Codon optimization:
Why & how to design DNA sequences for optimal soluble protein expression

Rachel Speer, Ph.D.

GenScript
Make Research Easy

1. Why do codons matter?
2. OptimumGene principles & performance
3. Case Studies
4. How to get better protein expression

Protein Expression Overview

Select/Design the end product (amino acid sequence)

↓

Choose expression system

↓

Design expression clone (DNA construct)

↓

Express the protein

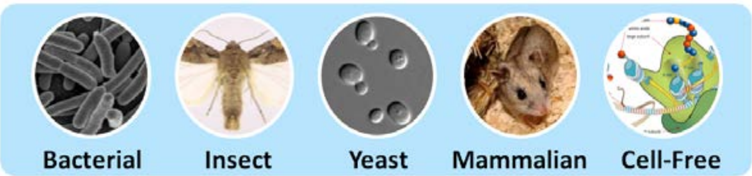
↓

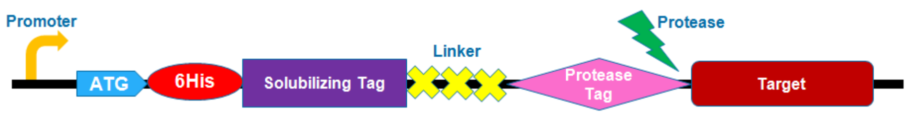
Purify the protein

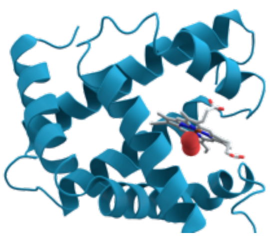
↓

Characterize the protein

MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLIC…







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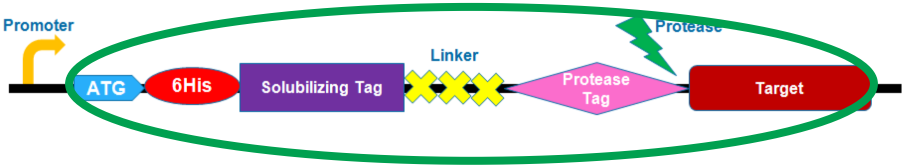
Codon Optimization



Why do Codons Matter? The Facts

- Redundancy in the genetic code
- Synonymous mutations affect protein expression rates up to 1000-fold.
- Synonymous mutations can also alter protein conformation, PTM, stability, and function.

Second Letter

1st letter	U		C		A		G		3rd letter
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA	Leu	UCA		UAA	Stop	UGA	Stop	A
	UUG		UCG		UAG	Stop	UGG	Trp	G
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

Codon Optimization:

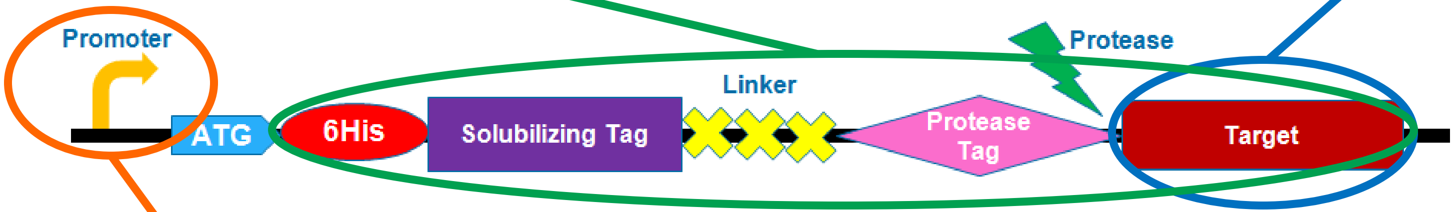
Introducing synonymous mutations that favor efficient soluble protein expression

```
Optimized AGTTTTCCAGGTTGAGGTCCGCCCGTT
          || || || || ||||| || || |||
Original  AGCTTCCCGGGATGAGGGCCCCCGGTT
```

What Codon Optimization is – and isn't

Codon Optimization:
Introducing synonymous mutations that favor efficient soluble protein expression

Protein Design:
changing the amino acid sequence



Expression strategy:
Selecting promoter, tags, etc.

Codon Bias

Observations:

- Species-specific bias in codon use and tRNA abundance
- Heterologous protein expression is often inefficient

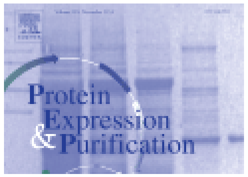
Theory:

- Rare codons reduce protein expression

Solutions:

- Express tRNA to remove bias in the host cells
- Alter the gene to replace rare codons with preferred ones:
  - site-directed mutagenesis
  - *de novo* gene synthesis with codon optimization

Codon Optimization Significantly Increases Protein Expression Levels



Codon optimization can improve expression of human genes in *Escherichia coli*: A multi-gene study.

