DNA toolkit: T7 terminator testing

Status	Design
Tags	
Researchers	Ⓒ Charlie Newell
Keywords	
Projects	Developer Cell: DNA Toolkit
Project Status	In Progress
Project Team	『ACJS』
Summary	The study quantifies the performance of T7 terminators in the PURE system using a dual-reporter construct to measure relative fluorescence signals. The T7 terminator collection offers a range of termination efficiencies, enhancing control over gene expression in multi-gene constructs. Testing reveals that the T7 terminator collection shows higher termination efficiencies in PURE compared to E. coli, with notable increases for certain variants, indicating potential for improved gene expression control in synthetic biology applications.

Overview

This work seeks to quantify the performance of the 'T7 terminator' collection in the PURE system. We will build a DNA construct encoding a dual-reporter system in which the T7 terminator is placed downstream of the first reporter and upstream of the second reporter enabling quantification of terminator strength through the relative fluorescence signals of each reporter.

Background

The native bacteriophage T7 terminator is a DNA sequence that transcribes into an RNA stem loop followed by a poly(U) tract to arrest T7 polymerase and to stop gene transcription. With a termination efficiency of only ~60%, this sequence often leads to read-through transcription and the unwanted expression of downstream genes. While transcriptional read-through can be a drawback for single-gene expression, it can be strategically harnessed in multi-gene constructs to tune expression levels. To provide greater control, the T7 terminator collection[1] offers a library of sequences with a 10-fold dynamic range of efficiencies. This versatility could enable the future design of complex multi-gene plasmids with inbuilt transcriptional isolation and precisely controlled gene expression levels.

The T7 terminator collection was first quantified through a dual-reporter system in which individual T7 terminator sequences were placed downstream of a first reporter and upstream of a second reporter enabling quantification of terminator strength through the relative fluorescence signals of each reporter. Whilst the collection was tested in E.coli and in an In vitro transcription system, the collection is yet to be tested in **PURE**.

We will build a dual-reporter PURE expression plasmid utilizing plamGFP and mScarlet. T7 terminators will be cloned in between reporters to enable their performance quantification.

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References

1. Diana G Calvopina-Chavez, Mikaela A Gardner, Joel S Griffitts, Engineering efficient termination of bacteriophage T7 RNA polymerase transcription, *G3 Genes|Genomes|Genetics*, Volume 12, Issue 6, June 2022, jkac070, https://doi.org/10.1093/g3journal/jkac070

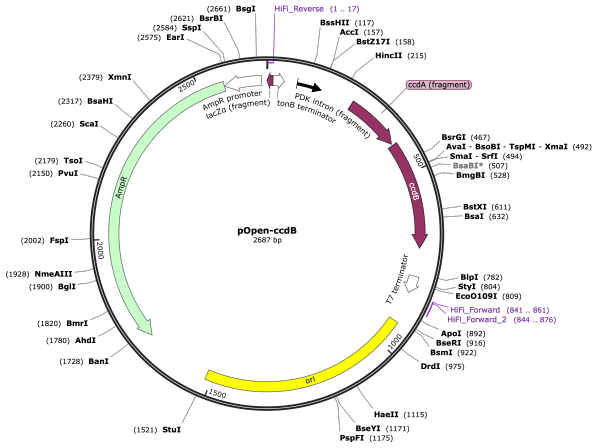
HiFi entry vector PCRs
@September 10, 2025: Gel electrophore and band extraction
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Dual-reporter design

