Computationally Optimised DNA Assembly of synthetic genes

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Abstract: Gene synthesis is hampered by two obstacles:

- improper assembly of oligonucleotides
- oligonucleotide defects incurred during chemical synthesis.

To overcome the first problem, we describe the employment of a Computationally Optimised DNA Assembly (CODA) algorithm that uses the degeneracy of the genetic code to design overlapping oligonucleotides with thermodynamic properties for self-assembly into a single, linear, DNA product. To address the second problem, we describe a hierarchical assembly strategy that reduces the incorporation of defective oligonucleotides into full-length gene constructs. The CODA algorithm and these biological methods enable fast, simple and reliable assemblies of sequence-correct full-length genes.

Keywords: synthetic biology; computational biology; DNA self-assembly; gene synthesis; Computationally Optimised DNA Assembly; CODA; bioinformatics.

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1 Introduction

It is often desirable to obtain a synthetic DNA molecule that encodes a protein of interest and has a sequence simultaneously optimised for several arbitrarily specified sequence properties, such as its own self-assembly, codon usage (Grosjean and Fiers, 1982) GC/AT ratio (Breslauer et al., 1986; Lio, 2002), translational kinetics (Sorensen et al., 1989), codon-pair bias (Gutman and Hatfield, 1989; Hatfield, 1993; Irwin et al., 1995) and other DNA structural scales (Seeman, 1998, 1999). Since synthesis of long gene sequences is not feasible because of cumulative chemical synthesis errors, largely point deletions (Smith et al., 2003), most synthetic genes are assembled from a large number of short partially overlapping complementary oligonucleotides (Stemmer et al., 1995). These overlapping oligonucleotides are assembled into long double-stranded DNA with ligation and polymerase extension reactions, either alone or in combination, in processes known variously as assembly PCR (Casimiro et al., 1997), splicing by overlap extension (Warrens et al., 1997), polymerase chain assembly (Smith et al., 2003), recursive PCR (Prodromou and Pearl, 1992), and others (Mandecki and Bolling, 1988; Theriault et al., 1988). However, all such approaches confront two major obstacles to accurate and reliable gene synthesis. Firstly, incorrect hybridisations during assembly produce an olio of DNA products of widely differing lengths predominantly arranged in the wrong order. Secondly, oligonucleotide synthesis defects produce frameshift (nonsense) mutations and other single-point errors. For example, with a 99.5% coupling efficiency, nearly 25% of chemically synthesised oligonucleotides 50 nucleotides in length contain an error.

Methods described in this paper address both problems by combining information technology with biological methods. Information technology is used to design a DNA sequence that encodes, simultaneously, the correct amino acid sequence, its own correct self-assembly from unpurified purchased oligonucleotides, a target expression organism, and other desired sequence properties. An efficient genetic screen to eliminate translational frameshifts caused by defective oligonucleotides also is described.

2 Methods

2.1 Materials

Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). PfuUltra High-Fidelity DNA Polymerase was purchased from Strategene (La Jolla, CA). The expression vector, pET-3a, was purchased from Novagen (Gibbstown, NJ). Restriction enzymes *Nde*I, *Bam*HI, *Eco*RI, Mung Bean Nuclease, and T4 DNA ligase were obtained from New England Biolabs. Zero Blunt TOPO PCR Cloning kit was purchased from Invitrogen. pGEM-3Z vector was obtained from Promega (Madison, WI).

2.2 Sequence optimisation engine

Each possible codon assignment influences several DNA sequence properties of interest, and all properties are optimised simultaneously. We define a general sequence objective function (figure of merit) called the Usability Codon Index, which is the harmonic mean of the desired properties, $N / \sum_{i=1}^{N} (1 / f_i)$, where each property f_i takes values in the half-open interval (0, 1] and higher values are better. The properties used here were:

- the melting temperature gap discussed below
- the codon adaptation index (Sharp and Li, 1987) for preference of codons of abundant tRNA isoacceptors (Boycheva et al., 2003; Gutman and Hatfield, 1989; Hatfield, 1993); the avoidance of consecutive rare codons (Hatfield, 1993; Moore and Maranas, 2002)
- codon pair statistics to minimise codon pairs with slow translational step-times (Gutman and Hatfield, 1989; Irwin et al., 1995).

