From Design to Protein: Cell-Free Protein Expression Using Twist Gene Fragments

ABSTRACT

A cornerstone of synthetic biology is designing DNA sequences and testing the characteristics and functions of the resultant proteins. Here, Twist Gene Fragments were used as DNA input of a cell-free protein expression study using multiple commercially available *in vitro* protein production kits. The results demonstrate that Twist Gene Fragments enable high-throughput protein expression and functional testing within a single day.

INTRODUCTION

In the design-build-test-learn cycle that underlies much of synthetic biology, an engineered gene sequence is often expressed as a functional protein within host cells (*in vivo*) or by using cell-free (*in vitro*) methods. Traditional *in vivo* protein expression workflows involve cloning the encoding DNA into an expression vector, transforming the vector into a host organism for protein synthesis, and then assessing the protein in either a crude cell lysate or following purification. Such methods can be time-intensive and laborious, and they can fail if the protein is toxic to the host cells.

In such situations, cell-free (*in vitro*) transcription/translation (TxTL) systems are both a practical option and an effective method for producing complex proteins more quickly. Cell-free protein expression systems use either a reconstituted mixture of recombinant enzymes or a cellular lysate to perform TxTL of input DNA. The input DNA is typically a sequence cloned into a plasmid or a linear double-stranded DNA (dsDNA, a PCR product or synthesized gene fragment). Though plasmids can protect gene sequences from degradation by nucleases (particularly in lysate-based systems), generating a clonal plasmid to encode a potentially toxic protein can be difficult. Cloning requires the replication of the plasmid within host cells, so transcription and expression of the cloned gene within those cells must be tightly controlled to prevent the production of harmful proteins. Synthetic DNA fragments, on the other hand, enable precise, rapid, high-throughput synthesis without the disadvantages of cloning.

Twist Bioscience offers custom synthesized gene fragments with industry-leading error rates of 1:3,000 bp for use in synthetic biology and other applications. These 300–1,800 bp linear dsDNA fragments include unique 23 bp adapter sequences on both ends that act as primer sites for robust amplification of a variety of templates with a single primer pair.

We examined the cell-free expression of Twist Gene Fragments using both a recombinant enzyme-based and a bacterial lysate-based TxTL kit. The wild-type and several variants of the Type II restriction endonuclease *Bam*HI, which is toxic to express *in vivo*, were used to demonstrate how Twist Gene Fragments enable effective cell-free protein expression within a single day.

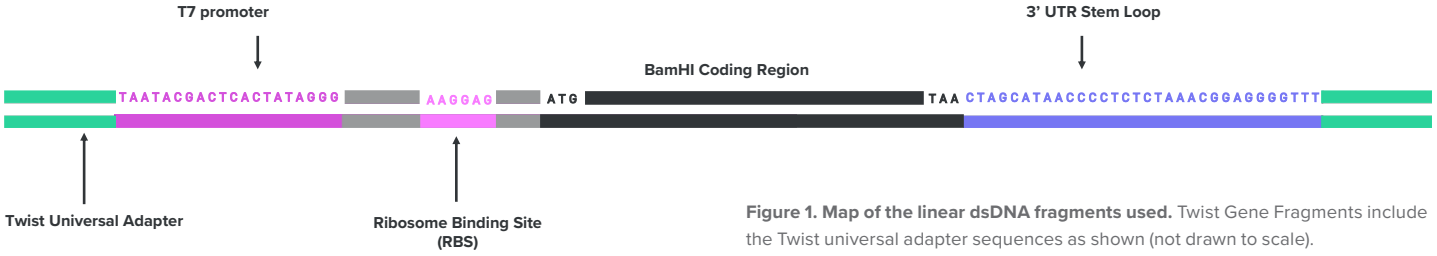

Figure 1. Map of the linear dsDNA fragments used. Twist Gene Fragments include the Twist universal adapter sequences as shown (not drawn to scale).