DNA parts

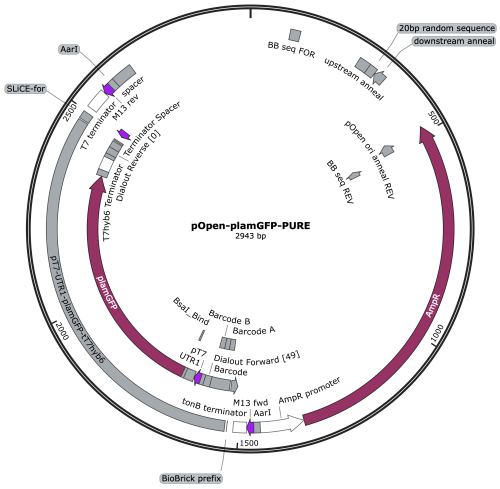
▼ Backbone

pOpen-ccdB

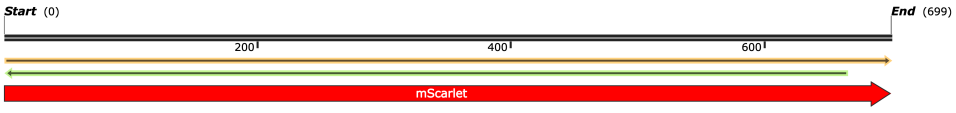


▼ Fluorescent proteins

▼ 1. plamGFP - Excitation: 502, Emission: 514



▼ 2. mScarlet - Excitation: 569, Emission: 594



mScarlet
699 bp

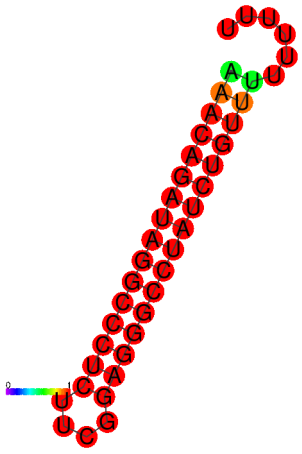
▼ Terminator sequences

▼ T7hyb1

Sequence

AAACAGATAGGCCCTCttcgGAGGGCCtatctgttTTTTTTT

Minimum free energy structure

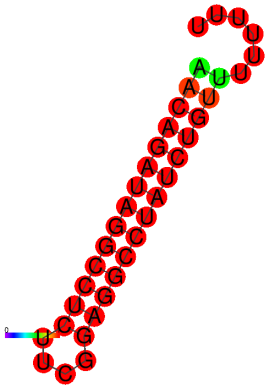


▼ T7hyb2

Sequence

AACAGATAGGCCTCttcgGAGGCCtatctgttTTTTTT

Minimum free energy structure

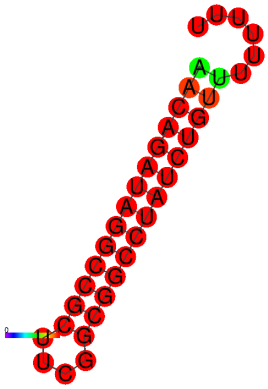


▼ T7hyb3

Sequence

AACAGATAGGCCGCttcgGCGGCCtatctgttTTTTTT

Minimum free energy structure

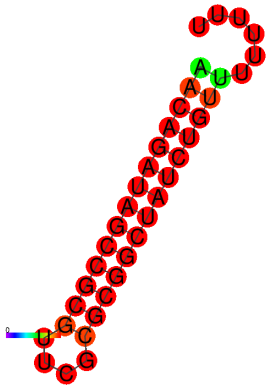


▼ T7hyb4

Sequence

AACAGATAGCCGCGttcgCGCGGCtatctgttTTTTTT

Minimum free energy structure

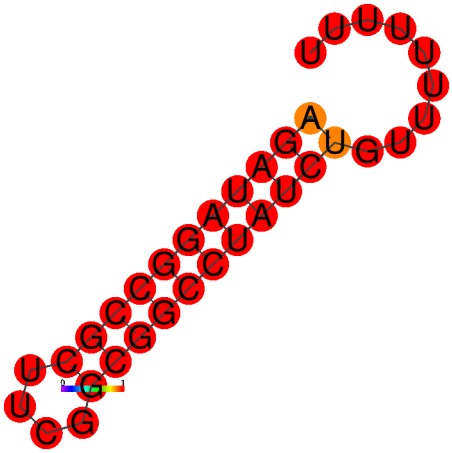


▼ T7hyb5

Sequence

AGATAGGCCGCttcgGCGGCCtatctgttTTTTTT

Minimum free energy structure

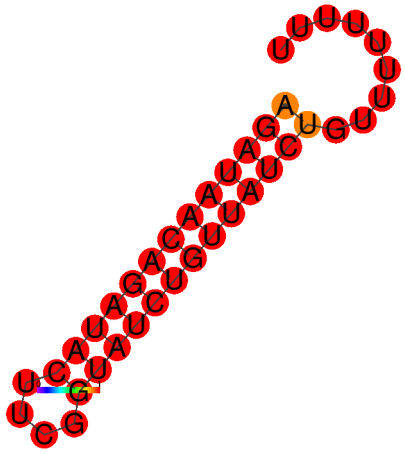


▼ T7hyb6

Sequence

AGATAACAGATACttcgGtatctgttatctgttTTTTTT

Minimum free energy structure

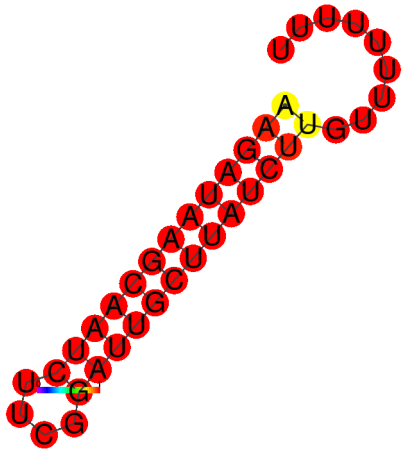


▼ T7hyb7

Sequence

AAGATAAGCAATCttcgGATTGCTTATCTTGTTTTTTTT

Minimum free energy structure

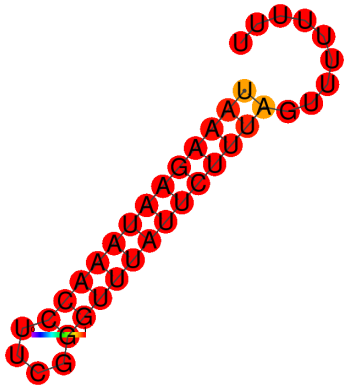


▼ T7hyb8

Sequence

TAAAGAATAAACCttcgGGTTTATTCTTTAGTTTTTTTT

Minimum free energy structure

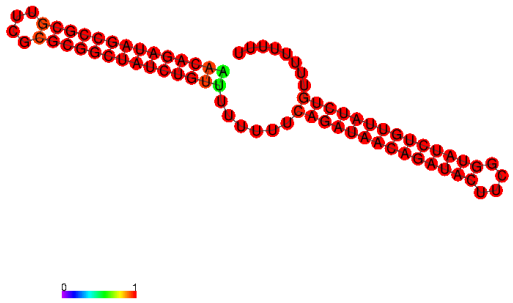


▼ T7hyb9

Sequence

AACAGATAGCCGCGttcgCGCGGCtatctgttTTTTTTCAGATAACAGATACttcgGtatctgttatctgttTTTTTT

Minimum free energy structure

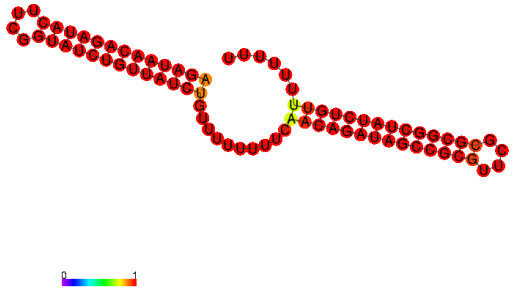


▼ T7hyb10

Sequence

AGATAACAGATACttcgGtatctgttatctgttTTTTTTcAACAGATAGCCGCGttcgCGCGGCtatctgttTTTTTT

Minimum free energy structure

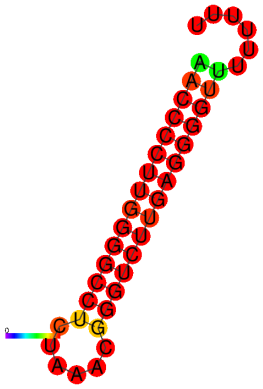


▼ T7nat

Sequence

AACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTTT

Minimum free energy structure

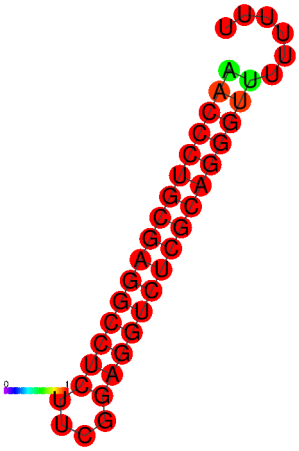


▼ T7mod

Sequence

AACCCTGCGAGGCCTCttcgGAGGTCTCGCAGGGTTTTTTTT

Minimum free energy structure



▼ T7pause

Sequence

CtatctgttatctgttC

Minimum free energy structure

N/A

Cloning strategy

- The dual reporter construct will be assembled in two cloning steps:
  1. Hifi assembly: pOpen-plamGFP-ccdB-mScarlet
  2. Golden gate assembly: pOpen-plamGFP-[T7_terminator]-mScarlet

HiFi assembly

- Entry vector: pOpen-ccdB
- Insert fragments: plamGFP and mScarlet

Note: To make use of ccdB counter-selection when identifying the Golden gate assembly clones, the ccdB gene will be maintained in the HiFi construct. To do this, ccdB will be amplified from pOpen-ccdB and essentially reinserted back into

the plasmid.

Assembly overview

All fragments have been designed with terminal overlaps (homology arms) to direct their assembly. Homology arms have been colour coded to reflect their complementarity.

Fragment type	Fragment name	Fragment source	Cloning identifier (backbone/insert)	5′ homology arm	3′ homology arm
Entry vector / backbone	pOpen	PCR	B1	F1	R1
Insert	plamGFP	gBlock	I2	F2	R2
backbone	ccdB	PCR	B3	F3	R3
Insert	mScarlet	gBlock	I4	F4	R4

Homology overview

—> F1 - R1 / F2 - R2 / F3 - R3 / F4 - R4 < —

Overlaps	Sequence (5′ —> 3′)	Length (bp)	Tm (˚C)
F1 / R4	caggtgatgacacacattaacaaatttcgtgaggag	36	69
R1 / F2	ctcccactccacctgaaccaagtcaaaagcc	31	72
R2 / F3	ggcttttagtagcgatggcgaagacta	27	68
R3 / F4	ccaagctctcaggaaacagctatgaccatgc	31	71

NEB recommends overlaps of 20-30 bp to increase assembly efficiency. 36bp may be slightly too long, however the Tm is in the range of a 20-30bp sequence.