Thus, each gene sequence is designed to encode its own correct self-assembly using synonymous codon substitutions for reliable expression in any in vivo or in vitro system of choice.

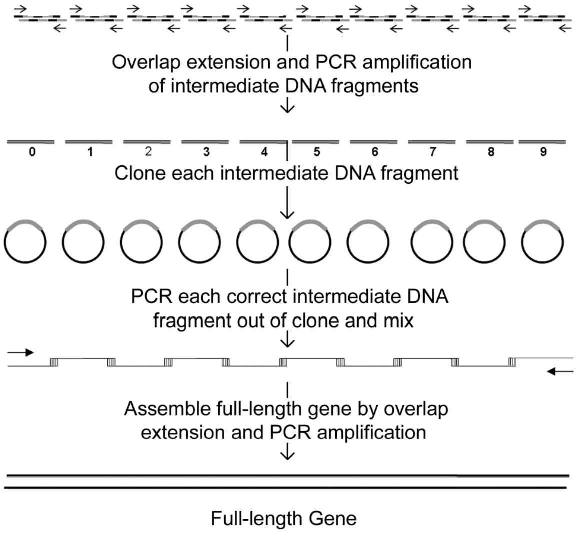
2.3 Computational infrastructure

The computational optimisation framework uses a multi-queue branch-and-bound algorithm (Lathrop et al., 2001). Allegro Common LISP was licensed from Franz, Inc. DNA secondary structure and melting temperatures were estimated using the Mfold software package (Zuker, 1989; Zuker et al., 1999) based on nearest-neighbour thermodynamic parameters (SantaLucia, 1998) Codon usage was estimated using the codon adaptation index (Sharp and Li, 1987) and codon pair statistics (Boycheva et al., 2003; Gutman and Hatfield, 1989; Moore and Maranas, 2002). Computations were performed on a 64-node cluster of 3 GHz Xeon dual processors.

2.4 Parallel system architecture

The system exploits both coarse- and fine-grained parallelism simultaneously on the same cluster. At a coarse-grain level, the design of a gene is divided into sub-designs for each intermediate DNA fragment. The sub-designs are run in parallel and recombined into an initial gene design which is then further refined. The coarse-grain level executes a general multi-queue branch-and-bound optimisation engine, written in LISP, consuming one cluster node for the design of each intermediate DNA fragment (Figure 1). At a fine-grain level, each step of sequence design requires estimating the melting temperatures of a large number of possible hybridisations. These are run in parallel and later recombined to yield an estimate of the melting temperature gap for that particular step of the design. The fine-grain level executes a series of PERL programs and Linux shell scripts that divide a large DNA melting run into individual Mfold thermodynamic calculations to be executed in parallel on the cluster.

Figure 1 Gene Assembly Schematics. Self-assembled overlapping oligonucleotides are extended by overlap extension and PCR to generate intermediate DNA fragments. The intermediate DNA fragments are cloned to be sequenced and PCR amplified from the selected vectors. Intermediate DNA fragments with correct DNA sequences are mixed together and extended by overlap extension and PCR amplification to generate the full-length gene


Overlap extension and PCR amplification
of intermediate DNA fragments
0
1
2
3
4
5
6
7
8
9
Clone each intermediate DNA fragment
PCR each correct intermediate DNA
fragment out of clone and mix
Assemble full-length gene by overlap
extension and PCR amplification
Full-length Gene

2.5 Assembly of intermediate DNA fragments

For each intermediate DNA fragment, the constituent oligonucleotide set was added to a primer extension reaction at a final concentration of 0.1 μM along with an excess (1 μM) of leader and trailer primer oligonucleotide (the most 5'- and 3'-distant oligonucleotides). Each oligonucleotide set was extended into an intermediate DNA fragment with 2.5 U of PfuUltra High-Fidelity DNA polymerase (Stratagene), 300 μM dNTPs (Roche Diagnostics, Indianapolis, IN), and 1X PfuUltra reaction buffer. These primer extension and PCR amplification reactions were performed in a MJ Research PTC-225 thermal cycler using the following calculated-control protocol: 1 min denaturation step at 95ºC, followed by 35 cycles of 10 s at 95ºC, 40 s at 60ºC, and 2 min at 72ºC, and a final step of 2 min at 72ºC.