Burgess-Brown NA *et al. Protein Expr Purif*. May 2008; 59(1): 94-102

Gene Name	Native			Synthetic			Expression	Solubility
	1	2	3	1	2	3	Syn vs Nat	Syn vs Nat
CBR1	Blue	Blue	Blue	Blue	Blue	Blue	▲	▲
CBR3	Blue	Blue	Blue	Blue	Blue	Blue	▲	▲
GMDS					Blue	Blue	▲	▲
HADH2		Green	Green		Blue	Green	▲	▲
HSD1 7B2	Green	Green		Green	Green		▲	
HSD17B4		Blue	Blue	Blue	Blue	Blue	▲	▲
MGC4172	Blue	Blue	Blue	Blue	Blue	Blue		
PECR				Blue	Blue	Green	▲	▲
RETSDR2	Green	Blue	Green	Green	Green	Green	▲	
SPR	Blue	Blue	Blue	Blue	Blue	Blue		

- Green: Expressed
- Blue: Expressed, Soluble and Purified
- White: Not Expressed

▲ Targets shown improvement of expression and/or solubility with synthetic gene after codon optimization

1. Total Cellular Protein
2. Soluble Fraction
3. Eluted Fraction

Codon Adaptation is not the most important factor for protein yield

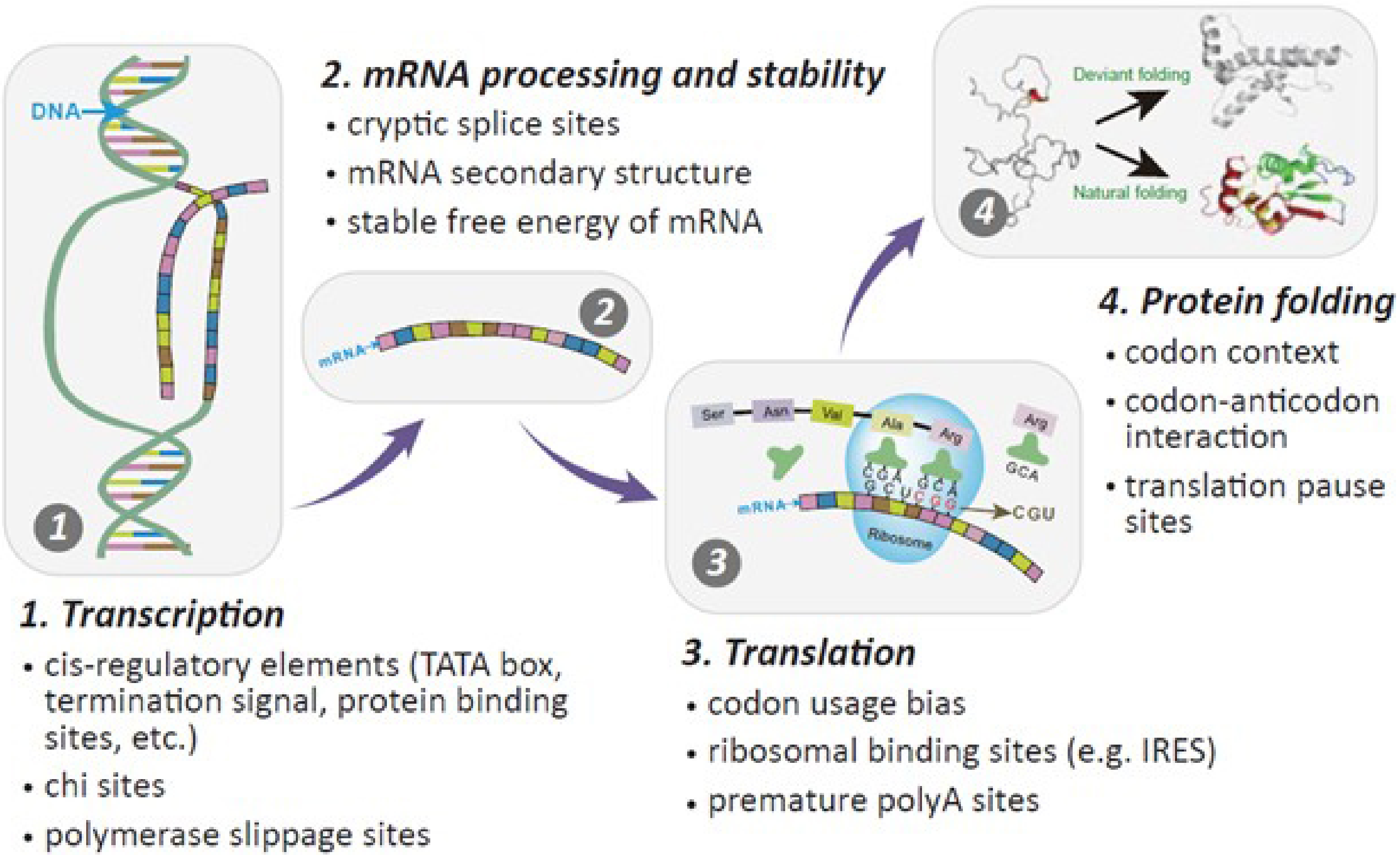
Coding-sequence determinants of gene expression in Escherichia coli.
Kudla G, Murray AW, Tollervey D, Plotkin JB. *Science.* 2009 Apr 10;324(5924):255-8.

- 154 synthetic GFP genes with random synonymous mutations
- 250-fold variation in fluorescence
- 44% of variation explained by 5' mRNA free energy (nt −4 to +37)

The anti-Shine-Dalgarno sequence drives translational pausing and codon choice in bacteria.
Li GW, Oh E, Weissman JS. *Nature.* 2012 Mar 28;484(7395):538-41.

