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ex vivo DNA cloning

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ACKNOWLEDGEMENTS

"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."
- Sir Winston Churchill, 1947

As I reach the conclusion of a graduate career that seems like an entire lifetime unto itself, there are numerous special people to recognize for making this experience not only possible but also successful. While not nearly adequate the best I can do is give my acknowledgement and thanks where it may be preserved and disseminated.

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ABBREVIATIONS

BCP = Blue Chromogenic Protein (also, *amil*CP)
BSA = Bovine Serum Albumin
CDS = Protein Coding Sequence
CFS = Cell-Free System
CFU = Colony-Forming Unit
DHAP = dihydroxyacetone phosphate
Dra = *Deinococcus radiodurans* lysate
DSB = Double-stranded break
dsDNA = double-stranded DNA
Eco = *Escherichia coli* lysate
EE = Early exponential
IDT = Integrated DNA Technologies
ISO = isothermal DNA assembly (also, Gibson assembly)
LB = Luria-Bertani media
LE = Late exponential
M9 = M9 minimal media
MAGE = Multiplex Automated Genome Engineering
NHEJ = Non-homologous end joining
PCA = Polymerase Chain Assembly
PCR = Polymerase Chain Reaction
Pfu = *Pyrococcus furiosus*
pUN = Plasmid Unique Neutral
RBS = Ribosome Binding Site
Sce = *Saccharomyces cerevisiae* lysate
SOB = Super Optiimal Broth media
SOC = Super Optimal Broth with Catabolite repression Media
SP = Stationary Phase
SSA = Single-strand annealing
ssDNA = single-stranded DNA
Taq = *Thermus acquaticus*
TAR = Transformation-associated recombination
TB = Terrific Broth media
UNS = Unique Neutral Site

ABSTRACT

Genetic engineering of microbes has developed rapidly along with our ability to synthesize DNA *de novo.* Yet, even with decreasing DNA synthesis costs there remains a need for inexpensive, rapid and reliable methods for assembling synthetic DNA into larger constructs or combinatorial libraries. While technological advances have resulted in powerful techniques for *in vitro* and *in vivo* assembly of DNA, each suffers inherent disadvantages. Here, an *ex vivo* DNA cloning suite using crude cellular lysates derived from *E. coli* is demonstrated to amplify and assemble DNA containing small sequence homologies. Further, the advantages of an *ex vivo* approach are leveraged to rapidly optimize several parameters of the *ex vivo* DNA assembly methodology testing lysates from different engineered strains of *E. coli*, with various buffer components and using titrations of purified cloning enzymes. Finally, in order to complete the cloning suite, a vector expressing the *Pyrococcus furiosis* (*Pfu*) DNA polymerase was designed, constructed and expressed in *E. coli* to create a 'functionalized lysate' capable of *ex vivo* PCR. Not only do we demonstrate *ex vivo* cloning methodology as a complete cloning package capable of replacing the expensive cloning reagents currently required by synthetic biologists, but also establish *ex vivo* as an overarching approach for conducting molecular biology.

Chapter 1. Introduction and Background

"What I cannot create, I do not understand"
- Richard Phillips Feynman, PhD

1.1. DNA Assembly and Synthetic Biology

Our capacity to (re)engineer living systems is linked to our ability to physically build specific DNA molecules that encode desired functionality and behavior. Thanks to major advances in DNA sequencing and DNA synthesis technologies, a renaissance in genetic engineering known as synthetic biology has sparked incredibly rapid progress in our ability to engineer life. Synthetic biology, metabolic engineering, systems biology and associated disciplines can be leveraged to not only access the estimated ~100 million biological compounds present on our planet, but create sophisticated biological entities as molecular machines capable of replication, catalysis, sensing and computational response[1]. The programmable chemistry potentiated by recombinant DNA technologies can be harnessed to address societal needs such as food scarcity; renewable fuel sources; environmental remediation and protection; medicinal (and veterinary) diagnostics and therapeutics; or defense against bioterrorism[2,3].

Due to difficulties stemming from the inherently 'analog' nature and complex interdependence of biochemical systems on top of the staggering permutations of even the smallest of genetic circuits,

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synthetic biologists have focused on the characterization, standardization and modularity of biological parts[4]. Yet, to achieve the overarching synthetic biology dream we must continue to optimize the design-build-test engineering loop until first principles of *de novo* genetic design are ascertained.

While the current bottleneck in the Synthetic biology engineering loop lies in the design portion of the loop (since most initial designs are not optimal and possibly not even functional), the only way to alleviate this design bottleneck is to continue to build and test many genetic designs. This is not just to identify adequate designs for the application at hand, but also to inform subsequent design efforts. The ensuing study not only presents an optimized DNA assembly methodology significantly less expensive and/or time-consuming than current methods, but also presents an overall approach to rapid phenotypic characterization, optimization and alternate route for laboratory molecular biology.

Although our ability to construct novel DNA has come a long way from Nirenberg's initial synthesis of a nucleic acid homopolymer[5], it still proves most cost effective to purchase DNA in small fragments or single-stranded oligomers to assemble into larger, gene-length products. This is particularly true when combinatorial libraries are being constructed. Indeed, synthetic biologists often rely on construction and thorough characterization of libraries of modular genetic parts. For instance, characterization of promoter regions and ribosome binding sites (RBS) has generated successful part libraries

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capable of tuning transcriptional and translational expression of downstream protein coding sequences[6-8]. Data collected from these experiments have even produced fairly robust models capable of rationally designing tunable parts, such as the RBS calculator[9]. Yet the interplay between DNA sequence and phenotype is full of nonlinear, epistatic and stochastic dynamics, minimizing the modularity and reliability of genetic parts. For example, combining a highly expressive promoter with a highly expressive RBS part may result in very low overall protein expression. To address these problems synthetic biologists must often rely on parts tested in a combinatorial manner. Beyond regulatory parts for modulating gene expression, encoding novel functions through protein engineering still remains an undertaking of brute force necessitating generation of extreme sequence diversity through directed evolution (requiring an appropriate screening approach), combinatorial domain-swapping or a synthetic metagenomics approach. In order to address the design bottleneck of the design-build-test loop, the build and test steps must be made extremely rapid.

Traditionally, recombinant DNA has been constructed using restriction cloning (i.e., cutting with an endonuclease and joining with a ligase) and DNA fragments isolated from natural sources by restriction digestion or polymerase chain reaction. In addition to restriction-ligation approaches, other site-specific recombination systems have been employed to assemble DNA fragments with great success[10]. However, genetic engineering has recently become more flexible with the use of

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'sequence-independent' approaches that take advantage of the decreasing cost of DNA synthesis[11-14]. In a perfect world, synthesis of DNA would be so cheap that all DNA constructs could be synthesized in their entirety, sequence-verified and delivered to researchers for testing. However, current gene synthesis rates are highly limiting often costing several thousand dollars to synthesize full-length genes. Instead, most DNA is ordered in more cost-effective fragments (as of this writing, single-stranded oligomers are $0.35 per base up to 60 bases and $120 for a 750 base pair double-stranded fragment through Integrated DNA Technologies® [IDT]) and then assembled using overlapping homologous sequences on the ends of the oligomers in a 'sequence-independent' fashion. For example, polymerase chain assembly (PCA), a variation on the general polymerase chain reaction (PCR), uses partially overlapping 60bp single-stranded oligomers alternating strands of an entire gene sequence in equimolarity as a template. These oligos are pooled and allowed to anneal and extend into longer and longer double-stranded sequences until a complete double-stranded gene sequence is generated. These 'synthons' can be quickly inserted into a plasmid backbone by traditional sequence-specific or sequence-independent methods.

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Enzyme	Function	ISO Example	Putative Wild-type Examples
	DNA unwinding and resection (chew-back)	T5 exonuclease	RecBCD (*E. coli*), RecJ and RecQ (*E. coli*, *D. radiodurans*), MRX and Sae2 (*S. cerevisiae*)
	DNA synthesis (polymerization)	Phusion/Q5 DNA polymerase (NEB)	Pol I/II (*E. coli*, *D. radiodurans*), Pol δ (*S. cerevisiae*)
	DNA ligation (sealing)	*Taq* DNA ligase (NEB)	LigA and LigB (*E. coli*, *D. radiodurans*), LIG4 (*S. cerevisiae*)

Figure 1.1 DNA Assembly by a Chew-back, Anneal and Repair Mechanism
Depicted are the steps employed by Gibson Isothermal assembly (ISO) and analogous putative steps for *in vivo* single-strand annealing predicted to be the active mechanism in *ex vivo* DNA assembly. In steps 1-3, an exonuclease (here 5'-to-3') chews back (resects) the double-stranded ends to expose terminal homologies (colored blue), which can anneal to each other in step 4. If there has been excessive resection as depicted in steps 5-6, a DNA polymerase is used to repair the exposed gaps. Finally, a DNA ligase acts to seal the DNA backbone (7), to yield a fully sealed, accurate assembly/repair. The table describes the tripartite mixture of enzymes in the ISO formulation and some analogous naturally-occuring enzymes from yeast, *E. coli* and *D. radiodurans*.

When trying to assess the function of DNA sequences in the cell, it is of great utility to have sequence-independent cloning techniques that do not constrain design (e.g., scars from restriction sites). Homologous recombination is the longest-tenured sequence-independent cloning method and the most widely utilized for integration onto the genome. Highly active homologous recombination systems native to *Bacillus subtilis* and *S. cerevisiae* make effective *in vivo* recombination systems, while in *E. coli*, the Lambda Red recombinase

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genes can greatly increase the rates of homologous recombination[15]. In fact, *S. cerevisiae* has been utilized to assemble and propagate entire bacterial genomes from very large overlapping fragments, through transformation-associated recombination (TAR)[16]. However, the assemblies must be designed to work with the organismal chassis and, unless the construct is to remain in the assembly host, must be designed to shuttle into the final host organism. The TAR cloning process can therefore take up to eight days to get a DNA construct into the terminal host. While monetarily inexpensive, these time expenses are often a disappointing attribute of *in vivo* methodologies.

In contrast to *in vivo* approaches, purified *in vitro* systems offer user-control, efficiency and time savings, but are often expensive to purchase or difficult to establish in-house due to complications in protein purification. Once established there is no denying the potency of these systems. In 2008, a completely synthetic Mycoplasma genome was assembled from chemically synthesized double-stranded DNA (dsDNA) fragments (5-7 kilobase pairs each) using an *in vitro* chew-back assembly method[16]. More recently, the mouse mitochondrial genome was reconstructed from overlapping synthetic single-stranded oligonucleotides (60 nucleotides each) using a three-enzyme *in vitro* isothermal DNA assembly method (ISO assembly, also known as Gibson assembly)[17]. Currently the most popular and efficient sequence-independent cloning method, Gibson and colleagues discovered an optimized mixture of a phage 5'-to-3' DNA exonuclease, DNA ligase, and heat-stable polymerase catalyzing highly efficient DNA end joining

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(Figure 1.1). As the current "gold-standard" for DNA assembly, Gibson assembly suffers from the disadvantages of most purified *in vitro* systems – it is highly expensive. Distributed as a master mixture from New England Biologicals® (through Synthetic Genomics®) each reaction costs $15 dollars. While very efficient and taking only an hour, if trying to assemble larger libraries of constructs this is not a cost-effective route.

1.2. The *ex vivo* approach

The terminology '*ex vivo*' – Latin for "out of the living" – is a common term in organismal biology, where tissue or even full organs are isolated from an organism with minimal perturbation of it's natural environment. This permits researchers more control over conditions and access to measurements not otherwise possible (or even ethical) in a living organism. In this manner, the "*ex vivo*" moniker describes an experiment combining the advantages of an *in vivo* approach and an *in vitro* approach.

While *ex vivo* experimentation is traditionally associated with organs and tissues, the approach has a storied history in microbiology and cellular biology. The first *ex vivo* reaction catalyzed by a microbial cell extract was performed by Eduard Buchner (1897) over 100 years ago, converting sugar to ethanol and carbon dioxide in a yeast extract, eventually winning the Nobel Prize in Chemistry (1907). Using lysate fractions derived through differential centrifugation, microsomes were discovered as catalysts of protein synthesis, leading

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to the identification of mRNA as the template for protein synthesis by Nirenberg and Matthaei (1961) in their own extract-based reaction[5]. As history and technology progressed the microbiology and cellular biology systems derived from cellular extracts have become popularly referred to as 'cell-free systems' (CFS). However, 'cell-free system' is an umbrella term giving very little detail to the composition of the final system. For instance, cell-free systems for protein synthesis range from crude extract to systems reconstituted *in vitro* with entirely purified components. For this reason, we propose *ex vivo* be used to describe a CFS which is minimally altered from its' *in vivo* origins by only very crude purification or manipulation (e.g., centrifugation of insoluble components).

1.3. Applications of Cell-Free Systems

Since their inception, CFSs have primarily been leveraged to probe the central processes of transcription and translation. Over the past ~25 years, building off work establishing coupled transcription-translation CFS[18], modern CFS have evolved into robust systems capable of high-level protein production. Recent work with *E. coli*-based extracts have garnished yields exceeding 2 mg/mL protein at scales exceeding 100 liters[19]. Complex eukaryotic proteins containing high-levels of post-translational modifications can be produced in cell-free extracts from wheat-germ, rabbit reticulocytes and insect cells at high titers. This is important, since CFSs obviate the need to perform *in vivo* manipulations that can be difficult or impossible in some eukaryotic cell lines. Cell-free protein production is therefore

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particularly attractive to industrial production of therapeutic peptides as they are commonly post-translationally modified, exist associated with the membrane or may exhibit toxicity (e.g. antimicrobial peptides).

For synthetic biology, CFSs have been cleverly employed to expedite the prototyping of genetic parts for gene expression. By monitoring the production of fluorescent proteins in a CFS the time investment and noise (i.e., plasmid copy number) associated with *in vivo* characterization is greatly reduced. Although not an absolute perfect measure of *in vivo* dynamics, a set of constitutive and inducible promoters tested in a CFS were shown to correlate well with measurements of *in vivo* gene expression and dose-response[20].

Cell-free protein synthesis has benefited greatly from advances in mimicking intracellular conditions, activating integrated biological processes and controlling cell-free metabolism. Control over metabolism in CFSs has motivated the development of CFSs as platforms for the synthesis of metabolites. Although most of these higher-functioning CFSs require further purification of the pathway constituents from the lysate, in a truly excellent demonstration of an *ex vivo* system, Panke and colleagues optimized the catalysis of dihydroxyacetone phosphate (DHAP) from glucose[21]. The authors were able to reach concentrations far beyond those documented *in vivo* (12 mM DHAP) by deletion of two genes in the production strain combined with supplementation of butanol and rabbit muscle aldolase to the reaction. DHAP is an unstable product, but the exogenous addition of the

aldolase and butanol allowed conversion to a more stable form. This is a perfect example of the simplicity and flexibility of CFS optimization, not only by modification of the host chassis, but also by circumventing the membrane to directly supply new components (here the small-molecule butanol and a non-bacterial enzyme) impossible in the same *in vivo* system.

The constant struggle between the engineering objective to optimize product yield and the cell's biological objective to optimize biomass production is remedied by an *ex vivo* approach. The functionalities of the cell remain intact, while removal of the cell encapsulation enables manipulation of the internal chemistries to activate, integrate and focus cellular resources towards a desired objective. Without compartmentalization there is the opportunity to greatly exaggerate substrate concentrations, remove product, and rapidly sample and monitor reaction conditions. In *in vivo* systems researchers and engineers must respect the constraints, time-scales and stochasticity inherent to life. On the other hand, purified *in vitro* systems provide exceptional flexibility and control, resulting in higher reaction efficiencies and better reproducibility. Yet, *in vitro* systems are low-throughput and orders of magnitude more expensive than their *in vivo* counterparts. This is clearly demonstrated in the production of cellulosic biofuels, where the primary cost-driver is the expense of purified enzymes for biomass pretreatment[22,23]. For investigators, *in vitro* systems sacrifice cellular context necessary to make conclusive inferences about *in vivo* phenomena. A simple, yet

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elegant compromise between *in vivo* and *in vitro* approaches, *ex vivo* systems enhance both engineering and investigatory freedom.

1.4. Double-strand Break Repair: Natural DNA Assembly

The end-to-end joining of free dsDNA fragments is a vitally important process for all cellular life in the repair of double-stranded DNA breakage. DNA double-stranded breaks (DSB) are ubiquitous across all kingdoms of life, occurring during normal housekeeping functions and in response to DNA damaging agents such as chemicals and irradiation[24,25]. Very simply, a DSB is a break in both strands of duplex DNA. A DSB is a particularly dangerous DNA lesion as these breaks can potentially disrupt gene expression, alter chromosome organization or provide substrates for single- or double-stranded exonucleases, catalyzing the deletion of genetic information. DSB are also notoriously difficult to repair. Where a single-stranded break can be easily repaired using the conserved information on the intact strand, generally DSB have no associated template at the free ends to guide repair. Due to these two reasons DSB repair is widely associated with the development of cancer and has, therefore, been the subject of extensive study[26,27]. There are two major mechanisms for DSB repair: end-joining and homologous recombination. The major distinction between the two being that the former requires no template to facilitate repair of the DSB. The presence of template DNA is, therefore, essential in the decision and mechanism of DSB repair pathways.

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1.4.1. Homologous Recombination

Homologous recombination generally leads to high-fidelity repair, making it the most ubiquitous and dominating DSB repair pathway across organisms. However, unlike end-joining, homologous recombination's requirements for a template means that a second copy of the region containing the DSB must exist in the cell. For most prokaryotes, this is not a problem as they often exist with multiple or, at least, partial copies of their genome. In eukaryotes, homologous recombination is reserved for the S and G_2 phases of the cell cycle when sister chromatids are present, acting as template for repair and meiotic crossing-over.

1.4.2. Non-homologous End-Joining

End-joining implies the linking of two free duplex DNA termini into a ligated product. Traditionally referred to as non-homologous end-joining (NHEJ), the relatively simple process relies on minor processing of DNA termini to prepare them for direct ligation in the absence of any required homologous template. While this means that repair can take place at any point during the cell cycle regardless of genome copy number, the fidelity of repair is relatively low. Errors are often propagated from loss-of-information during end processing (resection), explaining the relatively low occurrence in prokaryotes in comparison to homologous recombination. In higher eukaryotes, the error-prone nature of NHEJ has evolved into a highly coordinated system to generate sequence diversity in the differentiation of unique

B- and T-lymphocytes. More recent studies have revealed 'alternate end-joining' or 'microhomology-mediated end-joining' pathways utilizing repeat sequences at or near the terminus of DSBs, such as those that may occur in breaks near or within ribosomal operons of prokaryotes[28,29].

1.4.3. Alternative End-Joining

Alternative end-joining pathways appear to exist in a grey-area between the extremes of NHEJ and homologous recombination relying on combinations of cellular machineries from both processes, distinguished by the degree of available homologous template. Outside of rare circumstances, like the ribosomal operon described above, a DSB is very unlikely to form with significant end homologies to guide non-templated repair. Nevertheless, an alternative DSB repair pathway known as single-strand annealing (SSA) exists to join DSB with significant homologies at their termini[30]. As in the homologous recombination process, SSA involves an extensive end-resection to expose single-strand overhangs[31]. Yet, in contrast to homologous recombination, these ends subsequently anneal to the newly exposed homologies on the cognate DSB for a final step of ligation (similar to NHEJ). SSA is a mechanistic analog to the Gibson ISO assembly method (Figure 1.1), following the same chew-back (resection), annealing, polymerization and ligation steps, but occurs (albeit infrequently) in completely wild-type microbes. Hopefully, by freeing and concentrating the enzymes necessary for SSA end joining of overlapping DNA fragments will take place. *E. coli,* and *S. cerevisiae* make good chassis not only

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due to preliminary research suggesting they are capable of catalyzing end joining in an extract-based reaction, but their relatively easy culturing methods and availability to most labs[13,32]. *D. radiodurans* makes another excellent chassis for exDA, as it is known to have some of the most active DNA repair systems, making it the world's (current) most radioresistant organism[33].

1.5. The *ex vivo* Cloning approach

In the ensuing study, we endeavored to demonstrate the facilities of the *ex vivo* approach to demonstrate and subsequently investigate and optimize DNA assembly. Using our own assays of assembly we tested lysates prepared from different organisms and strains, under varying growth conditions and with variable buffer/reaction conditions. Titrations of common cloning enzymes were employed to investigate the underlying enzymatic mechanisms of DNA assembly. Applying genome engineering techniques we endeavored to engineer a new strain of *E. coli* lacking competing nucleases and capable of autolysis for *ex vivo* DNA assembly. We designed and constructed pUN ('plasmid unique neutral') a novel plasmid featuring a screenable blue chromogenic protein (BCP) insert and 'unique neutral sites' for facile cloning via *ex vivo* DNA assembly or other sequence-independent assembly methods. Lastly, using our new pUN plasmid we replaced the BCP insert with a cassette expressing a codon-optimized *Pyrococcus furiosus* (*Pfu*) DNA polymerase gene. This plasmid yields a lysate with high levels of the thermostable DNA polymerase now 'functionalized' to perform a high-fidelity *ex vivo* PCR reaction. With *ex vivo* methods for both DNA

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assembly and PCR we report the creation of the first all-encompassing

ex vivo Cloning pipeline.

Chapter 2. *ex vivo* DNA ASSEMBLY

Author Contributions:
George H. McArthur and Adam B. Fisher designed the experiments, which were carried out in the laboratory of Stephen S. Fong. Adam B. Fisher performed the experiments with Zachary B. Canfield and Laura C. Hayward. George H. McArthur, Adam B. Fisher, and Stephen S. Fong interpreted the data and wrote the manuscript. All authors discussed results and commented on the manuscript.

2.1. Initial 2-way *ex vivo* DNA Assemblies

To assess the ability of select cellular lysates to join together dsDNA, we first designed two amplicons with appropriate overlapping ends (26 and 30 bp overlaps) to be assembled into a circular plasmid (Figure 1.1A)[34]. Correctly assembled plasmids endow *E. coli* transformants with selective resistance to the antibiotic chloramphenicol and also visually screenable expression of a blue chromogenic protein (BCP) native to the coral *Acropora millepora* (Figure 1.1B)[35]. Template plasmids housing the *bcp* coding sequence (pSB1C3-K592009) and the appropriate antibiotic resistance and replication origin (pSB1C3-J04450) produce white and red/pink colonies, respectively, providing a convenient way to track transformation efficiencies (Figure 1.1B).

In this initial experiment, we used lysates of *S. cerevisiae*, *E. coli*, and *D. radiodurans* (hereafter *Sce*, *Eco*, and *Dra*, respectively) and a simple buffer containing ATP and $MgCl_2$ to attempt *ex vivo* DNA assembly.

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The two amplicons were incubated for 1 hour with each lysate. Samples of each reaction mixture were subsequently used to transform *E. coli* NEB10β.
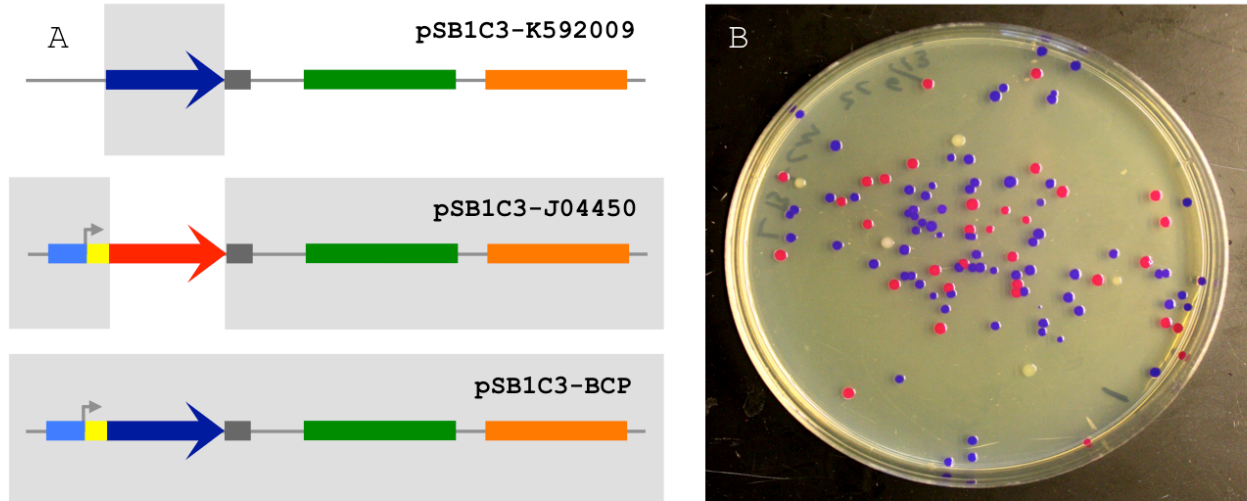
Figure 2.1 The 2-way Circular Assembly

(A) 2-way assembly was demonstrated by joining a coding sequence for blue chromogenic protein (BCP; a 698 bp segment of pSB1C3-K592009 colored in dark blue) and the majority of pSB1C3-J04450 (a 2446 bp segment), thereby replacing the RFP coding sequence with BCP (pSB1C3-BCP). The pMB1 origin is colored in green and the chloramphenicol resistance gene is colored orange. (B) Correctly assembled plasmids allow transformants to express BCP (blue colonies) while colonies containing carryover template plasmids appear either red (pSB1C3-J04450) or white (pSB1C3-K592009).

Interestingly, *Dra*-incubated DNA did not produce transformants, although DNA incubated with *Sce* or *Eco* successfully transformed *E. coli*. Further analysis by agarose gel electrophoresis showed significant degradation of the individual amplicons, indicating that a highly active exonuclease system might have prevented the assembly of DNA by *Dra* (Appendix B Figure B.1). Even expected background transformants resulting from lingering circular PCR template were absent, suggesting that endonuclease activity is also high in *Dra*. Indeed, after further investigation we found a consensus DrdI site

(GAC-N$_6$-GTC) between 1475 and 1486 (GACGCTCAAGTC) in the replication origin of our plasmids[36].

2.2. Testing the Magnesium:ATP Ratio with 2-way Linear Assemblies

We then explored ways to improve the efficiency of *ex vivo* DNA assembly with *Eco* and *Sce* by varying the relative composition of ATP and MgCl$_2$ in our buffer, adding NAD to the buffer (to power NAD+-dependent processes such as ligation), modulating the temperature at which the reaction mixture was incubated, increasing the amount of cellular lysate in our reaction, and increasing the duration of incubation. To directly assess the efficacy of *ex vivo* DNA assembly and avoid variation associated with transformation, we chose to visualize formation of a linear product (Figure 2.2A) from a pair of overlapping amplicons (28 bp overlap) via agarose gel electrophoresis of the *ex vivo* assembly reaction prior to transformation (Figure 2.2B). In this manner, we were able to compare different reaction conditions on their ability to join two overlapping dsDNA into one linear product.

Buffers were made of 1 mM DTT, 1 mM NAD, and varying concentrations of ATP and Mg^{+2}. A wide range of ATP:Mg^{+2} ratios were initially tested with 1-hour *Eco* reactions at 37°C: 1:5, 1:10, 1:20, 5:5, 5:10, 5:20, 10:5, 10:10, and 10:20 (mM:mM). Only three ATP:Mg^{+2} ratios were chosen for further reaction optimization: 10:5, 5:5, and 1:10 (mM:mM) (buffers 1-

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3 in Figure 2.2B, respectively). Linear products resulting from assembly by *Sce* were not at all visible in the gel, although bands of small pieces of DNA indicate that there is some nuclease activity under most conditions tested. On the other hand, *Eco* appears to have significant activity at 30°C and 37°C even without the addition of buffer. Previous studies have noted that the nucleolytic behavior of the RecBCD complex of *E. coli* changes based upon the relative amount of ATP to free Mg^{+2} *in vitro* [37]. We observed a similar trend in our lysates; the most efficient assembly reactions (for both 30°C and 37°C) were carried out under conditions of excess magnesium relative to ATP (Buffer 3 in Figure 2.2B), probably because ATP can chelate Mg^{+2} with high affinity under physiological conditions [38].
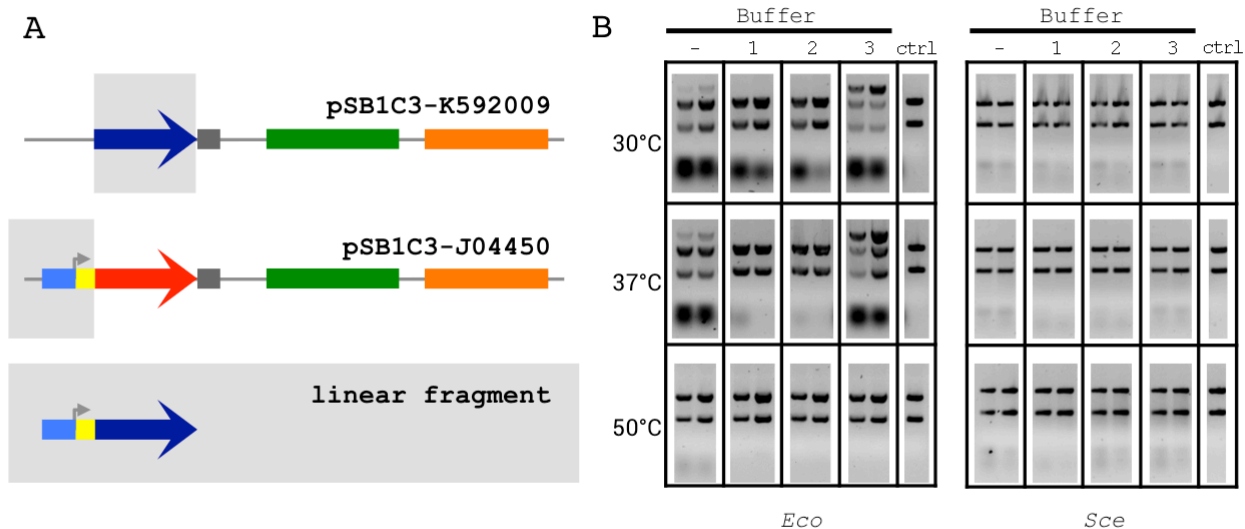
Figure 2.2 The 2-way Linear Assembly and Buffer Optimization
(A) Optimized *ex vivo* reaction conditions were identified by visualizing the joining of a 697 bp segment of pSB1C3-K592009 (BCP) and a 381 bp segment of pSB1C3-J04450 (Promoter) into a larger linear DNA molecule. **(B)** Reactions were run for 1 and 2 h (left and right in each gel, respectively), at three temperatures and four buffer compositions for each *Eco* and *Sce*. All buffers contained 1 mM NAD and 1 mM DTT. ATP and Mg2+ concentrations(mM:mM)varied as follows:(1)10:5,(2)5:5,(3) 1:10. The control lanes include a reaction with no supplemented buffer (-) and a negative control ("ctrl") DNA-only lane (no buffer or lysate).

Each *ex vivo* reaction condition was tested for both 1- and 2-hour incubations. Initial experiments indicated that greater incubation times (3-6 hours) do not improve *ex vivo* assembly yields (data not shown). For all successful assemblies, 2-hour reactions appear to generate more linear product than 1-hour reactions. The temperatures selected for *ex vivo* reaction optimization reflect cell culture conditions (30°C for yeast and 37°C for *E. coli*) and the temperature used for ISO assembly reactions (50°C). The 50°C reactions are considered a negative control since we do not expect the cellular machinery in *Eco* or *Sce* to be thermostable, although transient activity may occur initially. Based on the results of these

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experiments we selected a buffer composition of 1 mM ATP, 10 mM Mg^{+2}, 1 mM DTT, and 1 mM NAD+ (i.e., Buffer 3 in Figure 2.2B) incubated at 37°C for 2-hour as our optimized conditions for both *Eco* and *Sce*.

B

	2-way *ex vivo* circular DNA assembly			
Lysate	Reaction time	# Colonies	# Blue	# Red	# White
Eco	0 minutes	0			
Eco	120 minutes	429	423	0	6
Sce	0 minutes	54	51	0	3
Sce	120 minutes	125	124	0	1
None	0 minutes	63	62	0	1

C

	3-way *ex vivo* circular DNA assembly			
Lysate	Reaction time	# Colonies	# Blue	# Red	# White
Eco	0 minutes	0			
Eco	120 minutes	498	491	6	1
Sce	0 minutes	2	1	1	0
Sce	120 minutes	23	15	8	0
None	0 minutes	30	21	8	1

Figure 2.3 The 3-way Assembly and Colony Counts
(A) Three-way assembly was demonstrated by joining the BCP coding sequence (a 696 bp segment of pSB1C3-K592009), the majority of a plasmid carrying a different antibiotic resistance gene (a 2214 bp segment of pSB1K3-J04450) and a promoter-RBS region used to drive BCP expression (a 387 bp segment of pSB1C3-J04450), resulting in pSB1K3-BCP. **(B,C)** The pMB1 replication origin is colored in green, the chloramphenicol resistance gene is colored orange and the kanamycin resistance gene is colored purple. Colony counts for optimized two- and three-way assembly experiments. The negative control assembly reaction is labeled "None" meaning that these colonies result from *in vivo* assembly in *E. coli*.