2.6 Topo cloning and sequencing of intermediate DNA fragments

For each intermediate DNA fragment, 1 μL of the PCR product of the assembly reaction was used for cloning into a pCRII-Blunt-TOPO vector (Invitrogen (Carlsbad, CA)) according to the manufacturer's protocol. Reactions were incubated for 5 min at room temperature and 1 μL of each reaction was used for transformation of One Shot electrocompetent cells according to the manufacture's protocol. Following transformation, plasmid DNA was isolated for DNA sequencing.

2.7 Preparation and use of CODA blue vector for frameshift screening

pGEM-3Z plasmid DNA was digested with *Eco*RI and treated with Mung Bean Nuclease (New England Biolabs (Beverly, MA)) according to the manufacturer's protocol. The linearised plasmid was extracted and purified from an agarosegel with a Qiagen MinElute gel extraction kit and religated with T4 DNA ligase. The four bases (AATT) deleted from this plasmid result in *a* + 1 frameshift in the *α*-complementing subunit of *β*-galactosidase. Intermediate DNA fragments were cloned into the *Bam*HI site of the CODA Blue vector and transformed in *E. coli* JM109 and plated on LB agar plates containing 100 μg/ml ampicillin, 80 μg/ml 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside (X-Gal) and 0.5 mM Isopropyl-*β*-D-thiogalactopyranoside (IPTG). Blue colonies were chosen for plasmid DNA isolation and DNA sequencing.

2.8 Construction of the full-length Integrase (IN) gene

The 5'- and 3'-most distant oligonucleotides were used to PCR amplify each sequence verified intermediate DNA fragment with PfuUltra High-Fidelity DNA polymerase (Stratagene). Next, 1 μl of each intermediate DNA fragment PCR product was combined with an *IN* gene leader (1 μM) containing an *Nde*I site and a trailer (1 μM) containing a *Bam*HI site. The full-length *IN* gene was isolated by primer extension and PCR amplification with 2.5 U of PfuUltra High-Fidelity DNA polymerase (Stratagene), 300 μM dNTPs, and 1X PfuUltra buffer. These primer extension and PCR amplification reactions were performed in a MJ Research PTC-225 thermal cycler using the following calculated-control protocol: 5 min denaturation step at 95ºC, followed by 35 cycles of 30 s at 95ºC, 30 s at 68ºC, and 5 min at 72ºC, and a final step of 5 min at 72ºC. The PCR products were subjected to electrophoresis in 1% agarose gel. Full-length *IN* gene was inserted into the *Nde*I and *Bam*HI sites of the expression vector pET-3a (Novagen) and full-length *IN* sequence was verified by DNA sequencing and protein expression.

2.9 Probabilities

Sequencing of Intermediate DNA Fragments without Frameshift Screening. Let P_e be the per-base error rate, L the length of a clone in bases, N the number of clones sequenced, P_c the probability that any given clone is correct, and P_o the probability that at least one of the N sequenced clones is correct. In these cases, $P_c = (1 - P_e)^L$ and $P_o = (1 - (1 - P_c)^N)$. If the gene consists of M intermediate fragments, each of length L, and N clones are sequenced for each fragment, then the probability that every one of the M fragments has at least one correct clone is $P_g = P_o^M$.