- Variation in Translation Rate does not correlate with rare codon use
- Orthogonal ribosomes with altered anti-SD sequences: pausing results from hybridization between 16s rRNA and SD-like sequences in mRNA

Many sequence features influence protein expression



Evidence-Based Codon Optimization: OptimumGene

Transcriptional Efficacy:

- GC content
- CpG dinucleotides content
- Cryptic splicing sites
- Negative CpG islands
- SD sequence
- TATA boxes
- Terminal signal

Translation Efficiency:

- Codon usage bias
- GC content
- mRNA secondary structure
- Premature PolyA sites
- RNA instability motif (ARE)
- Stable free energy of mRNA
- Internal chi sites and ribosomal binding sites

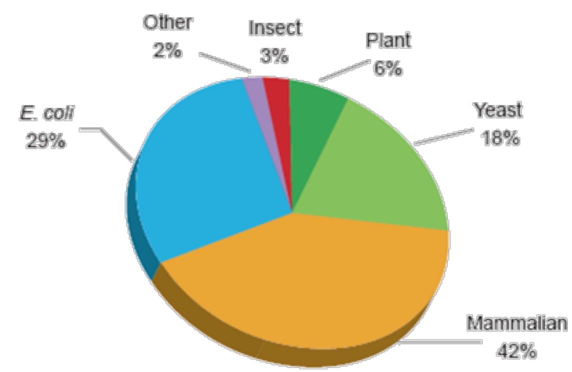
Protein Refolding:

- Codon usage bias
- Interaction of codon and anti-codon
- Codon-context
- RNA secondary structures

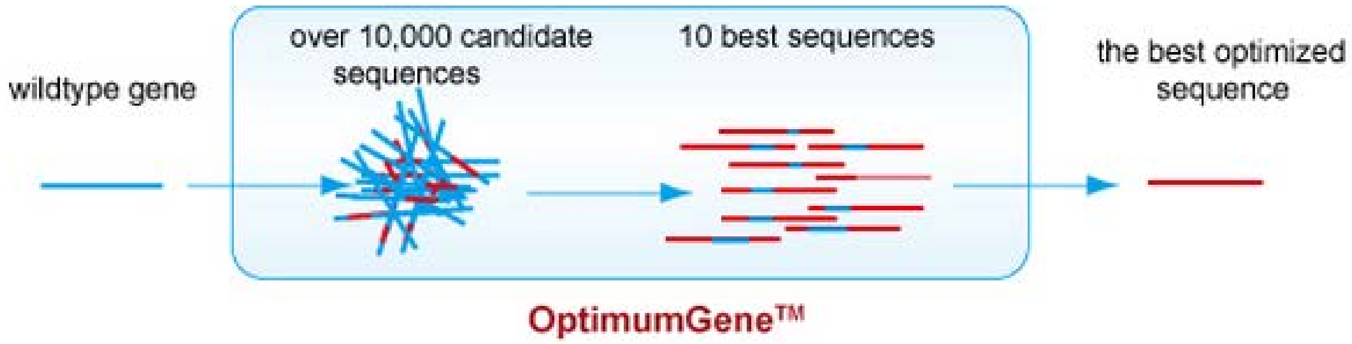
Flexibility to adjust the weight of different factors or add customized constraints:

- Filter out restriction sites
- Reduce similarity between library members
- Alternative codon tables / condition-specific codon preferences

GenScript has optimized over 50,000 sequences in all major expression systems.



Patented Bioinformatic Algorithm powers OptimumGene



Liu *et al.* **Method of sequence optimization for improved recombinant protein expression using a particle swarm optimization algorithm**. US Patent 8,326,547, issued December 4, 2012.

OptimumGene™ Improves Protein Expression Better that Competitors' Optimization

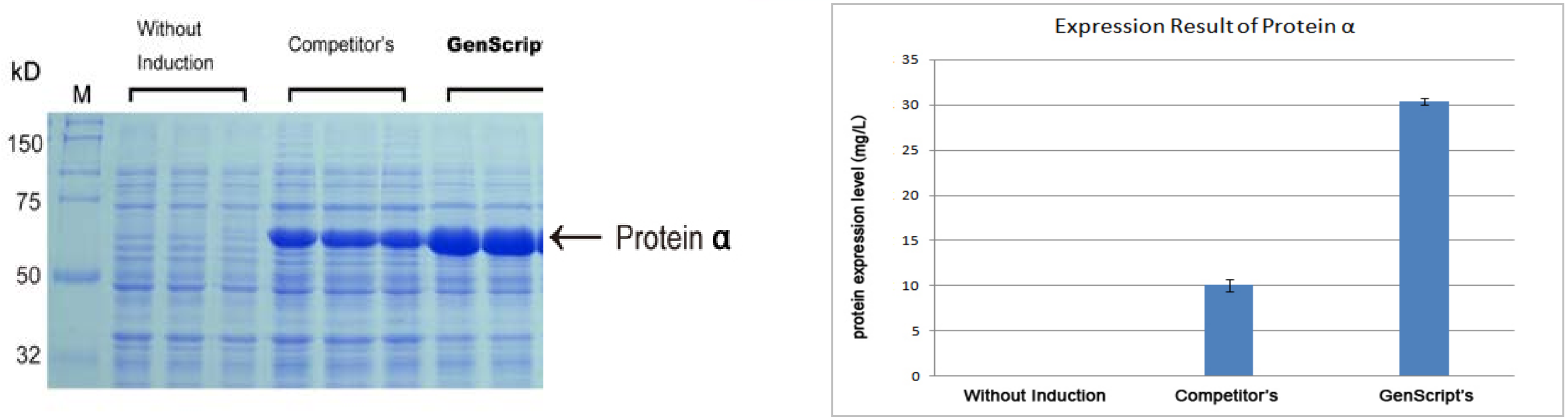


Fig. 1: Expression Result of Protein α after Codon Optimization. The expression level of Protein α using GenScript's OptimumGene™ Codon Optimization is **3** times more than that of competitor's.