2.3. Optimized Assemblies with Yeast and *E. coli* Lysates

Under optimized reaction conditions, *Eco* and *Sce* were used again to perform the two-way dsDNA assembly. In addition, we designed three overlapping amplicons (30, 29, and 26 bp overlaps) to demonstrate a three-way assembly, which is not only more complex but also more useful for generating combinatorial libraries (Figure 2.3A). For each of these assembly tests, lysate-incubated DNA was allowed to react for 2-hours before transformation of *E. coli*. Control conditions of zero

incubation time and reactions with no lysate added were also run to ensure that the lysate was indeed facilitating DNA assembly and not otherwise affecting the transformation process.

Our results (Figure 2.3B and Figure 2.3C) clearly indicate that DNA is indeed being assembled *ex vivo*. For two- and three-way assemblies, both *Eco* and *Sce*, transformation efficiencies significantly increase when the DNA to be assembled is allowed to incubate with the lysate. Unexpectedly, the "no lysate" negative control revealed that for two- and three-way assemblies the overlapping amplicons can be joined together into a circular plasmid *in vivo*, suggesting that a significant fraction of the *ex vivo* assembly reactions – and also *in vitro* reactions (e.g., ISO assembly) – actually occur inside *E. coli* after transformation (i.e., transformation-associated recombination cloning in *E. coli*). Although *Eco*-mediated assembly produced many blue colonies for both two- and three-way assemblies (423 and 491 colonies, respectively), *Eco*-lysate appeared to be detrimental to the overall transformation efficiency as when transformation reactions were spiked with *Eco*-lysate (but not allowed to incubate with the DNA amplicons) the presence of colonies was greatly diminished (See, Table 2.1).

2.3.1. Effect of Detergents on Transformants

To test whether or not the detergent-based lysis buffer used to produce *Eco* was affecting the transformation process, we carried out the assembly reactions and transformations in the presence of the lysis buffer but without cellular lysate. As summarized in Table 2.1,

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the bacterial lysis buffer inhibits transformation completely. Therefore, *Eco*-mediated assembly is likely much more efficient than we have observed and an alternative lysis method would probably increase overall transformation efficiency. The yeast lysis buffer does not appear to inhibit transformation of *E. coli*, which is not surprising because it is designed to lyse yeast cells. Interestingly, the number of colonies produced by DNA incubated with yeast lysis buffer was equivalent to the number of colonies produced by *Sce*-incubated DNA and that produced by the "no lysate" control. These data suggests that *Sce*-mediated DNA assembly was not observed but rather indicates that *in vivo* end joining in *E. coli* facilitates DNA assembly in this case. Interestingly, there would appear to be an increase in total colonies formed when the DNA is incubated with CelLytic Y. One might speculate that during incubation perhaps the buffer composition influences the DNA to adapt a more tightly supercoiled conformation as is seen in solutions with higher salt concentrations and is observed to greatly increase transformation efficiency[39,40]. However, without further experimentation and knowledge of the proprietary CelLytic Y formula this remains purely speculative.

Table 2.1. Effect of Detergents on Assembly and Transformation

2-way *ex vivo* circular DNA assembly

Lysis Buffer	Reaction time	# Colonies	# Blue	# Red	# White
CelLytic B	0 minutes	0	0	0	0
CelLytic B	120 minutes	0	0	0	0
CelLytic Y	0 minutes	42	41	0	1
CelLytic Y	120 minutes	150	140	0	10

3-way *ex vivo* circular DNA assembly

Lysis Buffer	Reaction time	# Colonies	# Blue	# Red	# White
CelLytic B	0 minutes	0	0	0	0
CelLytic B	120 minutes	0	0	0	0
CelLytic Y	0 minutes	15	8	7	0
CelLytic Y	120 minutes	27	18	9	0

Tabulation of colony counts for transformations that were carried out in the presence of lysis buffer and the appropriate DNA but without cellular lysate. No transformants were seen when the DNA was incubated with the bacterial lysis buffer (CelLytic B), suggesting that this particular buffer somehow inhibits transformation. On the other hand, the yeast lysis buffer (CelLytic Y) showed no inhibition of transformation, which may not be surprising since the bacterial lysis buffer is designed to act on bacteria such as *E. coli* NEB10β. However, it is also clear from these results that *Sce* does not offer any advantage over the yeast lysis buffer for DNA assembly and, therefore, it can be deduced that the transformants resulting from *Sce* incubation are actually a result of *in vivo* DNA assembly in *E. coli*.

2.4. Conclusions and Discussion

Through a series of experiments investigating circular and linear DNA assembly, we found that: 1) lysate derived from *E. coli* NEB10β, a RecA-deficient strain, was able to efficiently assemble dsDNA, 2) a fraction of the assembly takes places inside *E. coli* NEB10β post-transformation by *in vivo* DNA assembly, and 3) lysates derived from *D.*

radiodurans and *S. cerevisiae* were not able to join together dsDNA under the conditions we tested. In addition, it is worth noting that we attempted single-stranded DNA (ssDNA) assembly of synthetic 60-mer oligonucleotides (20 bp overlaps) that was accomplished by ISO assembly but was unsuccessful using our *ex vivo* approach (data not shown). Over the course of all of our experiments we found that the trends in efficiency of assembly held true across organisms independent of batch-to-batch variation.

Chapter 3. Optimization of *ex vivo* DNA Assembly

3.1. Lysate Preparation

Arguably, the most important step in any *ex vivo* investigation is the preparation of the lysate. Since this starting material may be used for many subsequent reactions and experiments it is critical to fully investigate the factors contributing to the suitability of resulting cell extracts prior to optimizing downstream reaction parameters. To investigate lysate preparation we tested the effects of varying media composition, harvesting at different points in the growth curve, the addition of cryopreservant (i.e., glycerol) and lysis method.

3.1.1. Harvest Phase

The typical growth curve of *E. coli* (and other prokaryotic bacteria) show an initial period of non-exponential growth (known as lag phase) in which the cells adjust their intracellular physiology in response to the change in extracellular conditions and the increase in available nutrients. This is followed by the exponential phase in which the cells reproduce at maximal capacity (given the external conditions) until the external environment becomes suboptimal (due to culture density, accumulation of waste/byproducts or depletion of nutrients). At this point the cells transition from exponential phase into a quiescent state known as stationary phase (SP). Reproduction is

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halted and metabolism is severely slowed as the cell allocates
resources to only the very most essential processes until new
resources become available and the whole grow curve can restart.
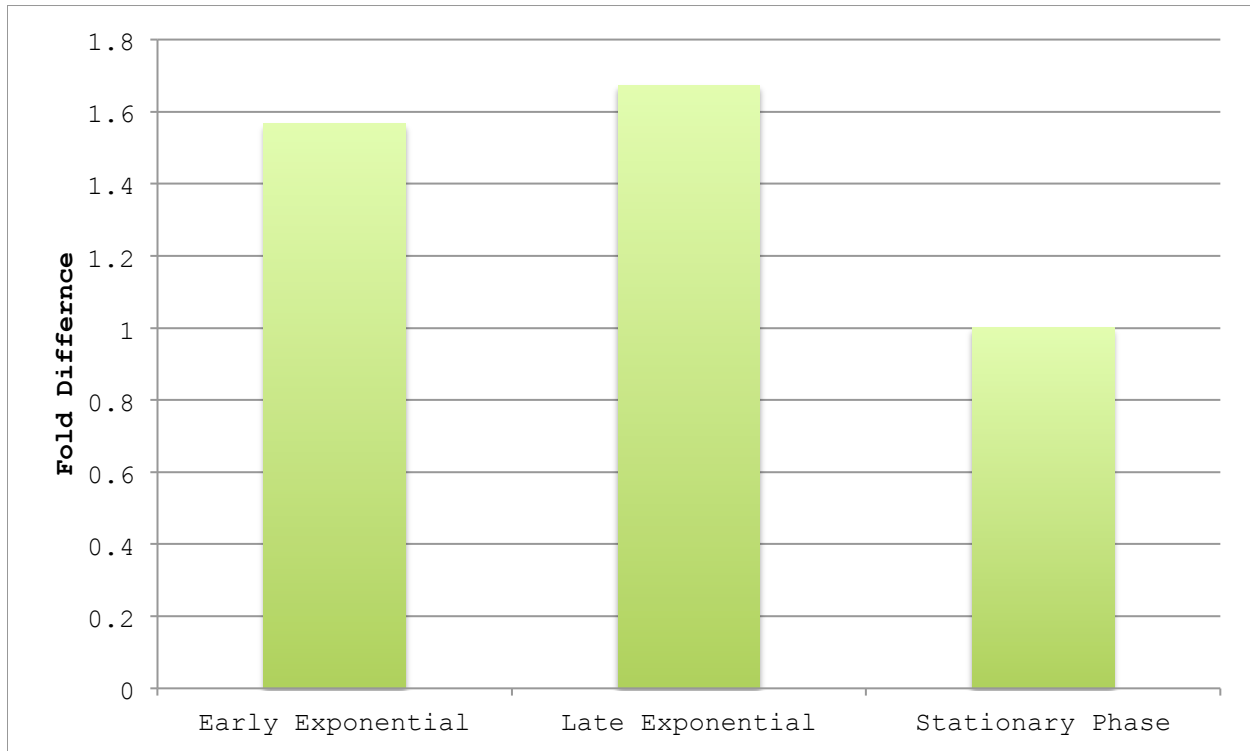
Figure 3.1 Harvested Growth Stage on Assembly Efficiency
NEB10β was grown in TB and harvested at various points along the growth curve. Growth
phase was judged by optical density at 600 nm, using absorbances of 0.4, 1.0 and >2.0
as markers of early exponential, late exponential and stationary phases, respectively.
Lysates were prepared as previously reported using detergents and two-way assemblies
performed. Fold difference represents the change in product band intensity of the
sample as compared to the intensity of Stationary Phase product band. Band intensities
are calculated relative to the BCP band of the negative control lane. Original gel
picture are shown in Figure B.2 and Figure B.3.

Initial experimentation with *ex vivo* DNA assembly grew the *E. coli*
cultures to saturation for two reasons. First, this is very convenient
for the researcher as the *E. coli* culture can be prepared as an
overnight culture and grown to saturation by the next day without the

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need to constantly monitor the progress of the culture. Second, during the later stationary phase of growth the cell and genome are bombarded by accumulated waste products (free radicals, high pH)[41,42]. The damage inflicted upon the cellular DNA may thereby cause the cell to produce DNA repair enzymes useful for DNA end joining. However, the highest levels of DNA replication occur during exponential phase and may in fact contain elevated concentrations of DNA repair enzymes useful to DNA assembly.

Based on previous observations of growth for NEB10β, we defined three different phases of growth, Early Exponential (EE), Late Exponential (LE) and Stationary phase (SP) represented by spectrophotometric measurements of optical density at 600 nm (OD600) of ~0.4, ~1.0 and >2.0, respectively. After harvesting the NEB10β grown to EE (OD600 = 0.46), LE (OD600 = 0.92) and SP (OD600 = 2.75) in previously detailed culturing conditions, lysates were prepared by resuspending in a 3:1 ratio of 2X CelLytic B (Sigma) detergent to pellet mass (e.g., 300 µL CelLytic for 100 mg of cell pellet), pelleting cell debris from lysis and diluting the soluble fraction 50% with glycerol.

The results of 2-way linear assemblies using these lysates displayed in Figure 3.1 shows an improvement in EE and LE harvesting over lysate prepared from SP. However, this improvement is very slight. Since harvesting in the latter stages of growth affords more biomass (and therefore lysate) and is convenient for the researcher we will continue to harvest in the stationary phases of growth.

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3.1.2. Rich (TB) versus Minimal (M9)

Cellular physiology is inextricably linked to the extracellular environment. In a laboratory setting the extracellular environment is established by the physical (e.g., temperature and pressure) and chemical conditions of the growth medium. Under optimal conditions, *E. coli* will consume metabolites to increase biomass and replicate as quickly as possible. Conversely, under nutrient-limited or suboptimal conditions *E. coli* will reproduce at a more conservative rate as resources are reallocated to deal with environmental challenges. The rate of growth and extracellular conditions directly impact the production of enzymes involved in DNA reproduction and repair – including those putatively involved in the *ex vivo* DNA assembly process.
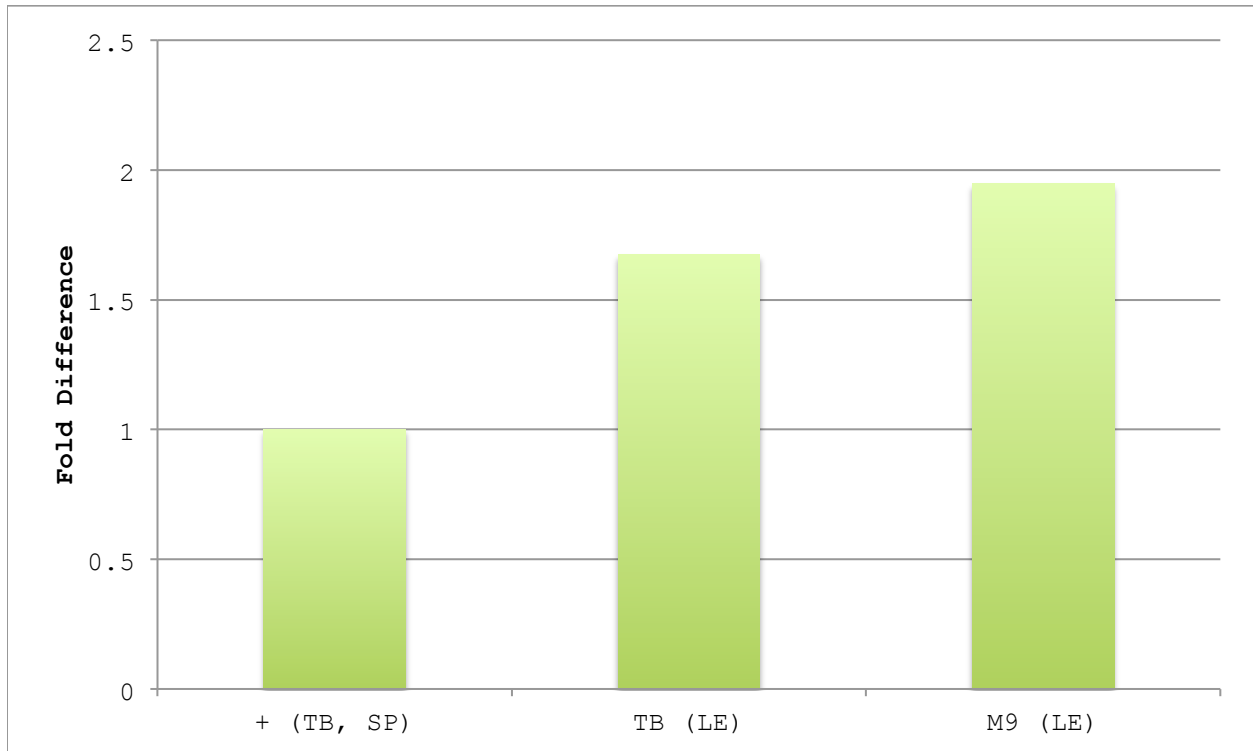
Figure 3.2 Rich (TB) versus Minimal (M9) media
Lysates prepared from cells grown in rich and minimal medias also show minimal difference in assembly efficiency, although minimal media showed a slightly larger increase in efficiency. Here, late exponential cultures had to be used because cultures grown in M9 media had trouble exceeding optical densities of 2.0 used as the marker of stationary phase. Fold difference represents the change in product band intensity as compared to Stationary Phase. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.2 and Figure B.3.

Previously, we grew our *E. coli* cultures to stationary phase (SP) in Terrific Broth (TB), a rich media very similar to the standard Luria-Bertani (LB) media but containing increased amounts of tryptone and yeast extract, glycerol as an added carbon source and a phosphate-based buffer system. This rich media allows growth to a much higher saturation than typical media such as LB. While we have used other rich media (LB, SOB, SOC; Data not shown) to prepare active lysates, here the activity of lysates prepared in TB were compared to those

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prepared in M9 minimal media with glucose. As its name implies, a minimal media contains only the minimum nutrients necessary for bacterial growth, usually salts providing essential elements (Magnesium, Sulfur, Phosphate, etc…), a carbon source (e.g., glucose, sucrose, succinate) and water. In a minimal media environment nothing is "supplemented" to the cell so that all the essential metabolic pathways are active.

Given that the NEB10β lysate appeared to have slight improvements in efficiency when harvested in LE and that when grown in M9 media NEB10β had trouble achieving cellular densities similar to that of our stationary phase cells grown in TB (OD600>2.0), we compared cells grown M9 to LE with cells grown in TB to LE. As in 3.1.1, the results of the 2-way linear assembly are interpreted as fold difference to lysates prepared in TB at SP. The lysates prepared in M9 media at LE show comparability with that of cells grown in TB to LE.

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3.1.3. Lysate Storage

Table 3.1 Characteristics of Protein Storage Methods

Storage Condition

	4°C	50% Glycerol Solution at -20°C	Frozen at -20°C or -80°C	Lysophilized (usually at -20°C or -80°C)
Typical Shelf Life	Days to several weeks	6 months to 2 years	Many years	Many years
Requires sterility or antimicrobial agent?	Yes	Usually	No	No
Number of times sample may be removed from storage	Many	Many	Once	Once

Proteins are a highly heterogeneous class of biological macromolecules with activities that are extremely dependent upon both their chemical composition and tertiary structure. Some proteins are even dependent upon their association with one or more other peptides. As such, maintaining the stability and activity of proteins outside of their native context can be challenging. If certain conditions are not adhered proteins may lose activity as a result of aggregation, proteolysis/microbial contamination and exposure to suboptimal thermochemistry. While the optimal conditions for storage vary between proteins[43], Table 3.1 highlights several general guidelines for protein storage and stability.
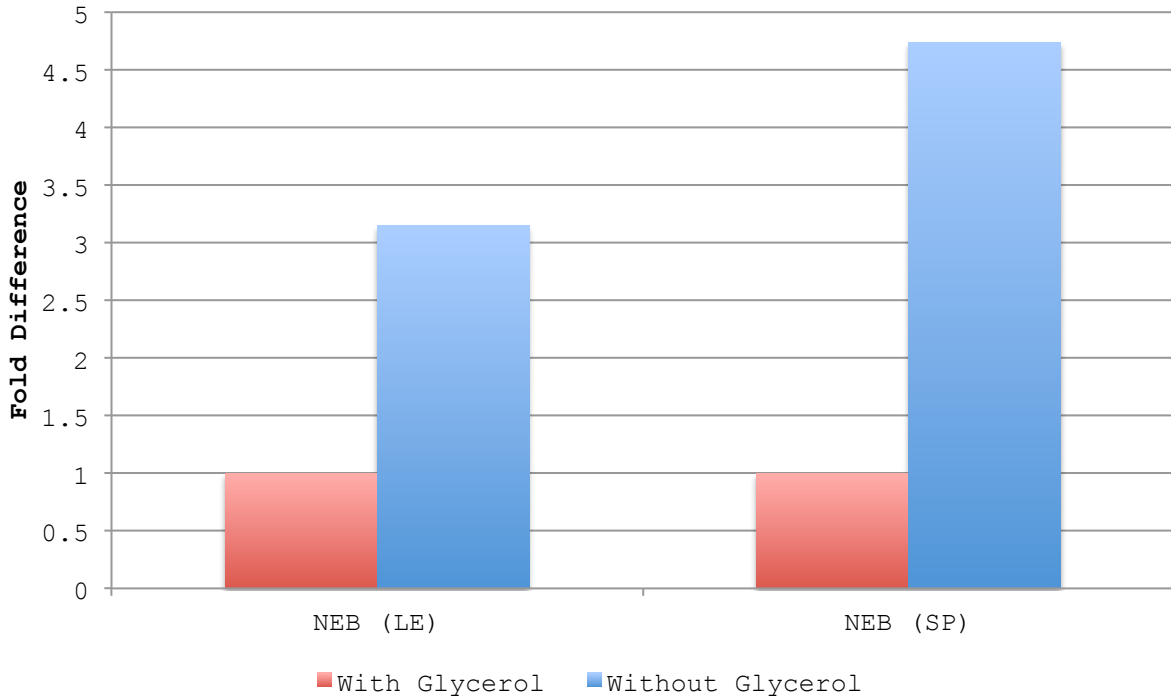
Figure 3.3 Glycerol negatively affects lysate activity
From the same NEB10β lysates prepared at various growth stages, aliquots were saved prior to glycerol dilution and instead diluted with water. These are compared with their glycerol counterparts and show a significant improvement in assembly efficiency. Fold difference represents the change in product band intensity of the non-glycerol sample as compared to its glycerol-containing counterpart. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.2 and Figure B.3.

For our purposes, once the soluble fraction of our crude lysate is isolated, we dilute with an equal volume of 100% glycerol and store the samples at -20°C. To evaluate the prudence of glycerol addition, Figure 3.3 shows the results of testing freshly prepared NEB10β lysates diluted with either an equal part glycerol or equal part water. Glycerol would appear to have a significant impact on assembly efficiency, decreasing the formation of linear product approximately four-fold.

While such a drastic effect on DNA end-joining might be attributed to enzymatic origins (reduction in exonuclease, polymerase or ligase activities), it is interesting to note that there does not seem to be a difference in the degradation bands formed with or without the presence of glycerol. This means that a roughly equivalent amount of substrate ends are formed by the action of exonucleases, but these compatible substrates are not incorporated into the final product band. Granted, it could be that while the total degradation is equivalent the exonuclease is functioning differently. For example, restriction enzymes are known to have reduced substrate specificity (so called, "star activity") for particular DNA sites in the presence of glycerol concentrations exceeding 5%[44]. It would appear that the glycerol might be interfering with the thermodynamics of base pairing between exposed homologous regions. This could make sense given that glycerol is able to decrease the hydration sphere around polynucleotides by competing with water to hydrogen bond with exposed base side groups and, perhaps for this reason, is used in electrophoretic buffers to help prevent DNA entanglement[45]. Indeed, prior investigation into ligases have shown that the electrostatic reduction of hydrogen bonding interactions experienced with increasing glycerol decrease the activity of the ligase but actually lead to an increase in the accuracy/specificity of the ends ligated together[46].

Although omitting the inclusion of glycerol is tempting due to it's clear detrimental effect on lysate activity, it's faculty as a cryopreservant provides convenience that – in our opinion – supersedes

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this disadvantage. For instance, if glycerol was to be omitted from the lysate there are a few alternatives. Lysate could be aliquoted into tubes, frozen and only thawed immediately prior to use. However, this may be very wasteful as only 2 µL of lysate is needed per reaction so there is either a waste of freezer space as small aliquots are frozen in individual tubes or a waste of lysate as leftover thawed lysate is discarded. Lysates could also be freshly prepared before all assembly reactions; however, this is laborious and requires a culture of *E. coli* to be prepared ahead of time. For these reasons we have decided to continue to utilize glycerol as a cryopreservant agent and maximize the efficiency of lysis in order to have a very concentrated lysate. This way the lysate may be diluted prior to the reaction and thereby the final concentration of glycerol is diluted, as well.

3.1.4. Detergents versus Autolysis

In the previous section we noted that ultimately, it is desirable to have a concentrated lysate stock, which can be diluted prior to the assembly reaction. In the previous context it was in reference to reducing glycerol contamination, but concentrated protein samples are also more resistant to deactivation and loss due to interactions with the storage vessel. Generating a highly concentrated cell extract is predicated upon an efficient lysis method.

Our current approach relying upon a chemical detergent means of lysis is efficient, compatible with small sample volumes and requires no specialized equipment. The detergents are non-toxic, non-denaturing and available as 1X, 2X and 10X concentrated formulations, so an

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increased concentration can be employed to make even more highly concentrated cell extracts. However, the detergents suffer from several major drawbacks including the expense of purchase, a lack of transparency to their proprietary formulation and most of all, extensive toxicity toward the fragile competent *E. coli* utilized for transformation (Table 2.1). To address this major dilemma, we decided to investigate alternative methods to lyse our *E. coli* cultures.

While a number of methods are commonly employed to lyse *E. coli* most are unsuitable. Thermochemical methods such as alkaline lysis and heat-lysis denature the necessary catalytic proteins and present conditions that may handicap downstream processes. On the other hand, physical methods of lysis using a bead basher, French press homogenizer or sonicator offer some of the highest lysis efficiencies with the greatest degree of flexibility in buffer composition, but may have minimum sample volumes, require vigilance to prevent denaturing of the sample and all require the purchase of specialized equipment.

In response to the pitfalls inherent in each of the previous lysis methods, Zymo Research™ has created autolytic strains of *E. coli*. These strains contain an ingenious genetic design drawing upon the lysogenic system of the *E. coli* λ bacteriophage. Briefly, during the lytic phase of the λ bacteriophage life cycle the bacteriophage expresses two proteins. One protein (protein 'R') is an endolysin/lysozyme capable of cleaving the peptidoglycan bounds found in the cell wall[47]. The other protein is a holin (protein 'S') that – as its name implies – punches holes in the cell membrane[48]. Not only

does this disrupt the osmotic balance maintained by the selective permeability of the cell membrane but also releases the endolysin 'R' protein into the extracellular space where it can act upon the cell wall. In the XJa design the autolysis cassette only contains the endolysin 'R' gene that is driven by the araB promoter from the arabinose degradation operon. By adding arabinose (at ~0.2%) to the growing culture, the endolysin is produced at high level within the intracellular milieu where it is prevent by the cell membrane from acting upon the cell wall. Once ready, the researcher can freeze and thaw the culture (resuspended in buffer of choice) in order to lyse the culture. During the freezing of the cell, intracellular water crystallization damages the cell membrane performing the functions typical of the holin and releasing the endolysin to degrade the cell wall and lyse the cell.

This autolysis cassette presents a unique approach to create a lysate in the buffer of choice, without expensive reagents or equipment and in a highly efficient fashion (Zymo reports lysis efficiencies >90% after one freeze-thaw cycle for the XJa strain)[49]. As such, we purchased the XJa strain that is derived from the ubiquitous cloning strain JM109. While this strain is genotypically different from NEB10β, from a phenotypic perspective as it relates to DNA, they are very similar. Like most cloning strains, JM109 (XJa) and NEB10β have mutations in *endA* and *recA* disrupting their endoplasmic DNA-specific endonuclease and homologous-recombination functions, respectively[50,51]. The *endA* mutation is important for protecting the integrity of any

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substrate DNA present in the lysate. In its natural context the *in vivo* secretion of EndA prevents the degradation of host episomal or genomic DNA, but once the membrane is lysed and removed in an *ex vivo* or *in vitro* context, any substrate DNA existing or subsequently added, assembled or circularized is an active substrate to be cleaved[52,53]. The *recA* mutation disrupts the cell's ability to perform homologous recombination between repetitive sequences. This way episomal (plasmid) DNA is maintained as intended rather than recombined with themselves, other plasmids or the genome[54,55]. In addition they both have their restriction systems removed. Strain genotypes are displayed in Table A.2 with an explanation in Table A.3.

To test the XJa autolysis for DNA assembly, we performed the growth experiments used for NEB10β and found that XJa performed similarly in 2-way linear experiments to NEB10β (Figure B.2 and Figure B.3). Based on the results of the lysate negative controls from Table 2.1 the effects of autolysis lysates on electroporation were studied first.
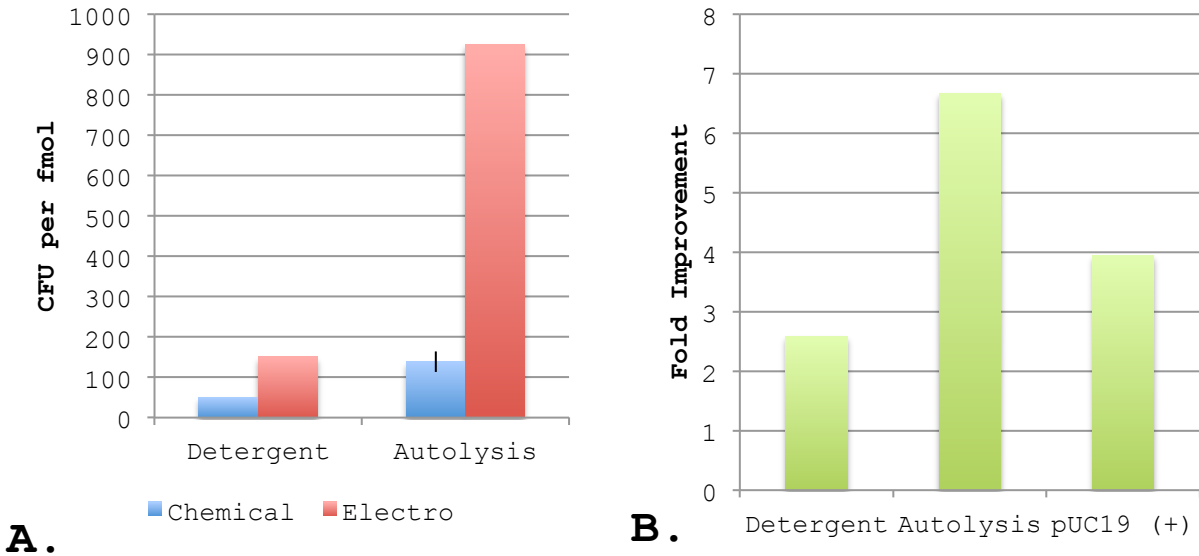
Figure 3.4 Detrimental effects of detergents on electroporation
3-way assemblies were performed using basic buffer and XJa autolysed or XJa detergent
lysed and transformed into in-house prepared chemicompetent or electrocompetent
NEB10β. **(A)** Colony forming units represent all colonies, regardless of color. **(B)** Fold
improvement between colonies formed using electroporation versus chemical
transformation. Error bars represent standard error of the mean for reactions
performed in triplicate (N=3).

Using two 3-way assemblies, one performed using a detergent-derived

XJa lysate and one using an autolysis-derived XJa lysate, the

resulting assemblies were transformed into in-house prepared

chemicompetent and electrocompetent NEB10β. The results of these

transformations, as well as transformations of pUC19 control DNA, are

depicted in Figure 3.4. The first thing to note about this experiment

is that the concentration of lysate in the transformation reactions is

significantly lower than those performed in Table 2.1 previously and

so detergent associated toxicity effects should be expected to be less

pronounced in turn. Still, there is clearly a dramatic difference

between electroporation efficiencies of lysates. Contrasting the

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chemical transformations in Figure 3.4.A, the autolysis reaction
appears to have been more efficient than the detergent reaction
(either in actual assembly or detrimental effects on transformation)
but the overall colony forming units (CFU) per femtomole appear to be
comparable. However, when looking at the electroporation results there
is a clear effect of detergents which is more succinctly stated in
Figure 3.4.B as fold difference between chemical transformation and
electroporation. It is also interesting that autolysis seems to have a
more pronounced difference in electro- versus chemical transformation
than that of the pUC19 positive control however, the pUC19 controls
may underestimate the fold improvement of electroporation. Typical
efficiencies reported by NEB for their commercially prepared NEB10β
chemicompetent and electrocompetent cells would place the improvement
at roughly ten-fold, matching the observed improvement seen for
autolysis transformation.

3.2. Optimizing the *ex vivo* DNA assembly reaction

The 'chew-back, anneal, repair' end joining mechanism hypothesized to
be the biochemical basis of the *ex vivo* reaction is an interestingly
complex optimization problem. While exonuclease activity is
imperative, overly active exonuclease activity can destroy the
substrate DNA. Additionally, since dsDNA exonuclease activity cannot
be constrained a competitive polymerization reaction must be
introduced to 'repair' uncontrolled exonuclease activity, but not so
much as to undo the requisite 'chew-back'. Several parameters of the

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ex vivo reaction were empirically investigated for their impact on this delicate balance.

3.2.1. Reaction Volumes and DNA Concentrations

In its original formulation, *ex vivo* DNA assembly was performed using 20 µL volumes with 20 ng/µL of the backbone (or larger BCP piece for linear assemblies) and a 6:1 molar ratio of insert fragments to backbone piece. While PCR and gel extraction easily generates enough substrate DNA for a couple reactions, each assembly reaction is consuming over a full microgram of DNA per reaction. Additionally – while able to distinguish background colonies by their red coloration, there was an exceptional amount of red colony formation for gel-purified substrates. To address these issues we made a concerted effort to not only reduce the DNA required for an *ex vivo* reaction and the amount of red background colonies.

First, the background was addressed by looking at our DNA preparation steps. The bands from our PCR reactions looked very strong and free of side-product formation yet somehow considerable template plasmid was co-purified with our backbone amplicon. Looking at our protocol for PCR we noted that for simplicity we had been adding 1 µL of pure plasmid mini-prep as template for a 50 µL reaction. Concentrations of our mini-preps usually range between 50-100 ng/µL for the high-copy plasmids used by *ex vivo* DNA assembly. Given that NEB protocols for Q5 DNA polymerase call for a maximum of 1 ng of plasmid template per 50 µL reaction we were adding 50 to over 100 fold excess template DNA necessary for PCR. In response, working dilutions of template plasmid

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at 1 ng/µL were prepared and PCR performed in the same fashion. Resultant gels still showed very bright banding indicative of successful PCR and eluents from gel extractions contained equivalent concentrations of DNA. However, assemblies and subsequent showed a drastic decline in the number of red colonies (~0-2 colonies per plate).