Sequencing of Intermediate DNA Fragments after Frameshift Screening. Let P_d be the per-base point deletion rate, P_i the per-base point insertion rate, and P_m the per-base point mutation rate. Assume $P_e = P_d + P_i + P_m$ and $1 >> P_d, P_i, P_m$ so that product terms may be neglected. Let $P_{[d=j]}$ be the probability of exactly j deletions in any given clone, $P_{[i=j]}$ of exactly j insertions, and $P_{[m=j]}$ of exactly j mutations. Then $P_{[d=j]} = (L_j)P_d^j(1-P_d)^{(L-j)}$, where $(^L{}_j) = L!/j!(L-j)!$ is the number of ways of choosing j of L objects. Similar expressions are obtained for $P_{[i=j]}$ and $P_{[m=j]}$. Let P_b be the probability that an insert in the CODA Blue plasmid turns the clone blue. A clone turns blue when the number of point insertions minus the number of point deletions is an integer multiple of three. Thus, $P_b = \sum_{((j-k \bmod 3) = 0)} P_{[i=j]}P_{[d=k]}$. The probability that any single clone is correct, given that it turns blue, is $P_{[c|b]} = P_{[d=0]}P_{[i=0]}P_{[m=0]}/P_b = P_c/P_b$. Probabilities corresponding to P_o and P_g above, but given blue clones, may be obtained by substituting $P_{[c|b]}$ for P_c. Thus, $P_{[o|b]} = (1 - (1 - P_{[c|b]})^{\mathrm{N}})$ and $P_{[g|b]} = P_{[o|b]}^{\mathrm{M}}$.

3 Results

In this paper, we describe the assembly of a 1,640 bp synthetic gene of the yeast, *Saccharomyces cerevisiae*, transposable element Ty3 Integrase (IN) protein optimised for expression in *E. coli.* Figure 1 illustrates the assembly process. The gene was divided into ten intermediate DNA fragments, each of which was subdivided into six or eight short overlapping and abutting oligonucleotides. DNA sequences for each oligonucleotide were designed computationally and purchased commercially. Self-assembled intermediate DNA fragments were isolated by polymerase extension and cloned. Each intermediate DNA fragment was amplified from selected clones and combined in another primer extension and PCR amplification reaction to generate the full-length *IN* gene.

3.1 Computationally Optimised DNA Assembly (CODA)

Given the amino acid sequence of the Ty3 IN protein, the CODA algorithm described in Methods generated a list of linearly overlapping and abutting oligonucleotides usually 45–50 nucleotides (nts) long, optimised for self-assembly and expression in *E. coli.* Correct self-assembly is achieved by a designed melting temperature gap, defined as [the minimum melting temperature of any correct hybridisation] minus [the maximum melting temperature of any incorrect hybridisation]. Correct hybridisations occur only at the overlap regions between adjacent segments when they are aligned to hybridise linearly across the entire extent of the overlap. Incorrect hybridisations occur as:

- hairpins, where an oligonucleotide folds back and hybridises to itself
- dimers, where an oligonucleotide is partially self-complementary
- inter-oligonucleotide mismatches, where one oligonucleotide is partially complementary to another
- shifted correct matches, where a misaligned overlap region is partially complementary to another region within the same overlap.

While previous approaches have removed local DNA structure such as hairpins and palindromes, the key extension here is to examine all possible incorrect hybridisations exhaustively.

A melting temperature gap. Different oligonucleotides possess different melting temperatures depending on their base composition and degree of base pairing complementarity (Breslauer et al., 1986; Mathews et al., 1999; SantaLucia, 1998). The CODA algorithm takes advantage of the degeneracy of the genetic code to assign codons so that correct hybridisations of the designed overlapping oligonucleotides have a high melting temperature and incorrect hybridisations have a low melting temperature. Thus, there exists a temperature gap within which, with high thermodynamic probability, correct hybridisations are annealed and incorrect hybridisations are melted. The graphs in Figure 2 show the computational melting temperatures for correct and incorrect hybridisations without (Figure 2(a)) and with (Figure 2(b)) melting temperature gap optimisations (CODA). In Figure 2(a), codons simply were chosen to be the most preferred codons in *E. coli* highly expressed genes, i.e., codons yielding a Codon Adaptation Index of 1.0. Not surprisingly, the melting temperature distributions of correct and incorrect hybridisations intersect when no CODA optimisation is performed. On the other hand, the melting temperature distributions of CODA designed sequences show a melting temperature gap of 18^{o}C between correct hybridisations and incorrect hybridisations (Figure 2(b)).

