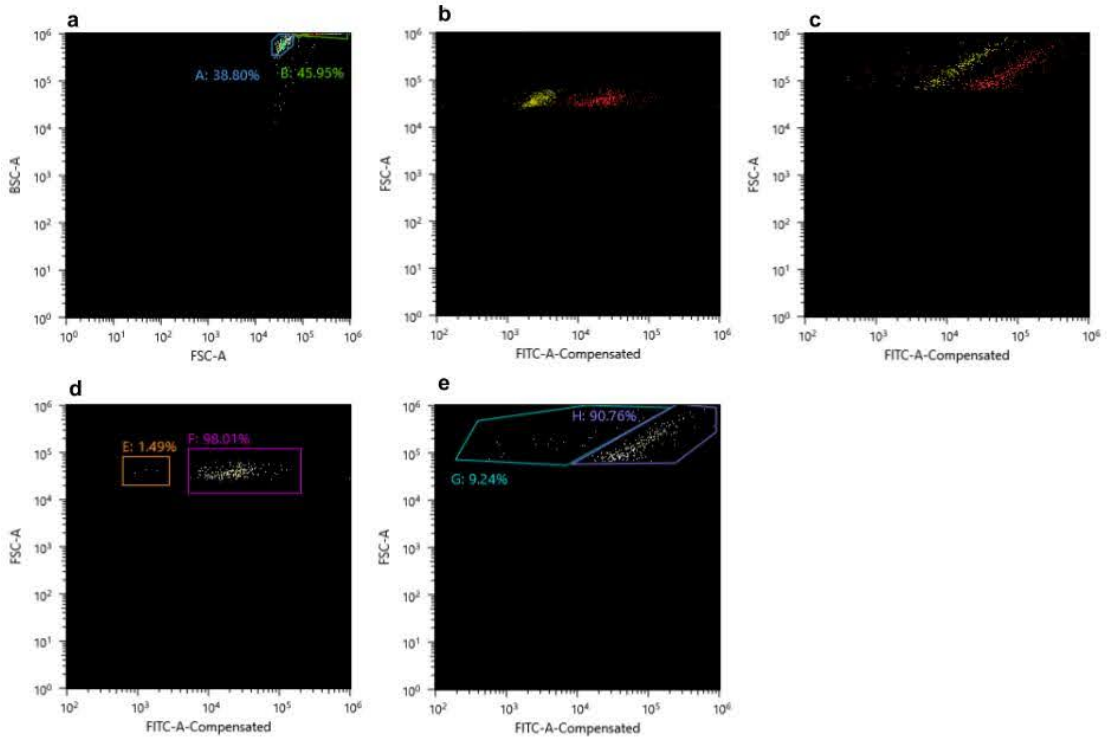

Figure 4.4: a: Display of the size(FSC-A) and surface structure (BSC-A) of the beads. The beads were divided into two groups based on size, A and B. **b&c:** Shift in fluorescence between beads without fluorescent primer (yellow) and beads with fluorescent primers (red) for the two size groups, A(Fig. b) and B(Fig. c). **d&e:** Showing only beads with fluorescent primers for the two size groups A (Fig. d) and B (Fig. e) where the groups to the left in both figures (E and G) indicate beads that were not successfully manufactured/fluorescent primers were not bound to the beads.

4.2.3 Evaluation of DropSynth ePCA Protocol

4.2.3.1 Initial Amplification and Nicking

When first starting the DropSynth protocol for ePCA it was found that the 15 bp primers for initial amplification of the oligo pool were nonfunctional. The primers were extended to overlap the cutting sites for BtsI, making them 21 and 22 bp long while still being universal for all oligos. The longer primers were found more effective and have been utilized continuously since their initial application. For the initial

amplification different polymerases were also tested as well as different annealing temperatures, where PrimeStar was proved the most effective and no difference could be found between the annealing temperatures (Fig. 4.5a). The same changes were made to the second step in the protocol, bulk amplification, where biotin is added to the 3' end of the top strand of each fragment.

The next step in the protocol is nicking with BspQI, which exposes the barcode and enables binding to the barcoded beads. In the protocol this reaction is done overnight, but we tested and compared with a one hour reaction and could not find a significant difference. We also added a checkpoint after the nicking reaction where the oligo pool (before nicking), nicking reaction and purified nicking (after removing biotinylated fragments with Streptavidin beads) are compared on agarose gel. The beads used to remove the biotinylated fragment can also be boiled and supernatant loaded on the gel as well. The oligo pool should be 300 bp, the nicking reaction should be both 300 bp (not nicked) and 277 bp (nicked), purified nicking should only contain nicked fragments of 277 bp. This is demonstrated for the PAZy pool in Fig. 4.5b, and it can be seen that the top strand of 300 bp has been removed in the purified sample. In Fig. 4.5c the same process is presented but for the 4 gene pool, where the boiled beads sample is included. As the design was changed for these fragments they are shorter than 300 bp, and not all the same length. Prior to nicking the fragments are around 270 bp, and nicked fragments around 256 bp. The boiled beads should contain fragments of 270 bp (not nicked oligos) and 26 bp (biotinylated fragments from nicked oligos). The 26 bp fragment is not visible on the gel but it was assumed that the nicking was successful based on the size difference between before and after nicking.