Entry vector / backbone

B1

pOpen-ccDB will be linearised to remove the ccdB gene to provide an entry vector for the HiFi assembly

▼ PCR

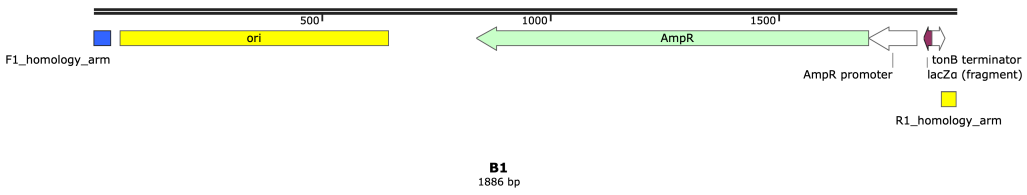
primers

Primer	Sequence	Tm (˚C)
F1	caggtgatgacacacattaacaaatttcg	66
R1	ctcccactccacctgaacc	68

Reaction conditions

Assembly	Fwd primer	Rev primer	Annealing temp (˚C)	Length (bp)	Extension time (secs)
B1	F1	R1	67	1886	40

▼ Product



B3

The ccdB gene will be amplified to then be reinserted into the entry vector between plamGFP and mScarlet

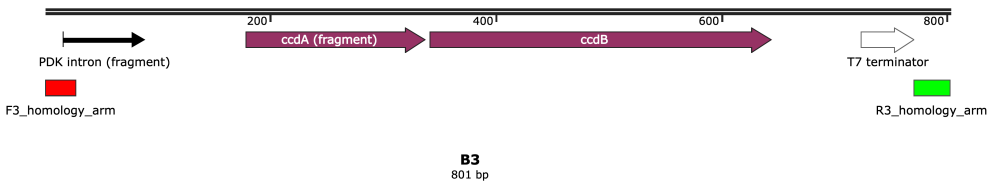
▼ PCR

Primer	Sequence	Tm (˚C)
F3	tagtcttcgccatcgctac	64
R3	ccaagctctcaggaaacagctatg	68

Reaction conditions

Assembly	Fwd primer	Rev primer	Annealing temp (˚C)	Length (bp)	Extension time (secs)
B3	F3	R3	65	801	20

▼ Product



Insert fragments

Insert fragments consist of a fluorescent protein with terminal homology arms and either a 3′ or 5′ BsmBI cut site to enable the golden gate driven removal of ccdB and the subsequent insertion of T7 terminator sequences.

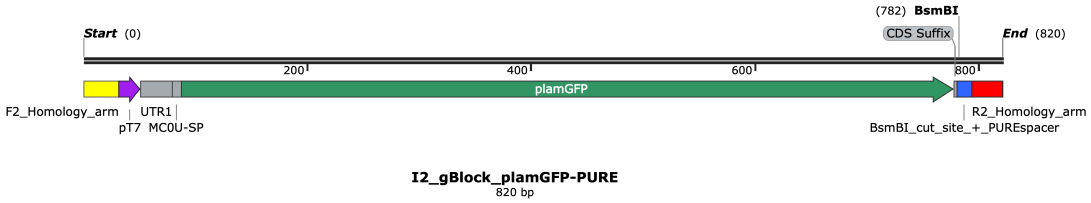
Note: I initially attempted to order plamGFP-ccdB-mscarlet as a single gBlock, but due to large homology between the FPs and other difficult to synthesise sequences, the most efficient way was to order the FPs separately.

I2

Structure

[Homology_arm]-[pT7]-[UTR1]-[spacer]-[plamGFP]-[BsmBI_cut_site]-[Homology_arm]

Note: I2 does not contain a T7_term to enable transcriptional read through.

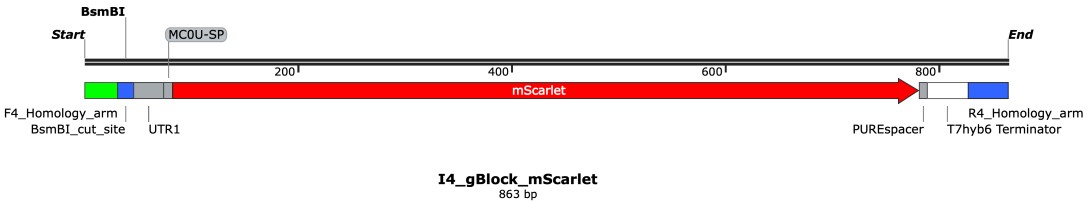


I4

Structure

[Homology_arm]-[BsmBI_cut_site]-[UTR1]-[spacer]-[mScarlet]-[spacer]-[T7_term]-[Homology_arm]

Note: I4 does not contain a T7 promoter to enable transcriptional read through from I2.

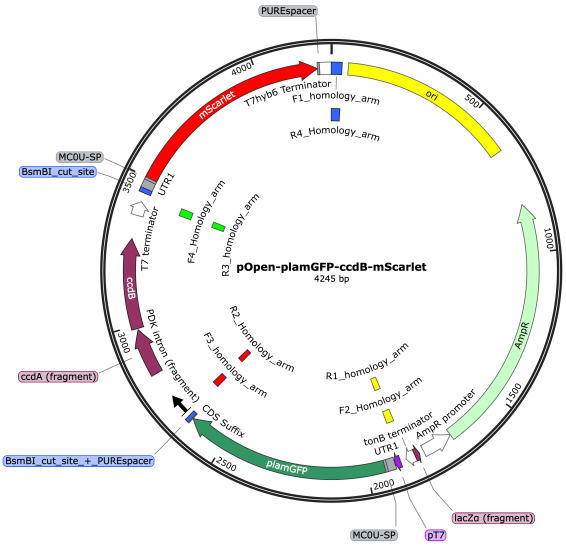


Assembly : pOpen-plamGFP-ccdB-mScarlet

Structure (Insert)

[pT7]-[UTR1]-[spacer]-[plamGFP]-[BsmBI_cut_site]-[ccdB]-[BsmBI_cut_site]-[UTR1]-[spacer]-[mScarlet]-[spacer]-[T7_term]

The fully assembled construct (4245 bp) consists of a pOpen backbone with a ccdB gene surrounded by plamGFP and mScarlet. The ccdB gene is flanked by BsmBI cut sites to enable the insertion of BsmBI digested terminator sequences.