Assembly of intermediate DNA fragments and full-length IN gene. The full-length *IN* gene was designed as ten separate intermediate DNA fragments overlapping by approximately 50 nts. Each intermediate DNA fragment was designed as a set of 6–8 oligonucleotides overlapping one another by 20–30 nts (fragment 0: 196 bp, 8 oligos; fragment 1: 224 bp, 8 oligos; fragment 2: 224 bp, 8 oligos; fragment 3: 223 bp, 8 oligos; fragment 4: 227 bp, 8 oligos; fragment 5: 223 bp, 8 oligos; fragment 6: 224 bp, 8 oligos; fragment 7: 172 bp, 6 oligos; fragment 8: 175 bp, 6 oligos; fragment 9: 174 bp, 8 oligos).

The electrophoretograms of Figure 2(c) and (d) show the results of gene assemblies from oligonucleotides with the melting temperature distributions of Figure 2(a) and (b), respectively. Intermediate DNA fragments assembled from oligonucleotides optimised only for codon usage, but not for self-assembly (Figure 2(a)), show a broad range of products both larger and smaller than expected (Figure 2(c), lanes 1–10). However, all intermediate DNA fragments assembled from CODA designed oligonucleotides (Figure 2(b)) resulted in single products of the expected length (Figure 2(d), lanes 1–10). In addition, a single full-length (1,640 bp) gene product was obtained from the assembly of ten CODA designed intermediate DNA fragments (Figure 2(d), lane 12), whereas no apparent full-length product was obtained from the assembly of fragments not CODA designed for self-assembly (Figure 2(c), lane 12).

3.2 Codon usage optimisation

In addition to optimising genes for self-assembly, the CODA algorithm also optimises genes for expression in an in vivo or in vitro system of choice. The Ty3 *IN* gene was optimised to be expressed in *E. coli.* The data plotted in Figure 3(a) compare codon usage in the CODA designed gene with genomic codon usage of highly expressed genes in *E. coli*. These data show that the *E. coli* codon usage of the CODA designed *IN* gene is comparable to the codon usage of highly expressed *E. coli* genes. Indeed, the codon optimised synthetic *IN* gene expressed from a pET-3a vector in *E. coli* strain BL-21 (DE3) produced more than 15% of the total cell protein (Figure 3(b)).

Figure 2 Hybridisation melting temperatures and assembly of the integrase (IN) gene of the S. cerevisiae transposable element Ty3. Computational melting temperatures without (A) and with (B) CODA melting temperature optimisations. Solid and dashed lines represent correct hybridisations of oligonucleotides and intermediate DNA fragments, respectively. Dot-dash and dotted lines represent corresponding incorrect hybridisations. Lanes 1-10, IN intermediate DNA fragments 1 through 10 assembled from oligonucleotides without (C) and with (D) CODA melting temperature optimisations. Lanes 11 and 13, molecular weight markers. Lane 12, corresponding assembly of full-length IN gene (1,640 bp)