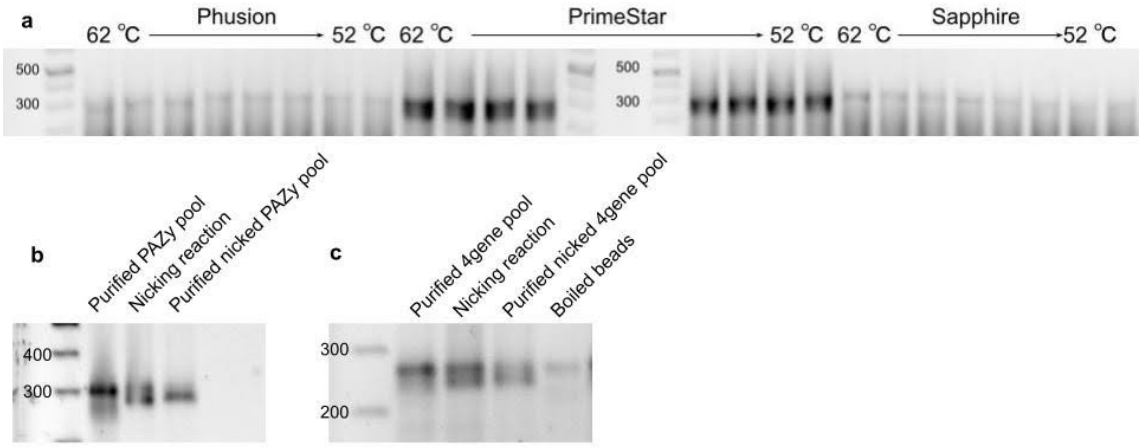

Figure 4.5: **a:** Annealing and polymerase test for initial amplification in the DropSynth ePCA protocol, correct amplified fragment should be 300 bp. **b:** nicking process for PAZy pool. **c:** Nicking process for 4gene pool.

4.2.3.2 Ligating with Barcoded Beads

After nicking the fragments can be bound to the barcoded beads through ligation with Taq DNA ligase. When boiling the beads after binding and measuring the

concentration of the supernatant of the binding reaction it was discovered that only a fraction of the nicked fragments are bound to the beads and several experiments were conducted both to confirm correct ligation and to explore if the efficiency of the ligation could be improved.

Firstly ligation experiments were carried out with the nicked fragments and the 52-bp barcoded fragment (not bound to beads). The efficiency of Taq DNA ligase and T4 DNA ligase was compared using overnight protocols. To verify presence of correctly ligated fragments, the ligation mix was used as template for PCR. A PCR control was added where the template was the nicked fragments but using the same primers and protocol as the PCR reactions for the ligated samples. The correctly ligated fragment should be around 287 bp which can be seen as a very weak band in ligation samples, and as a stronger band in the PCR samples in Fig. 4.6c. This confirms that the ligation is correct, even though the efficiency is low. There is no obvious difference in efficiency between T4 DNA ligase and Taq DNA ligase, rather that T4 DNA ligase also creates an unspecific band around 1000 bp. From this gel picture it also seems like the limiting factor in the ligation is the 52-bp barcoded fragment as it is not visible in any of the ligation samples.

To further test if the 52-bp barcoded fragment is the limiting factor a test was carried out with an increasing amount of barcoded fragment for both Taq, T4 and Hi-T4 DNA ligase. Taq and T4 were compared with up to four times the original amount of barcoded fragments, which resulted in increased ligation for both enzymes (Fig. 4.6a). All ligation reactions in this experiment were carried out for 1 hour instead of overnight and no significant difference could be identified, indicating that the ligation does not require a 12+ hour reaction. Taq DNA ligase was also compared with Hi-T4 DNA ligase with 16 (low-conc) and 32 (high conc) times the original amount of barcoded fragments (Fig. 4.6b). From the gel pictures it is clear that an increased amount of barcoded fragments does increase the ligation efficiency up to a certain point but not further, as some of the barcoded fragments are visible in the ligation samples. It is also clear that Taq DNA ligase, T4 DNA ligase and Hi-T4 DNA ligase works well for this ligation reaction. As no improvements could be identified for T4 or Hi-T4 DNA ligase, Taq DNA ligase was used for following ligation reactions.

As one hour reactions were sufficient for ligating the barcoded fragment and the nicked fragment in previous experiments this was also tested for ligating the nicked fragments to the barcoded beads. The reactions were prepared as described in the DropSynth protocol with the difference that one was incubated in 45 ℃ for one hour and the other was left overnight. This was performed for the 20 bp and 25 bp overlap pools. After binding, a fraction of beads from each reaction was heated to release the DNA bound to the beads. This was used as a template for PCR, as done in the previous ligation test. When loaded on the gel nothing was visible from the heated beads but in the PCR reactions a fragment of the correct size occurs. This fragment was sent for sequencing and correct ligation could be confirmed. No difference could be identified between the one hour reactions and the overnight reactions, suggesting

that binding for one hour is sufficient (Fig. 4.6d). An additional binding test was performed with double the amount of barcoded beads, to test the previous theory about concentration of barcoded fragments increasing ligation efficiency. However, no increase in efficiency could be confirmed (data not shown).