Next, the concentration of DNA used in a typical reaction was decreased along with the total volume of each reaction. Instead of a 20 µL volume, 20 ng/µL backbone and a 6:1 insert to backbone molar ratio, reactions were performed in 10 µL volumes using 5 ng/µL backbone (50 ng per reaction) and a 1.2:1 (2-way assemblies) or a 2:1.2:1 (3-way assemblies) molar ratio. This increased the number of reactions capable of being performed per preparation of DNA fragments by 4-fold (50 versus 200 ng backbone per reaction).

3.2.2. Calculating Protein Concentration

By reducing the DNA substrates used in each reaction and increasing our lysate concentrations (lysis efficiencies), we found many of initial reactions degraded most or all of the substrate DNA. The reduced substrate and increased catalyst concentrations had tipped the delicate balance between chew-back and repair making it clear adjustments to the lysate concentration were needed. Unfortunately, quantifying the "concentration" of the catalysts for *ex vivo* DNA assembly poses a challenge. First, the cellular extract is a highly heterogeneous environment with many species capable of interfering with quantification. Second, even if the responsible catalysts were

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capable of being specifically quantified their identities still remain largely unknown. Even so, measures of total protein concentration were employed as a proxy since the catalysts are almost certainly proteins.

The most common techniques for estimating the concentration of proteins are based on either a colorimetric reaction (e.g., Lowry[56], Bradford[57]) or UV absorbance spectrophotometry (absorbance at 280 nm)[58]. Deciding on the most appropriate method depends upon multiple factors including the concentration of protein in the sample, the amino acid composition of the peptide and the presence of interfering substances in the sample or buffer. For the *ex vivo* extracts Bradford colorimetric assays were compared with simple spectrophotometric absorbances of 280 nm for aromatic rings of amino acid side chains (mainly tyrosine and tryptophan), 260 nm for nucleic acids and/or 205 nm for peptide bonds.

Standard curves were generated for Bradford colorimetric assays using either Bovine serum albumin or lysozyme, at a minimum of three different quantities (along with blanks) in either water, the CelLytic detergent or the lysis/storage buffer matching the lysate samples used in the assay. While the Bradford reaction is typically incompatible with detergents, we found that the diluted samples still generated an acceptable standard curve. However, the concentrations of the lysates predicted by the Bradford (as could be expected) was dependent upon the protein used to calibrate the standard curve, the time allowed for color development and even the spectrophotometer used. Additionally,

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the assay requires the purchase of reagents and standards and a fairly time-consumptive, fickle protocol to measure samples and standards.

The difficulties posed by the Bradford assay prompted a simplified approach to concentration estimation. Peptide bonds can be detected in solution using absorbance at 205 nm[59], but when tested with lysate the assay was found to be overly sensitive, inviting error from extensive dilutions. Protein content can also be estimated using absorbance at 280 nm targeting the aromatic side chains of amino acids such as tryptophan and tyrosine. The challenge faced in a cellular extract context is interference from nucleic acids that also absorb in the 280 nm spectra. To correct for the presence of nucleic acids in the lysate absorbance at 260 nm was measured and a correction applied using the equation of Warburg and Christian[58]. While Warburg-Christian method is also susceptible to interference (e.g., NAD/NADP) it was found to be rapid, requiring minimal sample manipulation and having high reproducibility (for one sample diluted and measured in triplicate standard error of the mean = 0.012 mg/mL).
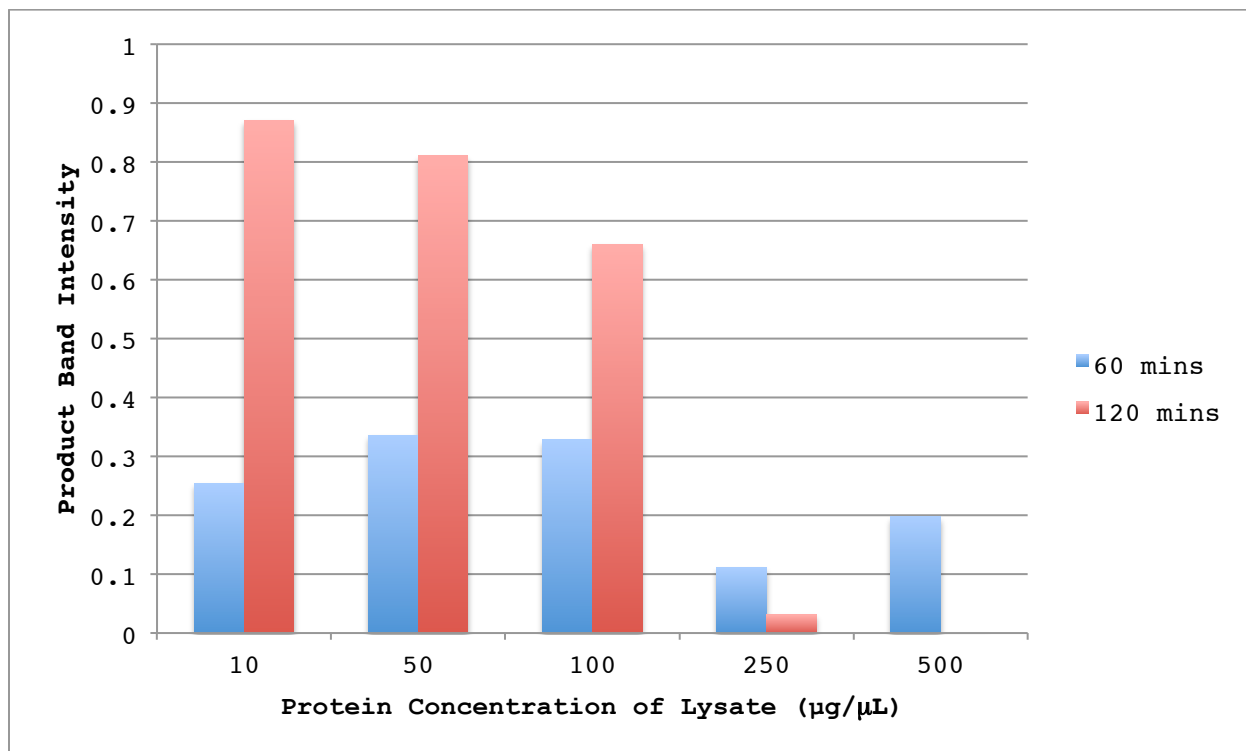
Figure 3.5 Concentration of Lysate
Here XJa lysate prepared by autolysis was measured via spectrophotometry and adjusted by the method of Christian-Warburg. Product Band intensities are calculated relative to the BCP band of the negative control lane. Gel picture for 60 minutes is in Figure B.4 and 120 minutes is in Figure B.5.

Applying the Warburg-Christian method to quantify our lysates a 2-way linear assembly was performed using titrations of autolysis-derived XJa lysate. Samples were removed at 60 minutes and after a full 120 minutes, visualized and interpreted in Figure 3.5 as the intensity of the product band. While in the first 60 minutes there is a product band formation at all tested concentrations, the second 60 minutes distinctly contribute a large portion of the final bands. This could be evidence that most of the "repair" activities do in fact precede in the second half of the 120-minute reaction. However, at higher concentrations of lysate the product bands produced in the initial 60 minutes are consumed, likely by the additional exonucleases present in

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the reaction. Looking at the results of the gel it would appear that the optimal final protein concentration of the autolysis lysates is between 10-50 µg/mL. Since our stocks of lysates produced via autolysis were measured at ~7-8 mg/mL, a 10X concentration could be reached by a 1/200 dilution of the lysate.

3.2.3. "Base" Buffer Optimization

In order to function many enzymes rely on the presence of cofactors and various energy sources. In section 2.2 the effects of Magnesium-to-ATP concentration were investigated and the results revealed an important relationship between the relative quantities of magnesium ions and available ATP. Beyond these two components of the reaction buffer, the other components were not varied but may illicit improvements in the overall reaction efficiency.

We tested the formulation of the *ex vivo* DNA assembly buffer with a few changes to more closely match buffer formulations optimal for purified *E. coli* enzymes, in particular for the *E. coli* DNA ligase (Eco Ligase). For all buffers tested the NAD+ was reduced to 50 µM (typically 26 µM for Eco Ligase) and Bovine Serum Albumin (BSA) at 50 µg/mL. Further the Magnesium and ATP levels were, once again, titrated between 4 mM Magnesium and 50 µM ATP.

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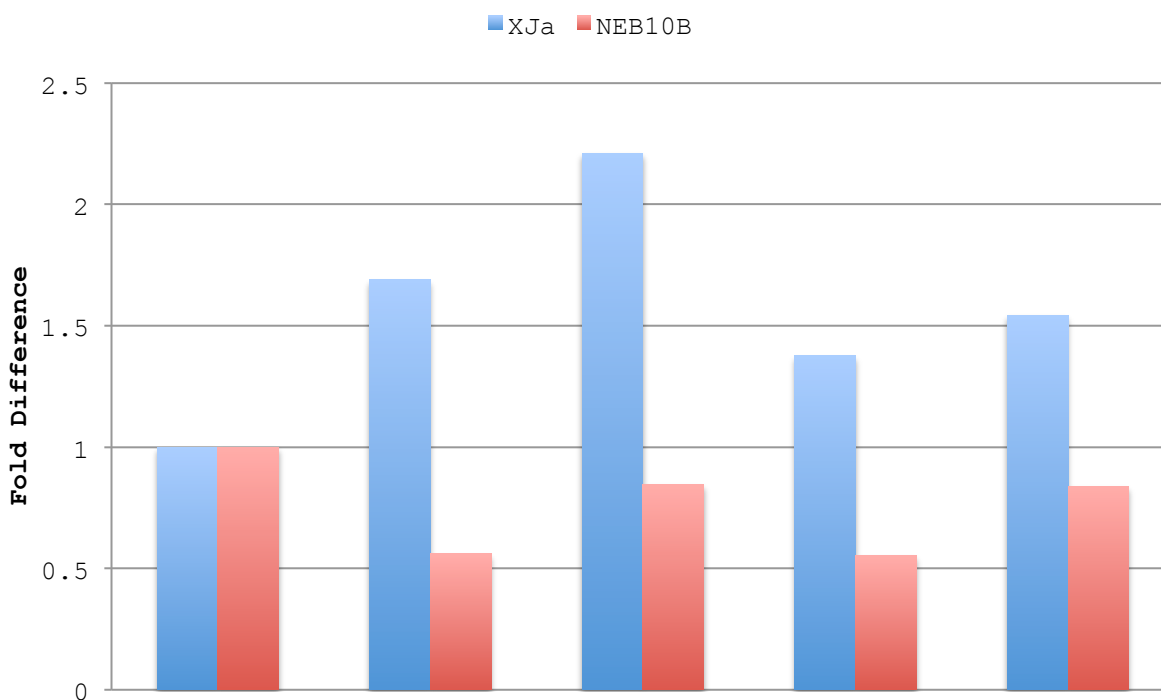
	+	1	2	3	4
Tris-HCl,	50 mM	50 mM	50 mM	50 mM	50 mM
DTT	1 mM	1 mM	1 mM	1 mM	1 mM
MgCl$_2$	10 mM	10 mM	**4 mM**	**4 mM**	10 mM
ATP	1 mM	1 mM	1 mM	**50 μM**	**50 μM**
βNAD	1 mM	**50 μM**	50 μM	50 μM	50 μM
BSA	0 μg/mL	**50 μg/mL**	50 μg/mL	50 μg/mL	50 μg/mL

Figure 3.6 Optimization of components of "Base" Buffer

The components of the base buffer were varied between their original values and values commonly used for the buffers of various molecular biology enzymatic reactions. Fold difference represents the change in product band intensity of the sample as compared to "+" sample of the same lysate type. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.6 and Figure B.7.

The NEB10β prepared by detergent lysis showed decreasing efficiencies

with the decrease in NAD+ and addition of BSA when compared to the

standard buffer. In contrast, the autolysis XJa showed a slight

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increase in assembly efficiency with the addition of BSA and decrease in NAD+. In fact, XJa showed an increase in all tested conditions over the standard buffer. Unfortunately, without additional replicates it is unclear whether the observed trend is due to differences in the XJa autolysis lysate versus the NEB10β detergent lysate or a function of fluctuations in the standard lane.

3.2.4. Divalent Metals Titrations

Throughout biochemistry metal ions play an integral role as enzymatic cofactors. In fact, it has been estimated that metal ion cofactors are needed in nearly one-third of enzyme-catalyzed reactions[60]. Most often these metals are coordinated within the active site of enzymes where they mediate redox (and sometimes non-redox) reactions by increasing the electrophilicity of the active site. Once a ligand is bound in the active site, the metal can act to remove electron density and cause polarization of reactive bonds in the ligand - usually C-O or P-O in nature[61].

As the most abundant divalent metal species in cellular contexts, magnesium is the most frequently utilized metal in active sites. As discussed previously, in physiologic environments magnesium forms stable chelates with molecules containing phosphate such as ATP, RNA, DNA and other nucleic acids. The tendency of magnesium to maintain elevated hydration states is the primary means by which it acts as a cofactor to bind and catalyze nucleic acid substrates[61].

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Due to its poor spectroscopic and crystallographic properties researchers have long employed other divalent metal analogues to study nucleic acid enzymology. While analogues such as zinc, calcium and most often manganese are acceptable for structural studies, these metals often display differing biochemistries to magnesium[62]. In fact, it is often observed that other transition metals, especially manganese, coordinate more tightly with active sites and thereby confer higher levels of activity. In other instances, the alternate metal can change the coordination geometry or structural conformation of the active site altering the substrate specificity, mode of action or completely inhibiting the protein.

49
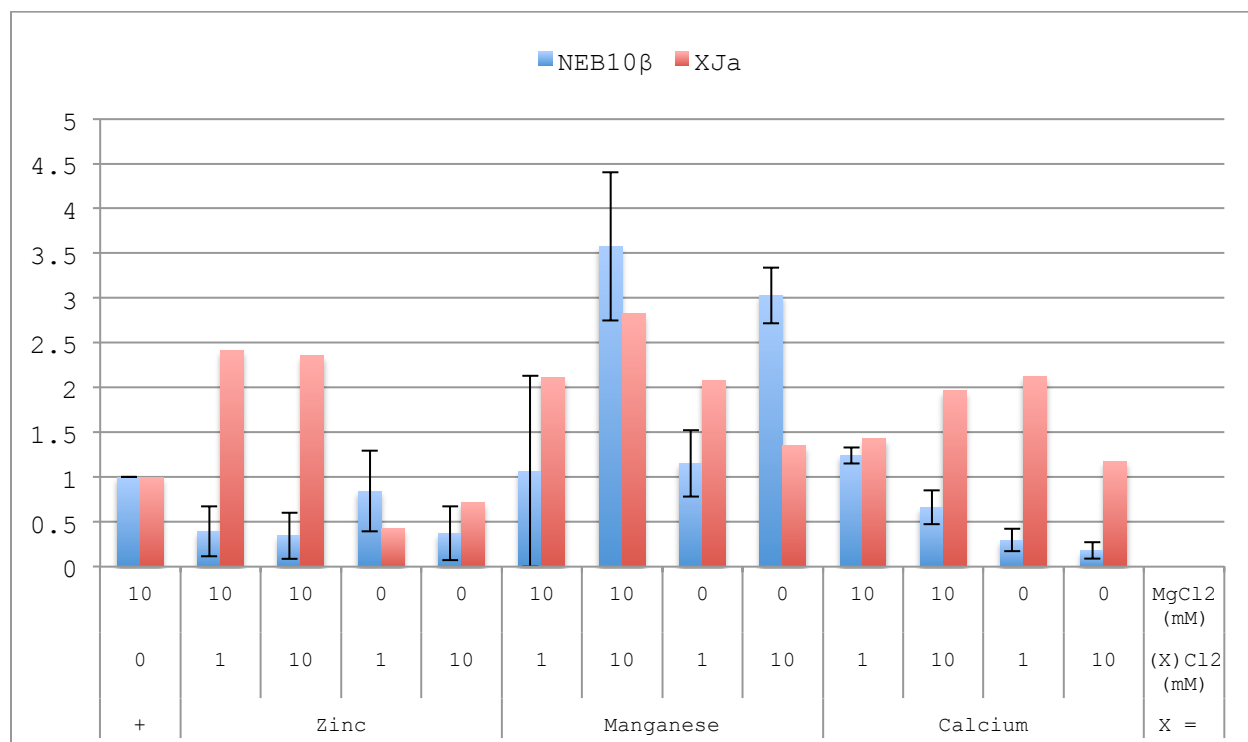
Figure 3.7 Titrations of Divalent Metal Cations
This figure shows the titration of Zinc Chloride, Manganese Chloride and Calcium Chloride with Magnesium Chloride and without Magnesium Chloride. Fold difference represents the change in product band intensity of the sample as compared to "+" sample (10 mM Magnesium) of the same lysate type. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.8, Figure B.9 and Figure B.10. NEB10β is the average of duplicate experiments and error bars represent standard error of the mean (N=2).

Previously, we established that the ratio of Magnesium-to-ATP in the *ex vivo* DNA assembly buffer was crucial for efficient end joining, most likely as a consequence of ATP's ability to chelate magnesium and the resulting effects on RecBCD activity. Although magnesium dominates as the native cofactor for nucleic acid-directed enzymes, we decided to test the effects of titrating zinc, manganese and calcium on the efficiency of end joining.

Using XJa (autolysis) and NEB10β (detergent) four formulations of each metal were tested with respect to magnesium (mM:mM): 1:10, 10:10, 1:0,

10:0. The results of this 2-way linear titration depicted in Figure 3.7 are compared to a control reaction with the *ex vivo* DNA assembly "base" buffer. Just as in the "base" buffer optimization in 3.2.3 there are clearly differing trends seen for the NEB10β/detergent and the XJa/autolysis lysates – although there is also some agreement between the two, as well. Both lysates responded negatively to Zinc in the complete absence of magnesium, while both responded positively to the addition of manganese – even in the complete absence of magnesium. XJa actually shows an increase in efficiency across all tested conditions so long as 10 mM of magnesium was present. Perhaps more surprising is that for both manganese and calcium XJa showed equivalent or higher activity even in the absence of magnesium. On the other hand, outside of manganese NEB10β clearly did not tolerate the omission of magnesium. The extremely pronounced response to manganese by both lysates prompted further investigation into the supplementation of manganese to the standard *ex vivo* DNA assembly buffer.

3.2.5. Manganese

The strong activation of DNA assembly imparted by manganese in the titrations of the previous section (3.2.4) prompted a more detailed study of manganese. Using a similar titration approach between magnesium and manganese, 2-way linear assemblies were performed using NEB10β lysate. Again, Figure 3.8 shows a comparison of the titrated samples relative to the intensity of the product band formed by the basic *ex vivo* DNA assembly buffer containing 10 mM magnesium and 0 mM

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manganese. In the experiment, total divalent species were held at 10-11 mM with the exception of the 0:2 and 0:6 magnesium:manganese (mM:mM) samples. In Figure 3.9, the results are displayed again, but showing the compositions of the lane by band (product band; BCP and Promoter substrate bands and; degradation band of lowest molecular weight) relative to the BCP band of the negative control lane (-).

MnCl2 (mM)	1	3	5	7	10	2	6	10	0
MgCl2 (mM)	10	7	5	3	1	0	0	0	10
									+
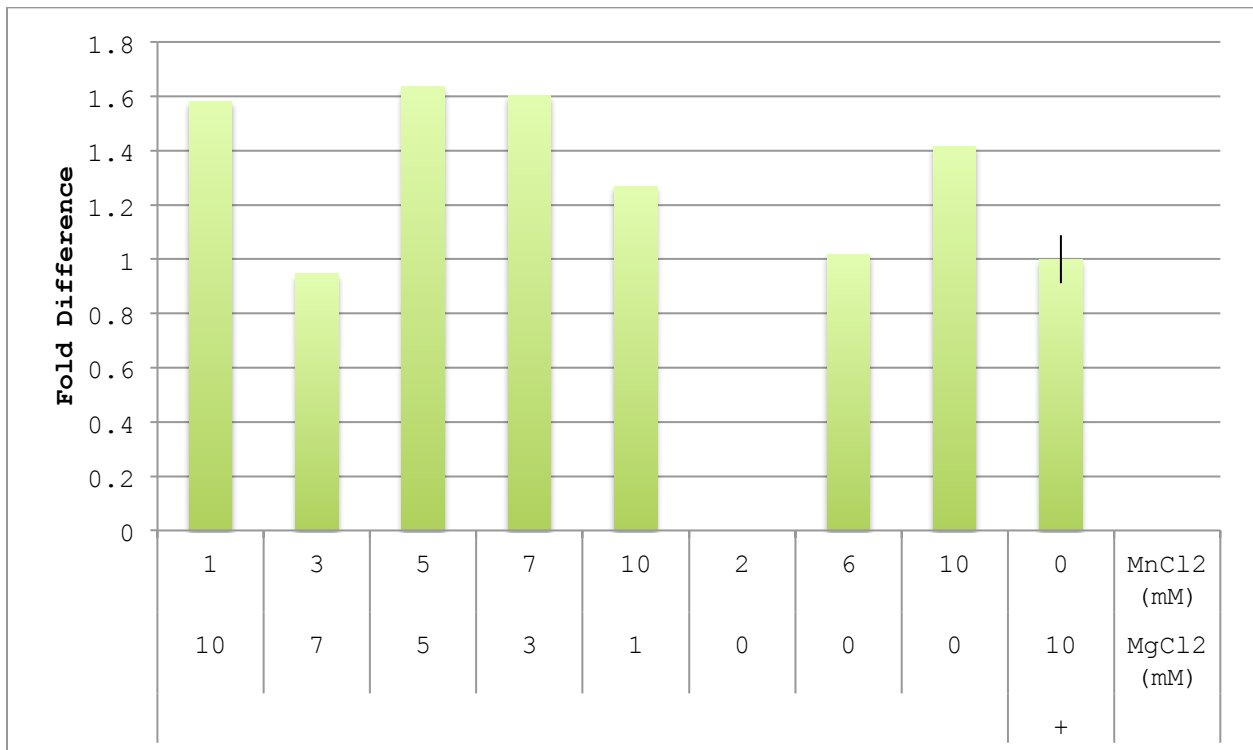
Figure 3.8 Titrations of Magnesium and Manganese

Using NEB10β lysate and the 2WL assembly, magnesium and manganese levels were titrated against one another. The ratio labels indicate the concentration of magnesium chloride (mM) to manganese chloride (mM) with the 10:0 ratio representing the original buffer constitution (+). Fold difference represents the change in product band intensity of the sample as compared to "+" sample of the same lysate type. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel picture is shown in Figure B.11.

								+	-	
10	7	5	3	1	0	0	0	10		MgCl2 (mM)
1	3	5	7	10	2	6	10	0		MnCl2 (mM)
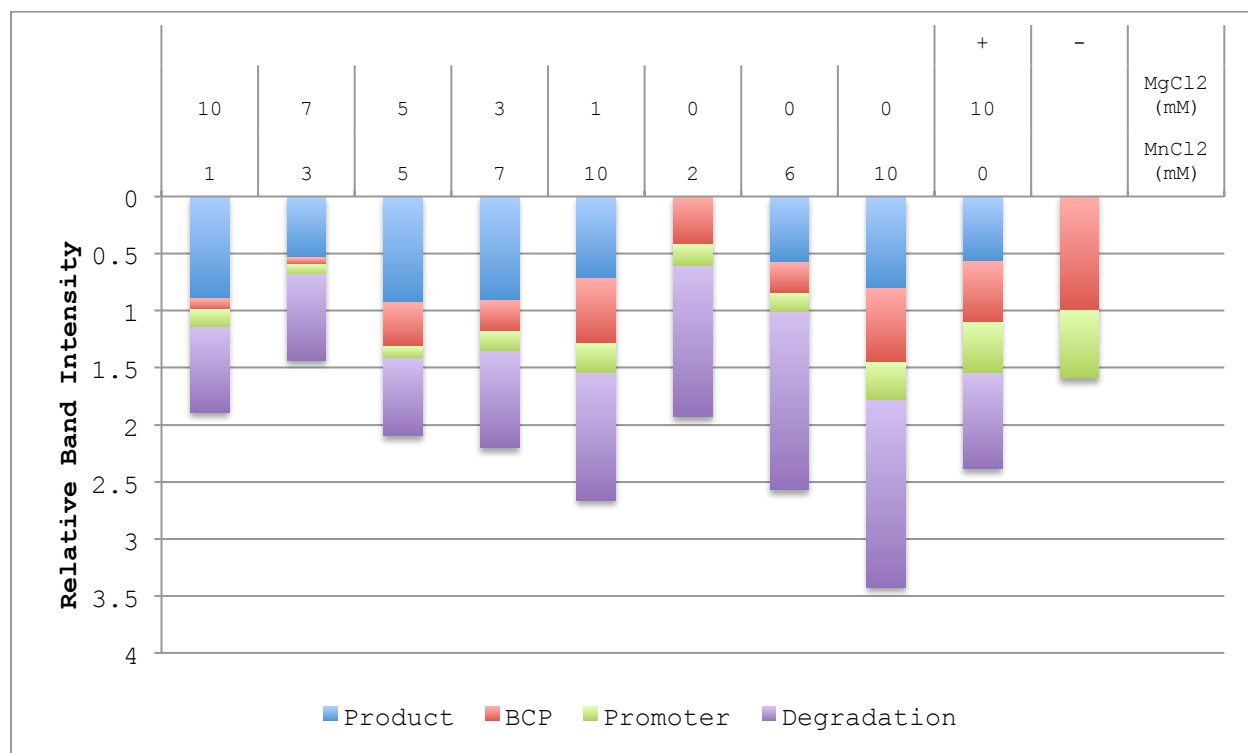
Figure 3.9 Lane composition of Manganese to Magnesium Titration

Chart shows the relative intensities of the product (Blue), BCP (Red) and Promoter (Green) substrate bands, as well as the low molecular weight band representing degradation products (Purple). Columns are displayed inverted to more closely represent the actual gel and the order in which the bands migrate. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.11.

The results of the 2-way assembly – albeit less pronounced - confirm the observed increase in assembly efficiencies previously observed in the metal titration experiment. The ratio of magnesium to manganese appeared to have minimal impact. In fact, even the 0:10 sample was able to catalyze assembly with at least equal efficiency to that of the positive control. Visualizing the lane compositions in Figure 3.9 provides more details into the possible mechanisms by which manganese may be enhancing assembly. For instance, the largest consumption of substrate DNA did not directly correlate with product formation as there was more remaining substrate DNA in the higher manganese ratio

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lanes containing the highest level of product. Further, both lanes
containing 10 mM manganese (1:10 and 0:10) appear to have the most
total DNA in the lane overall. This phenomenon is more than likely
attributed to increased DNA polymerase activity in the presence of
manganese. Indeed, the literature is ripe with evidence showing that
in the presence of manganese DNA polymerases (including the *E. coli*
PolA polymerase) experience a decrease in specificity of base
incorporation as well as 3'-5' proofreading functions. Such a decrease
in specificity may increase the polymerase activities not only to gap-
fill the product fragment, but also the chew-backed, partially
degraded substrate bands thereby preserving them.
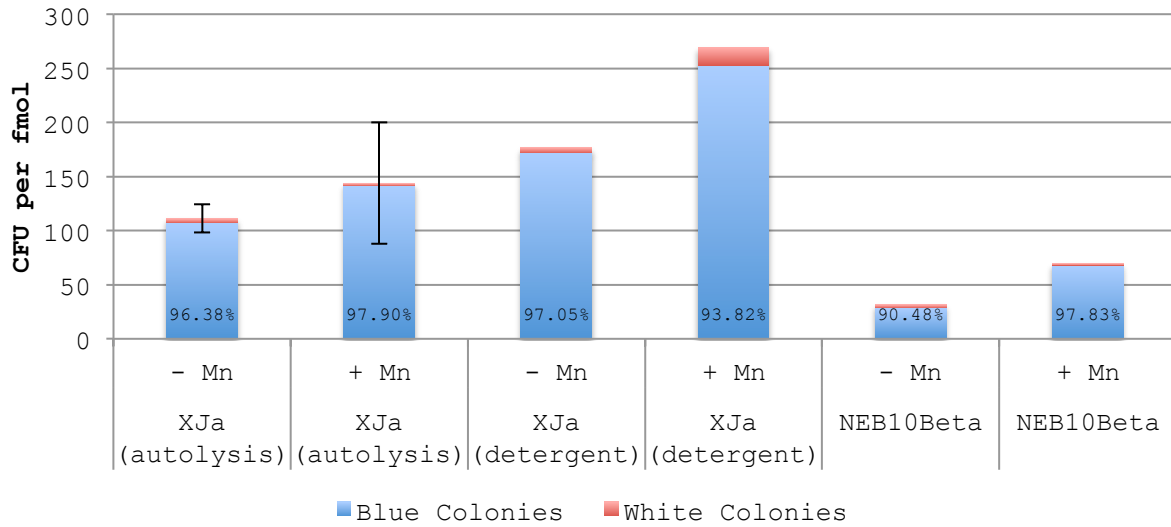
Figure 3.10 Effect of Manganese on Two-way Circular Assembly
Using XJa lysed with either CelLytic or using freeze-thaw autolysis, circular assemblies were performed in the original buffer (- Mn) or with the addition of 1 mM Manganese Chloride (10:1 ratio). Percentages represent Blue colonies to all colonies (blue and white). Error bars represent standard error of the mean for reactions performed in duplicate (N=2).

If the presence of the manganese is mutagenizing the recombined product then its addition – regardless of the improvements in apparent end joining – are undesirable for cloning. However, the 2-way linear assay has no way of revealing incorporated mutations. To reveal any increases in mutation rates as a result of manganese supplementation shows the results of 2-way circular assemblies with and without 1 mM manganese. The results still show an increase in total colonies formed across all lysates, but an increase in mutation rate is not immediately apparent. This does not necessarily mean there is not an increase in mutation rate from the addition of magnesium, because the number of white colonies falls outside the countable threshold (<30 CFU per plate) for XJa autolysis and NEB10β. For the XJa detergent

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sample that did have a countable number of white colonies there was a decrease in percentage of correct colonies.

Chapter 4. Probing *ex vivo* DNA Assembly with Enzymatic Titrations

The absence of the cell membrane allowed rapid prototyping of buffer compositions, substrate and catalyst concentrations, and titrations of small molecules. To uncover biochemical mechanisms typically enzymes are purified and titrated together in an *in vitro* study or knocked-out/overexpressed in an *in vivo* study. Both of these approaches can be leveraged in *ex vivo* experimentation to uncover underlying phenomena of the larger system.

4.1. Enzymatic Titrations

Tapping into the wealth of commercially purified cloning enzymes already available through molecular biology suppliers, the underlying biochemistry of *ex vivo* DNA assembly was probed using enzymatic titrations. Building on the premise that the *ex vivo* DNA assembly reaction proceeds via a chew-back, anneal and repair pathway similar to Gibson assembly, we purchased available exonucleases, polymerases and ligases (as well as RecA, a homologous recombination protein) native to *E. coli*. Table 3.1 gives details about each enzyme used in the titration experiment.

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Table 4.1 Enzymes used in Titration

	E. coli gene	Function	Product(s)
Exonuclease III	*xthA*	3' to 5' dsDNA exonuclease	ssDNA and dNMP
RecJ$_f$	*recJ*	5' to 3' ssDNA exonuclease	dNMP
Exonuclease I	*xonA/sbcB*	3' to 5' ssDNA exonuclease	dNMP and dinucleotide
Exonuclease T	*exoT*	3' to 5' ssDNA and RNA exonuclease	dNMP
Exonuclease V (RecBCD)	*recBCD*	dsDNA processive endonuclease	short oligos
E. coli DNA Ligase	*ligA*	DNA ligase	phosphodiester bonded DNA
DNA PolI	*polA*	DNA polymerase	DNA polymer
RecA	*recA*	ssDNA binding protein	N/A

The addition of exonucleases can help elucidate the possible overhangs generated from chew-back. For example, if homologous overhangs were generated as 5' overhangs, it would be expected that supplementing RecJ$_f$ would greatly decrease assembly efficiencies, as the homologous regions are substrates for degradation by RecJ. The three non-nucleases – E. coli Ligase, Polymerase I and RecA – are supplemented to see if they address a bottleneck or inefficiency.