Golden gate assembly

- Entry vector: pOpen-plamGFP-ccdB-mScarlet
- Insert fragments: T7 terminators

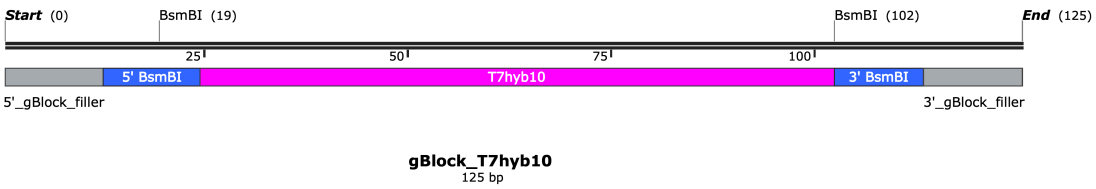
Note: Insert fragments were ordered as 125bp gBlocks containing BsmBI cut sites in a complementary orientation to the entry vector. Integrated across the 5′ entry vector and insert fragment cut sites is short ‘PURE_spacer’ sequence that will sit between the plamGFP stop codon and the beginning of the inserted terminator sequence.

Insert fragments

Structure

[5′_gBlock_filler]-[5′_BsmBI]-[T7_term]-[3′_BsmBI]-[3′_gBlock_filler]

Example (T7hyb10)



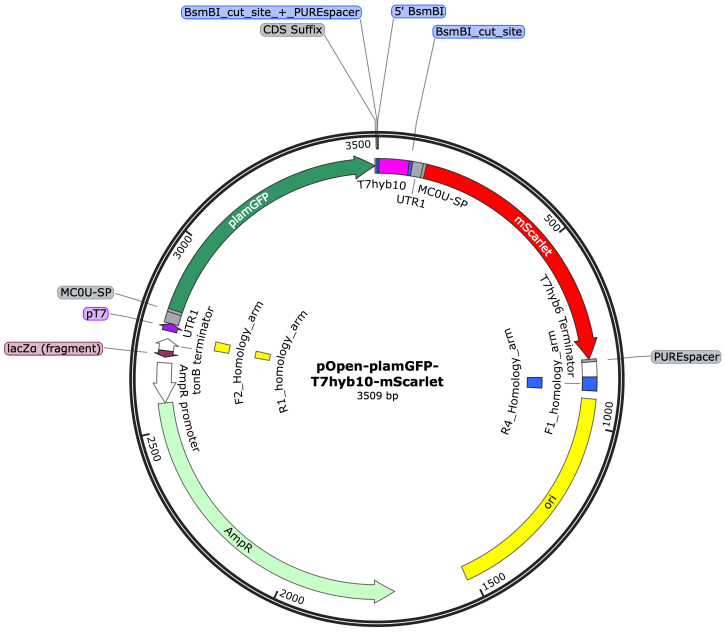
Assembly : pOpen-plamGFP-T7_term-mScarlet

Structure

[pT7]-[UTR1]-[spacer]-[plamGFP]-[T7hyb10]-[UTR1]-[spacer]-[mScarlet]-[spacer]-[T7_term]

The fully assembled dual reporter construct consists of pT7 controlled plamGFP gene followed by a T7 terminator and a downstream mScarlet gene. plamGFP and mScarlet possess separate ribosome binding sites facilitating the translation of both reporters from a single transcript. The relative production of reporters will therefore be determined by the strength of the central T7 terminator.

Example (pOpen-plamGFP-T7hyb10-mScarlet)



DNA build

@September 8, 2025 : HiFi entry vector PCRs

- B1 and B3 were prepared through linearisation of the pOpen-ccdB template.
- 100 µL reaction volumes, 4 ng template, 35 cycles.

Note: This was previously attempted @September 2, 2025 but the gel purification step failed. This was most likely due to a combination of smaller reaction volume (50 µL) and an expired gel purification kit.

▼ PCR

Sample	Fwd primer	Rev primer	Annealing temp (°C)	Length (bp)	Extension time (secs)
B1	F1	R1	67	1886	40
B3	F3	R3	65	801	20
Standard reaction					
	Component	**Final Concentration**	**Vol (µL)**	**Vol for 2.5x rxns (µL)**	
	Q5 High-Fidelity 2X Master Mix	1X	50	125	
	10 µM Forward Primer	0.5 µM	5	12.5	
	10 µM Reverse Primer	0.5 µM	5	12.5	
	Template	0.04 ng/ µL (5 ng total)	4	10	
	Nuclease-Free Water		36	90	
	Total		100	250	
Standard reaction (-primers)	**Component**	**Final Concentration**	**Vol (µL)**	**Vol for 2.5x rxns (µL)**	
	Q5 High-Fidelity 2X Master Mix	1X	50	125	
	Template	0.04 ng/ µL (5 ng total)	4	10	**Distribute 90 µL into 2x tubes**
	Nuclease-Free Water		36	90	
	Total		90	225	
Primer addition	**Sample**	**Vol Fwd primer (µL)**	**Vol Rev primer (µL)**	**Total (µL)**	
	B1	5	5	100	
	B3	5	5	100	
Template dilution (1ng / µL)	**Sample**	**V DNA (µL)**	**V H20 (µL)**	**Total**	
	pOpen-ccdB (25.1 ng / µL)	0.80	19.20	20.00	

@September 10, 2025: Gel electrophoresis and band extraction

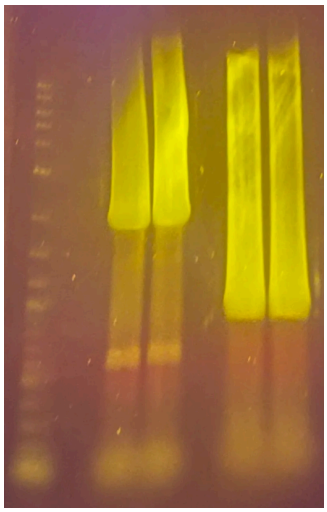
- B1 and B3 were run on 0.8 % agarose, 100v, 2hrs 15mins . 10 µL ladder.

  ▼ Sample prep

Sample	Conc (ng/µL)	**Vol remaining (µL**	**Mass remaining (ng)**	**V Dye**	**V DNA**	**V H20**	**Tota**
B1 (Extraction)	—	100	—	16.7	83.3	—	100
B3 (Extraction)	—	100	—	16.7	83.3	—	100

- Post-stained: 1x Sybr-gold, 30 mins.

- The thickest part of each band was excised and trimmed. Bands were stored in pre-weighed, sealed eppendorf tubes overnight at 4˚C.



Lanes (L→R)

1: Ladder

3-4: B1

6-7: B3

@September 11, 2025: Gel purification

- Samples were gel purified using a fresh Monarch DNA gel extraction kit.
  - **Note: It is important to ensure gel pieces do not exceed 400mg, and ideally, 300mg as the max addition of dissolving buffer is ~1250µL. B3 band 2 was split into B3 2.1 and 2.1 to adhere to this.**
  - After addition of dissolving buffer, samples were incubated at 55˚C with regular inversion to ensure gel pieces were completely dissolved.

▼ Sample prep / yield

Sample	Tube mass (g)	Tube + gel piece mass (g)	Gel piece mass (g)	Gel dissolving buffer (µL)	DNA conc (ng / µL)	Estimated DNA quantity (ng)
B1 #1	1.108	1.381	0.273	1092	209.9	2099
B1 #2	1.109	1.36	0.251	1004		
				0		
B3 #1	1.094	1.461	0.367	1468	168.4	1684
B3 #2.1	1.094	1.345	0.251	1004		
B3 #2.2	1.118	1.327	0.209	836		
				* 1200 µL was added to all samples <1400 µL	* Samples eluted in 6 µL and combined. Total:12 µL.	