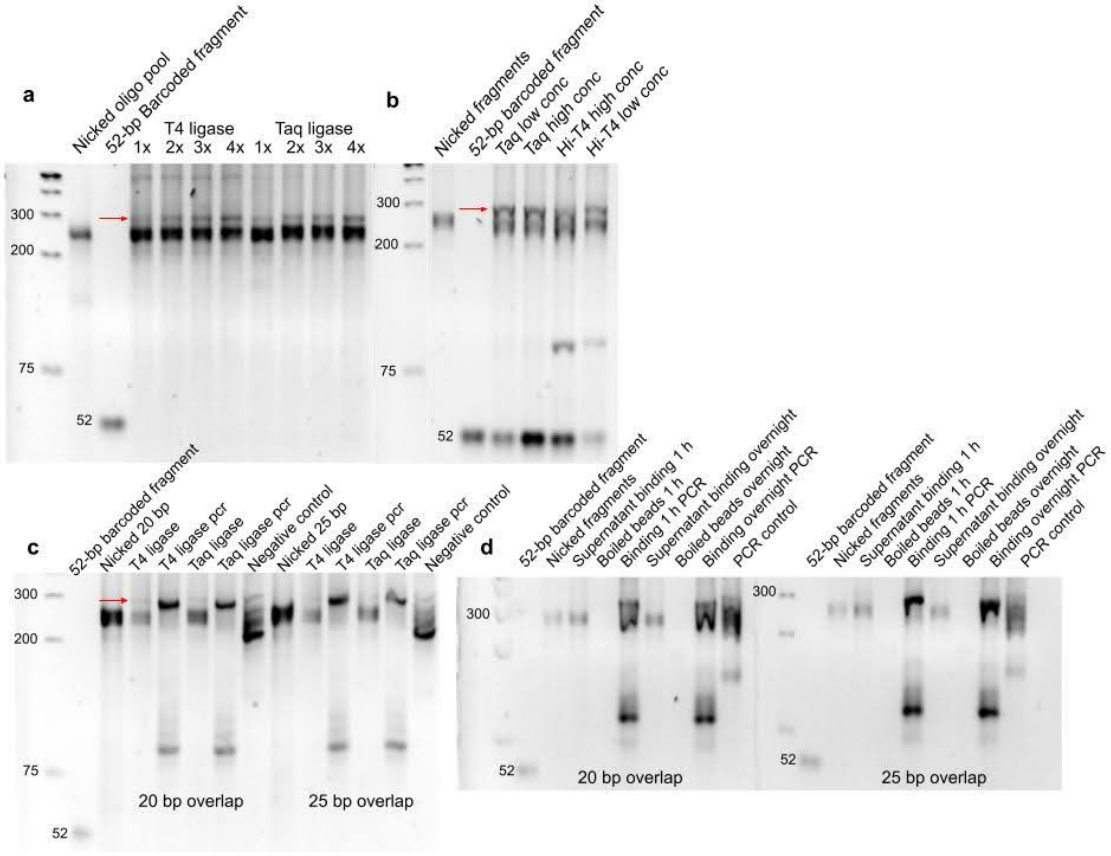

Figure 4.6: Ligation test of nicked fragments and barcoded fragments or barcoded beads where the red arrows indicate the size of correct ligation. **a:** Comparison of ligation with T4 DNA ligase and Taq DNA ligase with increasing concentration of barcoded fragments. **b:** Comparison of ligation with Taq DNA ligase and Hi-T4 DNA ligase with 16x (low) and 32x (high) concentrations of barcoded fragments. **c:** Comparison of T4 DNA ligase and Taq DNA ligase for 20 bp and 25 bp overlap pools where the ligation mixture was used as template for PCR to amplify and confirm correct ligation. Negative control was conducted by using the nicked fragments as template for the same PCR reaction as for the ligated samples. **d:** Ligation test of nicked fragments with barcoded beads for 20 bp and 25 bp overlap pools comparing 1 h and overnight reactions. After binding the supernatant was saved and loaded on the gel and the beads were washed and boiled and then used as a template for PCR.

4.2.3.3 ePCA Test

As it was confirmed that the nicked fragments can be ligated to the barcoded beads (Fig. 4.6d), a test assembly without emulsion was performed using fragments bound to beads for the 20 bp and 25 bp overlap pools. This was done to test if the problem with the initial PAZy assembly stems from the emulsion. The correct assembly should be around 750 bp, but nothing of this size could be identified on the gel

after PCA for either of the pools. Suppression PCR was also performed using the PCA as template and a vague band around 750 bp appeared for the 25 bp overlap assembly, but not for the 20 bp overlap assembly. The assembled mix was ligated with a pMD18-T vector and subsequently sequenced. It was found only two genes were partially assembled (3 out of 4 parts, and another one was missing) in the 25 bp pool, while none was assembled in the 20 bp pool.

Another ePCA test was performed with the 20 bp- and 25 bp-overlap pools, with a change of polymerase from PrimeStar to KAPA. Furthermore, two different cutting temperatures for BtsI were tested; 37 ℃ (recommended by NEB) and 55 ℃ (DropSynth protocol). When the assembly mixture was loaded on agarose gel a band around the correct size could be visualized for the 25 bp-overlap pools (lane 3 and 7 in Fig. 4.7c) but not for the 20 bp-overlap pools (lane 1 and 5 in Fig. 4.7c). After downstream processing of the ePCA samples, a sharper band could be visualized for the 25-bp overlap pools (lane 4 and 8 in Fig. 4.7c). The gel result is suggesting that the cutting temperature for BtsI did not make a significant difference (lane 4 and lane 8 in Fig 4.7c) but as the 55 ℃ samples showed higher concentration, these were chosen for further analysis. After ePCA two paths of downstream processing were chosen; one with only suppression PCR and one with gel extraction of assembly mixture followed by suppression PCR. The clean-up path proved more successful, as the correct fragment was amplified more distinctly, resulting in a sharper band on the gel with reduced smearing (4.7d).

Ligation with pMD18-T vector and gene-specific PCR (Fig. 4.2) could confirm 2 correctly assembled genes out of 4 in the 25 bp overlap pool but no correctly assembled genes in the 20 bp overlap pool. This result indicates that it was in fact the length of the overlaps in addition to the choice of polymerase in the ePCA reaction that was the issue for the first PAZy assembly.