METHODS

The 641 bp *Bam*HI sequence and four variants were synthesized using the Twist synthesis pipeline for dsDNA Gene Fragments. Because cell-free expression systems typically require input DNA to contain a promoter sequence, stop codon, and terminator sequence, each construct contained a T7 promoter sequence and ribosome-binding site upstream of the start codon, as well as a 3' UTR stem loop after the stop codon (**Figure 1**). The 23 bp Twist universal adapter sequences were included at both ends of each gene fragment.

The constructs were expressed and translated into protein using two commercially available cell-free kits:

- PURExpress® *In Vitro* Protein Synthesis Kit (New England Biolabs, Inc., catalog #E6800S) — reconstituted mixture of proteins
- *AccuRapid*™ Cell-Free Protein Expression Kit (Bioneer, catalog #K7250) — bacterial cell lysate

For each construct and kit, the following experimental workflow was followed:

1. **Amplification**: Following synthesis, each construct was subjected to PCR amplification to increase yield. PCR was performed using the KAPA HiFi PCR Kit (Roche) with 10 µM Twist universal primers and 100 pg template DNA. PCR products were purified using SPRI beads and eluted in 1 mM Tris buffer, pH 8.0.

   The following cycle conditions have been optimized for use with the Twist universal primer sequences:
   - Initial denaturation: 95°C for 3 min
   - Cycles (15): 98°C for 10 sec, 65°C for 15 sec, 72°C for 30 sec
   - Final extension: 72°C for 2 min
   - Hold: 4°C
2. **Expression**: DNA was transcribed and translated into protein according to the manufacturer's instructions for the PURExpress and *AccuRapid* kits. Before each reaction, the Twist Gene Fragment was added to a strip tube, lyophilized, and resuspended in molecular biology grade water. For the PURExpress kit, 100 ng DNA in a 10 µl reaction volume was used and with the *AccuRapid* kit, 500 ng DNA was used in a 45 µl reaction. For each kit, a control reaction with no input DNA was also performed.
3. ***Bam*HI functional assay**: *Bam*HI enzyme activity was evaluated in a restriction digest of a circularized plasmid (pUC19), which contains a single *Bam*HI site. In each assay, the plasmid (1 µg) was incubated at 37°C for 30 minutes (unless otherwise noted) in NEBuffer 3 (New England Biolabs, Inc.) with 1 µl lysate from each TxTL reaction, including the control. The reaction volume was 20 µl. Aliquots of each reaction mixture (5 µl) were then separated on a 1% agarose gel. Control assays included untreated pUC19, incubations of the pUC19 plasmid digested with Nt.BspQI, a nickase with a single cut site on pUC19 (New England Biolabs, Inc.), or recombinant, purified *Bam*HI (New England Biolabs, Inc.).
4. **TOPO® cloning**: Twist Gene Fragments were cloned using the Zero Blunt® TOPO PCR Cloning Kit (Invitrogen) and transformed via One Shot® TOP10 chemically competent *Escherichia coli* cells. They were then plated on LB-carbenicillin plates and Sanger sequenced.

RESULTS

Twist Gene Fragments encoding the restriction endonuclease *Bam*HI and four of its sequence variants were used for cell-free protein expression with two commercially available TxTL kits: the PURExpress *In Vitro* Protein Synthesis Kit (based on a reconstituted mixture of recombinant enzymes, New England BioLabs, Inc.) and the *AccuRapid* Cell-Free Protein Expression Kit (based on a bacterial cell lysate, Bioneer). The activities of the expressed *Bam*HI were then evaluated in a restriction digest of pUC19 plasmid DNA, which contains a single *Bam*HI restriction site. In this functional assay, *Bam*HI enzyme would be expected to digest the circular plasmid into a linear DNA molecule, a result that can be easily visualized on an agarose gel following electrophoresis (**Figure 2**).