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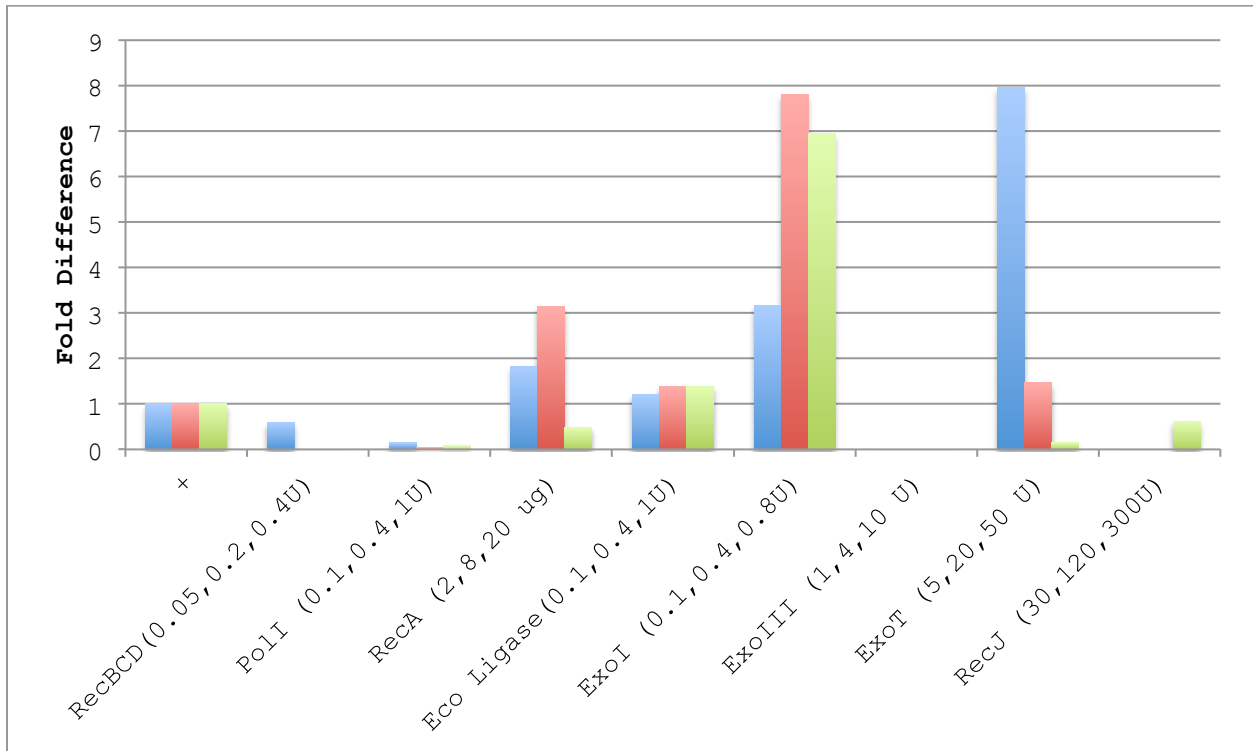
Figure 4.1 Titrations of Various DNA cloning Enzymes
The results of titratons of various purified enzymes in XJa lysates are shown. The bar colors reflect concentration of enzyme with blue, red and green reflecting the concentrations lowest to highest. Red represents units necessary to catalyze the formation of 10 nmol of product in a 20 μL reaction – except for RecA which had no unit definition. Unit definitions are available in Table A.4. Units were added as follows: RecBCD (.05, .2, .4); PolI (.1, .4, 1); RecA (2, 8, 20 μg); Eco Ligase (.1, .4, 1); ExoI (.1, .4, .8); ExoIII (1, 4, 10); ExoT (5, 20, 50); RecJ (30, 120, 300). Fold difference represents the change in product band intensity of the sample as compared to "+" sample (no enzyme). Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.12 and Figure B.13.

To determine relevant concentrations for each enzyme, the unit definitions provided by NEB were employed to calculate the number of units necessary to catalyze the formation of 10 nmol of product in a 20 μL reaction volume (5 ng/μL BCP is approximately 11 nmol). These values were used as a baseline to select three concentrations of each enzyme to test in conjunction with a 2-way linear assembly.

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Results of the assembly shown in Figure 4.1 were unexpected for several reasons. The most glaring outcome is the stimulating effect of ExoI and ExoT on assembly. It was not expected that any of the exonucleases would improve assembly as this has not presented as a rate-limiting step and can be a substrate sink if homologous ends are consumed. Clearly, for the 3'→5' ssDNA exonucleases ExoI and ExoT (at low quantities) the homologous overhangs are not being consumed. This is made clearer by Figure 4.2, which shows the lane composition of the titration. By looking at the lane composition and status of the substrate DNA, the activities of the various enzymes become clearer. For instance, it is very clear that the lack of product formation in the dsDNA exonuclease lanes is a direct result of consuming the substrate DNA. In contrast, RecJ appears inefficient at assembly not because it is consuming the substrate DNA, but for some other reason. It is very possible that the homologous overhangs produced during the *ex vivo* DNA assembly are 5' extensions. These extensions would be compatible substrates for degradation by RecJ resulting in a loss of the necessary homology and the creation of inert substrate DNA. However, it is also important to realize that these graphs are still numerical interpretations of the gel results. In the case of ExoT, without looking at the gel picture (Figure B.13) it would be nearly impossible to observe the decreasing resolution and molecular weight of the product band due to extensive degradation.

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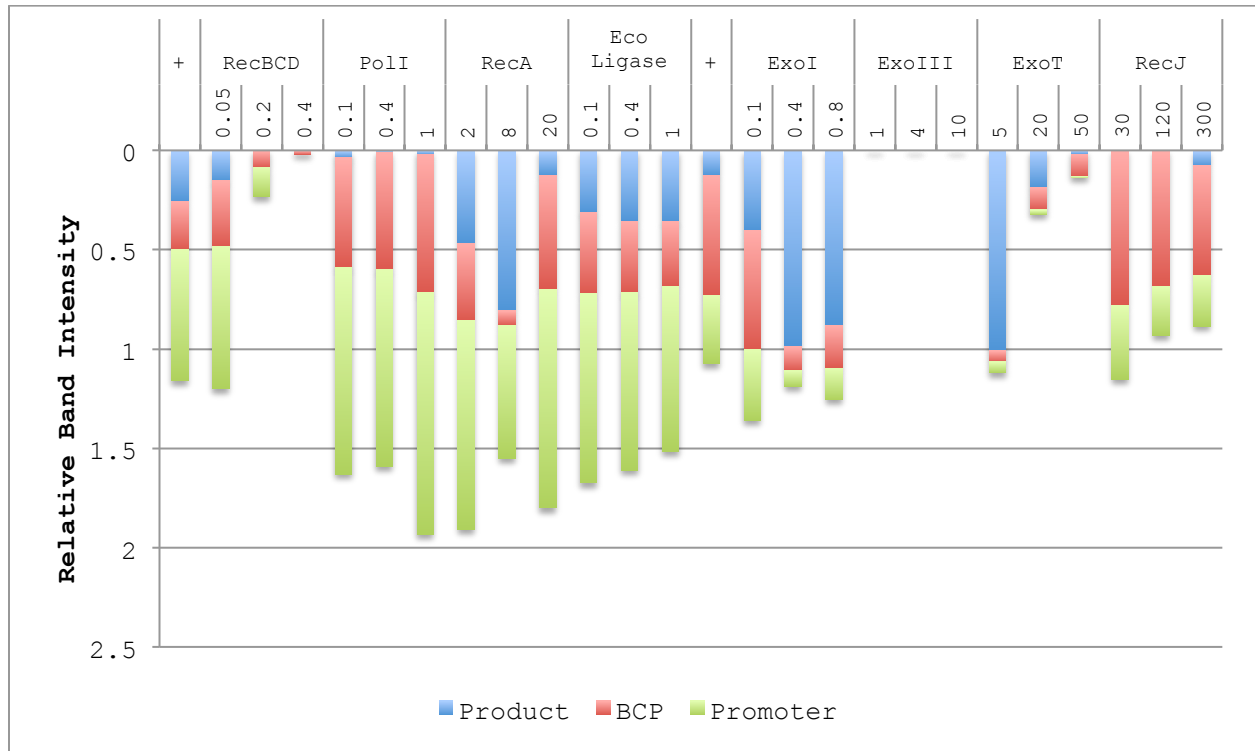
Figure 4.2 Lane Composition of Enzymatic Titration
Chart shows the relative intensities of the product (Blue), BCP (Red) and Promoter (Green) substrate bands, as well as the low molecular weight band representing degradation products (Purple). Columns are displayed inverted to more closely represent the actual gel and the order in which the bands migrate. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.12 and Figure B.13.

In the non-exonuclease category, improvements were observed using *E. coli* DNA ligase and RecA while addition of the DNA polymerase I inhibited assembly. Looking at the composition of the PolI lane the substrate fragments are maintained. The PolI protein has dual exonuclease activities: a 3'→5' proofreading activity typical of DNA polymerases and a 5'→3' exonuclease activity most likely used for nick translation. Similar to RecJ, the presence of substrate DNA and known 5'→3' activities suggests that PolI may be consuming the homology of the overhangs.

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4.2. RecA Titrations

Improvements in end joining in the presence of RecA was surprising as previous investigation revealed that recA+ strains of *E. coli* are suboptimal for *ex vivo* DNA assembly. RecA is a highly multifunctional protein *in vivo*, acting as an ssDNA binding protein[63], catalyzing strand exchange[64] and even acting as a highly specific protease (of the LexA protein) to signal the host SOS response to DNA damage[65,66]. Mechanistically, RecA performs its DNA recombination functions by binding tightly to ssDNA in long nucleoprotein filaments. Once bound to a ssDNA substrate (and ATP) RecA's secondary DNA binding site will bind to a dsDNA polymer and begin a "homology search" scanning across the dsDNA substrate for sequence matching the ssDNA. Once found, the RecA nucleoprotein filament can catalyze strand exchange and the formation of an intermediary triplex DNA[64]. Given the unique contradiction seen between *in vivo* produced RecA and *ex vivo* supplemented RecA further titration experiments were performed. Using the 2-way linear assembly, Figure 4.3 and Figure 4.4 show the results of RecA supplementation in a 20 µL reaction using XJa autolysis lysate.

Figure 4.3 Titrations of RecA
RecA protein was added to an XJa lysate in a 2-way linear assembly. Fold difference represents the change in product band intensity of the sample as compared to "+" sample (no enzyme). Band intensities are calculated relative to the BCP band of the negative control lane. Original gel picture is shown in Figure B.14
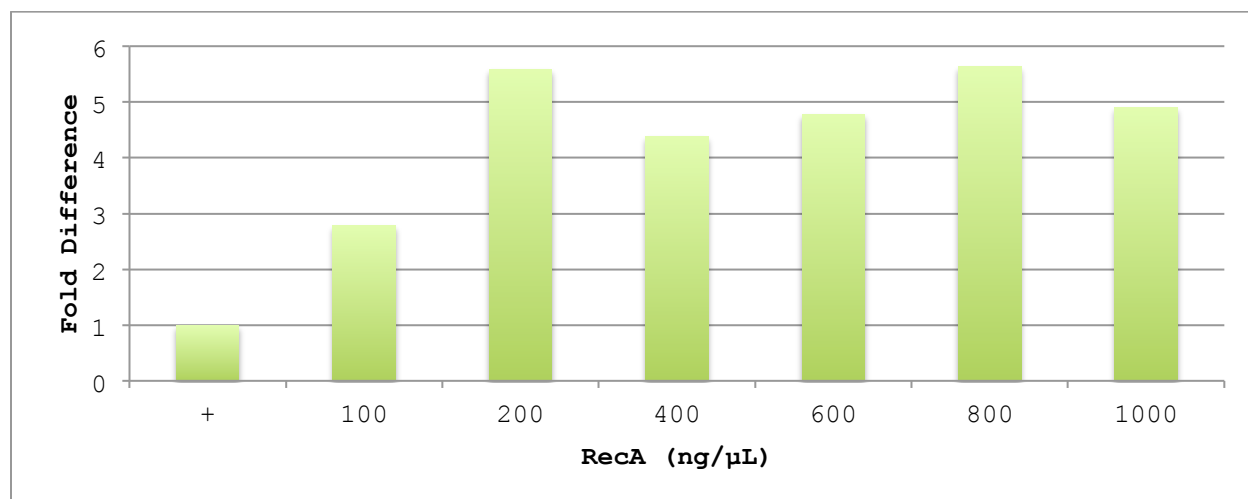
The repeated RecA titration showed a similar trend to that observed in the initial titration as additional RecA facilitates end joining in the two-way linear assembly. Although, it would appear that at a certain point the concentration of RecA becomes saturating – possibly even reducing assembly efficiency (as in Figure 4.1). The normal function of RecA to recombine ssDNA with a dsDNA substrate is not the same single-strand annealing method proposed earlier, but is clearly aiding in assembly. Again, looking at the lane compositions in Figure 4.4 it would appear that RecA helps to protect the substrate DNA from degradation as the total band intensity of the RecA lanes exceed that of the positive control lane.
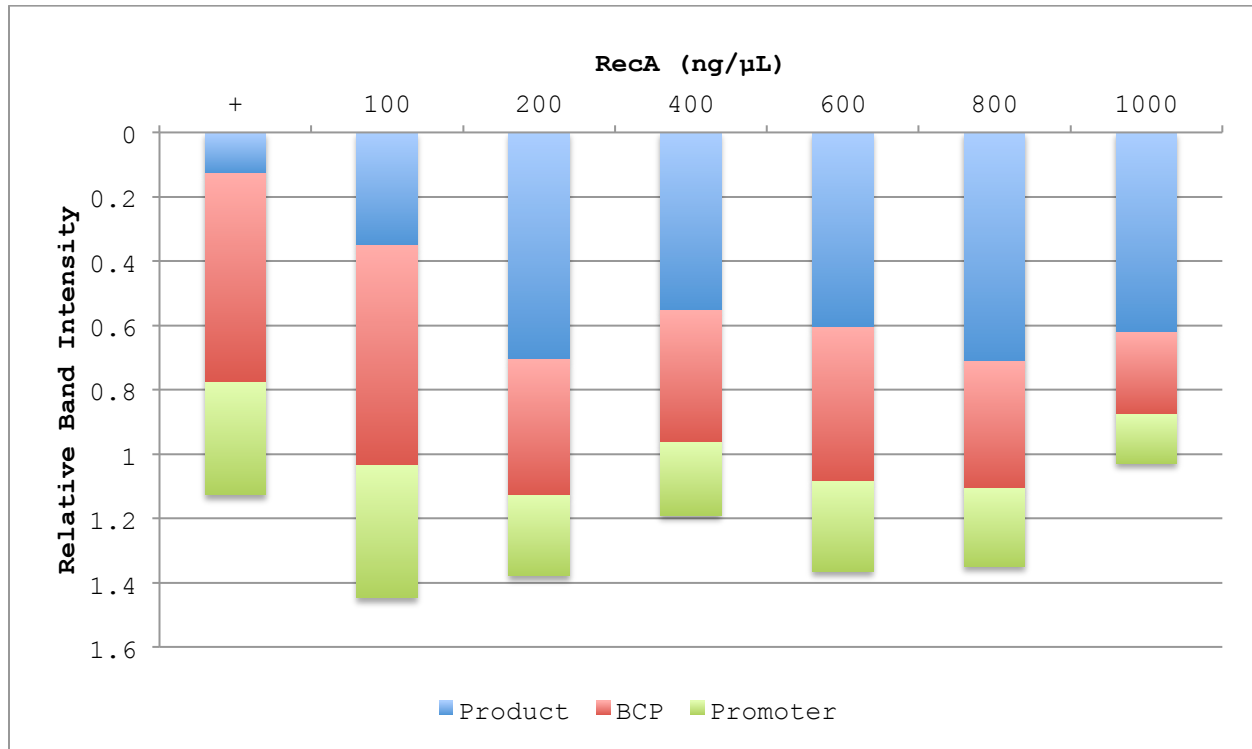
Figure 4.4 Lane composition of RecA titration
Chart shows the relative intensities of the product (Blue), BCP (Red) and Promoter (Green) substrate bands, as well as the low molecular weight band representing degradation products (Purple). Columns are displayed inverted to more closely represent the actual gel and the order in which the bands migrate. Band intensities are calculated relative to the BCP band of the negative control lane. Original gel pictures are shown in Figure B.12 and Figure B.13.

Finally, RecA was supplemented to 2-way circular assemblies, transformed and the resulting colony counts compiled into Figure 4.5. It would appear that there is another dynamic happening here – either during circularization or transformation – which decreases the efficiency of assembly for the circular assembly. Moreover, with "homology search" functions of RecA it was disappointing to find that RecA contributed nothing toward improving the mutation rate of assembly either.

www.manaraa.com
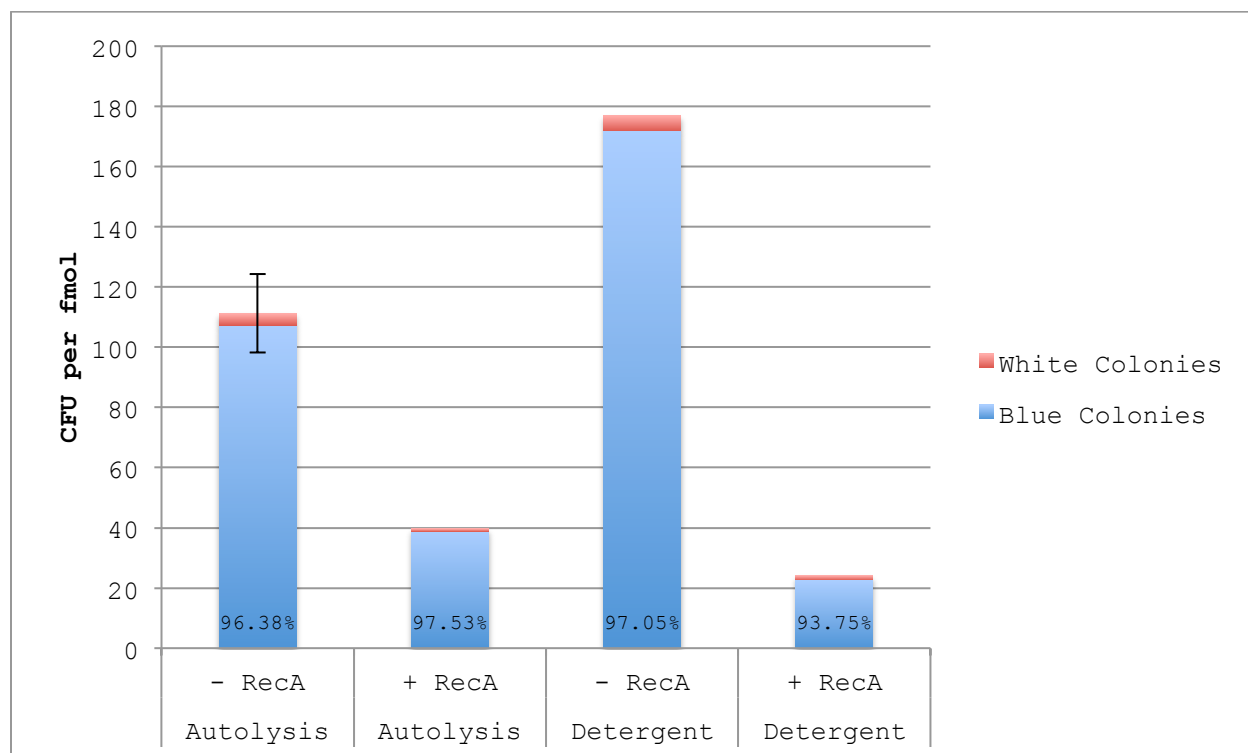
Figure 4.5 Colony counts of exDA reactions containing RecA
Assemblies were performed using basic buffer or supplemented 200 ng RecA using autolysed XJa or XJa lysed with detergents. Percentages represent Blue colonies to all colonies (blue and white). Error bars represent standard error of the mean for reactions performed in duplicate (N=2).

4.3. Strain Engineering (nuc4- and autolysis cassette)

Just as previously purified enzymes can be supplemented to the *ex vivo* reaction to synthetically test the effects of increased protein concentration, the genetic background of the strain can be altered to knockout the expression of native enzymes. Strain engineering or 'recombineering' (short for recombination-mediated genetic engineering) uses transformed DNA with homology to the host genome in order to target and integrate changes into the chromosome[67]. In *E. coli* this can be accomplished by the Lambda Red operon of bacteriophage

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lambda[68]. This three-protein operon encodes a 5'-to-3' exonuclease (alpha), a single-strand annealing protein (beta) and a protein, which binds and inhibits RecBCD activity (gamma). For both the native bacteriophage's linear dsDNA genome or transformed ds/ssDNA the Lambda Red system works by inhibiting RecBCD (preventing degradation of substrate DNA), converting the dsDNA to a fully single-stranded intermediate and annealing the subsequent ssDNA to homologous regions of the genome exposed as ssDNA during DNA replication[69,70]. Genomic changes can be selected if the targeting DNA integrates a selectable marker cassette (i.e., antibiotic resistance), otherwise colonies must be plated and screened via a high-throughput genotyping method like allele-specific PCR[71,72].

Given the promising applications of genome engineering considerable effort has been invested in optimizing Lambda Red and other methodologies (e.g., homologous recombination, RecET, CRISPR-Cas)[15,67,73-76]. The Church group pioneered a Lambda Red recombineering method to make many changes across the genome in an automated fashion known as 'Multiplex Automated Genome Engineering' (MAGE) using a closed-loop device to grow/recover cells, induce Lambda Red from a heat-inducible operon on the *E. coli* genome, and transform a solution of editing oligonucleotides[77-79]. In an effort to optimize MAGE this same group employed MAGE to knockout the most active *E. coli* ssDNA exonucleases (RecJ, ExoI, ExoVII and ExoX) creating a strain known as nuc4- (Genotype in Table A.2 and Table A.3), which they subsequently

deposited into the Addgene repository (Addgene bacterial strain #40803)[80].

We acquired the nuc4- strain from Addgene in order to test its suitability as a strain for *ex vivo* DNA assembly with the underlying assumption being that these exonucleases may compete to degrade exposed homologies. Since this strain contained the Lambda Red system we decided to integrate the lysis cassette from the XJa strain to create and autolysis version of the nuc4-, which we dubbed nuc4-.λR (for Genotype and description see Table A.2 and Table A.3; for oligo sequences see Table C.3).
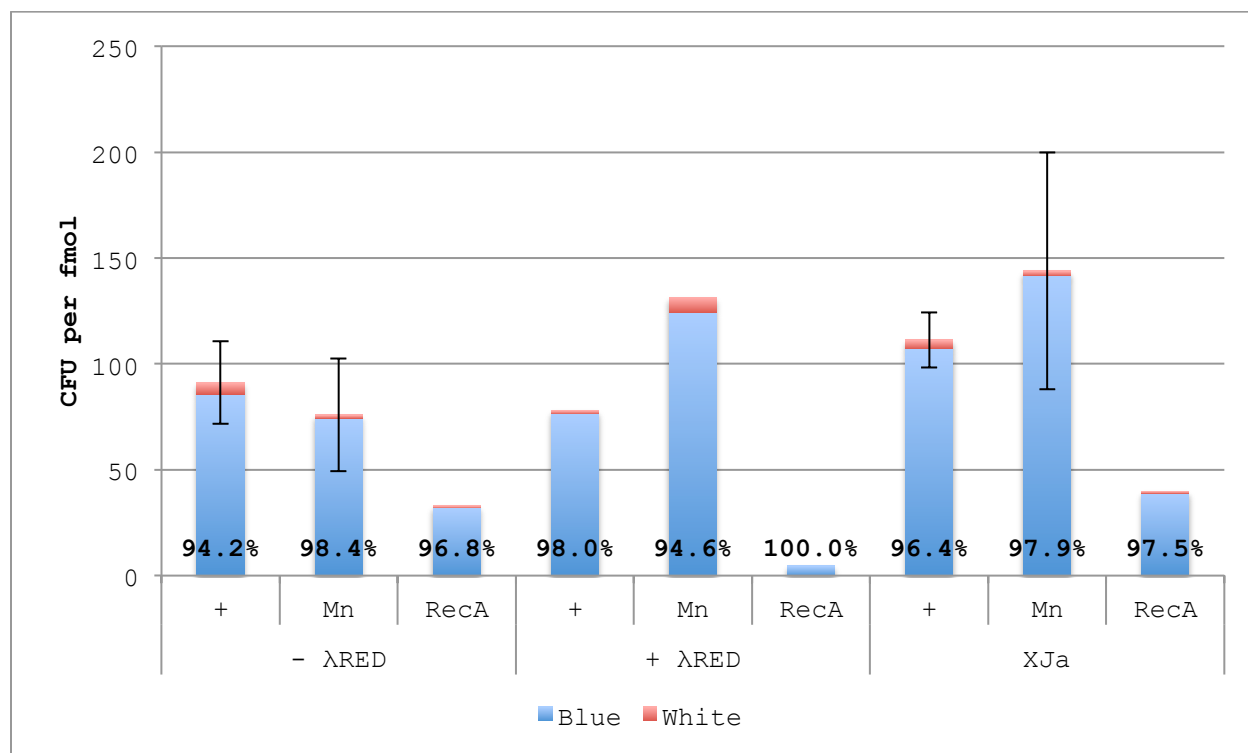
Figure 4.6 *ex vivo* DNA Assembly with nuc4-.λR and Lambda RED
Assemblies were performed using original buffer, supplemented with 1 mM Manganese or 200 ng RecA using autolysed nuc4-, nuc4- with λ Red or XJa. Percentages represent Blue colonies to all colonies (blue and white). Error bars represent standard error of the mean for reactions performed in duplicate (N=2).

Once isolated, the nuc4-.λR strain was used to create two lysates. One lysate was prepared by the same methods as XJa (-λRED), while the other was heat-shocked to induce production of the Lambda Red proteins (+λRED) as other studies have shown the Lambda RED system capable of facilitating DNA assembly[13,81]. The resulting lysates were used to perform 2-way assemblies with the standard buffer, with Manganese and with RecA (Figure 4.6). We were disappointed to find that our nuc4-.λR showed no improvement over the standard XJa reaction under any tested conditions with or without the inclusion of the Lambda RED system.

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Unfortunately, it is impossible to infer the direct relationship between the genotypic changes in nuc4-.λR and their impact on DNA assembly due to the absence of proper control strains. To clarify, we saw in the enzyme titration (Figure 4.1) that ssDNA exonucleases can have strong stimulating or inhibitory impact on DNA assembly. nuc4-.λR has four ssDNA exonucleases knockout, including one shown to stimulate DNA assembly (ExoI) and one shown to inhibit it (RecJ) in the enzyme titration experiments. Moreover, the parental strain (*E. coli* MG1655) that nuc4-.λR is derived from does not have the mutations in *endA* and *recA* like the cloning strains used throughout this study. While MAGE oligonucleotides were purchased to disrupt *endA* and *recA* via Lambda RED recombineering (sequences in Table C.3), we were never able to identify clones containing the mutant alleles. Without progressive knockouts of the ssDNA exonucleases with background mutations in the *recA* and *endA* loci to use as controls it is impossible to make direct conclusions about the effect of each knockout as it pertains to DNA assembly.

Chapter 5. *ex vivo* PCR and *ex vivo* Cloning

5.1. The pUN plasmid and *ex vivo* PCR

Using lysates derived from completely "wild-type" strains it is possible to perform sophisticated *in vitro* reactions such as protein expression or DNA assembly. As demonstrated in the previous section, the background genotype of the *E. coli* can be easily manipulated using modern genome editing tools to investigate genotype-phenotype relationships or create strains with phenotypic enhancements (i.e., autolysis). In a similar but perhaps simpler manner, expressional plasmids can be constructed and transformed into existing strains to produce one or more proteins (or even mRNA). Plasmid-based expression allows rapid introduction into the various strain backgrounds without the challenges required to integrate cassettes into the genome. We refer to lysates containing an (over)expressed protein(s) as a 'functionalized lysate', as a novel function has been imparted to the lysate. The following chapter demonstrates a functionalized lysate containing the *Pyrococcus furiosus* (*Pfu*) DNA polymerase that has the newly imparted ability to perform PCR. This functionalized lysate is then applied to amplify the fragments for 2- and 3-way assemblies, which are subsequently assembled and transformed using our *ex vivo* DNA assembly approach.

5.1.1. Design and construction of the pUN plasmid

Many plasmids have been developed and are available through numerous for- and not-for-profit distributors. These plasmids usually have features associated with specialized purposes such as clonal amplification, protein expression, interspecies transfer or phage production. Traditionally features are included which facilitate cloning/DNA assembly such as multiple cloning sites, integrase sites (Gateway cloning) or counter-selectable markers[82].

Up to this point, *ex vivo* assembly and its assays employed the commonly used high-copy number Biobrick plasmids, pSB1C3 and pSB1K3 to conduct assembly assays. These plasmids are freely distributed under a creative commons license and are fully compatible with the 'Biobrick Assembly™' cloning methodology. However, we decided to design and develop our own vector useful for expression and cloning via *ex vivo* DNA assembly and other sequence-independent cloning methods.

The plasmid backbone was derived from the pJ251-GERC vector available in the Addgene repository (Addgene plasmid #47441)[83]. The backbone fragment (amplified by pGERC_BB-f/-r; see Table C.2) contains the Kanamycin resistance selectable marker and the low copy number p15a origin of replication (~5-10 copies per cell). The second-half, 'insert' portion was synthesized as two linear fragments by Integrated DNA Technologies (IDT) and contains all the designed cloning and expressional features. The 'insert' design draws heavily upon the "Unique Neutral Sites" (UNS) developed by Torella, *et al*[84]. A UNS is a computationally derived 40 base pair site that meets several design

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criteria including: approximately 50% GC-content without homopolymeric runs; Unfavorable intramolecular (hairpins) and intermolecular (bonding between different UNSs) interactions; absence of common restriction/cloning sites; absence of start codons, promoter-like sequences or sequence homology to the *E. coli* MG1655 genome. The authors reported 10 UNSs that were subsequently demonstrated to assemble multiple repeated sequences via Gibson assembly and even provided functional insulation of promoter sequences from genetic context. In our design, some of these UNSs are incorporated throughout the insert separating each of the following features (in order from 5' to 3'): an upstream terminator; a T5 promoter and 5' untranslated region with a ribozyme; a newly optimized BCP cassette driven by a strong constitutive promoter and synthetic ribosome-binding site (RBS) and; two strong downstream terminators (sequence available in C.2.6).

The design of the insert was such that the new BCP cassette can be replaced with a desired RBS and protein coding sequence (CDS) and be driven by the strong upstream T5 promoter. Since the original BCP insert causes a very strong (and quicker developing) blue pigmentation, assemblies can be screened for the presence of background colonies. Unfortunately, we have found that the upstream promoter does not transcribe through the downstream UNS site so currently a promoter needs to be included with expression assemblies.

However, it is from the UNS sites that our plasmid derives its namesake, pUN (for "plasmid unique neutral"). The embedded UNS sites play a critical role not just for simplifying assembly and insulating

parts as demonstrated by the original authors, but as ideal priming sites for PCR. For all the reasons the UNS sites make excellent termini for assembly reactions, these sites are also ideal primer sequences for amplification or as 5' embedded overhangs. Indeed, we designed and synthesized forward and reverse primers for each UNS site (sequences available in Table C.2) and have performed PCR from all incorporated UNS sites without any preliminary optimization or troubleshooting.

5.1.2. Construction of pUN-PrhaBAD-Pfu

In Addgene there are two DNA polymerase-expressing vectors available: pAKTaq (Addgene plasmid #25712) that expresses the *Thermus aquaticus* (*Taq*) DNA polymerase and pET16b.Pfu (Addgene plasmid #12509) that expresses the *Pfu* DNA polymerase. While we were able to successful express the *Taq* protein and use it to perform *ex vivo* PCR, *Taq* is a less than ideal cloning polymerase. *Taq* does not create flush termini (leaves one 3' adenine residue overhang) and also lacks the proofreading activities typical of high-fidelity polymerases. On the other hand, *Pfu* has proofreading abilities and creates blunt-ended products ideal for cloning purposes, but the pET16b.Pfu construct is a nightmare for *E. coli* expression. The difficulty stems from the poor codon compatibility of the *Pfu* coding sequence with *E. coli* as the host. To circumvent the poor codon adaption a special strain of *E. coli* (BL21(DE3) Rosetta) is needed which hosts a vector overexpressing some of the less prevalent tRNAs along with the DE3 prophage (for T7 polymerase expression).

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In the spirit of open-access *ex vivo* DNA assembly we decided it was prudent to have the coding sequence codon optimized and synthesized so that the *Pfu* gene can be expressed in any *E. coli*. To perform codon-optimization the COOL codon optimization tool (http://bioinfo.bti.a-star.edu.sg/COOL/) was employed to simultaneously maximize codon context and codon adaptability for *E. coli*[85]. With the Ribosome Binding Site Calculator tool a synthetic ribosome-binding site was designed based on the codon-optimized *Pfu* gene and an upstream wild-type rhamnose-inducible promoter (PrhaBAD)[9,86]. The resulting cassette plus the flanking UNS sequences was synthesized by IDT as two overlapping fragments of 1144 and 1483 base pairs and subsequently assembled along with the backbone using *ex vivo* DNA assembly. Of the approximately 200 white transformants, 24 colonies were lifted (along with two blue colony controls) and used as template in colony PCR. The PCR amplified from UNSX across the entirety of the *Pfu* cassette to UNS9, and of the 24 clones all 24 showed amplicons at the expected size (~2800 bp; Blue colonies were ~1500) although the bands were faint. Of these 24, 3 clones were miniprepped the next day and sent for sequencing (Eurofins MWG). Of these three, one clone was identified as completely correct and used for subsequent procedures (pUN-PrhaBAD-Pfu sequence available C.2.7).