Samples underwent a further NEB DNA cleanup step to remove excess salt.

@September 15, 2025: HiFi reactions

- The NEB builder calculator was used to determine the HiFi assembly conditions. As this is a 4 part assembly, the quantity of input DNA was doubled to 0.5 pmols to increase the efficiency of the reaction.
    - **Note:** I2 and I4 were resuspended in IDTE to 50 ng / µL.

▼ Calculations

Sample	Fragment type	Size (bp)	Pmols	Mass (ng)	Fragment conc (ng / µL)	Vol required (µL) Cf = 10 µL
B1	Entry	1886	0.025	30.6	100.0	0.3
I2	Insert	820	0.025	13.3	50.0	0.3
B3	Entry	801	0.025	13.0	100.0	0.1
I4	Insert	863	0.025	14.0	50.0	0.3
			Maximised		Total:	1.0
			0.0625	76.6	100	0.77
			0.0625	33.3	50.0	0.67
			0.0625	32.5	100	0.33
			0.0625	35.1	50.0	0.70
					Total:	2.46

▼ Reactions

Component	Mass required (ng)	Fragment conc (ng / µL)	Vol required (µL)
B1	76.6	100	0.77
I2	33.3	50	0.67
B3	32.5	100	0.33
I4	35.1	50	0.70
H20	-	-	2.54
NEBuilder mix	-	-	5
Total:	-	-	10
Total check:	-	-	10.00

- Samples were incubated @ 50˚C for 1hr.
    - Assembly reactions (2 µL) were used to transform NEB Stable competent cells (50 µL) following 🧫 lysY/laclq Transformation and plated on LB-agar (1.5%, 1x carb).
        - Protocol deviations - Heat shock: 30 secs. Outgrowth: 14 mL culture tubes (NEB stable media). Dilutions: 40 µL, 20 µL, 10^{-1}:10^{-4}.

@September 17, 2025: HiFi assembly sequencing

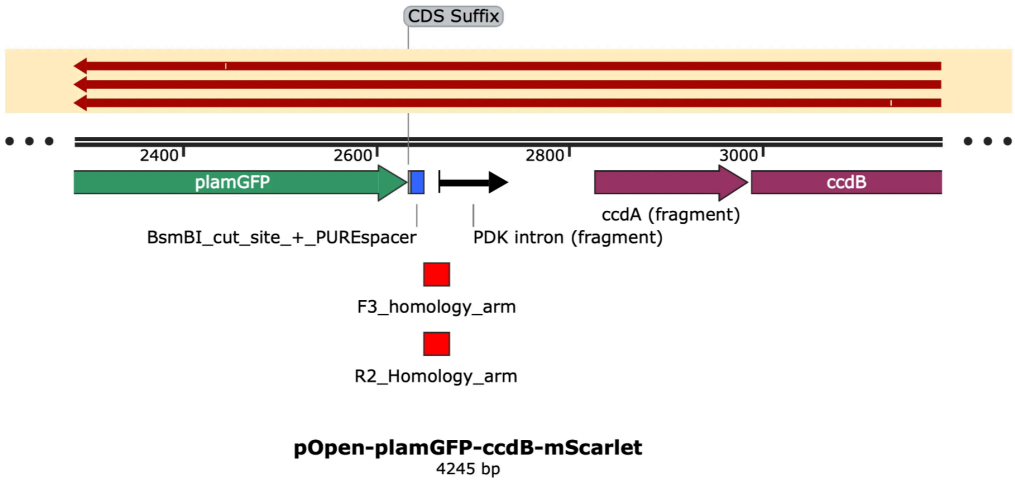
- @September 16, 2025: 3x colonies picked for outgrowth in 5 mL LB (1x carb) and incubated O/N.
- @September 17, 2025: Cultures miniprepped and sent for sequencing.

▼ Sequencing prep

Sample	DNA concentration (ng/µL)	V DNA for 10µL @ 25 ng / µL (µL)	V H20 for 10 µL @ 25 ng / µL (µL)
pOpen-plamGFP-ccdb-mScarlet #1	156	1.60	8.40
pOpen-plamGFP-ccdb-mScarlet #2	127.6	1.96	8.04
pOpen-plamGFP-ccdb-mScarlet	42.5	5.88	4.12

#2				

▼ Sequence alignments



- Colony 1: Mutation in plamGFP
- Colony 2: **Perfect alignment**
- Colony 3: Mutation in ccDB

Colony 2 to be used as the GG assembly entry vector.

@September 19, 2025: Golden gate assembly

- The NEBiocalculator was used to determine the golden gate assembly conditions. The entry vector and insert fragments are to be combined in a 1:2 ratio.
  - **Note:** The T7 terminator gBlock sequences were resuspended in IDTE to 4.5 ng/µL.

▼ Assembly calculations

Vector type	**mass required (ng)**	**Size (bp)**	Pmols
Entry	75	4245	0.027
Insert	**4.42**	125	0.054

▼ Assembly reactions

Assembly reactions							
	Sample	V Entry (75 ng/µL, µL)	V Insert (4.5 ng/µL, µL)	V T4 DNA ligase buffer (10x, µl)	NEBridge GG mix (µL)	V H2O (µL)	Total (µL)
	T7hyb1	1	1	2	1	15	20
	T7hyb2	1	1	2	1	15	20
	T7hyb3	1	1	2	1	15	20
	T7hyb4	1	1	2	1	15	20
	T7hyb5	1	1	2	1	15	20
	T7hyb6	1	1	2	1	15	20
	T7hyb7	1	1	2	1	15	20

	T7hyb8	1	1	2	1	15	20
	T7hyb9	1	1	2	1	15	20
	T7hyb10	1	1	2	1	15	20
	T7mod	1	1	2	1	15	20
	T7nat	1	1	2	1	15	20
	T7pause	1	1	2	1	15	20
Template dilutions							
	Sample	V DNA (µL)	V H20 (µL)	Total (µL)			
	Entry (75 ng / µL)	11.76	8.24	20.00			
	Insert (4.5 ng/ µL)	*	55.56	55.56			
		* gblocks @ 250ng requiring resuspension.					
Mastermix calculation	**Component**	**Volume (1 rxn, µL)**	**Volume (15 rxns, µL)**				
Scale factor	Entry (75 ng/ µL, µL)	1	15				
15	T4 DNA ligase buffer (10x, µl)	2	30				
	NEBridge GG mix (µL)	1	15	**Distribute 19 µL into 14x tubes and add 1 µL insert.**			
	V H2O (µL)	15	225				
	Total:	19	285				

- Samples incubated @ 42˚C for 5 mins and stored at -20˚C prior to transformation
  - @September 21, 2026 Assembly reactions (2 µL) were used to transform NEB DH5a competent cells (50 µL) following 💊 lysY/laclq Transformation and plated on LB-agar (1.5%, 1x carb).
    - Protocol deviations - Heat shock: 30 secs. Outgrowth: 14 mL culture tubes (NEB stable media). Dilutions: 20 µL, 10^{-1}:10^{-5}.