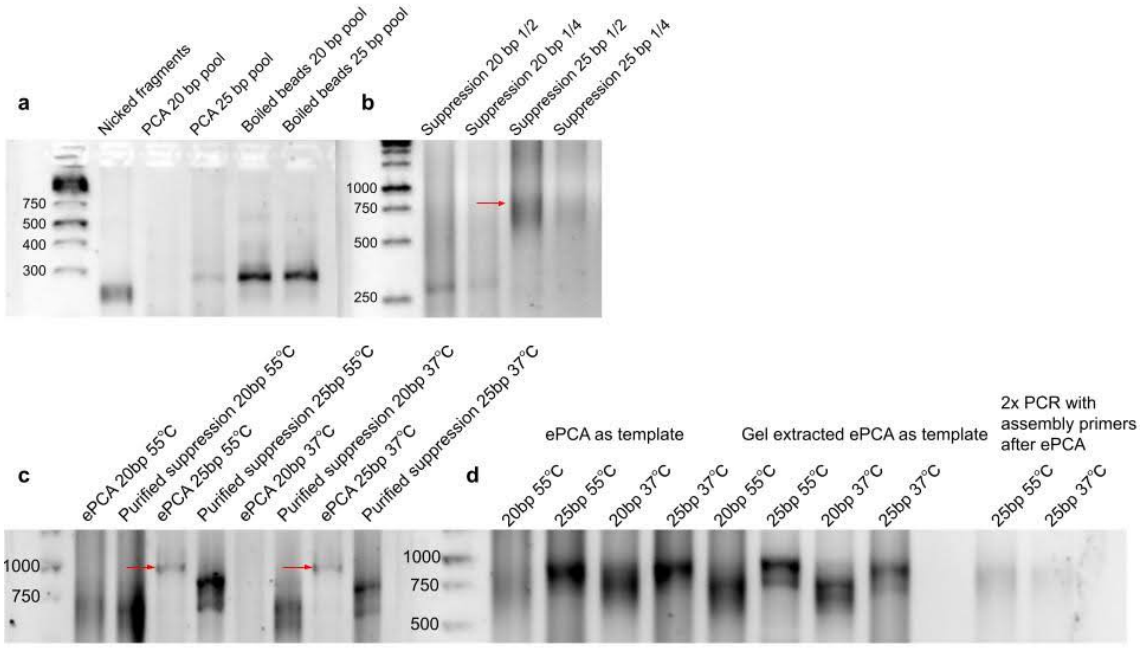

Figure 4.7: **a:** PCA using the fragments bound to barcoded beads, with the amplified fragments bound to beads as size reference. **b:** Suppression PCR using the PCA as template (diluted ½ and ¼). **c:** ePCA mix next to ePCA after clean-up and suppression PCR. **d:** Comparison of downstream processing after ePCA; ePCA mix as template for suppression PCR, gel extracted ePCA as template for suppression PCR, ePCA mix as template for PCR with 20 bp assembly primers.

4.2.3.4 Beads Distribution in Droplets

Based on the suggestion by (Hatori, Kim, and Abate 2018) that beads are randomly distributed within emulsions formed by vortexing, this was examined for our emulsions. Two samples with identical volumes were prepared in tubes of different sizes; 1.5 ml and 15 ml. The 1.5 ml sample was vortexed at maximum speed for 3 min (Fig. 4.8a), while 15 ml was vortexed at maximum speed for 6 min (Fig. 4.8b). Although the droplet sizes were not measured in detail, it was estimated that the majority of droplets containing beads had a diameter of approximately 40 μm. A total of 100 droplets from each sample were analyzed, and the number of beads per droplet was recorded. The result, presented in Fig. 4.8c, indicates that most droplets in both samples contain zero or one bead. However, a significant proportion of droplets contained more than one bead, with 48 % of the 1.5 ml sample and 39 % of the 15 ml sample exhibiting multiple beads per droplet.

