PPK Methods
DNA Work
Constructs for the cgPPK, PduD(1-20)_cgPPK_mCherry and PduD(1-20)_mCherry_cgPPK arrived as gBlocks® from IDT.

The pNIC28-bsa4 vector was chosen as an expression vector. To insert the constructs into pNIC28-bsa4, the vector was linearised by digestion using XbaI and XhoI and rSAP in Cutsmart™ buffer. The digestion proceeded for 2 hours at 37˚C followed by a heat inactivation step at 80˚C for 20 minutes. The Digestion products were separated by size via electrophoresis on a 1% TAE agarose gel with SYBRsafe stain at 100 V for 45 minutes. The 5 kb linerised fragment was excised from the gel and purified using a QIAquick Gel Extract kit (QIAgen) according to the manufacturers' instructions.
Each of the gBlock® constructs were digested with XbaI and XhoI for 90 minutes in Cutsmart™ buffer at 37 ˚C and heated to 80 ˚C for 20 minutes. The fragments were ligated with the linearised vector using NEB Quick Ligase™ and Quick Ligase buffer according to the manufacturer's instructions at room temperature for 20 minutes. The ligation product was transformed into NEB DH5α Ultra Competent cells by incubating a small volume of the ligation product with the cells on ice for 30 minutes prior to a 30 second heat shock at 42 ˚C. The heat shock was followed by a 5-minute incubation on ice and then a one hour outgrowth stage in SOC medium at 37 ˚C and 180rpm. The cells were immediately plated onto LB agar plates supplemented with 100mg/L kanamycin and incubated overnight at 37 ˚C.
Individual colonies from the transformation were selected for overnight 5ml liquid cultures in LB supplemented with 100mg/L kanamycin at 37 ˚C and 180 rpm. After 16 - 18 hours of growth, the cells were pelleted by centrifugation at 4,000 rpm for 10 minutes and the plasmid DNA was extracted and purified using a QIAprep Spin Miniprep Kit (QIAgen). The plasmid was sequenced using the TubeSeq Sanger Sequencing service (Eurofins) with the following sequencing primers:

Construct:	**Sequencing Primers used:**	**Sequence:**
pNIC28-bsa4_ PduD(1-20)_mCherry_cgPPK	**pET_Upstream**	ATGCGTCCGGCGTAGA
	CP Seq for	CGGCGAATTTATCTACAAAGTC
	CP Seq rev	CAAAATTTGGTCAATGAAATGCC

	T7 Term	CTAGTTATTGCTCAGCGGT
pNIC28-bsa4_ PduD(1-20)_cgPPK_ mCherry	**pET_Upstream**	ATGCGTCCGGCGTAGA
	PC Seq for	CGTTAGAAAAACCATCCCCG
	PC Seq rev	GCAGCTTGACTTTGTAGATA AATTC
	T7 Term	CTAGTTATTGCTCAGCGGT
pNIC28-bsa4_ cgPPK	**pET_Upstream**	ATGCGTCCGGCGTAGA
	T7 Term	CTAGTTATTGCTCAGCGGT

Sequence-confirmed constructs were retained for use in expression.

Expression of Constructs

Expression of the PPK constructs was based on the method described by (Lindner et al., 2007). BL21(DE3) cells were obtained from a parent stock from NEB, cultured in LB at 37 ˚C and 180 rpm and made competent with the transformation and storage solution (TSS) method described by (Chung, Niemela and Miller, 1989).

These cells were transformed with approximately 100ng of each construct using the same method used for the DH5α cells, transferred on LB agar plates supplemented with 100mg/L kanamycin and incubated overnight at 37 ˚C.

One monoclonal colony from each transformation was cultured in LB supplemented with 100mg/L kanamycin at 37 ˚C and 180 rpm for 16-18 hours. 500μl of each culture was used to inoculate 500ml of LB (supplemented with 100mg/L kanamycin) in shake flasks. Another 500μl of each culture was mixed with 500μl of a sterile 50% v/v glycerol in dH_2O and frozen at -80 ˚C for later use.

The liquid cultures were grown at 37 ˚C and 180 rpm until the OD_{600} reached 0.5 – 0.6, at which point Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5mM. The cultures were grown for a further 4 hours at 37 ˚C and 180 rpm before harvest by centrifugation at 5,000 rpm for 10 minutes. The cell pellets were frozen at -20 ˚C for later purification.