5.1.3. Expression and Preparation of *ex vivo* PCR lysate

As the original plasmid was transformed into NEB10β (and XJa/autolysis strains are a rarity in labs), expression and lysis was performed in NEB10β for all subsequent steps. Expression was performed by

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inoculating 24 mL of pre-warmed SOB media (in a 125 mL Erlenmeyer) with 200 µL of an overnight culture, allowing growth for approximately 2 hours, then adding filter sterilized 20% w/v rhamnose to a final concentration of 0.2% (~250 µL).

To assay the culture for the production of the *Pfu* DNA polymerase, aliquots were removed at 6, 9 and 24 hours post-induction and frozen until needed. The remainder of the culture was collected at 24 hours post-induction. These aliquots were lysed using CelLytic B, diluted and protein content quantified by Bradford assay. Each protein sample was prepared with 25 µg of total protein (along with a NEB10β negative control) and visualized using SDS-PAGE under denaturing conditions. In the resultant gel (Figure 5.1) there is clearly a strong band in all the sample lanes at roughly 90 kD that is absent in the negative control lanes. This size correlates with the predicted molecular weight of the *Pfu* DNA polymerase (90.11 kD). Interestingly, there appears to be little variation in the concentration of *Pfu* across the different harvesting times.
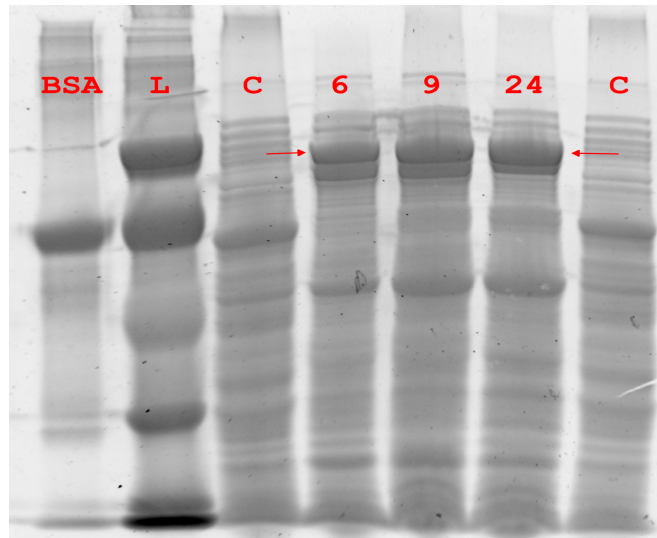
Figure 5.1 Expression of *Pfu* DNA Polymerase

Soluble lysate of NEB10β expressing *Pfu* was harvested at different time points and separated via denaturing SDS-PAGE. "C" is NEB10β without pUN plasmid, "L" is low-molecular weight ladder.

Given the encouraging results of the protein gel in Figure 5.1, the remaining pellets of the culture harvested at 24 hours were thawed and used to prepare lysates for *ex vivo* PCR. The lysis reaction was performed using CelLytic B in a 1.5:1 ratio and incubated at 30°C for 15 minutes and subsequently diluted with lysis buffer to a final volume-to-pellet mass ratio of 5:1. While most of the lysate was centrifuged to remove the cellular debris an aliquot was first removed which retained the total cellular extract. The soluble fraction and the total cellular extract were next incubated at 80°C for 30 minutes to kill any host proteins (such as nucleases). The lysates are given one last extended spin in the centrifuge to pellet any denatured insoluble host proteins and the supernatant aspirated and diluted by

half with 100% glycerol. As with the *ex vivo* DNA assembly lysates, these lysates are stored at -20°C and can be safely removed and replaced into the freezer many times.
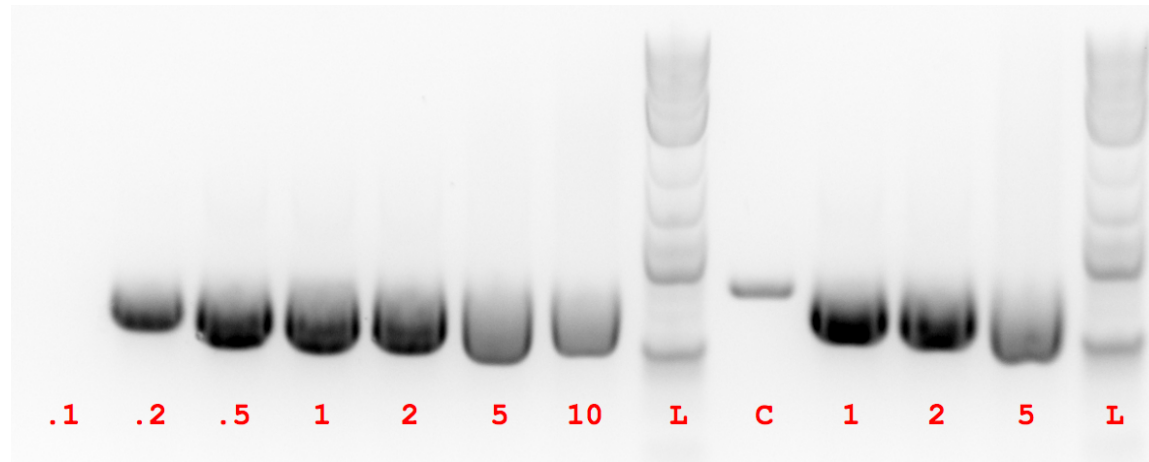
Figure 5.2 Volumetric titrations of *ex vivo* PCR

Functionalized lysates of *Pfu* were titrated to amplify the BCP band used in 2-way linear assemblies. Bands to the right were prepared with total lysate in the heat-kill process. The values below the bands indicate µL of lysate used per 50 µL reaction; "C" represents negative control of purified BCP fragment.

5.1.4. *ex vivo* PCR

Equipped with lysates containing the *Pfu* DNA polymerase, initial PCR reactions amplifying the 2-way circular BCP fragment were formulated using volumetric titrations of the extracts in a standard 50 µL *Pfu* PCR reaction. These titrations shown in Figure 5.2 exhibit a very nice trend with a peak at 1-2 µL of cell extract per 50 µL PCR. Comparing the bands on the left (heat-killed soluble fraction only) to the bands on the right (heat-killed total extract) it would appear that including the total cellular extract in the heat-kill step of lysate preparation increases the yield of *Pfu* in the final extract. This may be due to additional lysis of cells surviving the detergent treatment or may occur by freeing *Pfu* trapped in the insoluble fraction of the

extract prior to centrifugation. Second, it is clear that using excessive extract (or too little) causes an observable decrease in PCR efficiency. It is therefore advisable to perform a small titration experiment each time new lysates are prepared. While a slightly inconvenient consumption of materials and time, the long-term savings from the *ex vivo* approach are more than worth the investment.
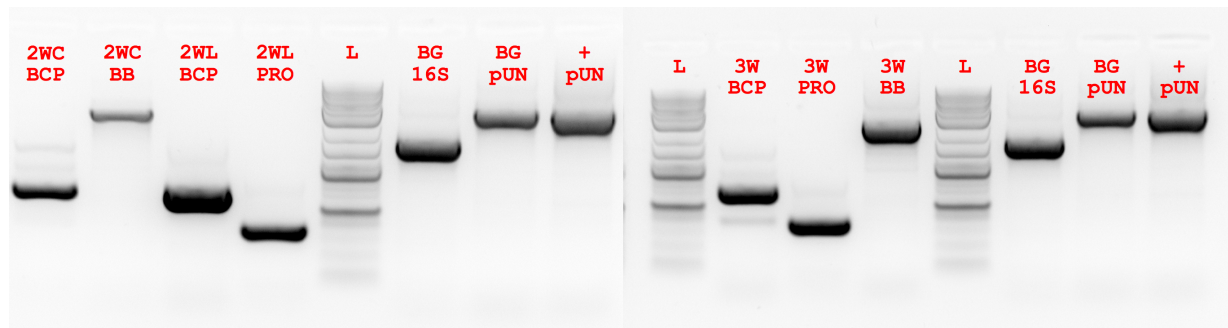
Figure 5.3 *ex vivo* PCR of 2-way and 3-way Fragments
Fragments for 2-way circular (2WC), 2-way linear (2WL) and 3-way circular (3W) were amplified using *ex vivo* PCR. In addition control PCRs were performed to detect the contribution of background genomic (BG 16S) and pUN-PrhaBAD-Pfu plasmid (pUN) DNA. The positive background (+ pUN) control had purified plasmid template added. There is a clear presence of background amplification and side amplification (see BCP lanes).

After constructing our *Pfu*-functionalized lysate and prototyping the *ex vivo* PCR reaction with volumetric titrations, *ex vivo* PCR was applied to generate the substrate DNA for 2-way linear, 2-way circular and 3-way circular assemblies. The analytical gels shown in Figure 5.3 demonstrate that *Pfu*-functionalized lysate was capable of synthesizing all the substrate DNA used in our assembly assays we decent efficiency. However, there is a fair amount of side-product formed in some of the reactions. The presence of high levels of contaminating host DNA may be the critical factor in the side-product formed. This is clearly evidenced by the strong amplicons formed in the background

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control lanes ("BG"). These lanes consisted of *ex vivo* PCR reactions using primers targeting the highly conserved 16S genomic loci ("16S") or the pUN-PrhaBAD-Pfu ("pUN") plasmid but without the addition of template. In the future the steps to remove host DNA contaminations would bolster *ex vivo* PCR fidelity. Fortunately, since the *Pfu* enzyme is thermostable, non-thermostable nucleases – such as DNase I or RecBCD – could be used during lysate preparation to destroy host DNA and then semi-selectively denature by a heat-kill before desired DNA substrates or products are introduced to the lysate.

5.2. *ex vivo* Cloning

Up to this point *ex vivo* DNA assembly has been demonstrated, investigated and optimized; tools such as clever assays for DNA assembly, self-lysing strains and a novel cloning plasmid were designed and developed; and the *Pfu* DNA polymerase was optimized, cloned and expressed to functionalize lysates with the ability to perform a high-fidelity polymerase chain reaction. Yet, the ultimate objective of the project was to merge these *ex vivo* tools and techniques into an encompassing *ex vivo*-based cloning suite. As with all cloning, this began with the generation and isolation of substrate DNA as the remainder of the 2-way and 3-way *ex vivo* PCRs from Figure 5.3 were purified by gel extraction. Subsequent *ex vivo* DNA assembly reactions were performed using our NEB10β detergent-based, XJa detergent-based and XJa autolysis-based lysates, with the basic *ex vivo* DNA assembly reaction buffer or with 1 mM Manganese supplemented. In-house formulated Gibson mastermix was used as a positive control

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and transformation of substrate DNA ('TAR') was used as a negative
control for background assembly.
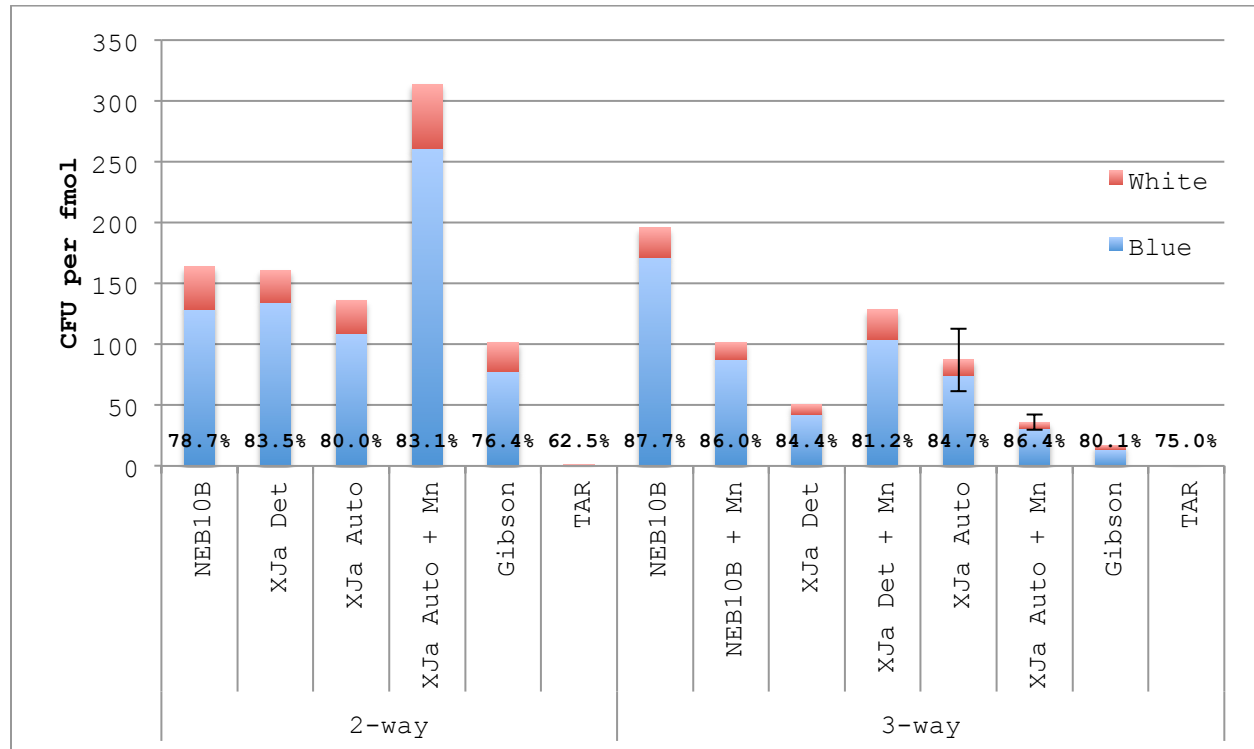
Figure 5.4 *ex vivo* Cloning
Substrate DNA amplified using *ex vivo* PCR was gel purified and used in *ex vivo* DNA
reactions. 2 µL of the 10 µL reactions were transformed using in-house prepared
chemicompetent NEB10β. Gibson lane represents a positive control using Gibson
isothermal assembly formulated in-house. TAR is a negative control consisting off DNA
fragments transformed directly into NEB10β without assembly reaction. Percentages
represent Blue colonies to all colonies (blue and white). Error bars represent
standard error of the mean for reactions performed in triplicate (N=3).

The most striking aspect of the *ex vivo* Cloning pipeline is the
elevated mutagenic rate in comparison with assemblies using Q5 DNA
polymerase (Figure 3.4). While the total (Blue and White) colony
forming units per femtomole are very comparable, the percentage of
white colonies-to-blue colonies changes by over 20% in some
circumstances. Even the Gibson assembly control shows a greatly
increased error rate over typical efficiency. As the only difference

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between these two tests are the manner by which the substrate DNA was prepared, it is convincing that the mutations are introduced during the PCR amplification of the substrates. However, this may not be indicative of significantly lowered fidelity of *Pfu* in comparison with Q5 polymerase, as misamplification products were observed in the *ex vivo* PCR reaction (Figure 5.3). Since the primers encode all the homology necessary for faithful assembly efficiencies of joining correct amplicons versus misamplified products would be approximately equivalent. If indeed this is the case, a simple post-lysis digestion using a non-thermostable nuclease (e.g.,DNase I) followed by the typical 80°C heat-treatment should alleviate the observed mutational rate of *ex vivo* Cloning.

Chapter 6. Conclusion and Future Directions

Responding to the needs of the synthetic biology community for inexpensive, rapid and efficient DNA manipulation we were able to uncover a unique approach to DNA assembly. Working on the observation that all of the enzymes used in prototypical *in vitro* manipulations of DNA are highly conserved, we incorporated an *ex vivo* approach to screen several candidate organisms for DNA assembly functions. To accomplish this we designed a very clever assay of DNA assembly.

Our circular assemblies provided a colorimetric output that allowed us to interpret the origins of each resulting transformant. In our intial pilot studies, we used two chloramphenicol plasmids in our two-way assembly and observed the growth of blue (correct), white (background) and red (colonies) – with many background colonies observable (Figure 1.1.B). While we realized that this would allow us to track transformation efficiencies by observing fluctuations in background, it was not until subsequent studies did we realize that if the donor plasmids were maintained on different antibiotics than the destination vector (like in the original 3-way assembly; see Figure 2.3), we could track mutational rates. Because our assemblies join the BCP coding sequence to the destination vector at a crucial junction for proper expression of the BCP protein (across Ribosome Binding Site and Start Codon), there is a low tolerance for error during end joining. Due to the low throughput of performing transformation to assess DNA assembly

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(~28 hours) we also designed a linear assembly assessed by gel electrophoresis (~3 hours) that greatly expanded our investigative capacities.

We leveraged this linear assembly to optimize and investigate *ex vivo* DNA further. During our titration of the assembly reaction with divalent metals we found contradictory results between our autolysis strain and detergent lysed strain. This could be a function of the composition of the detergent, as it could very well contain the strong chelators EDTA or EGTA in order to improve lysis efficieny. The presence of these chelators would alter the apparent divalent cation ratios between detergent and autolysis treated extracts. However, there was good agreement between both strains that manganese had a stimulatory effect on end joining, which we confirmed in follow up titrations. Similarly, we investigated the biochemical origins of the *ex vivo* DNA assembly by titrating putative enzymes.

In *E. coli*, DSB repair, dominated by homologous recombination, relies on the RecBCD complex to expose ssDNA ends and load RecA, forming the nucleofilament that subsequently directs a homology search and strand invasion of dsDNA[64] – but this is different phenomena than directly joining two ends of DNA together directly. Previously, it was shown that *recA-* strains of *E. coli* join linear dsDNA with homologous overhangs in lysate more efficiently than their RecA-expressing counterparts[13]. However, we observed an interesting dichotomy surrounding RecA. During our titrations using the linear assay, RecA showed consistent enhanced assembly properties, but when applied to

circular assemblies and transformations it showed a consistently negative effect. It could be that RecA plays a role preserving the ends of the linear DNA products, while the nucleases in *recA-* lysates are free to degrade the product (and substrate) DNAs[63]. Either way, as *recBCD-* strains have also been reported to direct accurate DNA end joining, *ex vivo* assembly reactions may involve mechanisms independent of homologous recombination machinery, perhaps by an alternative end-joining pathway[29,87]. We suspect that both *ex vivo* and *in vivo* DNA assembly is likely facilitated by multiple competing DNA repair mechanisms, but largely by RecBCD.

Exonuclease activity is required for DNA end resection, but is a balancing act. We saw with yeast very little exonuclease activity, which preserves the regions of homology, but may not expose complementary overhangs. Conversely, extreme exonuclease activities, like that of *Deinococcus*, may be resecting bi-directionally, deleting the necessary homologous regions. Again, knockouts of exonucleases implicated in deletion of exposed overhangs may greatly improve the efficiency of end joining[88]. In our own titration experiments we saw this effect with the addition of dsDNA exonucleases (RecBCD and ExoIII). Certain ssDNA exonucleases also showed detrimental effects on assembly (DNA PolI and RecJ), but there was significant substrate DNA remaining in the lanes supporting the hypothesis that they possess the ability to recess homology arms. In the cell ssDNA activity is actually beneficial as a sort of check-and-balance to ensure the cell can abort non-productive recombination events[88]. Previous studies have

shown ExoI and RecJ knockouts have elevated mutational rates due to the loss of this abortive activity[88]. These hypotheses would be very convincing if the complete opposite effect weren't observed in the exact same study. During the titration ExoI and – at its lowest levels – ExoT showed the ability to greatly enhance assembly, almost 8-fold in some circumstances. These both have opposite polarities to the inhibitory RecJ ssDNA exonuclease so there is clearly a preference for degrading the 3' extensions, rather than the 5' extensions. While we lacked the full library of strains needed to draw exquisite conclusions, the equivalency between nuc4-.λR and XJa highlights the complexity and redundancy of exonuclease activity and DNA repair pathways[89].

The final task at hand was to complete our *ex vivo*-based cloning pipeline. To do so we designed a new plasmid that incorporated features designed by the community (UNSs, riboJ), *de novo* designed parts (RBS, Codon-optimized gene) and the screenable BCP cassette from our own works. Using *ex vivo* assembly we recombined the plasmid backbone together with two larger synthetic fragments in a very effective manner. Based on 24 tested clones, all 24 contained the correct size inserts. Out of 3 sent for subsequent sequencing only one had no errors in the *Pfu* gene. This represents a clear win for *ex vivo* DNA assembly as a very practical cloning tool. The *Pfu* polymerase was at one time the highest-fidelity polymerase, though it has now been replaced by more engineered, more expensive polymerases like Q5. The assemblies showed a greatly elevated error rate based on an increased

white:blue colony ratio, but this might be corrected simply by removing the contaminating host DNA or performing PCR optimization (e.g., annealing temperature, buffer composition, etc…).

With the successful transformation of assembly reactions derived from both an optimized *ex vivo* DNA assembly and an *ex vivo* PCR, the project has successfully completed it's ultimate objective of establishing an *ex vivo* cloning suite. Yet, given the outcomes of the experimentation it is easy to instead focus on perceived shortcomings of the project. There were many questions that remained unanswered, conflicting results and components not carried to 'full completion' (i.e., generation of all nuclease-knockouts, optimization of *ex vivo* PCR). Even more egregious for some is the lack of data backed by extensive replicates. While understandable, this is simply a function of the biological systems being studied, the objectives of the study and even the *ex vivo* approach to the study.

Biological systems are inherently noisy and complex, meaning establishing definitive causalities is a difficult task often not accomplished in even the very deepest of investigations. Further, the particular subcellular system being probed – DNA maintenance and repair – is conserved amongst every living organism[25]. Such conservation is indicative of the "do-or-die" nature of this pathway and is reflected in the degree of redundancy and orchestration. By using an approach like *ex vivo* that combines the throughput of an *in vitro* system with endogenous complexity of an *in vivo* system, investigations into these mechanisms can generate results that quickly

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deviate the project from the original objective into a very large study all of it's own.

Continued development of faster and cheaper DNA assembly and genetic characterization methods, such as the *ex vivo* DNA cloning pipeline described here, will further accessibility and success of DNA-based studies and applications. As mentioned in detail, there were a number of surprising findings throughout the study that could be pursued. The first is the interesting case of ExoI. ExoI could be purchased and titrated into lysates with many different host background genotypes to investigate its contribution towards recombination/end joining. Alternatively, modifications could be employed to try and modulate native degradation. For example, using primers with non-standard phosphodiester analogues – such as the phosphorothioate bond – could be employed to halt degradation. This could be very helpful in the linear assembly when the product is formed and subsequently degraded from exposed termini before visualization. Building off the success of the *Pfu* functionalized lysate, a multienzymatic functionalized lysate featuring a ligase, polymerase and exonuclease could be constructed as an *ex vivo* mimic of the tripartite Gibson assembly. Moreover, functionalized lysates hold the potential to address some problems unsuitable to living cultures. Biocontainment has become a major point of contention for the employ of genetically modified organisms, particularly in an environmental context[90,91]. By substituting a functionalized lysate, the same systems can be applied as bioremediation treatment without the risk of spreading throughout the

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environment, and without the tremendous costs associated with bulk enzyme purification. While *ex vivo* engineering and *ex vivo* molecular biology may not be the "silver bullet" to solve every societal problem, there are many academic and industrial opportunities addressable by *ex vivo* and synthetic biology.

Chapter 7. Materials and Methods

7.1. Reagents, DNA and Enzymes

All chemical reagents were purchased from Sigma-Alrich. Divalent metals used in titration experiments were all complexed with chloride (i.e., MgCl2, MnCl2, CaCl2, ZnCl2) and formulated as 1 Molar Stock solutions. All enzymes used in the titration experiment were purchased from NEB, along with the Q5 polymerase used for PCR. All DNA synthesis of primers and synthetic fragments was performed by IDT. The plasmids used for 2-way and 3-way assembly experiments were obtained from the Registry of Standard Biological Parts. Sequences for all DNA used are provided in (DNA Sequences).

7.2. Generation of DNA Fragments for *ex vivo* DNA Assembly

The templates, primers and products used for the assembly assays are summarized in Appendix C.

7.2.1. PCR with Q5 DNA Polymerase

The DNA fragments used to demonstrate *ex vivo* DNA assembly were generated using standard PCR of parts of the following plasmids from the BioBricks registry: pSB1C3-J04450, pSB1C3-K592009, and pSB1K3-J04450. Primers used in this study (Table C.1) were generated using the j5 automated DNA assembly software[92-94]. Amplicons were generated by 100 μL PCR reactions with Q5 polymerase (NEB) under standard reaction

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conditions. These reactions were cycled at 98°C for 30 s; 98°C for 10 s, 50°C for 15 s, 72°C for 25 s (repeated for 25 cycles); 72°C for 2:00. Resultant PCRs were subsequently purified using agarose gel electrophoresis and extraction (Section 7.5).

7.2.2. *ex vivo* PCR with *Pfu* DNA polymerase

Pfu PCRs were formulated as follows (final concentration): 200 μM each dNTP, 1X Q5 reaction buffer (2 mM MgCl2), 500 μM each primer and ~2 ng template DNA per 50 μL reaction. These reactions were cycled differently for small fragments (<1 kb) and large fragments (>1 kb). For smaller pieces (i.e., BCP and Promoters): 95°C for 90 s; 95°C for 30 s, 55°C for 30 s, 73°C for 90 s (repeated for 28 cycles); 74°C for 3:00. For larger fragments (i.e., backbones, background controls): 95°C for 90 s; 95°C for 45 s, 55°C for 30 s, 73°C for 4 min (repeated for 30 cycles); 74°C for 6:00. Resultant PCRs were subsequently analyzed or purified purified using agarose gel (See, 7.5).

7.3. Preparation of Lysates

7.3.1. Initial Study

Cellular lysates were prepared from the following strains: *E. coli* NEB10β (NEB™ #C3019), *S. cerevisiae* BY4741 and *D. radiodurans* R1 (ATCC® 13939). *E. coli* was grown in Terrific Broth with glycerol (Sigma®) at 37°C with shaking at 250 rpm. *D. radiodurans* was grown in 123 TGY medium (5% Tryptone, 5% Yeast extract, 1% Glucose, 1% Potassium monophosphate) at 30°C and *S. cerevisiae* was grown with YPD media (Sigma™) at 30°C, both shaking at 250 rpm. The preparation of

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the bacterial (*E. coli* and *D. radiodurans*) and the *S. cerevisiae* lysates varied slightly: bacterial cultures were pelleted once OD600nm= 6.00-6.50, while the yeast cultures were pelleted once OD600nm= 4.00-4.50. Volumes of 4-6 mL of culture were centrifuged at 13,200 rpm at 4°C for 2 min, washed with 1 mL of Milli-Q H2O, centrifuged again and the wet pellet massed. 2X CelLytic B Lysis Reagent (Sigma™) was added to the bacterial cell pellets at 3 μL/mg of cells. 1X CelLytic Y Lysis Reagent (Sigma™) was added to the yeast pellets in the same ratio. After the addition of the lysis reagents, the cells were incubated at 37°C for 10 min shaking at 300 rpm. The lysed cells were centrifuged at 13,200 rpm for 15 min and a 20 μL sample of the supernatant (lysate) was mixed with 20 μL of 100% glycerol to yield 40 μL aliquots. All lysate aliquots were stored at −20°C.

7.3.2. Optimized Methods

Cultures of *E. coli* were grown under similar conditions to those reported in 7.3.1, with the exception that the cultures were inoculated from starter cultures. For cultures using the autolysis cassette, the media was supplemented with 3 mM L-arabinose and 10 mM magnesium chloride from a 500X stock. Cultures were grown into saturation (OD600nm= 2.0-4.0 after about 18 hours growth) and then pelleted at 4200RPM at 4°C for 20 minutes. From this point on cells were kept on ice. Media was aspirated, making sure to remove as much as possible using a pipette tip and the pellets were massed (~75 milligrams per 5 mL) in pre-massed tubes. We found that tubes can vary

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significantly and accuracy is best when each tube is massed initially. Cells were washed once with ice-cold 10% glycerol solution in order to prevent premature lysis in a hypotonic solution by the autolysis strains. For detergent lysis, cell pellets are resuspended in 1.5 volumes of CelLytic B 2X per milligram of pellet and incubated at 30°C with shaking for 15 minutes. The resulting lysate is then diluted with an additional 3.5 volumes of Lysis buffer (50 mM Tris-HCl, pH8; 0.2 mM EDTA; 2 mM DTT; 200 mM NaCl; 0.2% Triton X-100; 0.1 mM PMSF). Autolysis pellets are resuspended in 5 volumes of Lysis buffer and frozen at -80°C or in liquid nitrogen. Thawing the tube in a room temperature water bath lyses the autolysis strain. All lysates are then spun at the highest speed (20,000xg) for 20 minutes at 4°C. The resulting supernatant are very carefully aspirated into an equal volume of glycerol, mixed thoroughly (with pipette or vortex) and stored at -20°C.

7.3.3. *ex vivo* PCR Lysate

From an overnight culture, a SOB media (it is critical to use a medium without glucose) culture is inoculated (at a 10^{-2} dilution) and cultured at 37°C rotating at 250 RPM for about 2 hours. At this point the L-Rhamnose inducer is added to 0.2% (from a filter sterilized 20% solution). After another 3-24 hours the culture can be harvested at 8000xg and washed with 10% glycerol. The culture is lysed in 1.5 volumes of CelLytic B 2X and diluted to 5 volumes using lysis buffer (see above). The entire lysate is then incubated at 75°C for 30 minutes, centrifuged at the highest speed (16,000xg) for 20 minutes at

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4°C and the supernatant aspirated into an equal volume of glycerol, mixed thoroughly and stored at -20°C.

7.4. DNA Assembly Reactions

7.4.1. Initial Study

Buffers for assembly reactions were prepared from 100× stock solutions. Stock solutions were as follows: 100 mM NADβ, 100 mM ATP, 100 mM DTT, 1 M MgCl$_2$[32]. Tris-HCl was added to the buffer to 500 mM from a 1 M stock solution. A typical assembly reaction would include 6 µL of cellular lysate, 2 µL of 10× buffer, 2 µL of nuclease-free water and 10 µL of a DNA master mix. DNA master mixes contained all fragments needed for assembly and were formulated with 20 ng/µL of the backbone and 6:1 molar ratio of insert fragments to the backbone.

7.4.2. Optimized and Supplemented Conditions

Buffers were also formulated from 100X stocks with the expection of the divalent cation titrations. For these experiments a "base" buffer with all components except for magnesium and water was formulated and then aliquoted, then these aliquots were fully constituted with the various cations and water to a 10X concentration. A typical assembly optimized assembly was formulated as follows: 1 µL of 10X Buffer, 5 µL of a 2X DNA Mastermix, 1 µL of 10X Lysate (at ~200 µg/mL) and 3 µL of nuclease-free water. The DNA master mixes were formulated with 5 ng/µL of the backbone fragment (BCP band for 2-way linear) and molar ratios of 1.2:1 and 2:1.2:1 for 2-way (BCP:BB) and 3-way (Promoter:BCP:BB),

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respectively. These reactions were transferred immediately to the thermocycler and incubated at 37°C for 2 hours. Upon completion reactions were either used immediately for transformations or gel electrophoresis, or frozen at -20°C until used.

7.5. Agarose Gel Analyses and Extraction

7.5.1. Initial Study

Gel analysis was performed using 1.0-1.2% agarose-TAE gels containing GelRed (Biotium™) as the staining agent. All analytical gels were run at 100 V for 30-50 min and visualized with a Molecular Imager® Gel Doc™ XR+ Imaging System with Image Lab™ v4.0 software (Bio-Rad).

To purify PCR reactions, samples were run on 1.0% agarose-TAE gels stained with GelGreen (Biotium™). The gels were run at 100 V for 30 min and subsequently visualized under blue light excitation. The gels showed no side product formation and bands were excised and isolated using ZymoClean™ Gel DNA Recovery Kit (Zymo Research™).

7.5.2. Optimized Analytical Gels

In the optimized format, analytical gels for 2-way linear reactions were formulated at 1.8% agarose in TAE buffer. 3.5 µL of GelRed (Biotium™; 50,000X in Water) was added to the molten gel and once poured the gels were allowed to solidify for at least one hour. To each 10 µL assembly reaction, 3 µL of 5X Loading Dye was added and 5 µL loaded. For Ladders (NEB 100-bp, 1kb or 2-log Quick-load) 3.8 µL

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were added to each lane. The gel was run at 180V for 1 minute, the run at 90V for 35 minutes.

7.5.3. Gel Analysis

To analyze the gel they were visualized with a Molecular Imager® Gel Doc™ XR+ Imaging System (Bio-Rad). Using Image Lab™ v4.0 software (Bio-Rad) the lanes and bands were identified. The lane profile was used to manually adjust bands with exceptional tailing or false peaks. Using the Relative Quantity analysis tool, the BCP band of the negative control is selected as the internal reference. Band information was exported into a Comma Separated Values file for subsequent analysis. Pictures of all gels not included in the main text are provided in (Appendix B Gel Pictures).