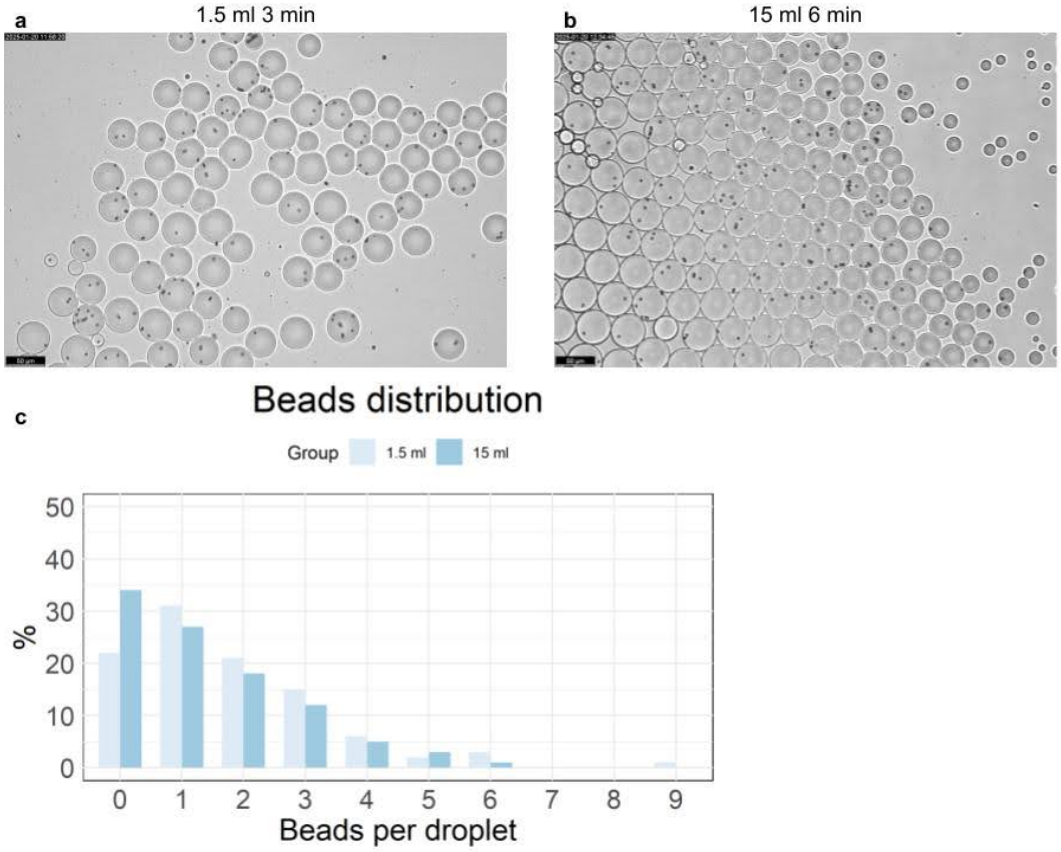

Figure 4.8: Distribution of beads inside droplets generated by vortexing. Beads can be seen as small black dots inside the droplets. **a:** Droplets generated in a 1.5 Eppendorf tube, vortexing 3 min. The scale bar on the lower left corresponds to 50 μm. **b:** Droplets generated in a 15 ml tube, vortexing 6 min. **c:** Distribution of beads in droplets for 1.5 ml and 15 ml sample, 100 droplets examined each.

4.3 DropGG Assembly of a Gene Library

To start the development of Droplet based Golden Gate assembly (DropGG) a few tests were performed with the gene mRFP to test the viability of Golden Gate assembly of oligos both without droplets and within droplets. Same as for the sfGFP test, mRFP was also split into 4 and 5 fragments independently, and a 4-oligo and 5-oligo pool were ordered accordingly. For the initial test without droplets two different programs were used, one with only one cycle (short program) and one with 30 cycles (long program). The pGGAselect vector provided by NEB was included in the assembly mixture to enable colony PCR to identify correct assemblies. 8 colonies each from the samples 4 oligo short program, 4 oligo long program and 5 oligos was selected for colony PCR.

As seen in Fig. 4.9a and 4.9b several of the colonies show correct insert size (810 bp), 6 for 4 oligo short (lane 1-8) and 2 for 4 oligo long (lane 9-16). No colonies of the 5 oligo assembly (lane 17-24) show the correct insert size. For further validation samples with correct insert size were sent for sequencing, confirming correct assembly

for all samples sent for sequencing.

For the second droplet-based test, only the 4-oligo pool combined with the short program was utilized, as this approach demonstrated higher effectiveness in prior experiments. Two different reagent mixtures were evaluated: a commercially available Golden Gate assembly mix from NEB and a self-prepared reagent mix. This comparison aimed to determine whether the self-prepared mix could improve assembly efficiency. In this test, no vector was included. Instead, the size of the assembled products was analyzed using agarose gel electrophoresis immediately following the assembly reaction and again after one round of PCR amplification to enrich for correctly assembled products. This approach allowed for an assessment of the presence of correct assemblies directly after the reaction.

For the droplet golden gate assembly the length of correct assemblies should be 737 bp and a very weak band (Fig. 4.9c) can be seen at this size before the PCR and after PCR it has been significantly amplified (Fig. 4.9d). There seems to be no difference in efficiency between the NEB mix (lane 3 in Fig. 4.9d) and our own mix of reagents (lane 4 in Fig. 4.9d). The samples were also sent for sequencing which confirmed that mRFP was correctly assembled in both samples.