@September 24, 2025: Golden gate assembly sequencing

- @September 23, 2025: 2x colonies from each terminator insert picked for outgrowth in 5.5 mL LB (1x carb) and incubated O/N.
- @September 24, 2025: Cultures miniprepped, and 0.5 mL combined with 0.5 mL 50% glycerol to prepare glycerol stocks stored in eppendorf tubes at -80˚C
  - **Note:** 26 minipreps performed simultaneously. **Importantly** lysis step performed in 4 batches (6, 6, 6, 8) to ensure samples have minimal exposure to lysis buffer. **Slight problem**, cultures overgrown so the neutralisation step involved 2x spins for 7 mins increasing the chance of salt carry over. Glycerol stocks should have been prepared in smaller volumes in 96 well plate.
- @September 25, 2025: Samples sent for sequencing.

▼ Sequencing results

Note: Interestingly, 5/26 samples weren't assembled. These only occurred in the even samples suggesting an issue at plasmidsaurus, especially as these didn't correlate to the samples that had minor salt carry over.

Sample	Conc (ng/µL)	Salt carry over (Y)	Sequence alignment (Y)
T7hyb1_1.1	68	-	**Y**
T7hyb1_1.2	62.7	-	No assembly
T7hyb2_1.1	47.9	-	**Y**
T7hyb2_1.2	37.1	-	**Y**
T7hyb3_1.1	111.9	-	**Y**
T7hyb3_1.2	79.6	-	**Y**
T7hyb4_1.1	40	**Y**	**Y**
T7hyb4_1.2	56.3	-	No assembly
T7hyb5_1.1	56.7	-	**Y**
T7hyb5_1.2	75	-	No assembly
T7hyb6_1.1	119.3	-	**Y**
T7hyb6_1.2	61.6	-	No assembly
T7hyb7_1.1	63.7	**Y**	- (plamGFP deletion)
T7hyb7_1.2	52.4	-	- (plamGFP deletion)
T7hyb8_1.1	47.5	**Y**	Y
T7hyb8_1.2	61.8	-	Y
T7hyb9_1.1	42.4	-	- (Deletion)
T7hyb9_1.2	54.2	-	No assembly
T7hyb10_1.1	66.3	-	- (Deletion)
T7hyb10_1.2	39.9	-	**Y**
T7nat_1.1	47.5	-	**Y**
T7nat_1.2	55.8	-	**Y**
T7mod_1.1	43.9	**Y**	**Y**
T7mod_1.2	50.8	-	**Y**
T7pause_1.1	53.7	-	**Y**
T7pause_1.2	55.8	-	**Y**

All samples except **T7hyb7** and **T7hyb9** contained a perfect colony.

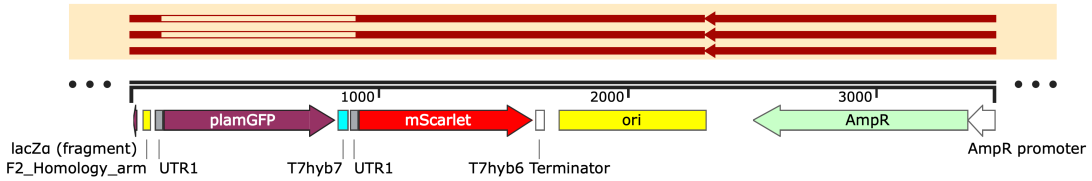
@September 28, 2025: T7hyb7 and T7hyb9 sequence verification

- @September 28, 2025: 3x colonies of T7hyb7 and T7hyb9 picked into 5.5 mL LB (1x carb) and incubated O/N
  - **Note:** Small colonies that are difficult to pick may result in slow growth of cultures
- @September 29, 2025: 2x T7hyb9 and 1x T7hyb7 cultures miniprepped and sent for sequencing.
  - **Note:** Some cultures had not grown by the next morning

▼ Sequencing results

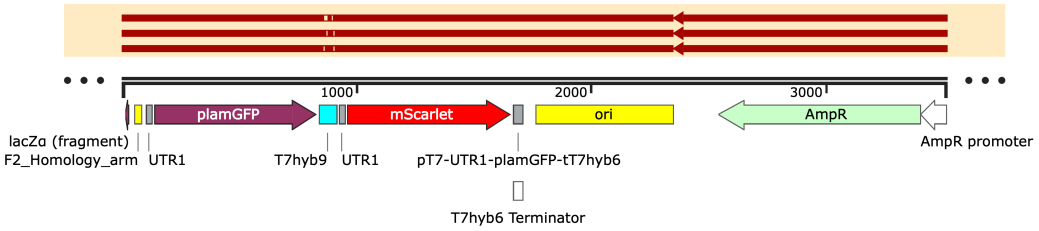
T7hyb7

- Colony 3 was a perfect match.
  - **Note:** Forgot to make glycerol stock. **Update:** @September 30, 2025 - One of the remaining T7hyb7 cultures was miniprepped and sent for sequencing. Sequence was a perfect match and a glycerol stock was prepared and stored at -80˚C.



pOpen-plamGFP-T7hyb7-mScarlet
3470 bp

T7hyb9



pOpen-plamGFP-T7hyb9-mScarlet
3509 bp

- No perfect match
  - **Note:** @September 30, 2025 - the remaining T7hyb9 cultures was miniprepped and sent for sequencing. Again, still mutated.

- @October 1, 2025 **T7hyb9 retransformed in DH5a and plated on Agar plate (20 mL, 1x carb) and incubated O/N.**
  - @October 2, 2025: 6x colonies picked in 5.5 mL LB (1x carb)

@September 29, 2025: TU isolation test

- Gradient PCR to test the TU isolation of the T7hyb10 dual-reporter construct.

▼ PCR conditions

Primers

Primer	Sequence	Tm (°C)
M13_fwd	gtaaaacgacggccagt	62
T7_term_rev	gcattcttctggagactc	59

Reaction conditions

Assembly	Fwd primer	Rev primer	Annealing temp (°C)	Length (bp)	Extension time (secs)
TU isolation	M13_fwd	T7_term_rev	57,**60**,63	~1700	40

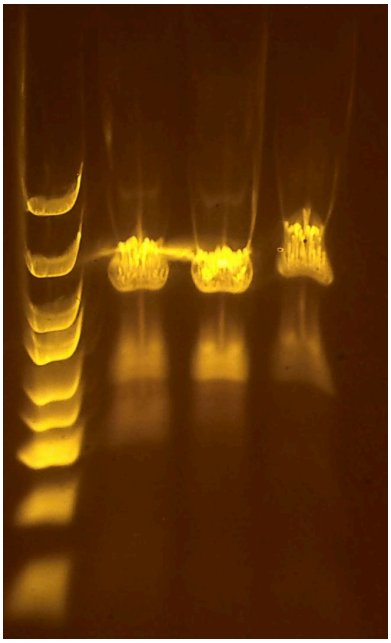
▼ PCR calculations

Scale factor	**Component**	**Final Concentration**	**Vol (µL)**	**Vol for 3.5x rxns (µL)**	