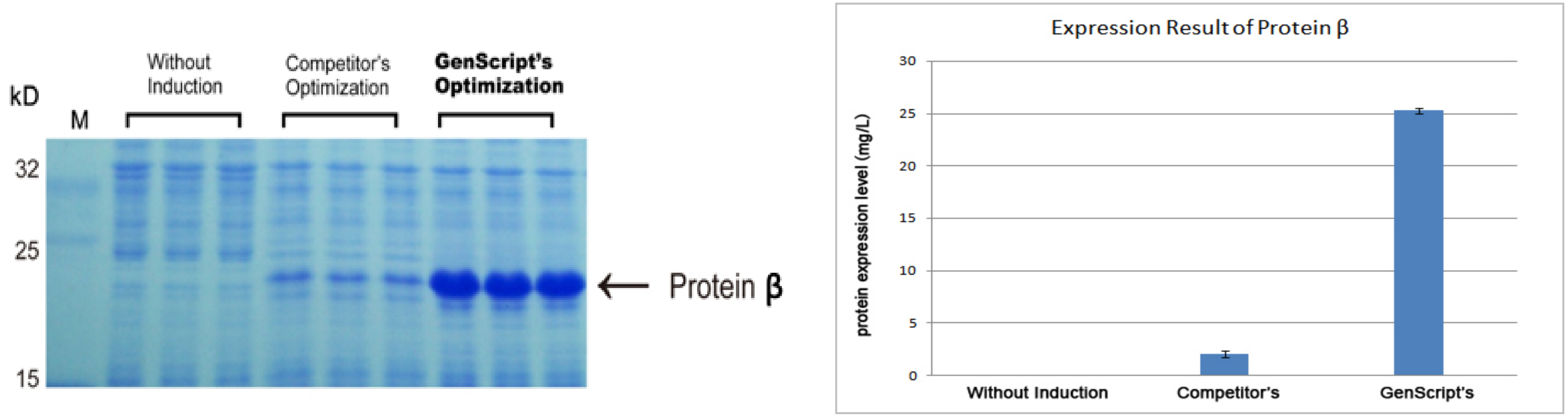
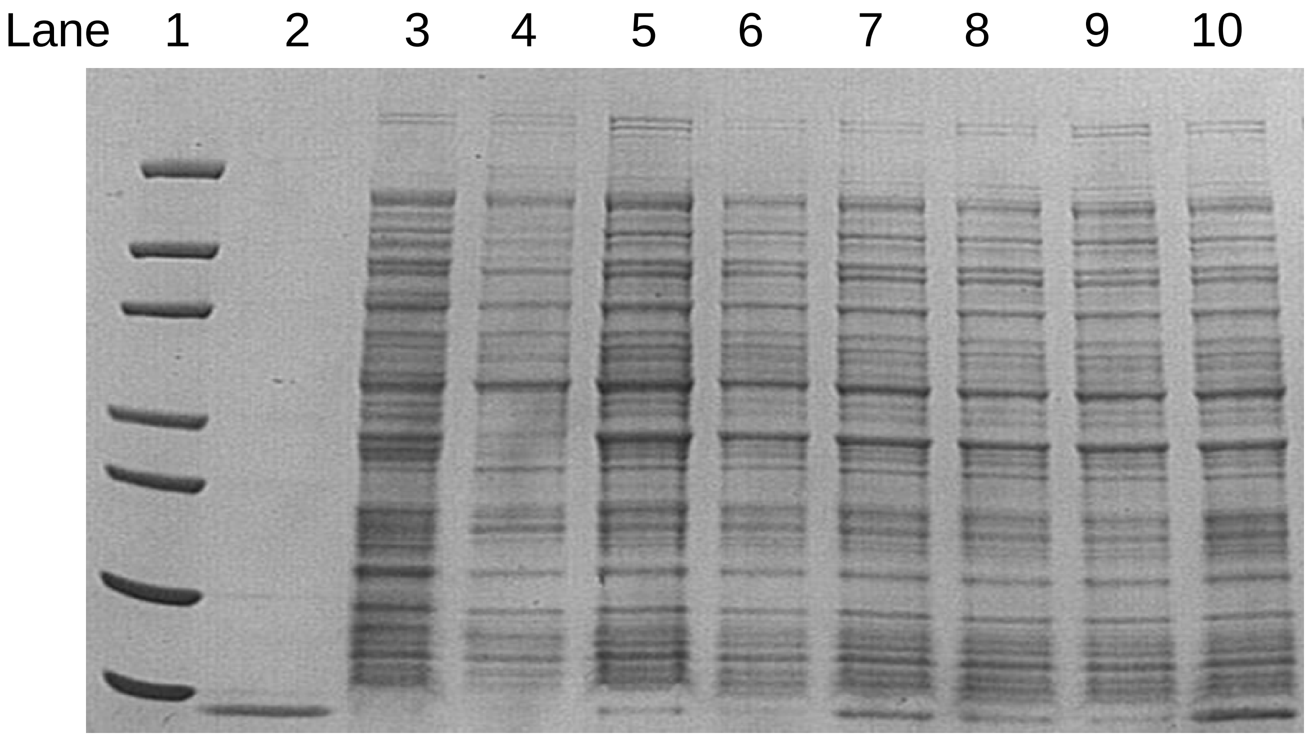


Fig. 2: Expression Result of Protein β after Codon Optimization. The expression level of Protein β using GenScript's OptimumGene™ Codon Optimization is **13** times more than that of competitor's.