7.6. Transformations and Preparation of Competent Cells

7.6.1. Initial Study

Transformations were performed using chemically competent *E. coli* NEB10β (NEB) according to manufacturer's recommendations. Briefly, 2 µL of reaction mixture (from *ex vivo* assemblies) or diluted DNA master mix (for *in vivo* assembly) were added to each transformation, these were incubated for 30 min on ice, heat shocked at 42°C, recovered in 950 µL SOC at 37°C for 60 min and 50 µL of culture was spread onto agar plates containing the appropriate antibiotic.

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7.6.2. Optimized Competence and Transformation

To prepare chemicompetent *E. coli* for chemical transformation the "Ultracompetent" method of Inoue, *et al* was followed closely[95]. As there is not a refrigerated incubator available in our lab to culture at 18°C the cultures were incubated at room temperature (~21°C).

To perform transformations, we incubated 48 µL of cells with 2 µL of DNA sample on ice for 30 minutes. The cells were heat-shocked in a 42°C water bath for 45 seconds and immediately transferred and swirled in an ice-water bath for 90 seconds. 950 µL of room temperature SOC was added to the tube and it was moved to the 37°C incubator with shaking at 250 RPM for 1 hour. Transformants were serially-diluted into sterile PBS and 100 µL plated onto LB with the appropriate antibiotic.

To prepare electrocompetent cells, we followed the protocol "Transformation of *E. coli* by Electroporation" in the Molecular Cloning Manual 4th ed. Electroporation reactions were carried out in a Bio-Rad Micropulser using the same volumes as used for chemical transformation above.

CFU per femtomole values were calculated by taking the raw counts of countable plates (30-300), multiplying by the dilution factor and dividing by the number of femtomoles of DNA transformed. Given the backbone of *ex vivo* assemblies as the limiting substrate at 5 ng/µL, 2 µL equates to approximately 6.6 femtomoles of 2-way and 7.3 femtomoles

of 3-way. 2 µL of a 50 pg/µL solution of pUC19 equates to 0.06 femtomoles.

7.7. Lambda RED Recombineering

Genomic manipulations of the nuc4- strains were performed using the protocols laid out by Wang, *et al*[77]. Summarizing, a single cycle of recombineering consisted of growing a culture of nuc4- in a 3 mL culture of LB + Ampicillin until OD600 = ~0.4, inducing lambda expression at 42°C for 15 minutes, washing 1 mL of the culture twice with ultrapure water, concentrating at ~50 µL and transforming 2 µL of a 10 µM solution of MAGE oligo(s)/λR cassette. In order to modify nuc4-, an already existing chloramphenicol cassette in the host genome was deactivated using the cat_fwd_stop oligo (see Appendix C) and the chloramphenicol mutant (nuc4-.Cm-) was isolated by replica plating. The λR autolysis cassette (including chloramphenicol resistance) was amplified out of XJa, gel extracted and transformed into nuc4-.Cm-. Unfortunately, there was so much homology between the chloramphenicol cassette in the λR autolysis cassette and the deactivated chloramphenicol loci that many, many false-positive chloramphenicol transformants were obtained (as confirmed by allele-specific PCR; Table C.3). Instead to identify the correct integrants, a screen approach utilizing Phenol Red Agar + 2% Arabinose was employed to identify colonies unable to utilize arabinose. A positive clone was isolated and the integration of the lysogen gene was confirmed by allele-specific genotyping and a lytic phenotype. Sequences of all oligos used for recombineering are provided in (Table C.3).

Chapter 8. References

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Appendix A. Tables

Table A.1 Summary of Assemblies

2- way Circular (2WC)

Part	Template	Forward Primer	Reverse Primer	Length
BCP	pSB1C3-K592009	exda1	exda2	698
Backbone	pSB1K3-J04450	exda3	exda4	2446

2-way Linear (2WL)

Part	Template	Forward Primer	Reverse Primer	Length
Promoter	pSB1C3-J00450	exda22	exda23	381
BCP	pSB1C3-K592009	exda12	exda13	697

3-way Circular (3WC)

Part	Template	Forward Primer	Reverse Primer	Length
Backbone	pSB1K3-J04450	exda3	exda19	2214
Promoter	pSB1C3-J00450	exda20	exda21	387
BCP	pSB1C3-K592009	exda12	exda2	696

Description of the DNA fragments used in each assembly assay. DNA sequences for primers, template plasmids and products of assemblies can be found in Appendix C.

Table A.2 Strain Genotypes

Strain	Parent Strain	Synonymous Strain	Growth (Celsius; Resistance)	Genotype
NEB10β	K-12	DH10Beta	37; Str	Δ(ara-leu) 7697 araD139 fhuA ΔlacX74 galK16 galE15 e14- φ80dlacZΔM15 **recA1** relA1 **endA1** nupG **rpsL (StrR)** rph spoT1 Δ(mrr-hsdRMS-mcrBC)
XJa	K-12	JM109	37; Cm	F`[traD36 proA+B+ laclq Δ(lacZ)M15] Δ(lac-proAB) glnV44 (supE44) e14- (McrA-) thi gyrA96 (NalR) **endA1** hsdR17(rK- mK+) relA1 **recA1 araB::λR, cat (CmR)**
nuc4-	EcNR2		32; Amp Cm	F- λ- ilvG- rfb-50 rph-1 **ΔmutS::cat** Δ(ybhB-bioAB)::[lcI857 **N(cro-ea59)::tetR-bla**] **xonA- recJ- xseA- exoX-**
nuc4-.Cm-	nuc4-		32; Amp	F- λ- ilvG- rfb-50 rph-1 **ΔmutS::cat-** Δ(ybhB-bioAB)::[**lcI857 N(cro-ea59)::tetR-bla**] **xonA- recJ- xseA- exoX-**
nuc4-.λR	nuc4-.Cm-		32; Amp Cm	F- λ- ilvG- rfb-50 rph-1 **ΔmutS::cat-** Δ(ybhB-bioAB)::[**lcI857 N(cro-ea59)::tetR-bla**] **xonA- recJ- xseA- exoX-** araB::λR, cat (CmR)

Genotypes of Strains used in the current study are presented above. Growth indicates temperatures used to culture strain and resistances available to strain. Relevant genotypes are indicated in bold and described below in Table A.3

2

Table A.3 Description of Relevant Genotypes

Genotype	Phenotypic Description
recA1	Disables homologous recombination pathways for reduced occurrence of unwanted recombination in cloned DNA; cells UV sensitive
endA1	For cleaner preparations of DNA and better results in downstream applications due to the elimination of non-specific digestion by Endonuclease I
rpsL	Mutation in ribosomal protein S12 conveying streptomycin resistance
ΔmutS::cat	Deletion of mutS used for mismatch repair for better Lambda Red recombination; Conveys Chloramphenicol Resistance
ΔmutS::cat-	Inactivation of Chloramphenicol cassette by insertion of stop codon
lcI857 N(cro-ea59)::tetR-bla	Lambda RED operon of alpha, beta and gamma proteins induced by heat-shock; Cells must be cultured at lower temperatures; Conveys Ampicillin resistance
araB::λR, cat (CmR)	Insertion of Lambda phage endolysin inducible by arabinose; Disrupts arabinose metabolism; Conveys Chloramphenicol resistance
xonA- recJ- xseA- exoX-	Inactivation of exonucleases (ExoI, RecJ, ExoVII, ExoX) by premature stop codon insertion

Descriptions of relevant genotypes found in Table A.2

Table A.4 Unit Definitions of Enzymes used in Titrations

Enzyme	Supplied Concentration (U/µL)	Temperature (Celsius)	Time	Buffer	[Substrate]	[Product]
RecBCD	10	37	30 min	NEB 4	Linear dsDNA (no concentration)	10 nmol (acid-soluble nucleotides)
ExoI	20	37	30 min	1X ExoI	170 ng/µL (ssDNA)	10 nmol
ExoIII	100	37	30 min	NEB 1	.15 mM (sonicated dsDNA)	1 nmol
ExoT	5	25	30 min	NEB 4	1 nmol (polythymidine)	.1 nmol (TCA soluble nucleotides)
RecJf	30	37	30 min	NEB 2	30 ng/µL (sonicated ssDNA)	.05 nmol (TCA soluble nucleotides)
DNA PolI	10	37	30 min	NEB 2	70 ng/µL SalmonSperm DNA; 33 µM dNTPs	10nmol (dNTPs -> acid insoluble material)
Eco Ligase	10	16	30 min	1X Eco Ligase Buffer	0.12 µM DNA termini (300 ng/µL)	.06 µM ligated ends

Unit definitions of enzymes supplied by NEB and used in titration experiments. For exonucleases, exonuclease activity is measured as formation of acid-soluble nucleotides. For DNA PolI polymerization activity is defined by formation of acid insoluble material from dNTPs. E. coli Ligase activity is measured by the formation of ligated ends from digested material. All reported unit definitions are for a 50 µL reaction volume.

4

Appendix B. Gel Pictures

Below are the original agarose gel pictures used in the analysis of *ex vivo* assembly. The higher molecular weight band (BCP band) in the control lane ("C") was used as the relative reference for band intensities.

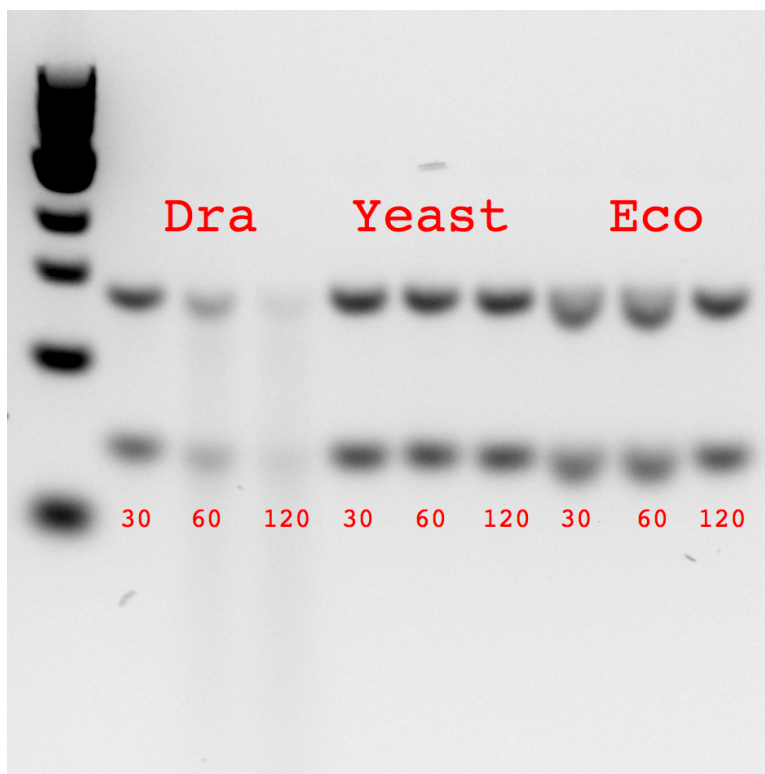## B.1.1 Time Course of *Deinococcus*, *Saccharomyces*, and *E. coli* lysates

Figure B.1 Dra, Sce and Eco Time Course

B.1.2 Lysate Preparation (Growth Phase, Media and Glycerol)
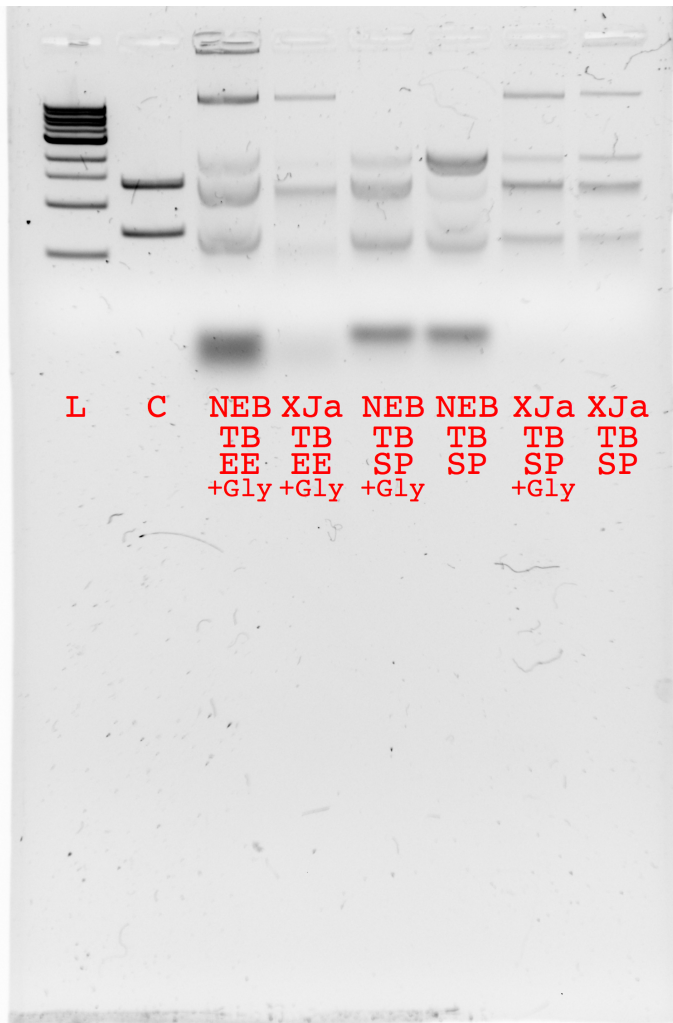

Figure B.2 Lysate Preparation (Gel 1)
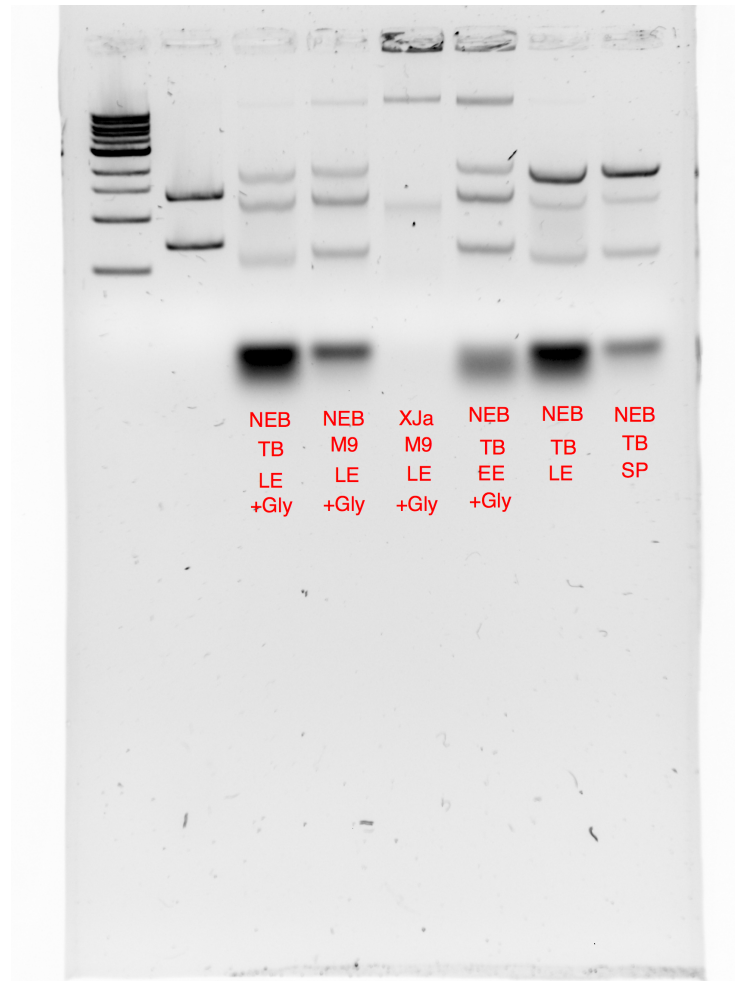
Figure B.3 Lysate Preparation (Gel 2)

B.1.3 Concentration of Lysates
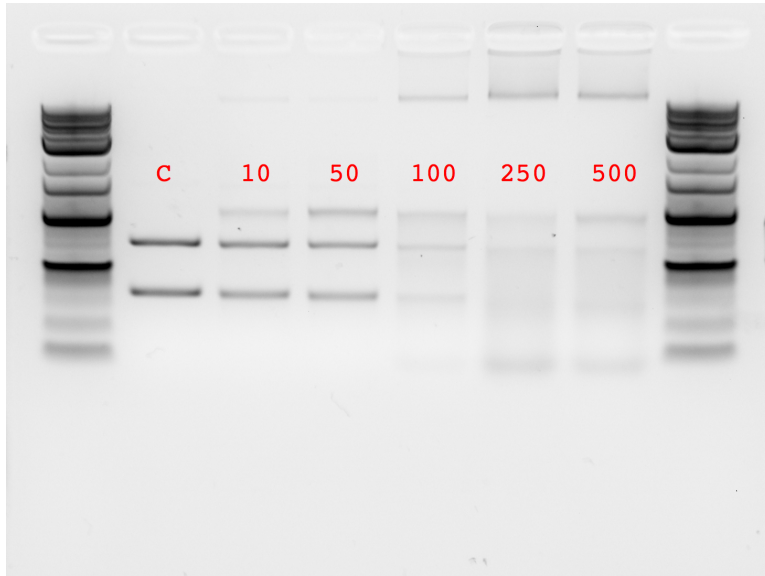
Figure B.4 Lysate Dilutions (60 minutes)
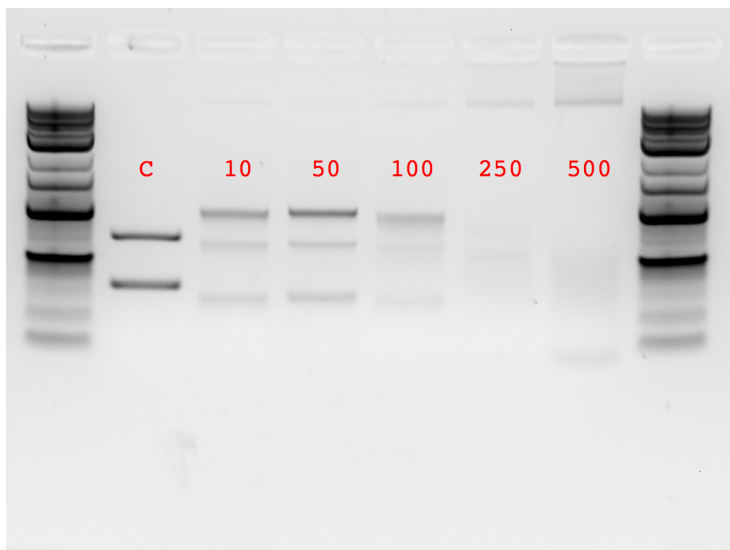
Figure B.5 Lysate Dilutions (120 minutes)

B.1.4 Buffer Optimization
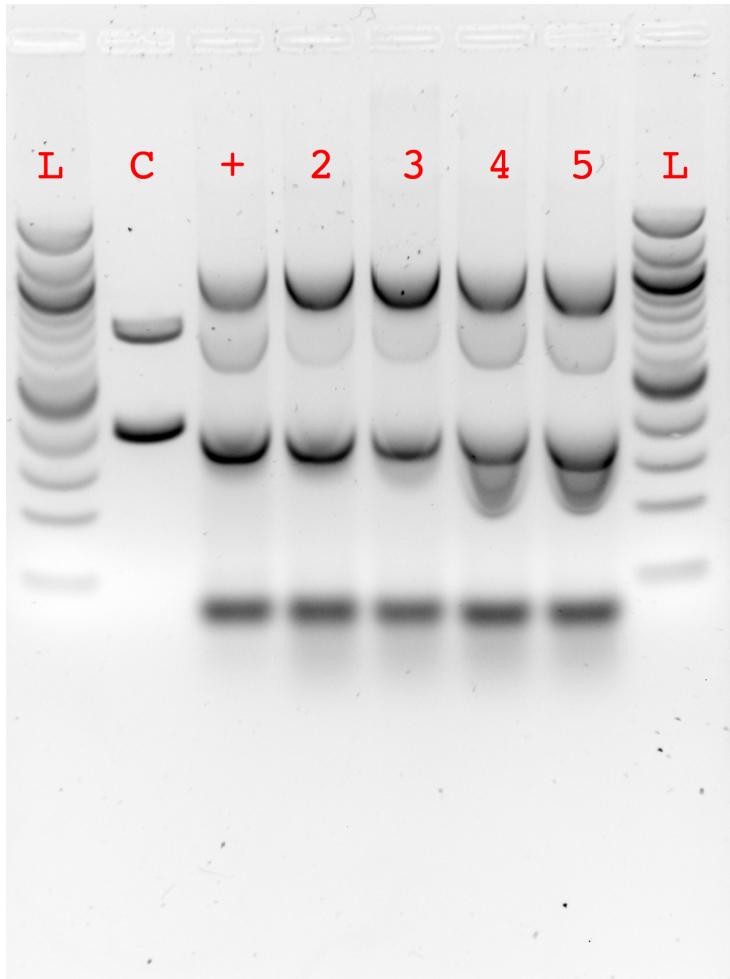
Figure B.6 Buffer Optimization (NEB10β)
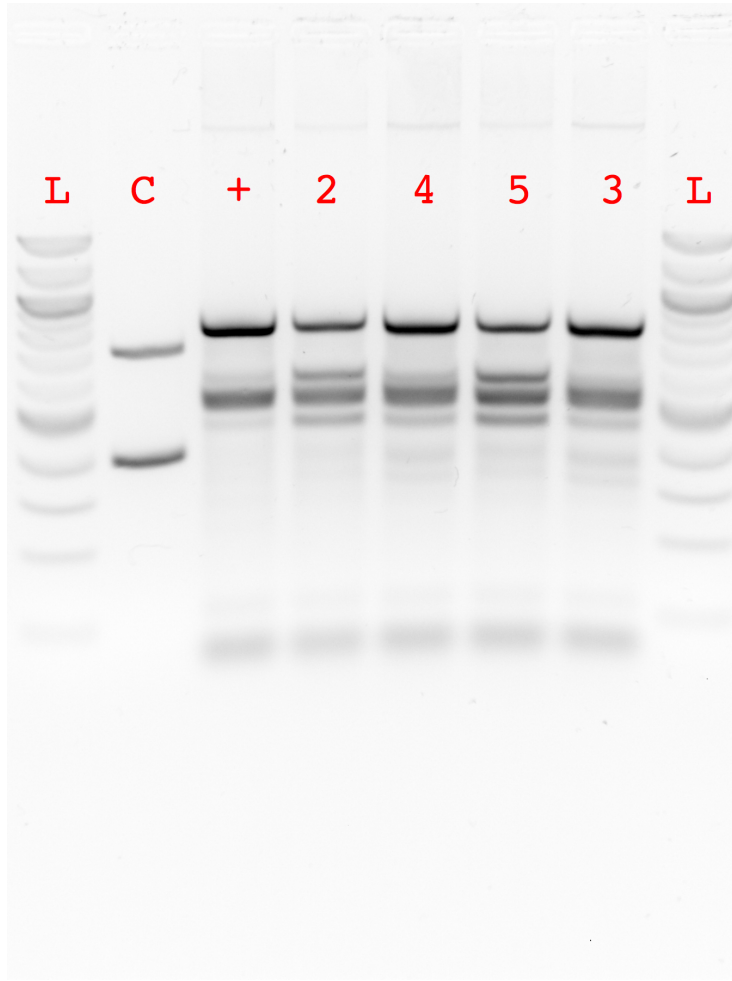
Figure B.7 Buffer Optimization (XJa Autolysis)

B.1.5 Buffer Titration
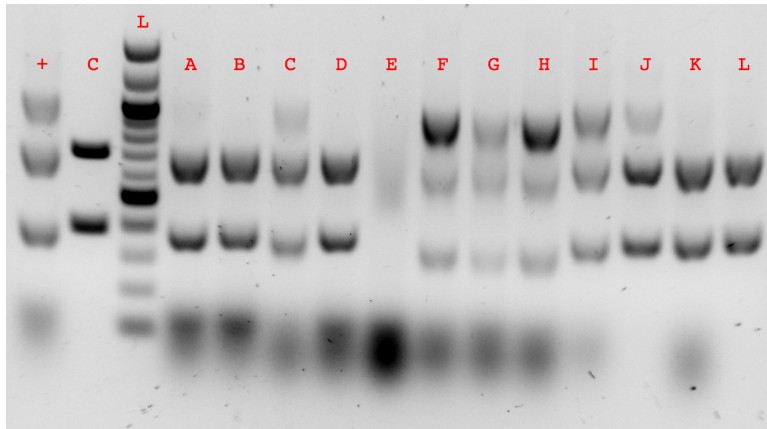
Figure B.8 Buffer Titration (NEB10β #1)
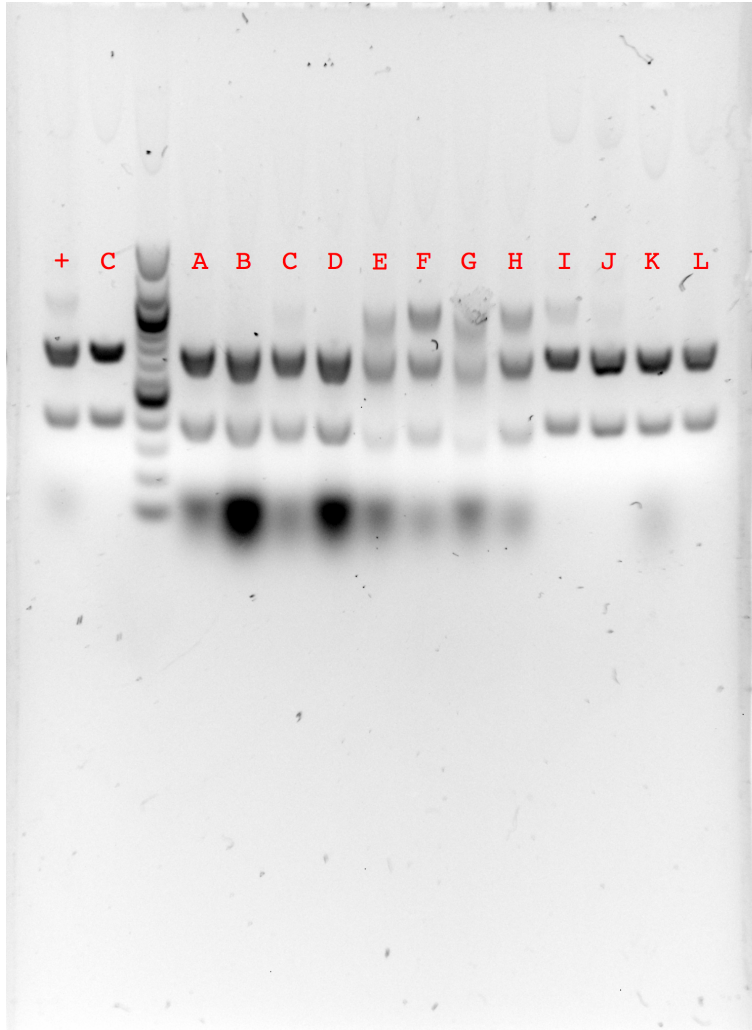
Figure B.9 Buffer Titration (NEB10β #2)
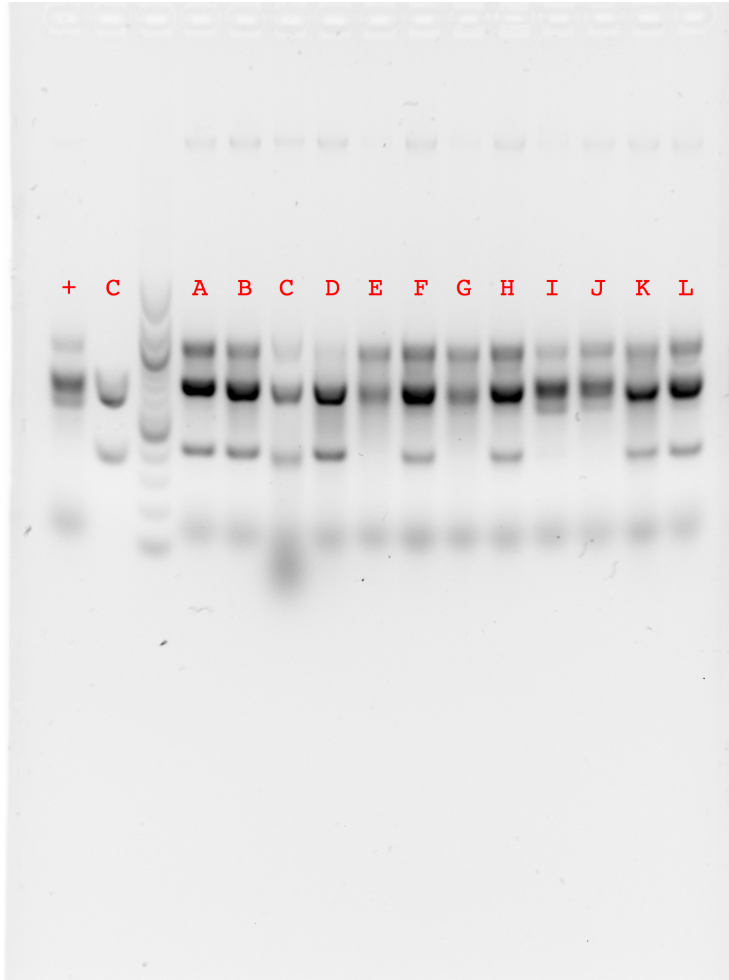
Figure B.10 Buffer Titration (XJa)

B.1.6 Manganese Titration
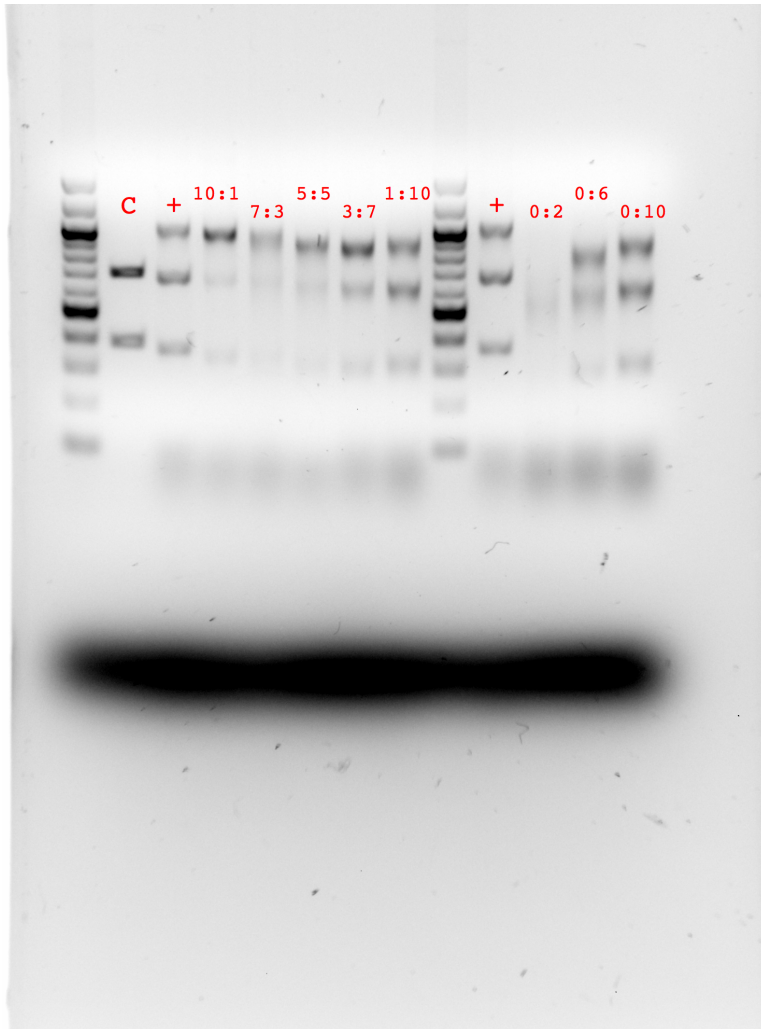
Figure B.11 Manganese Titration (NEB10β)