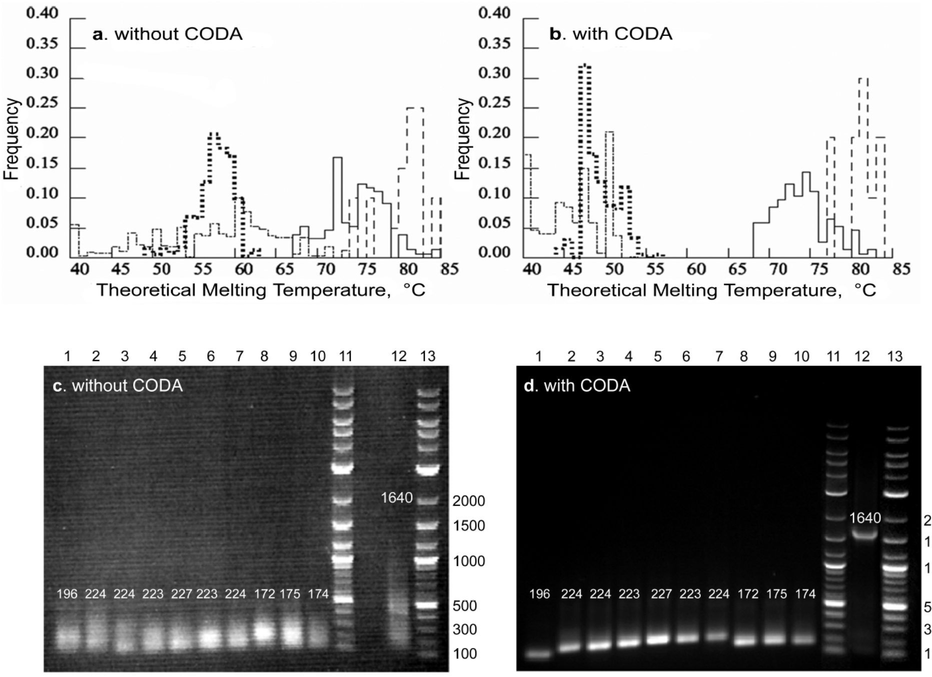
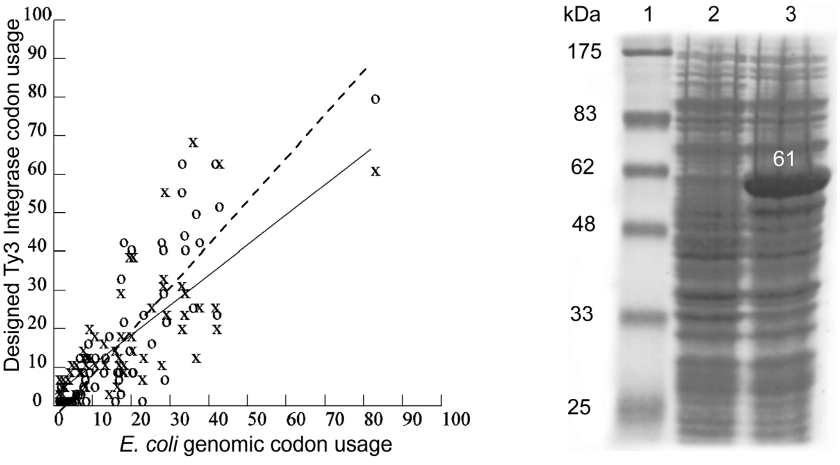


Figure 3 Codon usage in the CODA designed Ty3 IN gene vs. codon usage in E. coli highly expressed genes. A. X (solid regression line), CODA designed IN gene. O (dashed regression line), E. coli highly expressed genes. Each codon *x*-coordinate is usage per 1,000 in E. coli average genomic DNA; *y*-coordinate is in the CODA gene (X, solid line) or in highly expressed genes (O, dashed line). B. 10% SDS coomassie stained gel. Protein Marker (Lane 1). Protein products from uninduced (Lane 2) and induced (Lane 3) cells



(a) (b)

3.3 Errors in oligonucleotide synthesis

The chemical synthesis of oligonucleotides is never 100% efficient. Each base addition in the synthesis process is commonly 98–99.5% efficient, resulting in the formation of failed sequences with single-base deletions and other defects. Our prior sequencing results of several CODA synthetic genes, assembled from hundreds of commercially prepared, partially overlapping oligonucleotides, revealed an error rate per base in non-overlapping (single stranded) regions nearly twice as high as the error rate per base in overlapping (double stranded) regions. We reasoned that this difference was due to the selection for perfect complementarity in overlapping complementary regions. Therefore, to achieve a lower error rate in the gene assembly process, we have chosen to assemble genes with completely overlapping (abutting) oligonucleotides.

To obtain an estimate of the error rate per bp, we sequenced 8,300 bp from four independent clones of each of the ten integrase intermediate DNA fragments. The sequencing results show that, on average, we encounter an error every 218 bp (0.46%). Of these, we observe: a bp deletion every 553 bp (0.18%); a bp insertion every 638 bp (0.16%); and a bp substitution every 692 bp (0.12%).