We routinely test and improve our algorithm

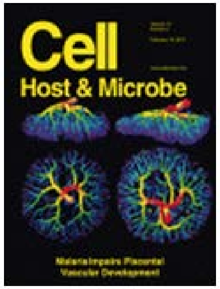


Lane	1	2	3	4	5	6	7	8	9	10
Sequence	MW marker	Purified hIGF-1 (PC)	BL21 cell lysate (NC)	WT (non-optimized) hIGF-1	Opti-0	Opti-1	Opti-2	Opti-3	Opti-4	Opti-5
Yield (mg/L)	--	--	--	Not detectable	7.7	3.1	18.5	11.4	5.4	28.5

Hundreds of papers cite GenScript for codon-optimized gene synthesis

"Humanization and optimization of codon usage was performed (GenScript) owing to **poor expression of the original zebrafish lyn in HEK293 cells**."

"…IFP1.4 gene was de novo synthesized by **GenScript** Company, based on the available protein sequence. The DNA sequence was **optimized with proprietary OptimumGene algorithm (GenScript)**..."



"...The following genes were **codon optimized and synthesized (Genscript):…"**

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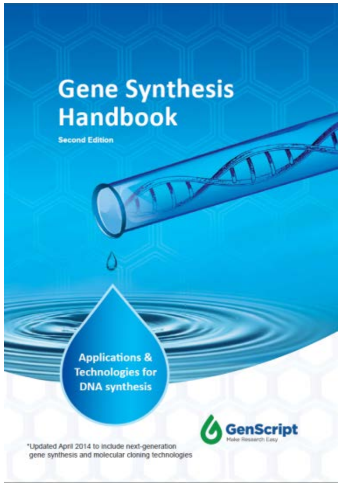


Case Study 1: Neurospora Circadian Clock

Non-optimal codon usage affects expression, structure and function of clock protein FRQ

Zhou M. *et al. Nature.* 2013 Mar;495 (7439); 111 – 5

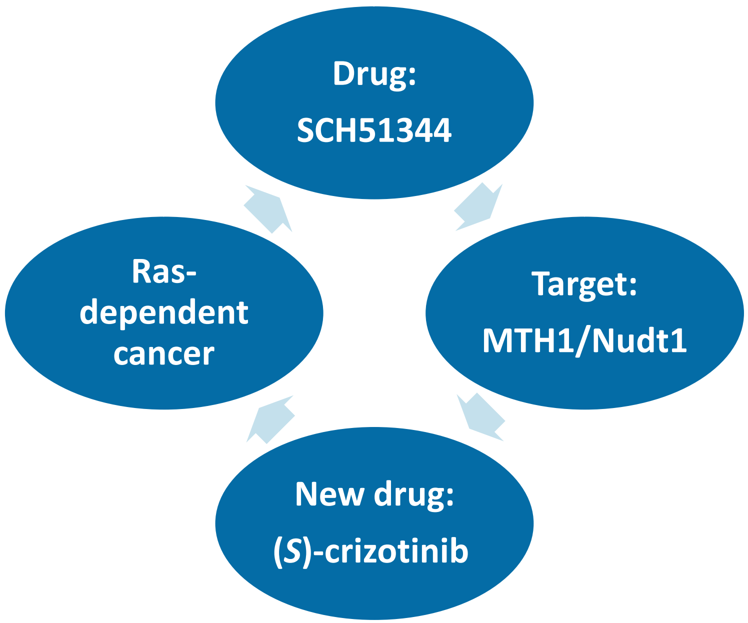
Codon Optimization performed in only specific regions of the gene:

"…**Optimized frq sequences (synthesized by Genscript)** …In the m1-frq construct, **only the codons upstream of the predicted intron branch point were optimized** as m-frq. For the m2-frq construct, **only the codons downstream of the intron 3' end were optimized** as m-frq..."

Case Study 2: Crystal Structure & PK/PD for Cancer Drug Discovery

Stereospecific targeting of MTH1 by (*S*)-crizotinib as anticancer strategy

Huber KV, et al. *Nature.* 2014 Apr 10;508(7495):222-7.



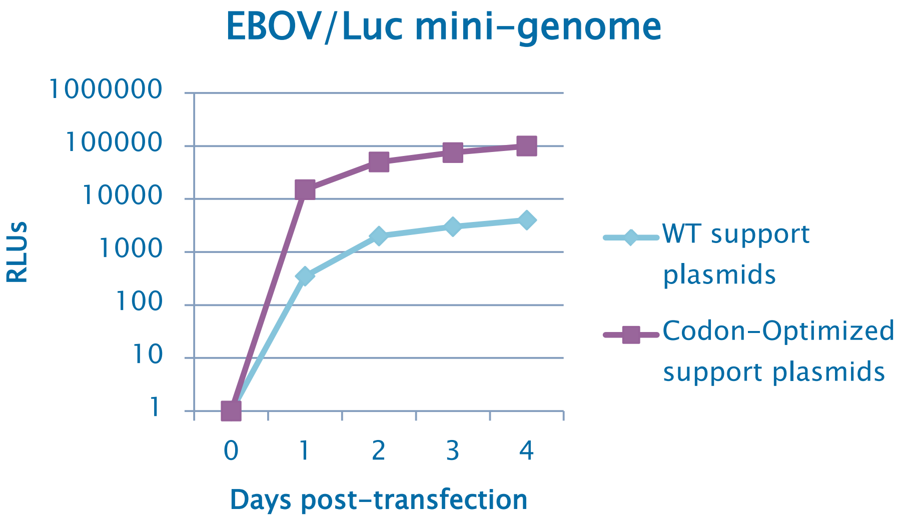
- **Codon-optimized gene synthesis from GenScript** was used to express Nudt1 for **enzymatic assays** and **crystallization studies.**

Case Study 3: Antiviral Drug Discovery for Ebola Virus

High-throughput, luciferase-based reverse genetics systems for identifying inhibitors of Marburg and Ebola viruses.