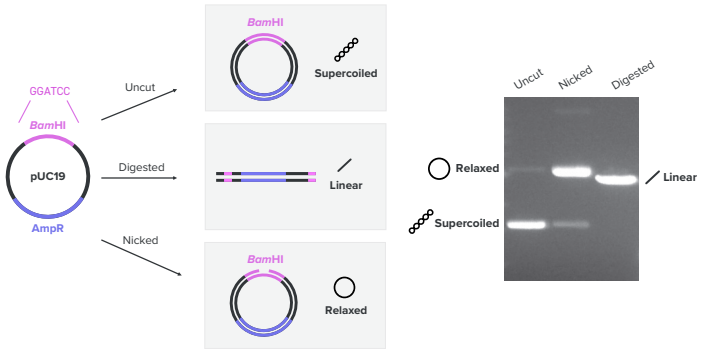

Figure 2. Schematic representation of the results expected from the functional assay of *Bam*HI activity. This assay included three controls: reactions with undigested plasmid (supercoiled, circular uncut plasmid DNA that migrates as two discrete bands), plasmid treated with nickase enzyme (relaxed, circular DNA), and plasmid digested with recombinant *Bam*HI (linear dsDNA).

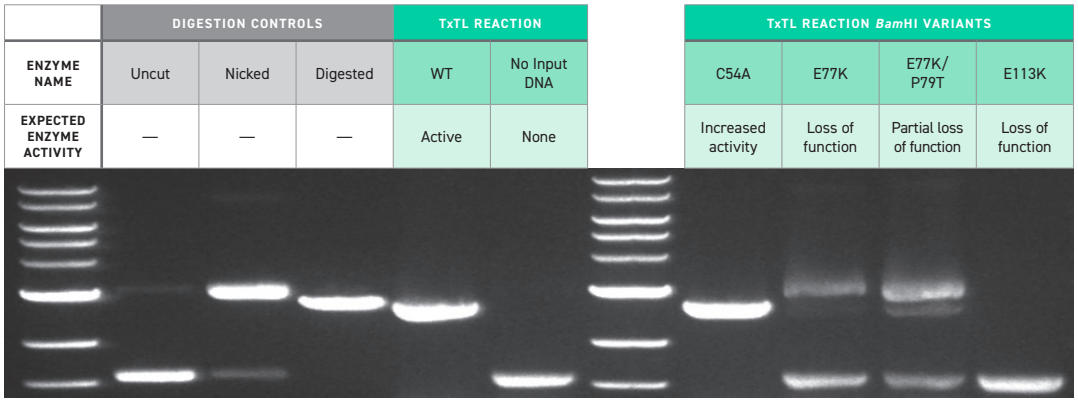

Figure 3. Functional assay of recombinant *Bam*HI expressed using the PURExpress system. The gel includes four controls: Uncut (untreated circular and plasmid DNA), Nicked (plasmid treated with nickase enzyme to be relaxed, circular DNA), Digested (plasmid treated with recombinant *Bam*HI to yield linear dsDNA), and No Input DNA (plasmid treated with the cell-free protein expression reaction containing No Input DNA). Note that the plasmid controls ran a bit more slowly in these gels; linear DNA from the WT reaction ran faster than that from the Digested control, and the undigested DNA from the No Input DNA reaction runs faster than the Uncut control due to the different buffer compositions of these samples.