3.5	Q5 High-Fidelity 2X Master Mix	1X	15	52.5	
	10 µM Forward Primer (M13_fwd)	0.5 µM	1.5	5.25	
	10 µM Reverse Primer (T7_term_rev)	0.5 µM	1.5	5.25	Distribute **25 µL** into 3x tubes
	Template	0.04 ng/ µL (5 ng total)	1	3.5	
	Nuclease-Free Water		11	38.5	
	Total		30	105	
Template dilution (1ng / µL)	**Sample**	**Sample Conc (ng / µL)**	**V DNA (µL)**	**V H20 (µL)**	**Total**
	pOpen-plamGFP-**T7hyb10**-mScarlet	39.90	1.00	39.00	40

▼ Gel QC

- 20 µL Samples (10 µL 0.5x PCR product, 10 µL Express running buffer) were run on an EX 1% gel for 10 mins.
- Estimated amplicon size = 1700 bp



Lanes

1: Ladder

2: 57˚C

3: 60˚C

4: 63˚C

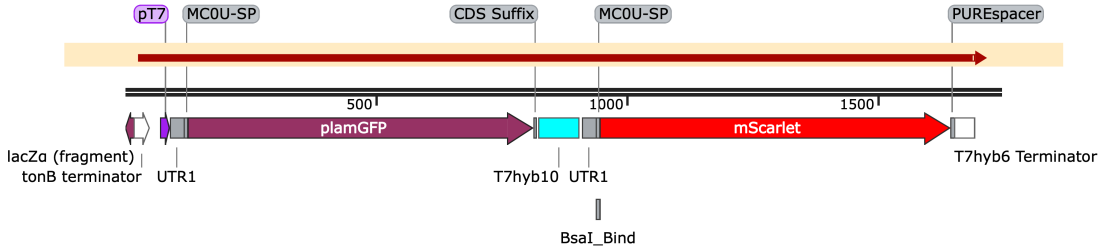
All samples indicate the production of an amplicon. Interestingly, the size of the amplicon appears to be much larger than expected. Also, there appears to be a reasonable amount of smaller contaminating PCR species - **Gel purification**

in future?

- **60˚C sample sent for amplicon sequencing to verify identity**

▼ Amplicon sequencing

- Sequence perfectly matched to expected T7hyb10 amplicon
  - **Note:** Point mutation at the end of the T7hyb6 terminator in a polyA region. No issues.



plamGFP-T7hyb10-mScarlet_TU
1743 bp

@September 30, 2025: TU scale up (-T7hyb9)

- TUs of all constructs (-T7hyb9) were PCR amplified. Reaction vol scaled to 100 µL.

▼ PCR conditions

PCR conditions	Sample	Fwd primer	Rev primer	Annealing temp (˚C)	Length (bp)	Extension time (secs)
	TU isolation	M13_fwd	T7_term_rev	**60**	~1700	40
Parameters	Sample	Initial denaturation 98˚C (secs)	Denaturation 98˚C (secs)	Annealing (secs) (57˚C, 60˚C, 63˚C)	Extension 72˚C (secs)	Final extension 72˚C (secs)
	TU isolation	30	10	30	40	120

▼ PCR calculations

Standard reaction	Component	Final Concentration	Vol (µL)			
	Q5 High-Fidelity 2X Master Mix	1X	50			
	10 µM Forward Primer (M13_fwd)	0.5 µM	5			
	10 µM Reverse Primer (T7_term_rev)	0.5 µM	5			
	Template	0.04 ng/ µL (5 ng total)	4			
	Nuclease-Free Water		36			
	Total		100			
Standard reaction	**Component**	**Final**	**Vol (µL)**	**Vol 13x (µL)**		

(- template)		**Concentration**				
Scale factor	Q5 High-Fidelity 2X Master Mix	1X	50	650		
13	10 µM Forward Primer (**M13_fwd**)	0.5 µM	5	65		
	10 µM Reverse Primer **(T7_term_rev)**	0.5 µM	5	65	**Distribute 916 µL in 12x tubes**	
	Nuclease-Free Water		36	468		
	Total		96	1248		
Template addition (plamGFP)	**Sample**	**V Template**	**Total (µL)**			
	T7hyb-1	4	100			
	T7hyb-2	4	100			
	T7hyb-3	4	100			
	T7hyb-4	4	100			
	T7hyb-5	4	100			
	T7hyb-6	4	100			
	T7hyb-7	4	100			
	T7hyb-8	4	100			
	T7hyb-10	4	100			
	T7hyb-Nat	4	100			
	T7hyb-Mod	4	100			
	T7hyb-Pause	4	100			
Template dilution (1 ng / µL)	**Sample**	**Conc (ng / µL)**	**V template (µL)**	**V H2O (µL)**	**Total**	
	T7hyb-1 (**1.1**)	68	1.47	98.53	100	
	T7hyb-2 (**1.1**)	47.9	2.09	97.91	100	
	T7hyb-3 (**1.1**)	111.9	0.89	99.11	100	
	T7hyb-4 (**1.1**)	11.1	1.80	18.20	20	
	T7hyb-5 (**1.1**)	56.7	1.76	98.24	100	
	T7hyb-6 (**1.1**)	119.3	0.84	99.16	100	
	T7hyb-7	27.2	3.68	96.32	100	
	T7hyb-8 (**1.2**)	61.8	1.62	98.38	100	
	T7hyb-10 (**1.2**)	39.9	2.51	97.49	100	
	T7hyb-Nat (**1.2**)	55.8	1.79	98.21	100	
	T7hyb-Mod (**1.2**)	50.8	1.97	98.03	100	
	T7hyb-Pause (**1.2**)	55.8	1.79	98.21	100	

- Reactions stored at -20˚C prior to DNA cleanup.

@October 15, 2025: Testing dual reporter constructs

- All constructs were expressed in triplicate using PURExpress. plamGFP and mScarlett signal was monitored throughout the duration of the reaction.

▼ PURExpress calculations

Standard reaction						
	Component	**Starting concentration**	**Final Concentration**	**Vol (µL)**		
	Solution A	2.5x	1X	14.00		
	Solution B	3.3x	1x	10.51		
	Murine RNAse inhibitor	40 U / µL	1 U / µL	0.88		
	DNA Template (100 ng / µL)	40 ng / µL	5 ng / µL	4.38		
	Nuclease-Free Water	—	—	5.24		
	Total			35		
Standard reaction (-DNA)	**Component**	**Starting concentration**	**Final Concentration**	**Vol_Standard (µL)**	**Vol_16x (µL)**	
	Solution A	2.5x	1X	14.0	224.0	
Scale factor	Solution B	3.3x	1x	10.5	168.2	
16	Murine RNAse inhibitor	40 U / µL	1 U / µL	0.88	14.0	**Distribute 30.63 µL into 14x tubes**
	Nuclease-Free Water	—	—	5.24	83.8	
	Total (check)			30.63	490.0	
Template addition	**Sample**	**V DNA**	**V H20**	**Total**		
	Negative	0	4.38	35.0		
	T7pause	4.38	0.00	35.0		
	T7nat	4.38	0.00	35.0		
	T7mod	4.38	0.00	35.0		
	T7hyb1	4.38	0.00	35.0		
	T7hyb2	4.38	0.00	35.0		
	T7hyb3	4.38	0.00	35.0		
	T7hyb4	4.38	0.00	35.0		
	T7hyb5	4.38	0.00	35.0		
	T7hyb6	4.38	0.00	35.0		
	T7hyb7	4.38	0.00	35.0		
	T7hyb8	4.38	0.00	35.0		
	T7hyb9	4.38	0.00	35.0		
	T7hyb10	4.38	0.00	35.0		
Template dilutions (40 ng/ µL)	**Sample**	**Conc (ng/µL)**	**V DNA**	**V H20**	**Total**	
	T7hyb1	230.50	2.60	12.40	15	
	T7hyb2	220.00	2.73	12.27	15	