Purification of Constructs

Cell pellets were resuspended in 35 ml of lysis buffer (**table 1**) on rollers at 4 ˚C before sonication with a Bandelin (**Model**) sonic probe on ice at 50% power and 4% pulse. The cell lysate was spun down at 10,000 rpm for 30 minutes and 4 ˚C. The supernatant for each construct was retained and passed through a 45μm filter to remove remaining

debris. The filtrate was mixed with 3 ml of QIAgen Ni-NTA agarose beads that had been cleaned with dH_2O and equilibrated with wash buffer 1 (**table 1**). The filtrate and the Ni-NTA agarose beads were mixed on rollers at 4 ˚C for 30 minutes before being loaded into a column. The column was washed with 10 column volumes (CVs) of wash buffer 1, 10 CVs of wash buffer 2 and then eluted with 5 CVs of elution buffer. Each fraction for each construct was run on a 10% SDS-PAGE gel (BioRad) at 200V for 20 minutes (**Figure 1**). Once the elution fractions were confirmed to contain protein of the expected size, they were dialysed into 2 litres of (25mM HEPES, 250mM NaCl, pH7) at 4°C with slow stirring. Every 30 mins the buffer was replaced with fresh buffer for three iterations. The protein was concentrated in two Vivaspin20 columns (MWCO 10kDa, Sigma). The final concentrations of each protein was determined using a Nanodrop 2000 (Thermo Fisher) and extinction coefficients calculated using the ExPasy Protparam tool based on the amino acid sequence of the constructs (**Table 2**). Glycerol was added to a final concentration of 5% v/v and 200µl aliquots were flash frozen in liquid nitrogen prior to storage at -80 ˚C.

Table 1: Buffer compositions used in construct purification

Buffer	**Component**	**Final Conentration**
Lysis Buffer (pH 8.0)	NaCl	250mM
	Tris base	25mM
	Imidazole	10mM
	Lysozyme	5 mg/L
	DNaseI	5 mg/L
	Roche cOmplete protease inhibitor	1 tablet per purification
Wash Buffer 1 (pH 8.0)	NaCl	250mM
	Tris base	25mM
	Imidazole	10mM

Wash Buffer 2 (pH 8.0)	NaCl	250mM
	Tris base	25mM
	Imidazole	50mM
Elution Buffer (pH 8.0)	NaCl	250mM
	Tris base	25mM
	Imidazole	200mM

Figure 1: SDS-PAGE gel images of each stage of the purification of the constructs. Arrows indicate the expected positions of bands for each construct. Ladder: **Precision Plus Protein™**

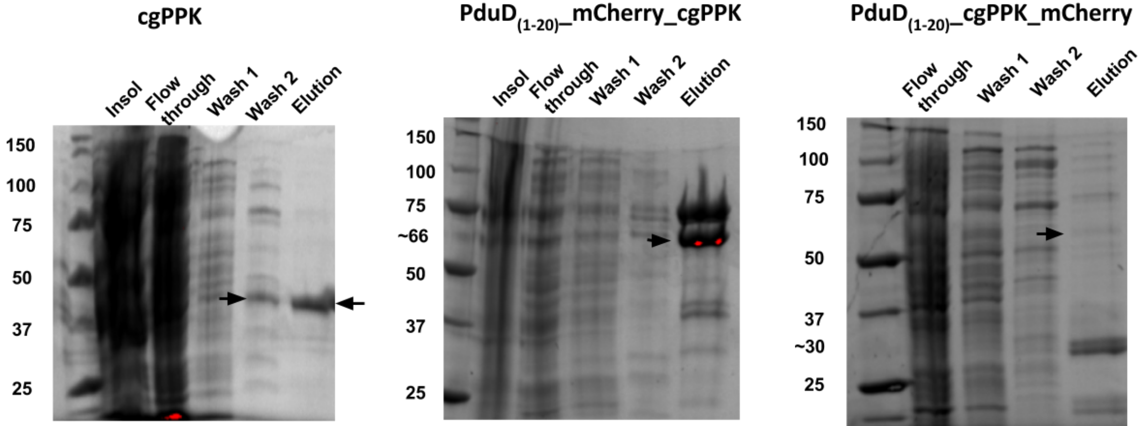

Table 2: Calculated extinction coefficients and calculated final concentrations of protein constructs

Construct	**Calculated extinction coefficient**	**Calculated final concentration**

pNIC28-bsa4_ PduD(1-20)_mCherry_cgPPK	100270	3.9mg/ml
pNIC28-bsa4_ PduD(1-20)_cgPPK_mCherry	100270	4.8mg/ml
pNIC28-bsa4_ cgPPK	65890	0.89mg/ml

References

Lindner, S., Vidaurre, D., Willbold, S., Schoberth, S. and Wendisch, V. (2007). NCgl2620 Encodes a Class II Polyphosphate Kinase in Corynebacterium glutamicum. *Applied and Environmental Microbiology*, 73(15), pp.5026-5033.

Chung, C., Niemela, S. and Miller, R. (1989). One-step preparation of competent Escherichia coli: transformation and storage of bacterial cells in the same solution. *Proceedings of the National Academy of Sciences*, 86(7), pp.2172-2175.