To reduce this error rate, we developed a simple genetic frameshift screen using the CODA Blue vector (Section 2). This vector was altered from pGEM-3Z (Promega) by introducing *a* + 1 frameshift, such that it is not able to restore β-galactosidase activity in an *E. coli* JM109 strain with a *lacZ* gene missing the α-fragment sequence. Therefore, cells containing intact CODA Blue plasmid grow as white colonies on LB agar plates containing IPTG and X-gal. The intermediate DNA fragments of *IN* gene were engineered to be 3N + 1 base pairs long and to contain flanking *Bam*HI sites. When these

*Bam*HI-(3N + 1)-*Bam*HI intermediate DNA gene fragments are cloned into the *Bam*HI site of the CODA Blue vector, *a* – 1 frameshift is created that restores the correct reading frame of the α-fragment coding sequence. Now the plasmid produces active, in frame, α-fragment that restores β-galactosidase activity. Thus, cells containing the CODA Blue plasmid with a *Bam*HI-(3N + 1)-*Bam*HI insert grow as easily identifiable blue colonies.

We cloned intermediate DNA gene fragments into the CODA Blue vector and picked blue colonies. This eliminated all point deletion or insertion errors except where the fragment length is altered by a multiple of three nucleotides. The sequence data from 8,300 bp, as four blue colonies from each of the ten intermediate DNA fragments, demonstrate that the frameshift screen nearly halves the total error rate per bp. The sequencing results show that, on average, we encounter an error every 417 bp (0.24%). Of these, we observe: a bp deletion every 750 bp (0.13%); a bp insertion every 4,165bp (0.024%); and a bp substitution every 1,190 bp (0.084%). The data in Table 1 show that, as expected, all deletions and insertions that escape the frameshift screen, in fact, are multiples of three.

Table 1 Observed and expected errors per intermediate DNA fragment. d, i, and s are the number of deletions, insertions, and substitutions in each intermediate DNA fragment. Integrase and Integrase blue are as in (a). # gives the observed number (and % the percentage) of fragments with each indicated condition (40 fragments of each were sequenced). Exp(.) is the expected number of fragments, based on the probabilistic formulae developed in the text with *Pd* = 0.18%, *Pi* = 0.16% and *Ps* = 0.12% taken from (a). *L* = 225, *M* = 10

Source	*Total #. bps sequenced*	*Deletions #. (Pd %)*	*Insertions #. (Pi %)*	*Substitutions #. (Ps %)*	*# errors total no. (Perr %)*
Integrase	8300	15 (0.18%)	13 (0.16%)	10 (0.12%)	38 (0.46%)
Integrase blue	8300	11 (0.13%)	2 (0.024%)	7 (0.084%)	20 (0.24%)

4 Discussion

In this report, we described the use of a CODA algorithm for the design of overlapping oligonucleotides that self-assemble into a single linear DNA product. We have employed this algorithm to construct synthetic genes that can be optimised for high-level protein expression in any standard in vivo or in vitro production system. The data in Figure 2(b) demonstrate that the CODA algorithm achieves a predicted melting temperature gap between the highest melting incorrect hybridisation and the lowest melting correct hybridisation for linear self-assembly of the overlapping oligonucleotides. Thus, at an annealing temperature for correct hybridisations, but far above that for all incorrect hybridisations, self-assembly into a single linear DNA product is of high thermodynamic probability (Figure 2(d)). In addition to optimising for self-assembly, the CODA algorithm also uses the degeneracy of the genetic code to maximise the use of abundant tRNA isoacceptors for frequently used codons (Figure 3(a)) (Boycheva et al., 2003; Gutman and Hatfield, 1989; Hatfield, 1993), to avoid consecutive rare codons (Hatfield, 1993; Moore and Maranas, 2002), and to use codon pair statistics (Gutman and Hatfield, 1989; Hatfield, 1993; Irwin et al., 1995) to minimise unfavourable codon pairs for high level protein expression (Figure 3(b)).