	T7hyb3	222.70	2.69	12.31	15	
	T7hyb4	230.00	2.61	12.39	15	
	T7hyb5	222.70	2.69	12.31	15	
	T7hyb6	217.00	2.76	12.24	15	
	T7hyb7	203.90	2.94	12.06	15	
	T7hyb8	234.60	2.56	12.44	15	
	T7hyb9	235.70	2.55	12.45	15	
	T7hyb10	221.30	2.71	12.29	15	
	T7nat	213.80	2.81	12.19	15	
	T7mod	218.40	2.75	12.25	15	
	T7pause	217.30	2.76	12.24	15	

▼ Platemap

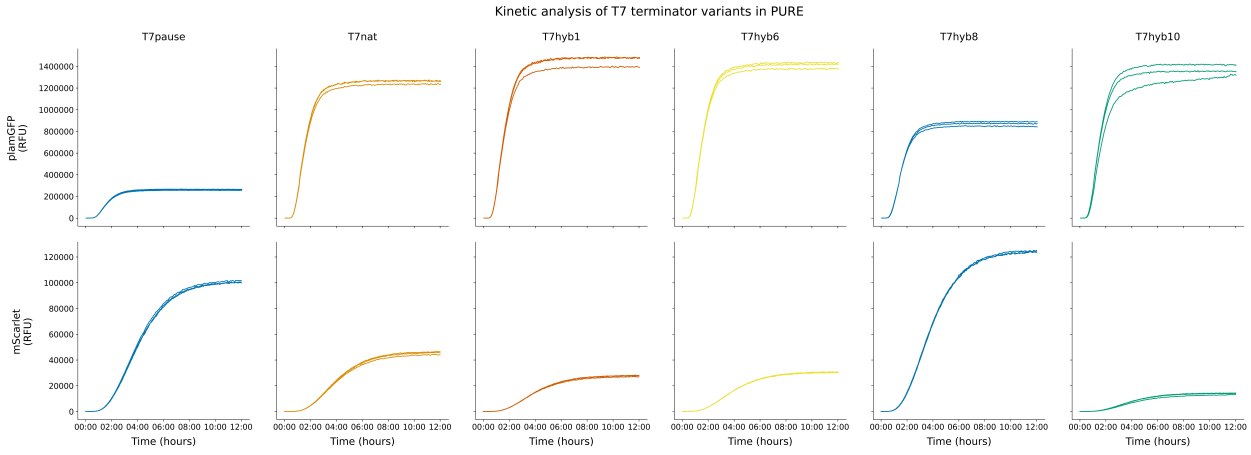
Well	Sample #	Name	Terminator
D3	1	Negative	
F3	1	Negative	2
H3	1	Negative	3
D5	2	T7pause	1
F5	2	T7pause	2
H5	2	T7pause	3
D7	3	T7nat	1
F7	3	T7nat	2
H7	3	T7nat	3
D9	4	T7mod	1
F9	4	T7mod	2
H9	4	T7mod	3
D11	5	T7hyb1	1
F11	5	T7hyb1	2
H11	5	T7hyb1	3
D13	6	T7hyb2	1
F13	6	T7hyb2	2
H13	6	T7hyb2	3
D15	7	T7hyb3	1
F15	7	T7hyb3	2
H15	7	T7hyb3	3
D17	8	T7hyb4	1
F17	8	T7hyb4	2
H17	8	T7hyb4	3
D19	9	T7hyb5	1
F19	9	T7hyb5	2
H19	9	T7hyb5	3
D21	10	T7hyb6	1
F21	10	T7hyb6	2
H21	10	T7hyb6	3
J3	11	T7hyb7	1

L3	11	T7hyb7	2
N3	11	T7hyb7	3
J5	12	T7hyb8	1
L5	12	T7hyb8	2
N5	12	T7hyb8	3
J7	13	T7hyb9	1
L7	13	T7hyb9	2
N7	13	T7hyb9	3
J9	14	T7hyb10	1
L9	14	T7hyb10	2
N9	14	T7hyb10	3

Data analysis

Kinetic data

- The below kinetic plot shows a subset of the T7 terminator collection (T7pause, T7nat, T7hyb1, T7hyb6, T7hyb8, and T7hyb10). This subset was chosen as it includes each type of variant within the collection: T7nat consists of a characteristic stem loop; T7hyb1 is a variant of T7nat with a stronger stem loop (T7mod) and an internal pause site; T7hyb6 is similar but with two overlapping pause sites; T7hyb8 is a mimic of T7hyb6 but with scrambled pause sites; and T7hyb10 contains tandem double stem loop structures. The figure shows both plamGFP and mScarlet plots for each variant. As the plamGFP gene is located at the 5′ end, and the mScarlet gene at the 3′ of the bicistron, it is expected that there would be lower GFP signal, and higher mScarlet signal in weaker terminators, and the opposite trend in stronger terminators. This pattern is present throughout the plot highlighted first by the T7pause variant displaying the weakest GFP signal and a strong mScarlet signal. Note, however, that despite being a stronger terminator than T7pause, T7hyb8 actually produces a greater mScarlet signal. We therefore have to look at the ratio of GFP/mScarlet signal to determine the true termination efficiencies of each variant. A possible reason for this may be due to varying amounts of DNA concentrations being present in the final reactions despite efforts to ensure that they were normalised.



Endpoint data and termination efficiencies

- By comparing our results with previously reported data, we found that the T7 terminator collection demonstrates higher absolute termination efficiencies across all variants in the PURE system. This is most notable in the T7nat sequence, which yielded an efficiency of 91% in PURE, compared to the 62% reported in *E. coli* and only 5% in simplified IVT systems. While the relative performance ranking remained consistent for T7nat, T7mod, and T7hyb1–6 across all biological contexts, the T7hyb7–10 variants presented notable exceptions. Although T7hyb7 and T8hyb8 exhibited the

lowest efficiencies in the collection as expected, they reached 70% and 63% in the PURE environment—a substantial increase and rank reversal over the 12% and 46% reported in *E. coli*. Similarly, T7hyb9 marginally exceeded the performance of T7hyb10 in PURE, whereas in *E. coli*, T7hyb9 showed a significantly weaker termination efficiency of 69% relative to the 98% achieved by T7hyb10.