Uebelhoer *et al. Antiviral Res*. 2014 Jun;106:86-94.

- **Codon-optimized EBOV gene provided by GenScript**
- **Codon-optimized support plasmids increased signal 2000-fold**



Case Study 4: Combinatorial Library for Human Genome Editing

nature biotechnology

A library of TAL effector nucleases spanning the human genome.

Kim Y, et al. *Nat. Biotechnol.* **31,** 251–258 (2013).

Table I. TALE specificity code

RVD	Nucleotide
NI	adenine
HD	cytosine
NG	thymine
NN	guanine

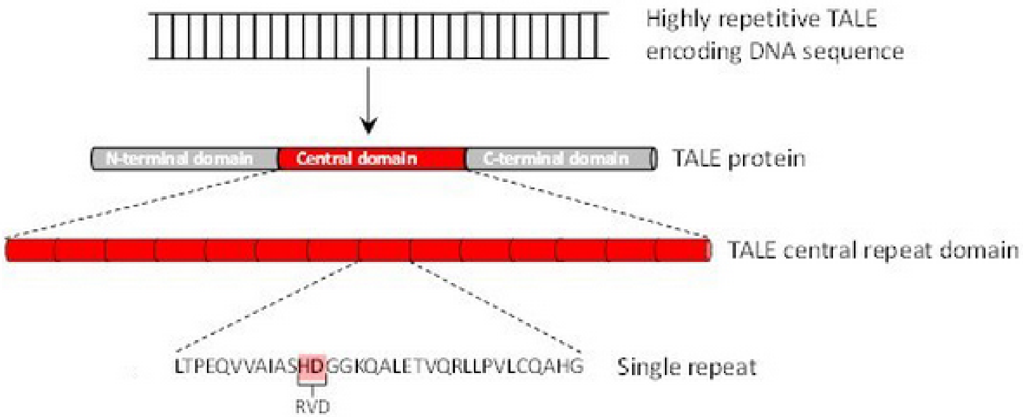


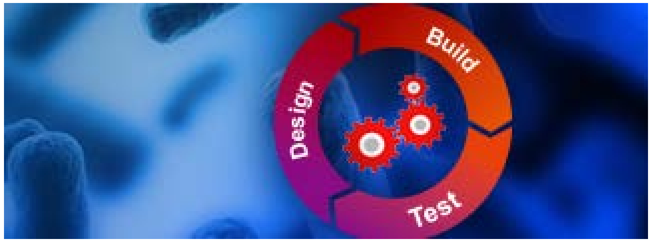
Fig 2. TALE protein organization

•**Codon Optimized Gene Synthesis from GenScript** was used to

1. Limit sequence similarity
2. Exclude rare codons
3. Guarantee accuracy of highly-repeated sequences

Case Study 5: Metabolic Engineering

Substantial improvements in methyl ketone production in E. coli and insights on the pathway from in vitro studies.
Goh EB *et al. Metab Eng.* 2014 Sep 18;26C:67-76.



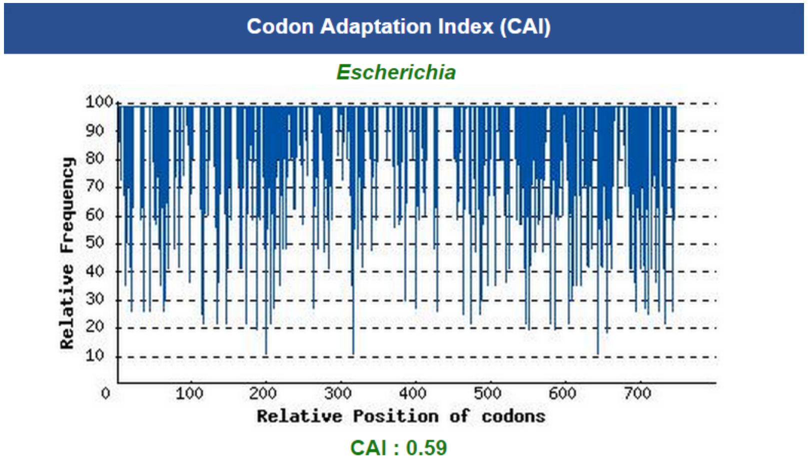
•**Codon Optimized Gene Synthesis from GenScript** was used to

1. improve metabolic pathway efficiency (↑substrate influx, ↓diversion)
2. improve GC content of gene from *M. luteus*, whose genome is 73% GC