B.1.7 Enzymatic Titrations
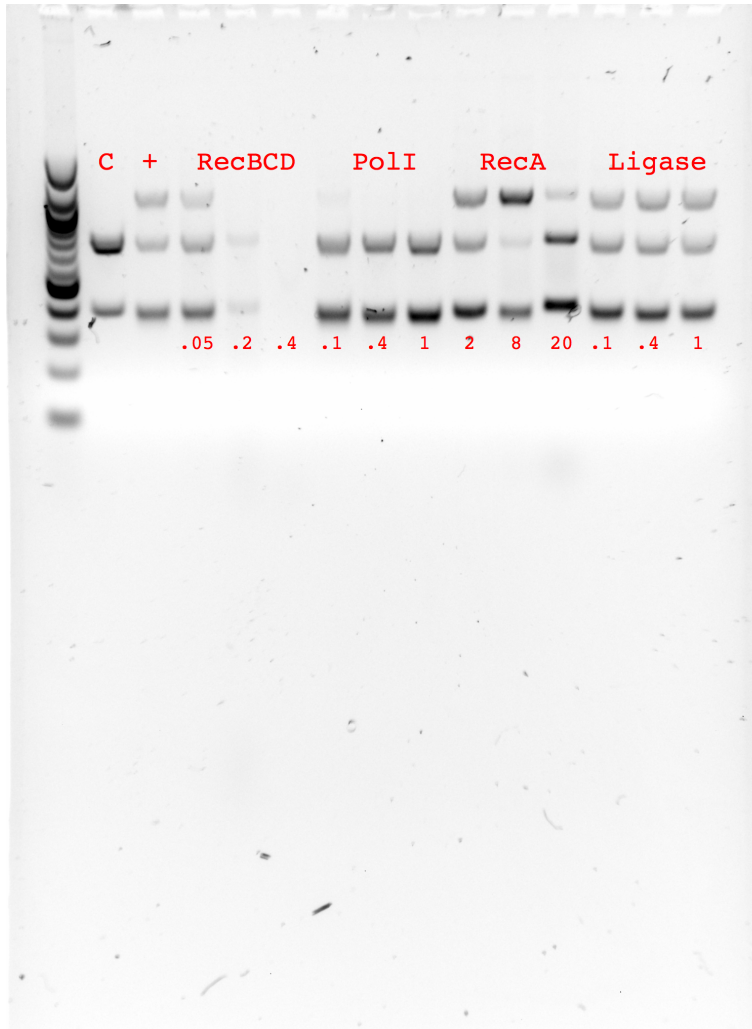
Figure B.12 RecBCD, PolI, RecA, Eco Ligase Titration
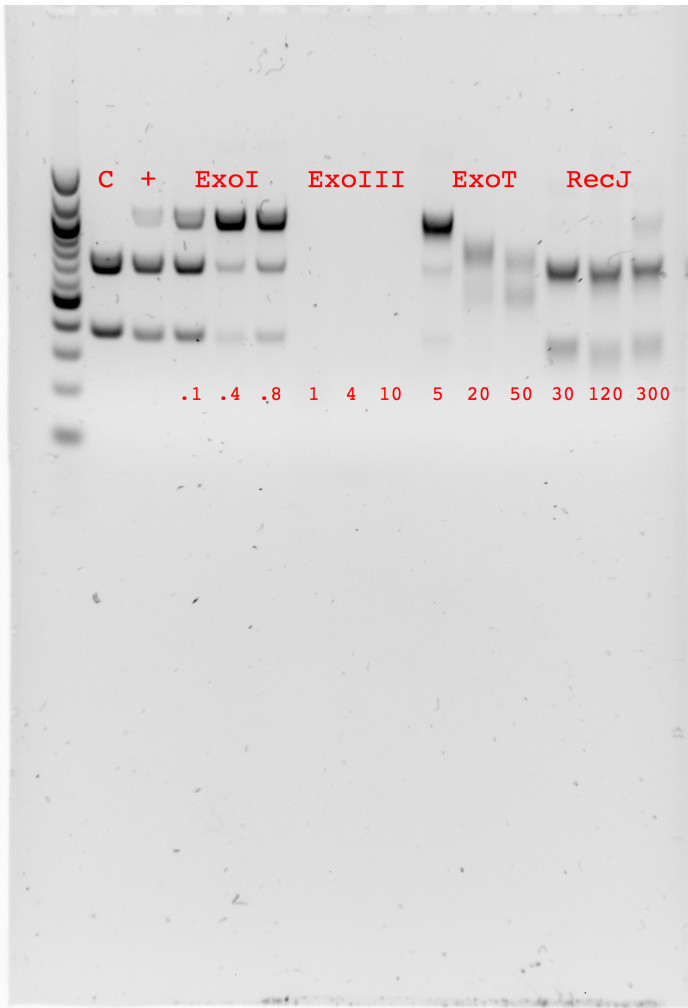
Figure B.13 ssDNA Exonucleases Titration
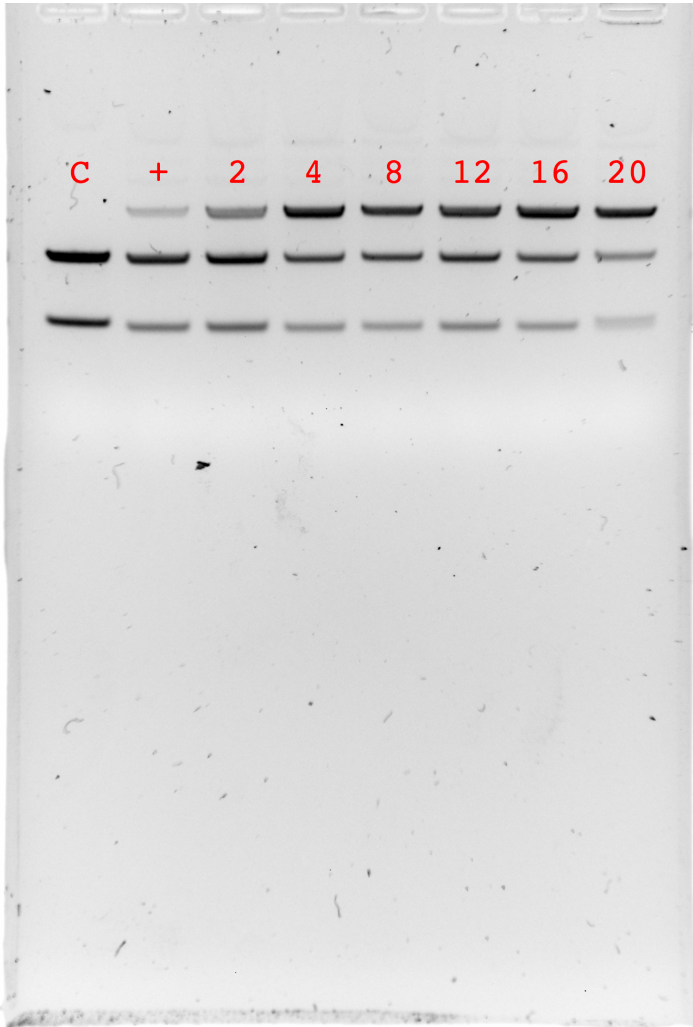
Figure B.14 RecA Titration (XJa Autolysis)

Appendix C. DNA Sequences

C.1 Primer Sequences

Table C.1 *ex vivo* Assembly Assay Primers

| Primer	Sequence	Length
exda1	AGAGAAAGAGGAGAAAATGAGTGTGATCGCTAAACAAATGACCTACAAGG	50
exda2	ATTTGATGCCTGGTTATTAGGCGACCACAGGTTTGCGTGC	40
exda3	GGTCGCCTAATAACCAGGCATCAAATAAAACGAAAGGCT	39
exda4	GCGATCACACTCATTTTCTCCTCTTTCTCTAGTATGTGTGA	41
exda12	AGGAGAAATACTAGATGAGTGTGATCGCTAAACAAATGACCTACAAGG	48
exda13	GCCTGGCTCTAGTATTATTAGGCGACCACAGGTTTGCGTGC	41
exda14	GGTCGCCTAATAATACTAGAGCCAGGCATCAAATAAAACG	40
exda19	TGGTTTCTTAGAAGCTGATCCTTCAACTCAGCA	33
exda20	TGAGTTGAAGGATCAGCTTCTAAGAAACCATTATTATCATGACATTAACC	50
exda21	AGCGATCACACTCATCTAGTATTTCTCCTCTTTCTCTAGTATGTG	45
exda22	TGAAGGATCAGCTTCTAAGAAACCATTATTATCATGACATTAACC	45
exda23	GCGATCACACTCATCTAGTATTTCTCCTCTTTCTCTAGTATGTG	44

18

Table C.2 pUN and UNS Primers

Primer	Sequence	Length
UNS1F	CATTACTCGCATCCATTCTCAG	22
UNS1R	GAGACGAGACGAGACAGC	18
UNS2F	GCTGGGAGTTCGTAGACG	18
UNS2R	GCTTGGATTCTGCGTTTGT	19
UNS3F	GCACTGAAGGTCCTCAATC	19
UNS3R	CGACCTTGATGTTTCCAGTG	20
UNS4F	CTGACCTCCTGCCAGC	16
UNS4R	GACTTTGCGTGTTGTCTTACT	21
UNS5F	GAGCCAACTCCCTTTACAAC	20
UNS5R	CTCTAACGGACTTGAGTGAGG	21
UNS6F	CTCGTTCGCTGCCACC	16
UNS6R	GTATGTGACCGTAGAGTATTCTTAG	25
UNS7F	CAAGACGCTGGCTCTGA	17
UNS7R	CGAGTAGTTCAGTAGCGGA	19
UNS8F	CCTCGTCTCAACCAAAGC	18
UNS8R	CCAGGTGGTTGATGGGT	17

UNS9F	GTTCCTTATCATCTGGCGAATC	22
UNS9R	CAGTGCTCTTGTGGGTC	17
UNSXF	CCAGGATACATAGATTACCACAAC	24
UNSXR	GGTGGAAGGGCTCGG	15
pGERC-BB_F	TCGAGAAGGACACGGTTA	18
pGERC-BB_R	AAAGCCTTGTATGCTTCTTT	20

Table C.3 Oligos and Primers used for Strain Engineering

Oligo Name	Sequence	Product Length	Purpose
cat_fwd_stop	G*C*A*T*CGTAAAGAACATTTTGAGGCATTTCAGTCAGTTGCTCAATGAACCTATAACCAGACCGTTCAGCTGGATATTACGGCCTTTTTAAA	NA	Introduces Stop Codon into cat gene of nuc4-
recA.KO	C*A*A*A*TTGTTTCTCAATCTGGCCCAGTGCTGCCGCCAACGCTcaCTaTTaGTTTTCGTCGATAGCCATTTTTACTCCTGTCATGCCGGGTA	NA	Introduces Stop Codon into recA gene
endA.KO	T*C*G*T*TTTAACACGGAGTAAGTGATGTACCGTTATTTGTCTATTGCTGCtGaGTGGTACTGAGCGCAGCATTTTCCGGCCCGGCGTTGGCC	NA	Introduces Stop Codon into endA gene
araB_lambdaR-f	CTTCGCCCGCAAAGCCGTAA	2066	Amplifies entire arabinose loci including endolysin and cat genes; Allele-specific PCR
araB_lambdaR-r	ACAGGTCGCTGAAATGCGGC	2066	Amplifies entire arabinose loci including endolysin and cat genes

21

asLambdaR-r	GGTCAGTTCGAGCATAAGGC	20	Targets LambdaR coding sequence; Allele-Specific PCR
endA.KO.v2_wt-f	CGTTATTTGTCTATTGCTGC<u>GG</u>	191	Allele-specifc PCR of endA
endA.KO*_mut-f	CCGTTATTTGTCTATTGCTGC<u>TGA</u>	191	Allele-specifc PCR of endA
endA.KO*-r	GCACGATTGCAGATCAACAACG	191	Allele-specifc PCR of endA
recA.KO.v2_wt-f	TGCCGCCAACGCTT<u>**T**</u>	474	Allele-specific PCR of recA
recA.KO.v2_mut-f	TGCCGCCAACGCTC<u>**A**</u>	474	Allele-specific PCR of recA
recA.KO.v3-r	GGTTTGAACGCGGATTTGTCAG	474	Allele-specific PCR of recA

C.2 Plasmid Sequences

Below are Genbank formatted files of the various plasmids used throughout the study, complete with annotations.

C.2.1 pSB1C3-J00450 (RFP expression)

```
LOCUS       pSB1C3-J00450           3139 bp ds-DNA    circular      22-APR-
2015

DEFINITION  .

ACCESSION   pSB1C3-J00450

VERSION     pSB1C3-J00450

FEATURES             Location/Qualifiers

   terminator      3099..3139

                   /label="B0012"

   terminator      3011..3139

                   /label="BBa_B0015"

   rep_origin      261..875

                   /label="pMB1 rep origin"

   terminator      1042..1147

                   /label="T0 terminator"

   RBS             2279..2290
```

23

```
                     /label="B0034"

misc_feature     3106..3125

                     /label="B0012 Stem-loop"

misc_binding     2049..2070

                     /label="BioBrick prefix"

primer_bind      12..33

                     /label="SB-prep-3P-1 primer site"

primer_bind      2031..2054

                     /label="SB-prep_2Ea primer site"

CDS              1160..1829

                     /label="CmR"

CDS              2297..3002

                     /label="E1010"

misc_feature     3114..3117

                     /label="B0012 Loop"

misc_binding     1..21

                     /label="BioBrick suffix"

misc_feature     3022..3065
```

```
                         /label="B0010 Stem-loop"

        terminator       3011..3090

                         /label="B0010"

        primer_bind      156..176

                         /label="VR primer site"

        primer_bind      1943..1962

                         /label="VF2 primer site"

        promoter         2071..2270

                         /label="R0010"

        terminator       22..93

                         /label="Eco His Term."

        misc_feature     3042..3045

                         /label="B1010 Loop"

ORIGIN

        1 tactagtagc ggccgctgca gtccggcaaa aaagggcaag gtgtcaccac cctgcccttt

       61 ttctttaaaa ccgaaaagat tacttcgcgt tatgcaggct tcctcgctca ctgactcgct

      121 gcgctcggtc gttcggctgc ggcgagcggt atcagctcac tcaaaggcgg taatacggtt

      181 atccacagaa tcaggggata acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc
```

```
 241 caggaaccgt aaaaaggccg cgttgctggc gttttttccac aggctccgcc cccctgacga

 301 gcatcacaaa aatcgacgct caagtcagag gtggcgaaac ccgacaggac tataaagata

 361 ccaggcgttt cccctggaa gctccctcgt gcgctctcct gttccgaccc tgccgcttac

 421 cggatacctg tccgccttc tcccttcggg aagcgtggcg ctttctcata gctcacgctg

 481 taggtatctc agttcggtgt aggtcgttcg ctccaagctg ggctgtgtgc acgaacccc

 541 cgttcagccc gaccgctgcg ccttatccgg taactatcgt cttgagtcca acccggtaag

 601 acacgactta tcgccactgg cagcagccac tggtaacagg attagcagag cgaggtatgt

 661 aggcggtgct acagagttct tgaagtggtg gcctaactac ggctacacta gaagaacagt

 721 atttggtatc tgcgctctgc tgaagccagt taccttcgga aaaagagttg gtagctcttg

 781 atccggcaaa caaaccaccg ctggtagcgg tggtttttttt gtttgcaagc agcagattac

 841 gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt tctacggggt ctgacgctca

 901 gtggaacgaa aactcacgtt aagggatttt ggtcatgaga ttatcaaaaa ggatcttcac

 961 ctagatcctt ttaaattaaa aatgaagttt taaatcaatc taaagtatat atgagtaaac

1021 ttggtctgac agctcgaggc ttggattctc accaataaaa aacgcccggc ggcaaccgag

1081 cgttctgaac aaatccagat ggagttctga ggtcattact ggatctatca acaggagtcc

1141 aagcgagctc gatatcaaat tacgccccgc cctgccactc atcgcagtac tgttgtaatt

1201 cattaagcat tctgccgaca tggaagccat cacaaacggc atgatgaacc tgaatcgcca

1261 gcggcatcag caccttgtcg ccttgcgtat aatatttgcc catggtgaaa acggggggcga
```

26

```
1321 agaagttgtc catattggcc acgtttaaat caaaactggt gaaactcacc cagggattgg

1381 ctgagacgaa aaacatattc tcaataaacc ctttagggaa ataggccagg ttttcaccgt

1441 aacacgccac atcttgcgaa tatatgtgta gaaactgccg gaaatcgtcg tggtattcac

1501 tccagagcga tgaaaacgtt tcagtttgct catggaaaac ggtgtaacaa gggtgaacac

1561 tatcccatat caccagctca ccgtctttca ttgccatacg aaattccgga tgagcattca

1621 tcaggcgggc aagaatgtga ataaaggccg gataaaactt gtgcttattt ttctttacgg

1681 tctttaaaaa ggccgtaata tccagctgaa cggtctggtt ataggtacat tgagcaactg

1741 actgaaatgc ctcaaaatgt tctttacgat gccattggga tatatcaacg gtggtatatc

1801 cagtgatttt tttctccatt ttagcttcct tagctcctga aaatctcgat aactcaaaaa

1861 atacgcccgg tagtgatctt atttcattat ggtgaaagtt ggaacctctt acgtgcccga

1921 tcaactcgag tgccacctga cgtctaagaa accattatta tcatgacatt aacctataaa

1981 aataggcgta tcacgaggca gaatttcaga taaaaaaaat ccttagcttt cgctaaggat

2041 gatttctgga attcgcggcc gcttctagag caatacgcaa accgcctctc cccgcgcgtt

2101 ggccgattca ttaatgcagc tggcacgaca ggtttcccga ctggaaagcg ggcagtgagc

2161 gcaacgcaat taatgtgagt tagctcactc attaggcacc ccaggcttta cactttatgc

2221 ttccggctcg tatgttgtgt ggaattgtga gcggataaca atttcacaca tactagagaa

2281 agaggagaaa tactagatgg cttcctccga agacgttatc aaagagttca tgcgtttcaa

2341 agttcgtatg gaaggttccg ttaacggtca cgagttcgaa atcgaaggtg aaggtgaagg
```

27

```
2401 tcgtccgtac gaaggtaccc agaccgctaa actgaaagtt accaaaggtg gtccgctgcc

2461 gttcgcttgg gacatcctgt ccccgcagtt ccagtacggt tccaaagctt acgttaaaca

2521 cccggctgac atcccggact acctgaaact gtccttcccg gaaggtttca aatgggaacg

2581 tgttatgaac ttcgaagacg gtggtgttgt taccgttacc caggactcct ccctgcaaga

2641 cggtgagttc atctacaaag ttaaactgcg tggtaccaac ttcccgtccg acggtccggt

2701 tatgcagaaa aaaaccatgg gttgggaagc ttccaccgaa cgtatgtacc cggaagacgg

2761 tgctctgaaa ggtgaaatca aaatgcgtct gaaactgaaa gacggtggtc actacgacgc

2821 tgaagttaaa accacctaca tggctaaaaa accggttcag ctgccgggtg cttacaaaac

2881 cgacatcaaa ctggacatca cctcccacaa cgaagactac accatcgttg aacagtacga

2941 acgtgctgaa ggtcgtcact ccaccggtgc ttaataacgc tgatagtgct agtgtagatc

3001 gctactagag ccaggcatca aataaaacga aaggctcagt cgaaagactg ggcctttcgt

3061 tttatctgtt gtttgtcggt gaacgctctc tactagagtc acactggctc accttcgggt

3121 gggcctttct gcgtttata
```

//

C.2.2 pSB1C3-K592009 (Contains BCP Coding Sequence)

```
LOCUS       pSB1C3-K592009       2739 bp ds-DNA    circular    27-JUN-
2015

DEFINITION  .

COMMENT     ApEinfo:methylated:1

FEATURES             Location/Qualifiers

    misc_binding    670..690

                    /label="BioBrick suffix"

    primer_bind     825..845

                    /label="VR primer site"

    CDS             1..669

                    /label="amilCP"

    terminator      691..762

                    /label="Eco His Term."

    primer_bind     2700..2723

                    /label="SB-prep_2Ea primer site"

    rep_origin      930..1544

                    /label="pMB1 rep origin"

    primer_bind     2612..2631
```

29

```
                      /label="VF2 primer site"

      primer_bind      2640..2666

                       /label="pSBamil_rev"

      CDS              1829..2498

                       /label="CmR"

      terminator       1711..1816

                       /label="T0 terminator"

      misc_binding     2718..2739

                       /label="BioBrick prefix"

      primer_bind      81..102

                       /label="pSBamil_for"

      primer_bind      681..702

                       /label="SB-prep-3P-1 primer site"

   ORIGIN

        1 atgagtgtga tcgctaaaca aatgacctac aaggtttata tgtcaggcac ggtcaatgga

       61 cactactttg aggtcgaagg cgatggaaaa ggtaagccct acgaggggga gcagacggta

      121 aagctcactg tcaccaaggg cggacctctg ccatttgctt gggatatttt atcaccacag

      181 tgtcagtacg gaagcatacc attcaccaag taccctgaag acatccctga ctatgtaaag
```

30

```
 241 cagtcattcc cggagggcta tacatgggag aggatcatga actttgaaga tggtgcagtg

 301 tgtactgtca gcaatgattc cagcatccaa ggcaactgtt tcatctacca tgtcaagttc

 361 tctggtttga actttcctcc caatggacct gtcatgcaga agaagacaca gggctgggaa

 421 cccaacactg agcgtctctt tgcacgagat ggaatgctgc taggaaacaa ctttatggct

 481 ctgaagttag aaggaggcgg tcactatttg tgtgaattta aaactactta caaggcaaag

 541 aagcctgtga agatgccagg gtatcactat gttgaccgca aactggatgt aaccaatcac

 601 aacaaggatt acacttcggt tgagcagtgt gaaatttcca ttgcacgcaa acctgtggtc

 661 gcctaataat actagtagcg gccgctgcag tccggcaaaa aagggcaagg tgtcaccacc

 721 ctgccctttt tctttaaaac cgaaaagatt acttcgcgtt atgcaggctt cctcgctcac

 781 tgactcgctg cgctcggtcg ttcggctgcg gcgagcggta tcagctcact caaaggcggt

 841 aatacggtta tccacagaat caggggataa cgcaggaaag aacatgtgag caaaaggcca

 901 gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg tttttccaca ggctccgccc

 961 ccctgacgag catcacaaaa atcgacgctc aagtcagagg tggcgaaacc cgacaggact

1021 ataaagatac caggcgtttc cccctggaag ctccctcgtg cgctctcctg ttccgaccct

1081 gccgcttacc ggatacctgt ccgcctttct cccttcggga agcgtggcgc tttctcatag

1141 ctcacgctgt aggtatctca gttcggtgta ggtcgttcgc tccaagctgg gctgtgtgca

1201 cgaacccccc gttcagcccg accgctgcgc cttatccggt aactatcgtc ttgagtccaa

1261 cccggtaaga cacgacttat cgccactggc agcagccact ggtaacagga ttagcagagc
```

31

```
1321 gaggtatgta ggcggtgcta cagagttctt gaagtggtgg cctaactacg gctacactag

1381 aagaacagta tttggtatct gcgctctgct gaagccagtt accttcggaa aaagagttgg

1441 tagctcttga tccggcaaac aaaccaccgc tggtagcggt ggtttttttg tttgcaagca

1501 gcagattacg cgcagaaaaa aaggatctca agaagatcct ttgatctttt ctacggggtc

1561 tgacgctcag tggaacgaaa actcacgtta agggattttg gtcatgagat tatcaaaaag

1621 gatcttcacc tagatccttt taaattaaaa atgaagtttt aaatcaatct aaagtatata

1681 tgagtaaact tggtctgaca gctcgaggct tggattctca ccaataaaaa acgcccggcg

1741 gcaaccgagc gttctgaaca aatccagatg gagttctgag gtcattactg gatctatcaa

1801 caggagtcca agcgagctcg atatcaaatt acgccccgcc ctgccactca tcgcagtact

1861 gttgtaattc attaagcatt ctgccgacat ggaagccatc acaaacggca tgatgaacct

1921 gaatcgccag cggcatcagc accttgtcgc cttgcgtata atatttgccc atggtgaaaa

1981 cggggggcgaa gaagttgtcc atattggcca cgtttaaatc aaaactggtg aaactcaccc

2041 agggattggc tgagacgaaa aacatattct caataaaccc tttagggaaa taggccaggt

2101 tttcaccgta acacgccaca tcttgcgaat atatgtgtag aaactgccgg aaatcgtcgt

2161 ggtattcact ccagagcgat gaaaacgttt cagtttgctc atggaaaacg gtgtaacaag

2221 ggtgaacact atcccatatc accagctcac cgtctttcat tgccatacga aattccggat

2281 gagcattcat caggcgggca agaatgtgaa taaaggccgg ataaaacttg tgcttatttt

2341 tctttacggt ctttaaaaag gccgtaatat ccagctgaac ggtctggtta taggtacatt
```

```
2401 gagcaactga ctgaaatgcc tcaaaatgtt ctttacgatg ccattgggat atatcaacgg

2461 tggtatatcc agtgattttt ttctccattt tagcttcctt agctcctgaa aatctcgata

2521 actcaaaaaa tacgcccggt agtgatctta tttcattatg gtgaaagttg gaacctctta

2581 cgtgcccgat caactcgagt gccacctgac gtctaagaaa ccattattat catgacatta

2641 acctataaaa ataggcgtat cacgaggcag aatttcagat aaaaaaaatc cttagctttc

2701 gctaaggatg atttctggaa ttcgcggccg cttctagag
```

//

C.2.3 pSB1K3-J00450 (RFP expression with Kanamycin Resistance)

```
LOCUS        pSB1K3-J04450            3273 bp ds-DNA    circular     21-APR-
2015

DEFINITION  .

FEATURES             Location/Qualifiers

    stem_loop        30..64

                     /label="Stem loop"

    CDS              2431..3136

                     /label="E1010"

    misc_feature     3240..3259

                     /label="B0012 Stem-loop"

    primer_bind      complement(157..176)

                     /label="Verification reverse (VR) primer binding site"

    gene             2205..3273

                     /label="BBa_J04450"

    misc_feature     1..21

                     /label="DNA: BioBrick suffix"

    misc_feature     2183..2204
```

34

```
                       /label="Biobrick Prefix Standard (RFC10)"

     terminator       3233..3273

                       /label="B0012"

     misc_difference complement(1880..1880)

                       /label="Silent Mut: G->C mutation to remove XhoI site"

     terminator       3145..3224

                       /label="B0010"

     promoter         2205..2404

                       /label="R0010"

     primer_bind      2067..2086

                       /label="Verification forward (VF2) primer binding site"

     terminator       3145..3273

                       /label="BBa_B0015"

     misc_feature     3248..3251

                       /label="B0012 Loop"

     stem_loop        22..93

                       /label="E. coli his operon terminator"

     misc_feature     complement(2017..2060)
```

35

```
                         /label="repeat region misc"

misc_feature    3156..3199

                         /label="B0010 Stem-loop"

RBS             2413..2424

                         /label="B0034"

misc_feature    complement(261..875)

                         /label="rep (pMB1) misc"

misc_feature    3176..3179

                         /label="B1010 Loop"

misc_feature    276..276

                         /label="ORI misc"

stem_loop       complement(2143..2146)

                         /label="end of terminator"

stem_loop       complement(2156..2175)

                         /label="Stem loop"

misc_feature    1..21

                         /label="Biobrick Suffix (RFC10)"

CDS             complement(1097..1912)
```

/label="Kanamycin resistance marker CDS"

ORIGIN

        1 tactagtagc ggccgctgca gtccggcaaa aaagggcaag gtgtcaccac cctgcccttt

       61 ttctttaaaa ccgaaaagat tacttcgcgt tatgcaggct tcctcgctca ctgactcgct

      121 gcgctcggtc gttcggctgc ggcgagcggt atcagctcac tcaaaggcgg taatacggtt

      181 atccacagaa tcaggggata acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc

      241 caggaaccgt aaaaaggccg cgttgctggc gttttttccac aggctccgcc cccctgacga

      301 gcatcacaaa aatcgacgct caagtcagag gtggcgaaac ccgacaggac tataaagata

      361 ccaggcgttt cccctggaa gctccctcgt gcgctctcct gttccgaccc tgccgcttac

      421 cggatacctg tccgccttc tcccttcggg aagcgtggcg ctttctcata gctcacgctg

      481 taggtatctc agttcggtgt aggtcgttcg ctccaagctg ggctgtgtgc acgaacccc

      541 cgttcagccc gaccgctgcg ccttatccgg taactatcgt cttgagtcca acccggtaag

      601 acacgactta tcgccactgg cagcagccac tggtaacagg attagcagag cgaggtatgt

      661 aggcggtgct acagagttct tgaagtggtg gcctaactac ggctacacta gaagaacagt

      721 atttggtatc tgcgctctgc tgaagccagt taccttcgga aaaagagttg gtagctcttg

      781 atccggcaaa caaaccaccg ctggtagcgg tggttttttt gtttgcaagc agcagattac

      841 gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt tctacggggt ctgacgctca

      901 gtggaacgaa aactcacgtt aagggatttt ggtcatgaga ttatcaaaaa ggatcttcac

37

```
 961 ctagatcctt ttaaattaaa aatgaagttt taaatcaatc taaagtatat atgagtaaac

1021 ttggtctgac agctcgagtc ccgtcaagtc agcgtaatgc tctgccagtg ttacaaccaa

1081 ttaaccaatt ctgattagaa aaactcatcg agcatcaaat gaaactgcaa tttattcata

1141 tcaggattat caataccata tttttgaaaa agccgtttct gtaatgaagg agaaaactca

1201 ccgaggcagt tccataggat ggcaagatcc tggtatcggt ctgcgattcc gactcgtcca

1261 acatcaatac aacctattaa tttcccctcg tcaaaaataa ggttatcaag tgagaaatca

1321 ccatgagtga cgactgaatc cggtgagaat ggcaaaagct tatgcatttc tttccagact

1381 tgttcaacag gccagccatt acgctcgtca tcaaaatcac tcgcatcaac caaaccgtta

1441 ttcattcgtg attgcgcctg agcgagacga atacgcgat cgctgttaaa aggacaatta

1501 caaacaggaa tcgaatgcaa ccggcgcagg aacactgcca gcgcatcaac aatattttca

1561 cctgaatcag gatattcttc taatacctgg aatgctgttt tcccggggat cgcagtggtg

1621 agtaaccatg catcatcagg agtacggata aaatgcttga tggtcggaag aggcataaat

1681 tccgtcagcc agtttagtct gaccatctca tctgtaacat cattggcaac gctacctttg

1741 ccatgtttca gaaacaactc tggcgcatcg ggcttcccat acaatcgata gattgtcgca

1801 cctgattgcc cgacattatc gcgagcccat ttatacccat ataaatcagc atccatgttg

1861 gaatttaatc gcggcctgga gcaagacgtt tcccgttgaa tatggctcat aacacccctt

1921 gtattactgt ttatgtaagc agacagtttt attgttcatg atgatatatt tttatcttgt

1981 gcaatgtaac atcagagatt ttgagacaca acgtggcttt gttgaataaa tcgaactttt
```

38

2041 gctgagttga aggatcagct cgagtgccac ctgacgtcta agaaaccatt attatcatga

2101 cattaaccta taaaaatagg cgtatcacga ggcagaattt cagataaaaa aaatccttag

2161 ctttcgctaa ggatgatttc tggaattcgc ggccgcttct agagcaatac gcaaaccgcc

2221 tctccccgcg cgttggccga ttcattaatg cagctggcac gacaggtttc ccgactggaa

2281 agcgggcagt gagcgcaacg caattaatgt gagttagctc actcattagg caccccaggc

2341 tttcacttt atgcttccgg ctcgtatgtt gtgtggaatt gtgagcggat aacaatttca

2401 cacatactag agaaagagga gaaatactag atggcttcct ccgaagacgt tatcaaagag

2461 ttcatgcgtt tcaaagttcg tatggaaggt tccgttaacg gtcacgagtt cgaaatcgaa

2521 ggtgaaggtg aaggtcgtcc gtacgaaggt acccagaccg ctaaactgaa agttaccaaa

2581 ggtggtccgc tgccgttcgc ttgggacatc ctgtccccgc agttccagta cggttccaaa

2641 gcttacgtta aacacccggc tgacatcccg gactacctga aactgtcctt cccggaaggt

2701 ttcaaatggg aacgtgttat gaacttcgaa gacggtggtg ttgttaccgt tacccaggac

2761 tcctccctgc aagacggtga gttcatctac aaagttaaac tgcgtggtac caacttcccg

2821 tccgacggtc cggttatgca gaaaaaaacc atgggttggg aagcttccac cgaacgtatg

2881 tacccggaag acggtgctct gaaaggtgaa atcaaaatgc gtctgaaact gaaagacggt

2941 ggtcactacg acgctgaagt taaaaccacc tacatggcta aaaaaccggt tcagctgccg

3001 ggtgcttaca aaaccgacat caaactggac atcacctccc acaacgaaga ctacaccatc

3061 gttgaacagt acgaacgtgc tgaaggtcgt cactccaccg gtgcttaata acgctgatag

39

```
3121 tgctagtgta gatcgctact agagccaggc atcaaataaa acgaaaggct cagtcgaaag

3181 actgggcctt tcgttttatc tgttgtttgt cggtgaacgc tctctactag agtcacactg

3241 gctcaccttc gggtgggcct ttctgcgttt ata
```

//

C.2.4 Circular Assembly Product (in Kanamycin Backbone)

```
LOCUS        pSB1K3-J04450          3273 bp ds-DNA     circular     21-APR-
2015

DEFINITION  .

FEATURES             Location/Qualifiers

    stem_loop        30..64

                     /label="Stem loop"

    CDS              2431..3136

                     /label="E1010"

    misc_feature     3240..3259

                     /label="B0012 Stem-loop"

    primer_bind      complement(157..176)

                     /label="Verification reverse (VR) primer binding site"

    gene             2205..3273

                     /label="BBa_J04450"

    misc_feature     1..21

                     /label="DNA: BioBrick suffix"

    misc_feature     2183..2204

                     /label="Biobrick Prefix Standard (RFC10)"
```