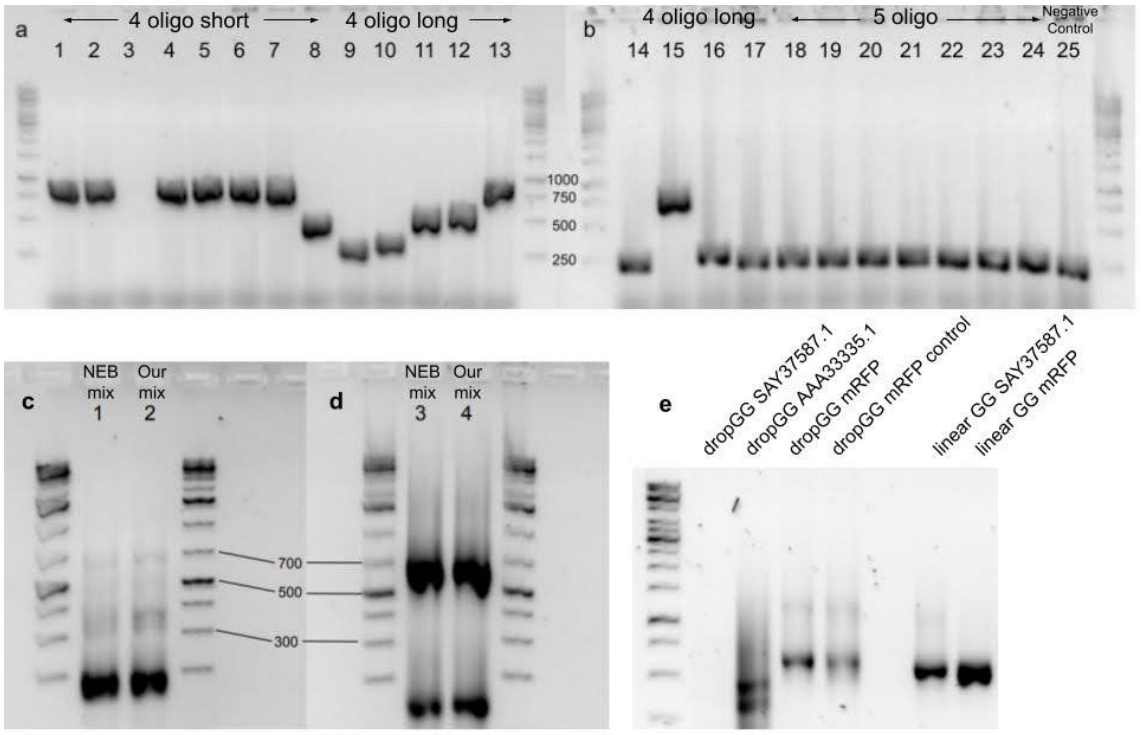

Figure 4.9: a&b: Colony PCR of Golden Gate assembly of mRFP for 4 oligos and 5 oligos. Lane 1-8: 4 oligo short program, lane 9-16: 4 oligo long program, lane 17-24: 5 oligo, lane 25: negative control. **c&d:** Droplet Golden Gate assembly of mRFP before PCR (c) and after PCR (d) where lane 1 and 3 are NEB Golden Gate mix and lane 2 and 4 are our own mix. **e:** Golden Gate assembly in droplets compared with separate linear Golden Gate assembly.

As the linear golden gate assembly in droplets for mRFP was successful as well as the ePCA using the barcoded beads, a new experiment of Golden Gate assembly in droplets (DropGG) for multiple genes using the barcoded beads was planned. For this three out of the four genes from the ePCA experiment was chosen, as well as mRFP. As previously done the design of the oligos were changed using oligo-specific primers for PCR to change the overhangs to 4 bp as well as adding flanking recognition sites for BsaI. Additional PCR reactions were performed to add the sequence for barcodes and nicking sites, to enable binding to the barcoded beads. As the amplification failed for two of the oligos one gene had to be removed from the experiment and one would be assembled with three oligos instead of four. The prepared oligos were pooled, nicked and bound to the barcoded beads according to our own new workflow (used shorter reaction times for binding). An additional pool with only oligos for mRFP was prepared as a positive control. The dropGG was prepared with T4 DNA ligase and BsaI-HFv2 and incubated at 37 ℃ overnight before the emulsion was broken and the assembly mixture retrieved. Gene specific primers were used to amplify the genes separately using the assembly mixture as template. As seen on the gel in Fig. 4.8e the gene with three oligos as well as mRFP

was assembled, which was also confirmed with sequencing. The result confirms that multiplexed Golden Gate assembly in droplets works.

4.4 Comparison of DropSynth and DropGG

A comparative analysis of the results obtained from the DropSynth and dropGG revealed that 66% of genes were successfully assembled using dropGG, compared to 50% using DropSynth (Fig. 4.10b). The dropGG assembly included 3 genes, of which 2 were successfully assembled, while the DropSynth assembly included 4 genes, of which 2 were successfully assembled (Fig. 4.10a). Gene assembly outcomes from both workflows were validated through gene-specific PCR, followed by sequencing confirmation. Additionally, the post-assembly processing time was significantly reduced in the dropGG workflow compared to ePCA, highlighting an efficiency advantage of dropGG (Fig. 4.10c).

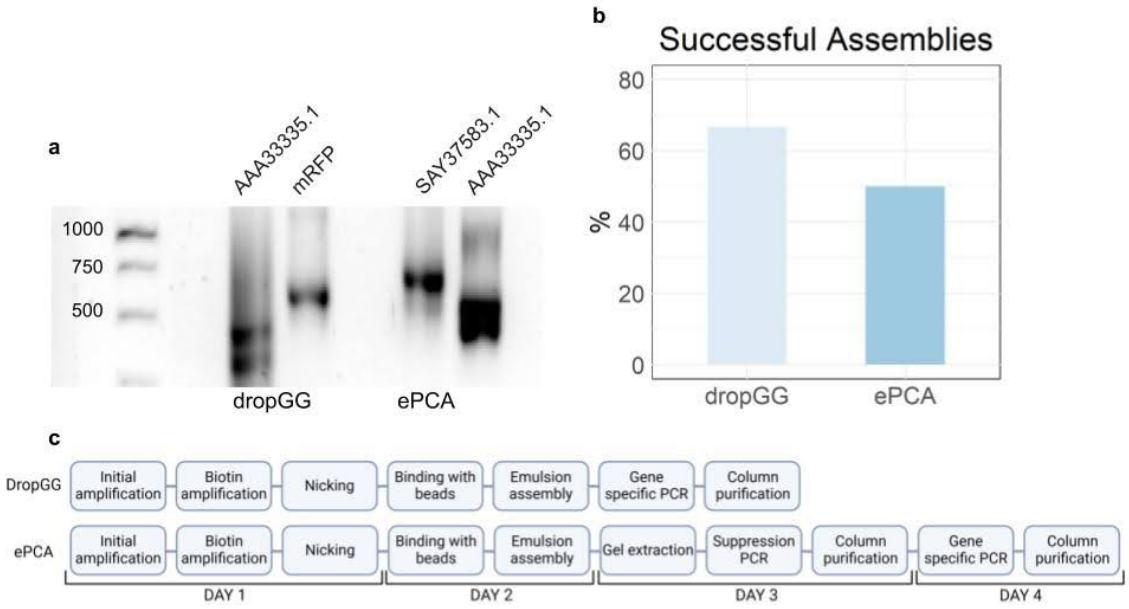

Figure 4.10: Comparison of dropGG and ePCA. **a:** Successfully assembled genes from ePCA and dropGG (AAA33335.1 (690 bp), mRFP(675 bp), SAY37583.1(777 bp)). **b:** Percentage of successful assemblies from dropGG and ePCA. **c:** Comparison of workflow for dropGG and ePCA.