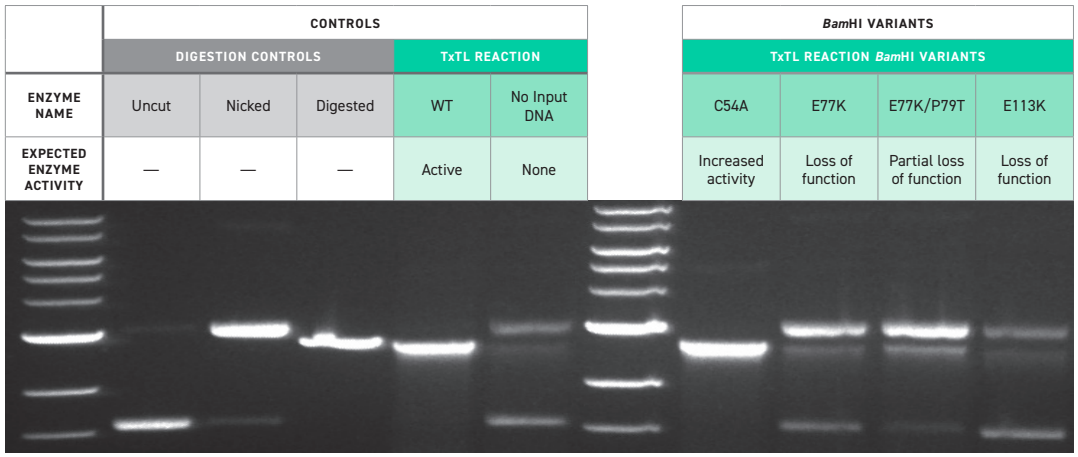

Figure 4. Functional assay of recombinant *Bam*HI expressed using the *AccuRapid* system. The gel includes four controls: Uncut (untreated circular and plasmid DNA), Nicked (plasmid treated with nickase enzyme to be relaxed, circular DNA), Digested (plasmid treated with recombinant *Bam*HI to yield linear dsDNA), and No Input DNA (plasmid treated with the cell-free protein expression reaction containing No Input DNA). Note the band migrating as a "nicked" plasmid, resulting from endogenous endonuclease activity in the cell-free reaction mixture.

Functional Expression with the PURExpress System (Recombinant)

Figure 3 shows the *Bam*HI activities obtained following expression of the Twist Gene Fragments (linear dsDNA) with the PURExpress kit. In this functional assay, the Twist Gene Fragment encoding wild-type (WT) *Bam*HI exhibited restriction endonuclease activity that was consistent with the activity of the purified, control *Bam*HI enzyme. These two restriction digests both produced a linear DNA molecule, whereas the negative control PURExpress sample (without input DNA) yielded only the circular, supercoiled DNA.

Expression of the four *Bam*HI variants also yielded anticipated results. Loss-of-function variants E77K and E113K cleave at less than 0.1% the rate of WT (Xu and Schildkraut 1991a), and in the assay produced no (or very little) linear dsDNA after digestion. The E77K/P79T double mutant can rescue the E77K loss-of-function mutation (Xu and Schildkraut 1991b), and accordingly, in the assay, the double mutant produced linear DNA, though at a lower rate than WT.

Functional Expression with the AccuRapid System (Bacterial Lysate)

Figure 4 displays the results of expression with the *AccuRapid* Cell-Free Protein Expression Kit (Bioneer). The *AccuRapid* kit is derived from a cell lysate, which contains endogenous nucleases, so the manufacturer recommends using plasmid input DNA. **Figure 5** shows, however, that significant enzyme activity can be obtained with this kit using Twist linear dsDNA fragments with universal adapters.

As with the PURExpress kit, WT *Bam*HI generated with the *AccuRapid* kit exhibited activity consistent with that of the purified, control *Bam*HI enzyme and the activities of the *Bam*HI variants were also consistent with those from the PURExpress kit. In this case, however, the digest performed with the cell-free reaction mixture lacking input DNA (No Input DNA) generated a band consistent with that produced with the nickase control — a single band migrating as a larger, relaxed conformation. This band was also detected in all the digests with the loss-of-function variants, a result consistent with the presence of endogenous nucleases in the cell-free reaction mixture.

Among the *Bam*HI variants examined, C54A exhibits an increased K_{cat} (Mukhopadhyay and Roy 1998), which was examined in the experiment depicted in **Figure 5**. In this experiment, the *AccuRapid* WT and C54A cell-free reactions were serially diluted (1:9 and 1:27) and incubated with pUC19 for 5 minutes.