41

```
terminator       3233..3273

                 /label="B0012"

misc_difference  complement(1880..1880)

                 /label="Silent Mut: G->C mutation to remove XhoI site"

terminator       3145..3224

                 /label="B0010"

promoter         2205..2404

                 /label="R0010"

primer_bind      2067..2086

                 /label="Verification forward (VF2) primer binding site"

terminator       3145..3273

                 /label="BBa_B0015"

misc_feature     3248..3251

                 /label="B0012 Loop"

stem_loop        22..93

                 /label="E. coli his operon terminator"

misc_feature     complement(2017..2060)

                 /label="repeat region misc"
```

```
misc_feature      3156..3199

                  /label="B0010 Stem-loop"

RBS               2413..2424

                  /label="B0034"

misc_feature      complement(261..875)

                  /label="rep (pMB1) misc"

misc_feature      3176..3179

                  /label="B1010 Loop"

misc_feature      276..276

                  /label="ORI misc"

stem_loop         complement(2143..2146)

                  /label="end of terminator"

stem_loop         complement(2156..2175)

                  /label="Stem loop"

misc_feature      1..21

                  /label="Biobrick Suffix (RFC10)"

CDS               complement(1097..1912)

                  /label="Kanamycin resistance marker CDS"
```

43

ORIGIN

```
  1 tactagtagc ggccgctgca gtccggcaaa aaagggcaag gtgtcaccac cctgcccttt

 61 ttctttaaaa ccgaaaagat tacttcgcgt tatgcaggct tcctcgctca ctgactcgct

121 gcgctcggtc gttcggctgc ggcgagcggt atcagctcac tcaaaggcgg taatacggtt

181 atccacagaa tcaggggata acgcaggaaa gaacatgtga gcaaaaggcc agcaaaaggc

241 caggaaccgt aaaaaggccg cgttgctggc gtttttccac aggctccgcc cccctgacga

301 gcatcacaaa aatcgacgct caagtcagag gtggcgaaac ccgacaggac tataaagata

361 ccaggcgttt ccccctggaa gctccctcgt gcgctctcct gttccgaccc tgccgcttac

421 cggatacctg tccgcctttc tcccttcggg aagcgtggcg ctttctcata gctcacgctg

481 taggtatctc agttcggtgt aggtcgttcg ctccaagctg ggctgtgtgc acgaacccccc

541 cgttcagccc gaccgctgcg ccttatccgg taactatcgt cttgagtcca acccggtaag

601 acacgactta tcgccactgg cagcagccac tggtaacagg attagcagag cgaggtatgt

661 aggcggtgct acagagttct tgaagtggtg gcctaactac ggctacacta gaagaacagt

721 atttggtatc tgcgctctgc tgaagccagt taccttcgga aaaagagttg gtagctcttg

781 atccggcaaa caaaccaccg ctggtagcgg tggttttttt gtttgcaagc agcagattac

841 gcgcagaaaa aaaggatctc aagaagatcc tttgatcttt tctacggggt ctgacgctca

901 gtggaacgaa aactcacgtt aagggatttt ggtcatgaga ttatcaaaaa ggatcttcac

961 ctagatcctt ttaaattaaa aatgaagttt taaatcaatc taagtatat atgagtaaac
```

44

```
1021 ttggtctgac agctcgagtc ccgtcaagtc agcgtaatgc tctgccagtg ttacaaccaa

1081 ttaaccaatt ctgattagaa aaactcatcg agcatcaaat gaaactgcaa tttattcata

1141 tcaggattat caataccata tttttgaaaa agccgtttct gtaatgaagg agaaaactca

1201 ccgaggcagt tccataggat ggcaagatcc tggtatcggt ctgcgattcc gactcgtcca

1261 acatcaatac aacctattaa tttcccctcg tcaaaaataa ggttatcaag tgagaaatca

1321 ccatgagtga cgactgaatc cggtgagaat ggcaaaagct tatgcatttc tttccagact

1381 tgttcaacag gccagccatt acgctcgtca tcaaaatcac tcgcatcaac caaaccgtta

1441 ttcattcgtg attgcgcctg agcgagacga atacgcgat cgctgttaaa aggacaatta

1501 caaacaggaa tcgaatgcaa ccggcgcagg aacactgcca gcgcatcaac aatattttca

1561 cctgaatcag gatattcttc taatacctgg aatgctgttt ccccggggat cgcagtggtg

1621 agtaaccatg catcatcagg agtacggata aaatgcttga tggtcggaag aggcataaat

1681 tccgtcagcc agtttagtct gaccatctca tctgtaacat cattggcaac gctacctttg

1741 ccatgtttca gaaacaactc tggcgcatcg ggcttcccat acaatcgata gattgtcgca

1801 cctgattgcc cgacattatc gcgagcccat ttatacccat ataaatcagc atccatgttg

1861 gaatttaatc gcggcctgga gcaagacgtt tcccgttgaa tatggctcat aacacccctt

1921 gtattactgt ttatgtaagc agacagtttt attgttcatg atgatatatt tttatcttgt

1981 gcaatgtaac atcagagatt ttgagacaca acgtggcttt gttgaataaa tcgaactttt

2041 gctgagttga aggatcagct cgagtgccac ctgacgtcta agaaaccatt attatcatga
```

45

```
2101 cattaaccta taaaaatagg cgtatcacga ggcagaattt cagataaaaa aaatccttag

2161 ctttcgctaa ggatgatttc tggaattcgc ggccgcttct agagcaatac gcaaaccgcc

2221 tctccccgcg cgttggccga ttcattaatg cagctggcac gacaggtttc ccgactggaa

2281 agcgggcagt gagcgcaacg caattaatgt gagttagctc actcattagg caccccaggc

2341 tttcactttt atgcttccgg ctcgtatgtt gtgtggaatt gtgagcggat aacaatttca

2401 cacatactag agaaagagga gaaatactag atggcttcct ccgaagacgt tatcaaagag

2461 ttcatgcgtt tcaaagttcg tatggaaggt tccgttaacg gtcacgagtt cgaaatcgaa

2521 ggtgaaggtg aaggtcgtcc gtacgaaggt acccagaccg ctaaactgaa agttaccaaa

2581 ggtggtccgc tgccgttcgc ttgggacatc ctgtccccgc agttccagta cggttccaaa

2641 gcttacgtta aacacccggc tgacatcccg gactacctga aactgtcctt cccggaaggt

2701 ttcaaatggg aacgtgttat gaacttcgaa gacggtggtg ttgttaccgt tacccaggac

2761 tcctccctgc aagacggtga gttcatctac aaagttaaac tgcgtggtac caacttcccg

2821 tccgacggtc cggttatgca gaaaaaaacc atgggttggg aagcttccac cgaacgtatg

2881 tacccggaag acggtgctct gaaaggtgaa atcaaaatgc gtctgaaact gaaagacggt

2941 ggtcactacg acgctgaagt taaaaccacc tacatggcta aaaaaccggt tcagctgccg

3001 ggtgcttaca aaaccgacat caaactggac atcacctccc acaacgaaga ctacaccatc

3061 gttgaacagt acgaacgtgc tgaaggtcgt cactccaccg gtgcttaata acgctgatag

3121 tgctagtgta gatcgctact agagccaggc atcaaataaa acgaaaggct cagtcgaaag
```

46

```
3181 actgggcctt tcgttttatc tgttgtttgt cggtgaacgc tctctactag agtcacactg

3241 gctcaccttc gggtgggcct ttctgcgttt ata
```

//

C.2.5 2-way Linear Product

```
LOCUS        2-Way Linear Product    1050 bp ds-DNA      linear        27-JUN-
2015

DEFINITION  .

FEATURES             Location/Qualifiers

    misc_feature    120..141

                    /label="Biobrick Prefix Standard (RFC10)"

    misc_feature    1..13

                    /label="exda22 5'overhang"

    misc_feature    1..381

                    /label="2WL-Promoter"

    misc_feature    354..1049

                    /label="2WL-BCP"

    misc_feature    354..381

                    /label="2WL-overlap"

    promoter        142..341

                    /label="R0010"

    primer_bind     448..469
```

48

```
                        /label="pSBamil_for"

    stem_loop       complement(80..83)

                        /label="end of terminator"

    CDS             368..1036

                        /label="amilCP"

    misc_feature    354..365

                        /label="exda12 5'overhang"

    stem_loop       complement(93..112)

                        /label="Stem loop"

    misc_feature    complement(371..381)

                        /label="exda23 5'overhang"

    RBS             350..361

                        /label="B0034"

ORIGIN

      1 TGAAGGATCA GCTTCTAAGA AACCATTATT ATCATGACAT TAACCtataa aaataggcgt

     61 atcacgaggc agaatttcag ataaaaaaaa tccttagctt tcgctaagga tgatttctgg

    121 aattcgcggc cgcttctaga gcaatacgca aaccgcctct ccccgcgcgt tggccgattc

    181 attaatgcag ctggcacgac aggtttcccg actggaaagc gggcagtgag cgcaacgcaa
```

49

```
 241 ttaatgtgag ttagctcact cattaggcac cccaggcttt acactttatg cttccggctc

 301 gtatgttgtg tggaattgtg agcggataac aatttcaCAC ATACTAGAGA AAGAGGAGAA

 361 ATACTAGATG AGTGTGATCG CTAAACAAAT GACCTACAAG Gtttatatgt caggcacggt

 421 caatggacac tactttgagg tcgaaggcga tggaaaaggt aagccctacg aggggagca

 481 gacggtaaag ctcactgtca ccaagggcgg acctctgcca tttgcttggg atattttatc

 541 accacagtgt cagtacggaa gcataccatt caccaagtac cctgaagaca tccctgacta

 601 tgtaaagcag tcattcccgg agggctatac atgggagagg atcatgaact ttgaagatgg

 661 tgcagtgtgt actgtcagca atgattccag catccaaggc aactgtttca tctaccatgt

 721 caagttctct ggtttgaact ttcctcccaa tggacctgtc atgcagaaga agacacaggg

 781 ctgggaaccc aacactgagc gtctctttgc acgagatgga atgctgctag gaaacaactt

 841 tatggctctg aagttagaag gaggcggtca ctatttgtgt gaatttaaaa ctacttacaa

 901 ggcaaagaag cctgtgaaga tgccagggta tcactatgtt gaccgcaaac tggatgtaac

 961 caatcacaac aaggattaca cttcggttga gcagtgtgaa atttccattG CACGCAAACC

1021 TGTGGTCGCC TAATAATACT AGAGCCAGGC

//
```

C.2.6 pUN Plasmid

```
LOCUS       T5-J23100-modBCP-GERC    4348 bp ds-DNA     circular     10-FEB-
2015

DEFINITION   .

FEATURES             Location/Qualifiers

    terminator       complement(1813..1866)

                     /label="putative terminator"

    misc_RNA         244..250

                     /label="cleaved 5'UTR"

    promoter         complement(3771..3799)

                     /label="AmpR promoter"

    misc_feature     1275..1314

                     /label="UNS9"

    promoter         383..417

                     /label="J23100 (strong promoter)"

    terminator       3910..4067

                     /label="rrnB terminator"

    misc_feature     2086..2915
```

51

```
                     /label="p15A origin"

  terminator         complement(4255..4295)

                     /label="Sal2 terminator"

  misc_binding       2754..2765

                     /label="Bsp24I site"

  misc_binding       1907..1912

                     /label=""

  terminator         4182..4226

                     /label="RNAI terminator"

  misc_feature       244..318

                     /label="RiboJ"

  primer_bind        119..158

                     /label="UNS7"

  misc_feature       complement(1125..1164)

                     /label="UNS5"

  terminator         complement(1441..1483)

                     /label="L17 terminator"

  CDS                complement(2920..3729)
```

```
                         /label="KanR"

        terminator       1328..1381

                         /label="R2-17 terminator"

        terminator       41..118

                         /label="B0055 Terminator (NCBI:AY643800)"

        misc_feature     319..358

                         /label="UNS6"

        enhancer         359..382

                         /label="Full UP Element"

        misc_feature     204..243

                         /label="UNS4"

        misc_feature     1..40

                         /label="UNSX"

        promoter         159..203

                         /label="T5 promoter"

        CDS              456..1124

                         /label="modified amilCP CDS"

ORIGIN
```

```
   1 ccaggataca tagattacca caactccgag cccttccacc aaggaatatt cagcaatttg

  61 cccgtgccga agaaaggccc acccgtgaag gtgagccagt gagttgattg ctacgtaaca

 121 agacgctggc tctgacattt ccgctactga actactcgTC ATAAAAAATT TATTTGCTTT

 181 GTGAGCGGAT AACAATTATA ATActgacct cctgccagca atagtaagac aacacgcaaa

 241 gtcagctgtc accggatgtg ctttccggtc tgatgagtcc gtgaggacga aacagcctct

 301 acaaataatt ttgtttaaCT CGTTCGCTGC CACCTAAGAA TACTCTACGG TCACATACGG

 361 AAAATTTTTT TAAAAAAAAA ACTTTACAGC TAGCTCAGTC CTAGGTATTA TGCTAGCTAC

 421 ATCCAATCGC CACTCAAATA AGGAGCACGT AAACAATGAG CGTTATCGCT AAACAAATGA

 481 CCTACAAGGT TTATATGTCA GGCACGGTCA ATGGACACTA CTTTGAGGTC GAAGGCGATG

 541 GAAAAGGTAA GCCCTACGAG GGGGAGCAGA CGGTAAAGCT CACTGTCACC AAGGGCGGAC

 601 CTCTGCCATT TGCTTGGGAT ATTTTATCAC CACAGTGTCA GTACGGAAGC ATACCATTCA

 661 CCAAGTACCC TGAAGACATC CCTGACTATG TAAAGCAGTC ATTCCCGGAG GGCTATACAT

 721 GGGAGAGGAT CATGAACTTT GAAGATGGTG CAGTGTGTAC TGTCAGCAAT GATTCCAGCA

 781 TCCAAGGCAA CTGTTTCATC TACCATGTCA AGTTCTCTGG TTTGAACTTT CCTCCCAATG

 841 GACCTGTCAT GCAGAAGAAG ACACAGGGCT GGGAACCCAA CACTGAGCGT CTCTTTGCAC

 901 GAGATGGAAT GCTGCTAGGA AACAACTTTA TGGCTCTGAA GTTAGAAGGA GGCGGTCACT

 961 ATTTGTGTGA ATTTAAAACT ACTTACAAGG CAAAGAAGCC TGTGAAGATG CCAGGGTATC

1021 ACTATGTTGA CCGCAAACTG GATGTAACCA ATCACAACAA GGATTACACT TCGGTTGAGC
```

54

```
1081  AGTGTGAAAT  TTCCATTGCA  CGCAAACCTG  TGGTCGCCTA  ATAACTCTAA  CGGACTTGAG

1141  TGAGGTTGTA  AAGGGAGTTG  GCTCCTCGGT  ACCAAATTCC  AGAAAAGAGG  CCTCCCGAAA

1201  GGGGGGCCTT  TTTTCGTTTT  GGTCCGGGAG  ACCAGAAACA  AAAAAAGGCC  GCGTTAGCGG

1261  CCTTCAATAA  TTGGGTTCCT  TATCATCTGG  CGAATCGGAC  CCACAAGAGC  ACTGtcgaga

1321  aggacacggt  taatactagg  cctgctggct  ggtaatcgcc  agcaggcctt  tttatttggg

1381  ggagagggaa  gtcatgaaaa  aactaacctt  tgaaattcga  tctccaccac  atcagctctg

1441  aagcaacgta  aaaaaacccg  ccccggcggg  ttttttttata  cccgtagtat  ccccacttat

1501  ctacaatagc  tgtccttaat  taatctagaa  aaatgaaggg  aagttcctat  actttctaga

1561  gaataggaac  ttctataggg  agtcgaataa  gggcgacaca  aaaggtattc  taaatgcata

1621  ataaatactg  ataacatctt  atagtttgta  ttatattttg  tattatcgtt  gacatgtata

1681  attttgatat  caaaaactga  ttttcccttt  attattttcg  agatttattt  tcttaattct

1741  ctttaacaaa  ctagaaatat  tgtatataca  aaaaatcata  aataatagat  gaatagttta

1801  attataggtg  ttcatcaatc  gaaaaagcaa  cgtatcttat  ttaaagtgcg  ttgctttttt

1861  ctcatttata  aggttaaata  attctcatat  atcaagcaaa  gtgacaggcg  cccttaaata

1921  ttctgacaaa  tgctctttcc  ctaaactccc  cccataaaaa  aacccgccga  agcgggtttt

1981  tacgttattt  gcggattaac  gattactcgt  tatcagaacc  gcccaggatg  cctggcagtt

2041  ccctactctc  gccgctgcgc  tcggtcgttc  ggctgcggga  cctcagcgct  agcggagtgt

2101  atactggctt  actatgttgg  cactgatgag  ggtgtcagtg  aagtgcttca  tgtggcagga
```

55

2161 gaaaaaaggc tgcaccggtg cgtcagcaga atatgtgata caggatatat tccgcttcct

2221 cgctcactga ctcgctacgc tcggtcgttc gactgcggcg agcggaaatg gcttacgaac

2281 ggggcggaga tttcctggaa gatgccagga agatacttaa cagggaagtg agagggccgc

2341 ggcaaagccg tttttccata ggctccgccc ccctgacaag catcacgaaa tctgacgctc

2401 aaatcagtgg tggcgaaacc cgacaggact ataaagatac caggcgtttc cccctggcgg

2461 ctccctcgtg cgctctcctg ttcctgcctt tcggtttacc ggtgtcattc cgctgttatg

2521 gccgcgtttg tctcattcca cgcctgacac tcagttccgg gtaggcagtt cgctccaagc

2581 tggactgtat gcacgaaccc cccgttcagt ccgaccgctg cgccttatcc ggtaactatc

2641 gtcttgagtc caacccggaa agacatgcaa aagcaccact ggcagcagcc actggtaatt

2701 gatttagagg agttagtctt gaagtcatgc gccggttaag gctaaactga aaggacaagt

2761 tttggtgact gcgctcctcc aagccagtta cctcggttca aagagttggt agctcagaga

2821 accttcgaaa aaccgccctg caaggcggtt ttttcgtttt cagagcaaga gattacgcgc

2881 agaccaaaac gatctcaaga agatcatctt attaagcttt tagaaaaact catcgagcat

2941 caaatgaaac tgcaatttat tcatatcagg attatcaata ccatattttt gaaaaagccg

3001 tttctgtaat gaaggagaaa actcaccgag gcagttccat aggatggcaa gatcctggta

3061 tcggtctgcg attccgactc gtccaacatc aatacaacct attaatttcc cctcgtcaaa

3121 aataaggtta tcaagtgaga aatcaccatg agtgacgact gaatccggtg agaatggcaa

3181 aagtttatgc atttctttcc agacttgttc aacaggccag ccattacgct cgtcatcaaa

56

```
3241  atcactcgca  tcaaccaaac  cgttattcat  tcgtgattgc  gcctgagcga  ggcgaaatac

3301  gcgatcgctg  ttaaaaggac  aattacaaac  aggaatcgag  tgcaaccggc  gcaggaacac

3361  tgccagcgca  tcaacaatat  tttcacctga  atcaggatat  tcttctaata  cctggaacgc

3421  tgttttttccg  gggatcgcag  tggtgagtaa  ccatgcatca  tcaggagtac  ggataaaatg

3481  cttgatggtc  ggaagtggca  taaattccgt  cagccagttt  agtctgacca  tctcatctgt

3541  aacatcattg  gcaacgctac  ctttgccatg  tttcagaaac  aactctggcg  catcgggctt

3601  cccatacaag  cgatagattg  tcgcacctga  ttgcccgaca  ttatcgcgag  cccatttata

3661  cccatataaa  tcagcatcca  tgttggaatt  taatcgcggc  ctcgacgttt  cccgttgaat

3721  atggctcata  ttcttccttt  ttcaatatta  ttgaagcatt  tatcagggtt  attgtctcat

3781  gagcggatac  atatttgaat  gtatttagaa  aaataaacaa  ataggggtca  gtgttacaac

3841  caattaacca  attctgaaca  ttatcgcgag  cccatttata  cctgaatatg  gctcataaca

3901  cccccttgttt  gcctggcggc  agtagcgcgg  tggtcccacc  tgaccccatg  ccgaactcag

3961  aagtgaaacg  ccgtagcgcc  gatggtagtg  tggggactcc  ccatgcgaga  gtagggaact

4021  gccaggcatc  aaataaaacg  aaaggctcag  tcgaaagact  gggcctttcg  cccgggctaa

4081  ttaggggggtg  tcgcccttat  tcgactctat  agggaagttc  ctattctcta  gaaagtatag

4141  gaacttctga  aggggggctc  gagcggccgc  aaaaggaaaa  gatccggcaa  acaaaccacc

4201  gttggtagcg  gtggtttttt  tgtttggatc  gacaatcttc  gtaagcgtca  tcaataagcg

4261  taaaaaaacc  gggcaatgcc  cggttttttta  atgagaaatt  ttacctgtcg  tagccgccac
```

57

```
4321 catccggcaa agaagcatac aaggcttt

     //
```

C.2.7 *Pfu* expression plasmid for *ex vivo* PCR

```
LOCUS          pUN-rhaBp-Pfu          5887 bp ds-DNA     circular     27-JUN-
2015

DEFINITION  .

FEATURES                Location/Qualifiers

     -10_signal     280..285

                        /label="-10 region"

     primer_bind    119..158

                        /label="UNS7"

     RBS            302..335

                        /label="synRBS (60K TIR)"

     terminator     complement(2980..3022)

                        /label="L17 terminator"

     CDS            complement(4459..5268)

                        /label="KanR"

     protein_bind   159..180

                        /label="CRP-cAMP binding site"

     misc_binding   4293..4304
```

59

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                  /label="Bsp24I site"

terminator        2867..2920

                  /label="R2-17 terminator"

misc_feature      1..40

                  /label="UNSX"

misc_feature      3625..4454

                  /label="p15A origin"

terminator        complement(5794..5834)

                  /label="Sal2 terminator"

misc_feature      2814..2853

                  /label="UNS9"

-35_signal        259..264

                  /label="-35 region"

terminator        41..118

                  /label="B0055 Terminator (NCBI:AY643800)"

misc_signal       292..292

                  /label="TSS"

misc_feature      1221..1262
```

60

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                              /label="gBlock overlap"


protein_bind      244..260

                              /label="RhaS binding site"


promoter          159..301

                              /label="rhaBADp"


misc_feature      complement(2664..2703)

                              /label="UNS5"


CDS               336..2663

                              /label="PfuDNApol"


terminator        5721..5765

                              /label="RNAI terminator"


protein_bind      189..210

                              /label="CRP-cAMP binding site"


promoter          complement(5310..5338)

                              /label="AmpR promoter"


terminator        5449..5606

                              /label="rrnB terminator"


misc_binding      3446..3451
```

```
                /label=""

     misc_signal    264..269

                /label="EcoRI site"

     protein_bind   211..227

                /label="RhaS binding site"

     terminator     complement(3352..3405)

                /label="putative terminator"

ORIGIN

     1 ccaggataca tagattacca caactccgag cccttccacc aaggaatatt cagcaatttg

    61 cccgtgccga agaaaggccc acccgtgaag gtgagccagt gagttgattg ctacgtaaca

   121 agacgctggc tctgacattt ccgctactga actactcgcg gtgagcatca catcaccaca

   181 attcagcaaa ttgtgaacat catcacgttc atctttccct ggttgccaat ggcccatttt

   241 cctgtcagta acgagaaggt cgcgtattca ggcgcttttt agactggtcg taatgaaatt

   301 cAATAAAGCA AGCAAACACT ATAAGGAGTC CGCACATGAT CCTGGATGTC GATTACATCA

   361 CCGAAGAAGG CAAACCGGTG ATCCGTCTGT TCAAAAAAGA AAATGGTAAA TTCAAGATAG

   421 AACACGATCG CACCTTTCGC CCTTATATTT ATGCACTGTT ACGCGATGAC AGCAAAATCG

   481 AAGAAGTCAA AAAGATCACC GGCGAACGCC ACGGTAAAAT CGTGCGCATT GTTGATGTTG

   541 AAAAAGTGGA GAAAAAATTC CTGGGCAAGC CAATTACTGT CTGGAAGCTC TACCTGGAAC
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62

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 601 ATCCGCAGGA TGTGCCGACG ATTCGTGAAA AGGTGCGTGA GCATCCCGCG GTGGTGGATA

 661 TTTTTGAATA TGACATTCCG TTTGCCAAAC GGTATCTGAT TGATAAAGGG CTGATCCCGA

 721 TGGAAGGTGA AGAGGAACTG AAAATTCTGG CATTTGATAT CGAAACGCTG TATCACGAAG

 781 GTGAGGAGTT CGGCAAAGGC CCGATCATCA TGATCAGCTA CGCTGATGAA AATGAAGCAA

 841 AAGTAATTAC CTGGAAAAAC ATCGACCTGC CGTATGTCGA AGTTGTTTCT TCAGAGCGCG

 901 AAATGATTAA ACGCTTTTTA CGGATTATTC GCGAAAAAGA CCCAGACATC ATTGTCACCT

 961 ACAACGGTGA CAGTTTCGAT TTCCCGTACC TTGCGAAGCG TGCAGAAAAA CTCGGCATTA

1021 AACTGACCAT TGGTCGTGAC GGCAGCGAGC CAAAAATGCA GCGTATTGGC GATATGACCG

1081 CCGTAGAAGT AAAAGGCCGT ATCCATTTTG ACCTTTATCA TGTTATCACT CGCACGATCA

1141 ACCTGCCAAC ATACACCCTC GAGGCGGTTT ATGAAGCGAT CTTCGGTAAG CCGAAAGAAA

1201 AAGTGTATGC CGATGAAATT GCGAAAGCGT GGGAATCCGG CGAGAATCTG GAACGTGTTG

1261 CCAAATACAG CATGGAAGAT GCCAAAGCTA CTTACGAATT GGGAAAGAG TTTTTGCCAA

1321 TGGAGATTCA GCTGAGCCGC CTGGTTGGTC AGCCGCTGTG GGATGTATCC CGTTCTTCGA

1381 CGGGTAACCT GGTGGAATGG TTTCTCTTGC GTAAGGCTTA TGAGCGTAAC GAAGTGGCGC

1441 CGAACAAACC TTCTGAAGAA GAGTATCAGC GCCGTTTGCG CGAGAGTTAT ACCGGTGGTT

1501 TTGTGAAAGA ACCGGAGAAA GGATTATGGG AAAACATTGT CTATCTCGAC TTCCGCGCGC

1561 TGTATCCGTC TATTATTATC ACCCACAACG TGTCACCGGA TACCCTGAAC CTCGAAGGCT

1621 GTAAAAATTA CGATATTGCC CCGCAAGTCG GTCACAAATT TTGCAAAGAT ATCCCCGGAT
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63

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1681  TTATTCCTTC GCTGCTGGGG CATCTGCTGG AAGAGCGGCA AAAAATTAAA ACGAAAATGA

1741  AAGAAACACA AGATCCGATT GAGAAGATTT TGCTCGATTA TCGCCAGAAA GCAATAAAAT

1801  TGCTGGCGAA CAGCTTCTAC GGCTACTACG GTTATGCGAA AGCCCGCTGG TATTGTAAGG

1861  AGTGCGCCGA AAGTGTCACT GCCTGGGGCC GCAAATATAT TGAACTGGTA TGGAAAGAGC

1921  TGGAAGAGAA GTTTGGTTTT AAAGTTCTTT ACATCGACAC CGATGGTCTG TACGCCACGA

1981  TTCCCGGCGG CGAAAGCGAA GAAATTAAGA AGAAAGCACT TGAATTTGTC AAGTATATCA

2041  ATTCGAAACT GCCTGGCCTG CTGGAGCTTG AGTACGAGGG GTTTTATAAA CGCGGTTTCT

2101  TTGTTACCAA AAAACGTTAT GCTGTGATTG ACGAAGAAGG GAAAGTCATT ACCCGCGGCT

2161  TAGAGATCGT TCGTCGCGAC TGGAGCGAAA TCGCCAAGGA AACTCAGGCG CGCGTGCTGG

2221  AAACCATCTT AAAACACGGC GACGTTGAGG AAGCCGTTCG CATTGTGAAG GAAGTGATTC

2281  AAAAGCTTGC CAACTACGAA ATCCCGCCGG AAAAACTGGC CATCTATGAA CAGATTACGC

2341  GTCCGCTGCA TGAATACAAA GCGATTGGGC CGCATGTCGC AGTGGCAAAA AAGCTGGCGG

2401  CAAAAGGTGT GAAAATCAAA CCGGGCATGG TGATCGGCTA TATCGTACTG CGTGGTGATG

2461  GCCCAATCAG TAATCGTGCG ATACTGGCTG AAGAATACGA CCCGAAGAAA CATAAATATG

2521  ATGCGGAATA TTACATTGAA AACCAGGTTC TGCCGGCGGT ATTACGTATT CTGGAGGGCT

2581  TTGGCTACCG TAAAGAGGAT CTGCGCTATC AAAAAACCCG TCAGGTGGGA CTGACGAGCT

2641  GGCTGAATAT CAAAAAAAGC TAACTCTAAC GGACTTGAGT GAGGTTGTAA AGGGAGTTGG

2701  CTCCTCGGTA CCAAATTCCA GAAAAGAGGC CTCCCGAAAG GGGGGCCTTT TTTCGTTTTG
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64

2761 GTCCGGGAGA CCAGAAACAA AAAAAGGCCG CGTTAGCGGC CTTCAATAAT TGGGTTCCTT

2821 ATCATCTGGC GAATCGGACC CACAAGAGCA CTGtcgagaa ggacacggtt aatactaggc

2881 ctgctggctg gtaatcgcca gcaggccttt ttatttgggg gagagggaag tcatgaaaaa

2941 actaaccttt gaaattcgat ctccaccaca tcagctctga agcaacgtaa aaaaacccgc

3001 cccggcgggt tttttatac ccgtagtatc cccacttatc tacaatagct gtccttaatt

3061 aatctagaaa aatgaaggga agttcctata ctttctagag aataggaact tctataggga

3121 gtcgaataag ggcgacacaa aaggtattct aaatgcataa taaatactga taacatctta

3181 tagtttgtat tatattttgt attatcgttg acatgtataa ttttgatatc aaaaactgat

3241 tttccctta ttattttcga gatttatttt cttaattctc tttaacaaac tagaaatatt

3301 gtatatacaa aaaatcataa ataatagatg aatagtttaa ttataggtgt tcatcaatcg

3361 aaaaagcaac gtatcttatt taaagtgcgt tgcttttttc tcatttataa ggttaaataa

3421 ttctcatata tcaagcaaag tgacaggcgc ccttaaatat tctgacaaat gctctttccc

3481 taaactcccc ccataaaaaa acccgccgaa gcgggttttt acgttatttg cggattaacg

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3781 cggtcgttcg actgcggcga gcggaaatgg cttacgaacg gggcggagat ttcctggaag

65

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3841 atgccaggaa gatacttaac agggaagtga gagggccgcg gcaaagccgt ttttccatag

3901 gctccgcccc cctgacaagc atcacgaaat ctgacgctca aatcagtggt ggcgaaaccc

3961 gacaggacta taaagatacc aggcgtttcc ccctggcggc tccctcgtgc gctctcctgt

4021 tcctgccttt cggtttaccg gtgtcattcc gctgttatgg ccgcgtttgt ctcattccac

4081 gcctgacact cagttccggg taggcagttc gctccaagct ggactgtatg cacgaacccc

4141 ccgttcagtc cgaccgctgc gccttatccg gtaactatcg tcttgagtcc aacccggaaa

4201 gacatgcaaa agcaccactg gcagcagcca ctggtaattg atttagagga gttagtcttg

4261 aagtcatgcg ccggttaagg ctaaactgaa aggacaagtt ttggtgactg cgctcctcca

4321 agccagttac ctcggttcaa agagttggta gctcagagaa ccttcgaaaa accgccctgc

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4441 gatcatctta ttaagctttt agaaaaactc atcgagcatc aaatgaaact gcaatttatt

4501 catatcagga ttatcaatac catatttttg aaaaagccgt ttctgtaatg aaggagaaaa

4561 ctcaccgagg cagttccata ggatggcaag atcctggtat cggtctgcga ttccgactcg

4621 tccaacatca atacaaccta ttaatttccc ctcgtcaaaa ataaggttat caagtgagaa

4681 atcaccatga gtgacgactg aatccggtga gaatggcaaa agtttatgca tttctttcca

4741 gacttgttca acaggccagc cattacgctc gtcatcaaaa tcactcgcat caaccaaacc

4801 gttattcatt cgtgattgcg cctgagcgag gcgaaatacg cgatcgctgt taaaaggaca

4861 attacaaaca ggaatcgagt gcaaccggcg caggaacact gccagcgcat caacaatatt
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66

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4921 ttcacctgaa tcaggatatt cttctaatac ctggaacgct gttttttccgg ggatcgcagt

4981 ggtgagtaac catgcatcat caggagtacg gataaaatgc ttgatggtcg gaagtggcat

5041 aaattccgtc agccagttta gtctgaccat ctcatctgta acatcattgg caacgctacc

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5881 aggcttt
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