How to get codon-optimized genes

- Online Tools to identify rare codons

- Rare Codon Analysis Tool
- Codon Frequency Tables



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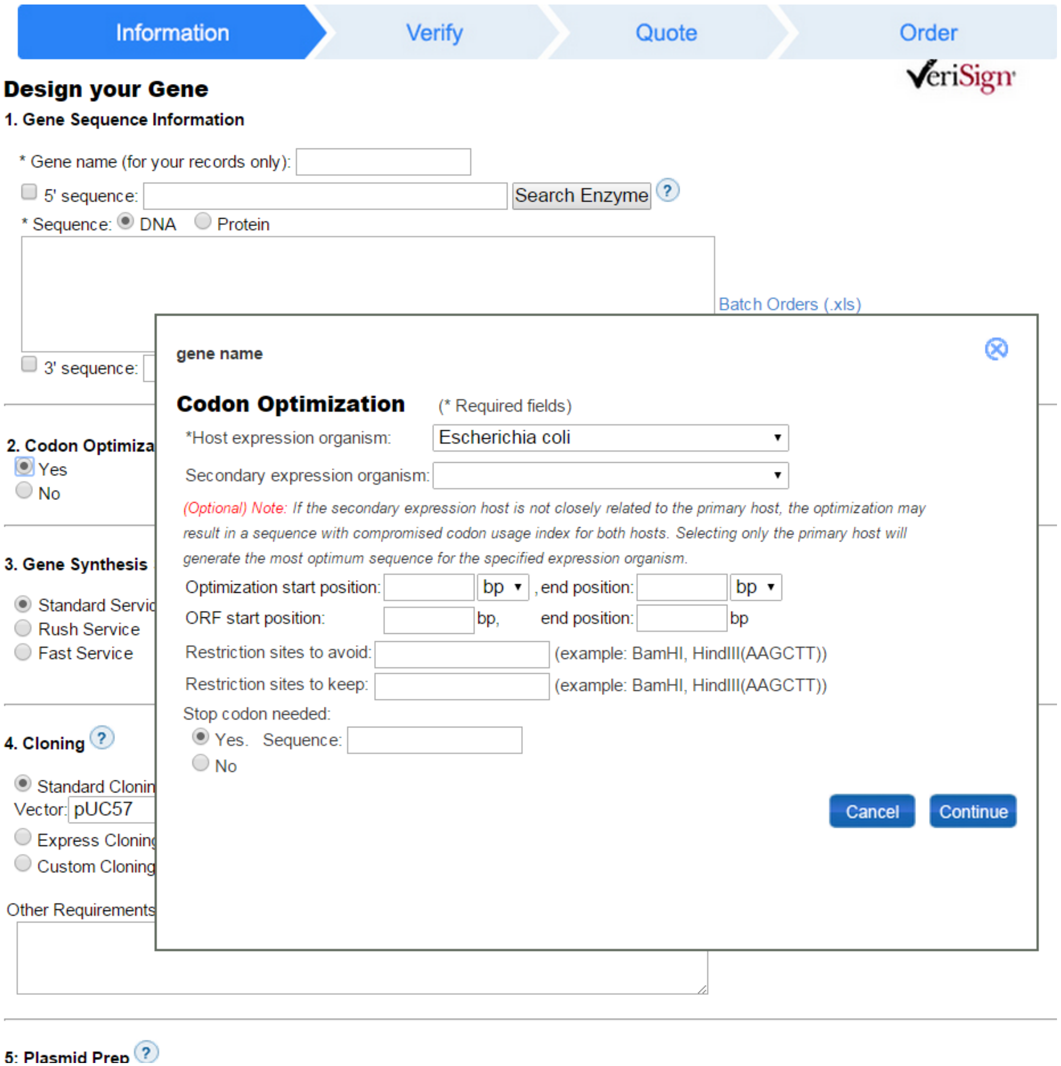

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Phone: 1-877-436-7274 (Toll-Free)

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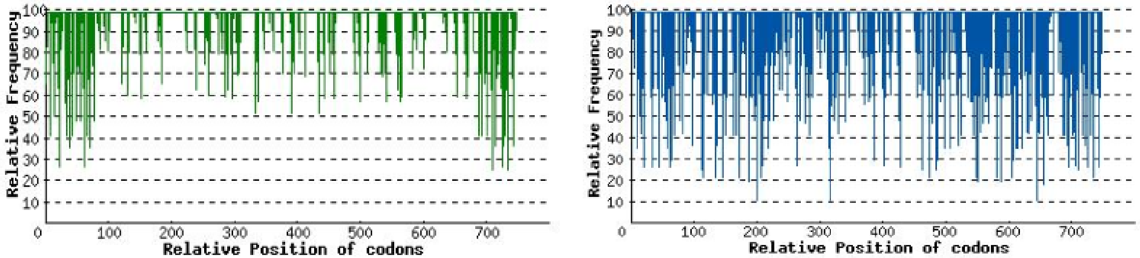
Codon Adaptation Index (CAI)

After OptimumGene™ Optimization

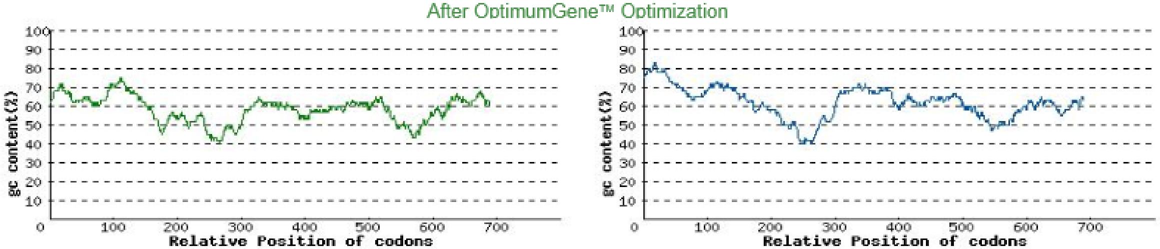


0.83 CAI: 0.59

Figure 1a. The distribution of codon usage frequency along the length of the gene sequence. A CAI of 1.0 is considered to be perfect in the desired expression organism, and a CAI of > 0.8 is regarded as good, in terms of high gene expression level.