While our results demonstrate that it is possible to design oligonucleotides that cleanly self-assemble into a single DNA product, it is not possible to chemically synthesise these oligonucleotides without errors. The sequencing results of intermediate DNA fragments that were not screened by CODA Blue vector show that the cumulative error of all bp substitutions, insertions and deletions is 0.46%. This means that a sequence error is encountered about every 200 bp. Therefore, if all 74 of the overlapping ~45 bp oligonucleotides for the Ty3 integrase gene were combined at once to produce a full-length gene, it would be littered with mutations. This motivates the hierarchical assembly strategy described in this report (Figure 1). The probability of incorporating a defective oligonucleotide into a self-assembled DNA molecule is a function of the error rate per bp and the length of the molecule. Furthermore, Table 2 shows that if intermediate DNA fragments 200–250 bp in length are assembled and screened with the CODA Blue frameshift vector, then the probability of obtaining a correct sequence is high (~90 to 95%) if only two CODA Blue inserts for each intermediate DNA fragment are sequenced. Thus, building the gene in a hierarchical fashion from verified correct intermediate DNA fragments enables the rapid assembly of a sequence-correct, full-length gene. The efficacy of the CODA Blue frameshift screen is illustrated in Figure 4. For example, if four clones for each intermediate DNA fragment are sequenced, these data predict a greater than 90% chance of obtaining at least one correct sequence for each of the ten integrase intermediate DNA fragments for the assembly of a correct full-length gene. In the absence of this screen this chance drops to less than 20%.

The hierarchical assembly described here is facilitated by oligonucleotides that are CODA designed for self-assembly into a single product (Figure 2(d)). Otherwise, even with the CODA Blue frameshift screen, many clones of widely varying lengths would need to be screened and sequenced to obtain one of the correct length and sequence (Figure 2(c)). This addresses the problem of how to assemble and PCR amplify a full-length DNA molecule from the mixture of randomly hybridised oligonucleotides (Stemmer et al., 1995) while avoiding mutations incorporated with defective oligonucleotides into the full-length gene, even when a stringent biological selection such as that described by Smith et al. (2003) is not possible.

Figure 4 Probability of Obtaining at Least One Correct Sequence for each of the Intermediate DNA Fragments. *Pg* (light bars) is the probability that every one of the M intermediate DNA fragments has at least one clone with the correct sequence. *P*[*g*|*b*] (dark bars) is the probability that every one of the M intermediate DNA fragments has at least one clone with the correct sequence given that only pCODAblue screened blue clones are considered

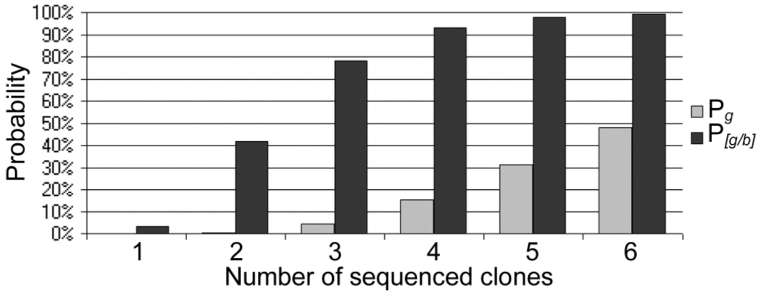


Table 2 Probabilities for obtaining error-free intermediate DNA fragments. The error per base values are from Table 1. *M* = 10

	Intermediate DNA fragment clone length (*L*)	*Probability of one given correct individual clone* (*Pc*)	*Probability of one correct individual clone if two clones are sequenced* (*Po, N = 2*)	*Probability of one correct individual clone if four clones are sequenced* (*Po, N = 4*)	*Probability of one correct individual clone if six clones are sequenced* (*Po, N = 6*)
Without CODA Blue Screen	200	0.398	0.637	0.868	0.952
	250	0.316	0.532	0.781	0.897
	300	0.251	0.439	0.685	0.823
With CODA Blue Screen	200	0.747	0.936	0.9959	0.9997
	250	0.672	0.893	0.988	0.9987
	300	0.593	0.834	0.973	0.9955

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