5

Discussion

The primary objective of this study was to explore and adapt the DropSynth methodology for in-house gene synthesis and to develop a droplet-based Golden Gate (DropGG) assembly technique to overcome the inherent limitations of DropSynth, particularly its constraint to gene lengths of approximately 1 kbp. The results provide critical insights into the potential and limitations of these approaches.

5.1 Adoption of DropSynth

The successful assembly of sfGFP using PCA demonstrated that this technique can reliably synthesize genes of up to 750 bp from both 4 and 5 oligos 4.6. Incorporating droplets into the PCA did not seem to affect the assembly process negatively, showing that the results are consistent across different PCR strategies. However, the lower efficiency observed with the 5-oligo pool suggests that fewer oligos per gene may yield higher assembly efficiency, possibly due to reduced complexity in the assembly process.

Initially ePCA for the PAZy library faced significant challenges. The lack of correct assembly in the initial experiments was traced to design issues, specifically the lengths and Tm of overlaps between the oligos. The redesign of the overlaps to 20 bp and 25 bp revealed that longer overlaps significantly improved assembly efficiency, as evidenced by the correct assembly of the two genes in the 25 bp overlap pool. This underscores the importance of optimizing overlap length and Tm for successful ePCA in high-complexity libraries. Additionally, switching from PrimeStar to KAPA polymerase played a critical role in overcoming previous assembly failures, suggesting that enzyme choice is a key parameter in ePCA.

The failure to confirm assembly of the remaining two genes in the redesigned 4 gene PAZy pool likely stemmed from insufficient primer differentiation, which led to the amplification of a non-target gene. Sequencing analysis of the assembled fragments revealed that the amplified product in both samples for gene SAY37584.1 and SAY37587.1 corresponded to gene SAY37583.1. Consequently, the assembly of genes SAY37584.1 and SAY37587.1 could not be confirmed. This issue highlights the need for careful primer design, particularly in multiplexed assemblies. Time constraints prevented further troubleshooting, but these findings establish a clear pathway for further optimization.

5.2 Development of DropGG

Initial tests with mRFP demonstrated that linear Golden Gate assembly could successfully be performed in droplets for genes of 750 bp from four oligos. When applied to multiple genes using barcoded beads, DropGG successfully assembled mRFP and gene 4. As the third gene included in the pool was successfully assembled in a separate linear Golden Gate assembly, the issue most likely does not stem from the overlap design but rather from failure to correctly add the nicking sites and barcode with PCR. This causes the oligos not being able to bind with the barcoded beads and therefore not being included in the assembly reaction. Looking forward this will not be an issue as the designed oligos will contain all necessary parts and will not need to be changed with PCR.

The DropGG process also simplifies the downstream processing after assembly as there is no need for gel extraction, suppression PCR and purification before using the assembly as a template for the gene specific PCR.

5.3 Assembly of Genes of Arbitrary Length

Due to time constraints, genes longer than approximately 750 bp were not tested for either ePCA or dropGG in this study. Previous data from DropSynth demonstrated successful assembly of genes up to 1 kbp but not beyond. Similarly, TerMaat et al. 2009 reported challenges in assembling genes exceeding 1 kbp using the integrated PCA-PCR approach. In contrast, Golden Gate assembly has been shown to successfully construct a 40 kbp genome in a single reaction [Pryor et al., 2022], highlighting its greater potential for assembling genes longer than 1 kbp. Based on this it has been decided to continue the optimization of in-house gene synthesis using the Golden Gate approach.

5.4 Beads Distribution in Droplets

It was discovered that a substantial amount of droplets in the vortexed emulsions contained more than one bead. The presence of multiple beads within a single droplet increases the risk of oligos from different genes being mixed in the assembly reaction, thereby raising the likelihood of erroneous assembly. In the emulsion analysis conducted by [Plesa et al., 2018] the reported droplet median was <5 μm. However, their included images showed the presence of droplets with diameters up to approximately 40 μm. This analysis was performed in the absence of beads, and thus, no data was provided on bead distribution within droplets in their study. Based on our findings, it can be assumed that a considerable number of droplets in their emulsions may also have contained multiple beads. Regardless, they achieved 75 % perfect assembly rate in a pool of 384 genes, suggesting that the presence of multiple beads in one droplet may not be as limiting as initially assumed.

To promote a more uniform bead distribution, alternative approaches can be con-

sidered. One potential strategy is to use larger beads. [Hatori et al., 2018] proposed that droplets generated through vortexing tend to form around particles present in the solution. While this phenomenon may not apply effectively to the 2.8 μm beads used in this study, beads with a diameter of 10 μm may influence droplet formation more significantly. This could potentially reduce the occurrence of droplets containing multiple beads.

Alternatively, microfluidics could be employed for droplet formation, ensuring monodispersed droplets and preventing the encapsulation of multiple beads within individual droplets. However, the use of microfluidics entails certain trade-offs, including the need for larger sample volumes and a more time-consuming emulsion preparation process.

5.5 Development of a Workflow for 96-gene Assembly using DropGG

Following a comprehensive evaluation of the DropSynth protocol for ePCA, a workflow was created to facilitate 96-gene assembly using the dropGG method. Firstly the target genes are bioinformatically split into oligos using NEB's NEBridge Split-Set® Lite API, which will create an optimal set of 4 nt-overhangs for each gene. The following design of the oligos will be generated using an altered version of DropSynth script. Each oligo is designed to include universal primer binding sites, BspQI nicking sites, BsaI restriction sites, and a gene specific barcode. Additionally, the first and last oligos of each gene are designed with sequences for gene-specific primers, restriction sites for BsmBI, and overhangs compatible with the pGGAselect vector, enabling downstream cloning post-assembly. The oligos will be ordered in pool-format. The barcoded beads will be prepared according to the DropSynth protocol in 96x format.