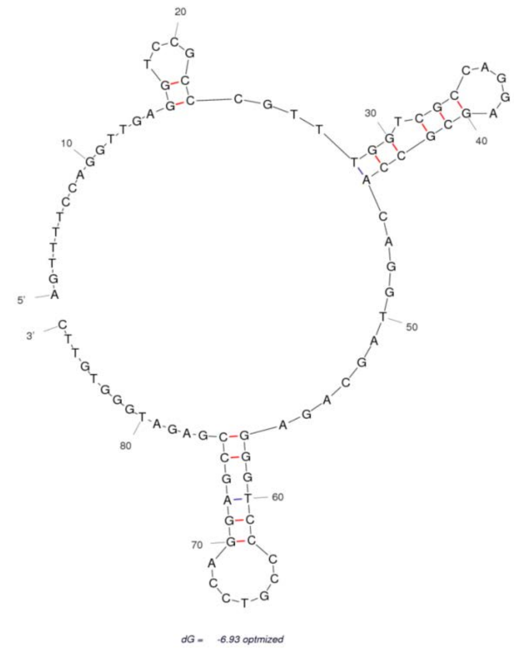
GC Content Adjustment

After OptimumGene™ Optimization



58.21 Average GC content: 61.82

Figure 2. The ideal percentage range of GC content is between 30-70 %. Peaks of %GC content in a 60 bp window have been removed.



Helices in structure (all)

Helix	ΔG (kcal/mol)	Length	Position
1	-4.74	5	64-->68 ; 85<--81
2	-4.08	3	16-->18 ; 42<--40
3	-3.12	3	19-->21 ; 38<--36
4	-2.17	2	69-->70 ; 76<--75
5	-2.17	2	24-->25 ; 33<--32
6	-1.84	2	53-->54 ; 59<--58
7	-1.84	2	43-->44 ; 49<--48

Hairpins in structure (all)

Hairpin	ΔG (kcal/mol)	Length	Position
1	3.10	10	24-->...<--33
2	2.50	8	69-->...<--76
3	1.50	7	53-->...<--59
4	1.50	7	43-->...<--49

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GenScript Toolkit For Improving Protein Expression

Select/Design the end product (amino acid sequence)	GenPlus™ high-throughput gene synthesis Gene Variant Library services
↓	
Choose expression system	PROTential™ protein expression evaluation service
↓	
Design expression clone (DNA construct)	Codon-Optimized Gene Synthesis
↓	
Express the protein	BacPower™ YeastHIGH™ MamPower™ InsectPower™ FragPower™ Recombinant Antibody
↓	
Purify the protein	FoldArt™ Refolding ToxinEraser™ Endotoxin Removal
↓	
Characterize the protein	Protein Characterization Services

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Select/Design the end product (amino acid sequence)

↓

Choose expression system

↓

Design expression clone (DNA construct)

↓

Express the protein

↓

Purify the protein

↓

Characterize the protein

Mutant library for protein engineering?
Combinatorial Library?
Truncation Variants?

Gene variant libraries: design, construction, and research applications

Presented by: Rachel Speer, Ph.D.
Originally aired May 21st and June 18th, 2014

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(amino acid sequence)

↓

Choose expression system

↓

Design expression clone
(DNA construct)

↓

Express the protein

↓

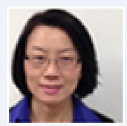
Purify the protein

↓

Characterize the protein

- Pros and Cons of different expression hosts
- Techniques for protein re-folding, protection from rapid degradation

Recombinant protein expression & purification: challenges and solutions



Presented by: Liyan Pang, Ph.D.
Originally aired June 11th and June 12th, 2014

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Select/Design the end product (amino acid sequence)

↓

Choose expression system

↓

Design expression clone (DNA construct)

↓

Express the protein

↓

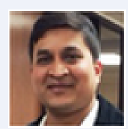
Purify the protein

↓

Characterize the protein

- Fusion partners/epitope tags
- e. coli strain, induction conditions, etc

Optimizing conditions for recombinant soluble protein production in *E. coli*



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Select/Design the end product (amino acid sequence)

↓

Choose expression system

↓

Design expression clone (DNA construct)

↓

Express the protein

↓

Purify the protein

↓

Characterize the protein

- maximizing purity and yield

Identify the optimal protein purification strategy for your recombinant protein production

Presented by: Liyan Pang, Ph.D.

November 12, 2014 8:00 am	November 12, 2014 2:00 pm
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References

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Goh EB et al. **Substantial improvements in methyl ketone production in E. coli and insights on the pathway from in vitro studies.** *Metab Eng.* 2014 Sep 18;26C:67-76.

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Kramer G, Boehringer D, Ban N, Bukau B. **The ribosome as a platform for co-translational processing, folding and targeting of newly synthesized proteins.** *Nat Struct Mol Biol.* 2009;16:589–597.

Kudla G, Murray AW, Tollervey D, Plotkin JB. **Coding-sequence determinants of gene expression in Escherichia coli.** *Science.* 2009 Apr 10;324(5924):255-8. Free Full Text

Li GW, Oh E, Weissman JS. **The anti-Shine-Dalgarno sequence drives translational pausing and codon choice in bacteria.** *Nature.* 2012 Mar 28;484(7395):538-41. Free Full Text

Mellitzer A. *et al.* **Synergistic modular promoter and gene optimization to push cellulase secretion by Pichia pastoris beyond existing benchmarks**. *J. Biotechnol.* (2014), http://dx.doi.org/10.1016/j.jbiotec.2014.08.035

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Shine J, Dalgarno L. **The 3′-terminal sequence of Escherichia coli 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites.** *Proc. Natl Acad. Sci. USA* 1974;71:1342-1346. Free Full Text

Uebelhoer *et al.* **High-throughput, luciferase-based reverse genetics systems for identifying inhibitors of Marburg and Ebola viruses.** *Antiviral Res*. 2014 Jun;106:86-94.

Welch M et al. **Design parameters to control synthetic gene expression in Escherichia coli**. *PLoS One*. 2009 Sep 14;4(9):e7002. doi: 10.1371/journal.pone.0007002.

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