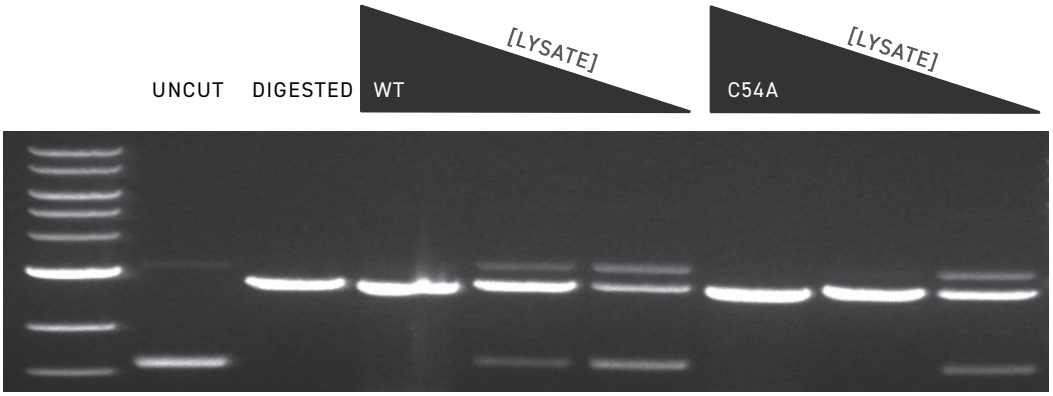

Figure 5. Functional assay of WT *Bam*HI and the C54A variant expressed using the *AccuRapid* system. This assay included two controls: Uncut DNA, (supercoiled, circular uncut plasmid DNA) and a Digested DNA, (linear dsDNA). WT and C54A variants were incubated with pUC19 for 5 minutes as undiluted cell-free reaction mixtures and as 1:9 and 1:27 dilutions. Note the more prominent bands from the 1:9 and 1:27 C54A variant solutions.

The WT *Bam*HI exhibited incomplete digestion at the 1:9 dilution, and only a fraction of the pUC19 was digested at 1:27 dilution. As expected, the most dilute preparation of C54A exhibited more activity (generated more of the linear pUC19) than the similar dilution of the WT *Bam*HI (complete digestion of pUC19 at the 1:9 dilution and more significant activity at 1:27).

Determining the Error Rate of Twist Gene Fragments

To confirm the connection between gene synthesis and enzymatic function of *Bam*HI, Twist Gene Fragments encoding wild-type *Bam*HI were TOPO-cloned and sequenced. Each construct sequenced, however, contained a frameshift mutation causing an inactive mutant. This result was inconsistent with the functional activity observed above, and it highlights the difficulties associated with subcloning genes encoding toxic proteins. *E. coli* selects against any construct encoding a functional enzyme because even basal levels of expression result in protein that digests genomic DNA, killing the cell.

CONCLUSION

Obtaining functional data for proteins that are toxic to expression hosts can challenge researchers studying sequence-function relationships. Creative solutions are often required to sequester the protein within cellular compartments or have it secreted into solution. Developing these solutions complicates and prolongs the design-build-test cycle. Cell-free protein expression (TxTL) kits are emerging as robust solutions for rapid functional testing of protein variants, and as a testament to their utility, the number of commercially available TxTL kits is growing.

As shown here, Twist Gene Fragments serve as effective input DNA for both types of commercially available TxTL kits: those using a reconstituted mixture of reconstituted proteins, as well as those based on cellular lysates. Cell-free protein expression from the linear dsDNA Twist Gene Fragments yielded significant enzyme activity with both types of kits, directly from the cell-free reaction mix (no purification was required). Furthermore, the impact of sequence variations on enzyme activity was easily observed using these systems.

Compatible with many downstream cloning methods, Twist Gene Fragments are a uniquely effective high-throughput solution for *in vitro* protein expression. Twist synthetic DNA offers precision and scalability traditional cloning methods cannot match:

- Scalable orders of one to one million Gene Fragments
- Industry-leading error rate of 1:3,000 bp
- Competitive pricing

Twist's Online Ordering Platform also facilitates ordering and includes a codon-optimization tool that detects and fixes potentially problematic DNA sequences before ordering. The 5' and 3' universal adapter sequences further streamline the design-build-test cycle by allowing efficient scale-up of each sequence. Taken together, with Twist Gene Fragments and *in vitro* protein expression kits, proteins can be expressed and functionally tested in a single work day. To order Twist Gene Fragments online, visit twistbioscience.com/shop

REFERENCES

Mukhopadhyay P, Roy KB (1998) Protein engineering of *Bam*HI restriction endonuclease: replacement of Cys54 by Ala enhances catalytic activity. Protein Eng. 11(10): 931–935.

Xu SY, Schildkraut I (1991a) Isolation of *Bam*HI variants with reduced cleavage activity. J. Biol. Chem. 266: 4425–4429.

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