The workflow is divided over three days where day one contains preparatory tasks, day two preparation and assembly and day three post-assembly processing (Fig. 5.1) Firstly the oligo pool is amplified, purified and diluted to the desired concentration. A second amplification step is performed using a biotinylated forward primer. The amplified fragments are nicked overnight and the next day the biotinylated fragments are removed and the nicked fragments are bound to the barcoded beads. The dropGG reaction is prepared and performed overnight. The assembly mixture serves as a template for gene-specific PCR amplification in a 96x format, ensuring the separation and amplification of all target genes. Amplification is verified via quantitative PCR (qPCR) using a fluorescent dsDNA-binding dye. The amplified genes are purified using magnetic beads, yielding a final product of purified genes arranged in the 96-plate. This workflow provides a streamlined and high-throughput method for 96-gene assembly using the dropGG system.

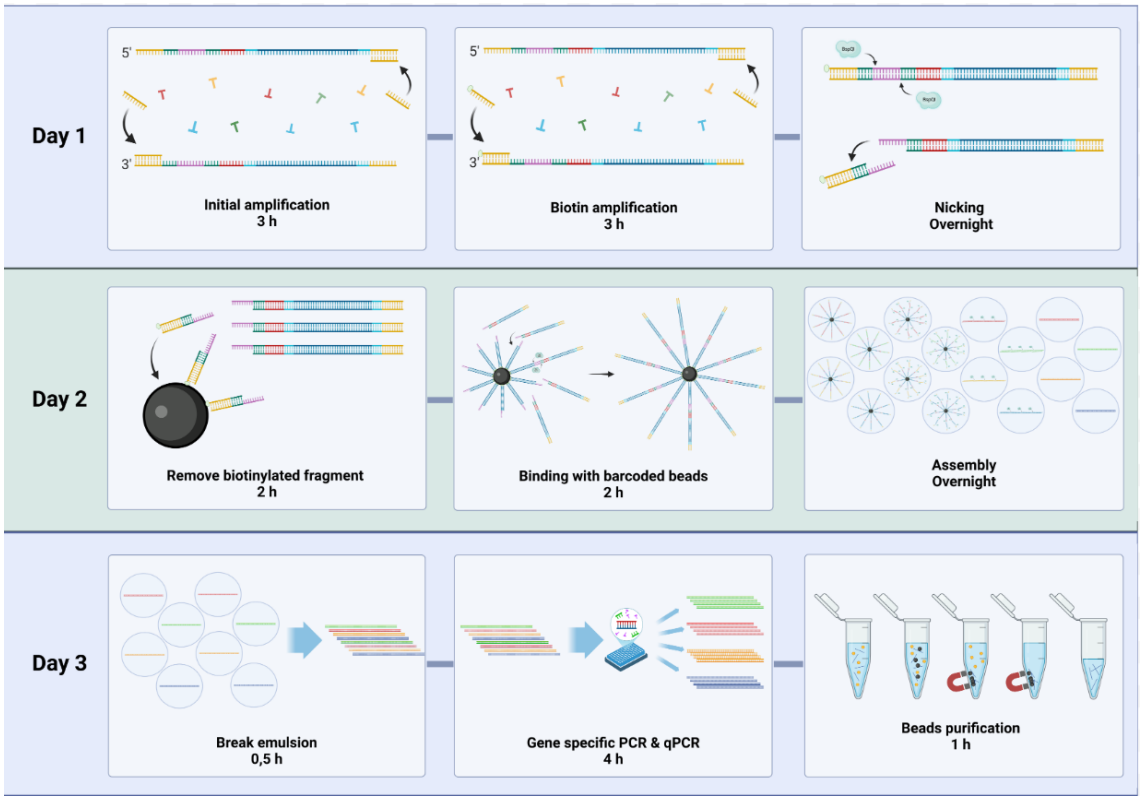

Figure 5.1: Overview of the DropGG workflow. **Day 1:** Initial amplification of oligo pool, biotin amplification using a biotinylated forward primer and nicking overnight with BspQI to expose the barcode. **Day 2:** The nicked biotinylated fragments are removed using Streptavidin beads, purified nicked fragments are bound to the barcoded beads, emulsion assembly reaction prepared and placed in PCR tubes to be incubated overnight. **Day 3:** Emulsion is broken and assembly mix recovered, assembly mix is used as template for gene specific PCR on 96-well plate, amplification are confirmed using qPCR and purified using magnetic beads.

6
Conclusion

This study successfully adapted the DropSynth methodology for in-house gene synthesis and developed the droplet-based Golden Gate (DropGG) assembly technique. The findings demonstrate that droplets can reliably support both PCA and Golden Gate assembly, enabling synthesis of genes up to 750 bp while highlighting critical factors that may influence assembly efficiency, such as overlap design, polymerase choice, and bead distribution within droplets. Overall, this work establishes a foundation for further optimization of droplet-based gene synthesis techniques, demonstrating their feasibility for multiplexed gene assembly while providing practical insights into overcoming key challenges.

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Supplementary Material

- Oligos, genes primers (supplementary tables) https://docs.google.com/spreadsheets/d/1ngBDAY6eCZ544eYgjcc5TKsWpJuY-kOhHRgqlVS5C8w/edit?usp=sharing
- DropSynth protocols
  https://www.dropsynth.org/

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