Rinehart lab reagents for *improved* expression of recombinant phosphoproteins.

You will need to purchase the following:
- **Addgene 52055**: Bacterial strain EcAR7 (*1*)
- **Addgene 52054:** pKD –SepRS-EFSep-5x tRNASep (B40 OTS) plasmid in bacterial strain Top10 (*unpublished*)
- **Addgene 52053:** pCRT7 NT Topo tetR/pLtetO Amp- WT sfGFP plasmid in bacterial strain DH5α

Note: The Top10 and DH5α strains are for plasmid preps only and are not to be used for recombinant expression of Phosphoserine containing proteins.

* Throughout the protocol the pKD –SepRS-EFSep-5x tRNASep (B40 OTS) plasmid will be referred to only as B40 OTS.

EcAR7 Growth
- Grow the EcAR7 strain at 30 °C in LB supplemented with 0.08% glucose (no antibiotics).
- The EcAR7 strains normally take 1.5-3 days to grow, at 30 °C (*1*), after transformation or streaking the strain on an agar plate (with the appropriate antibiotics).
- The ECAR7 strain has very low level resistant to chloramphenicol; therefore, no plasmids using CAM as a selectable marker can be used in this strain (*1*). Do not use chloramphenicol for a selectable marker when growing the strain, it makes the cells extremely sick, and impairs their growth. The plasmids we use for expressing our proteins of interest are generally either AMP or Zeocin resistant.
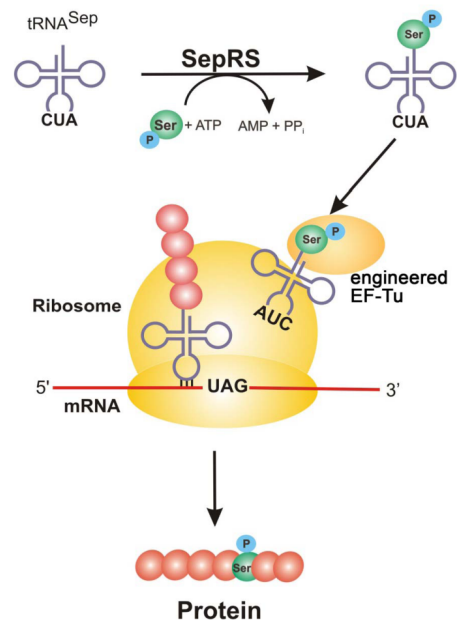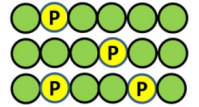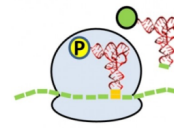
EcAR7 Competent cells

We make the EcAR7 strains chemically competent using a standard $RbCl_2$ method listed below. Other methods might result in poor transformation efficiencies. Electroporation conditions might work, but we don't routinely use them for EcAR7. (*see (2) for possible electroporation conditions for the EcAR7 genetic background .*)

Making competent cells for ECAR7
1.   Start a 30 mL culture of cells in LB media supplemented with 0.08% glucose and grow at 30 °C, 230 rpm overnight
2.   In the morning, dilute the 30 mL culture to 500 mL, grow at 30 °C, 230 rpm until OD600 ~0.6
3.   Spin down cells at 4,000 rpm for 10 min at 4 °C
4.   Discard the supernatant
5.   Resuspend the pellet with 150 mL TFB1, and incubate on ice for 15 min
6.   Spin down at 4,000 rpm for 10 min at 4 °C
7.   Resuspend the pellet with 15 mL TFB2, and incubate on ice for 30 min
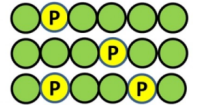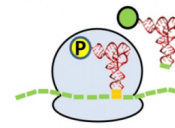8.   Make 100 uL aliquots and freeze at -80 °C

Buffers
TFB1 buffer
8.463g RbCl (MW 120.92)
6.926g MnCl2 (MW 197.9)
2.058g Potassium Acetate (MW 98.14)
1.029g CaCl2 (MW 147.02)
105 mL glycerol
Add ddH20 to 700 mL
Adjust pH to 5.8
Sterile filter, and store at 4 °C

TFB2 buffer
209mg MOPS (MW 209.3)
121mg RbCl
1.1025g CaCl2
15 mL glycerol
Add ddH20 to 100 mL
Adjust pH to 6.8
Sterile filter and store at 4 °C

EcAR7 Transformation

1) 100 uL aliquot of comp cells
1) add 200-300 ng of each plasmid and incubate on ice for 30 min
2) Heat shock for 40 sec at 42 °C, then incubate on ice for 2 min
3) Add 700 uL of SOC media
4) Incubate/shake at 30 °C, 230 rpm for at least 2hrs
5) Plate 10 uL, 100 uL and spin down to check for transformation efficiency with each combination of plasmids

* The ECAR7 strain is resistant to chloramphenicol. The plasmids we use for expressing our proteins of interest are generally either AMP or Zeocin resistant.

*The pCRT7 pLtetO tetR plasmid and the B40 OTS can be transformed simultaneously, and colonies harboring resistance to both AMP and KAN should be selected.

* If three plasmids are going to be transformed, it is recommended to first transform the plasmids that are being used to express the proteins of interest. These transformed cells, will then need to be made chemically competent as described above. The new competent cells will then be used to transform the B40 OTS plasmid.

Grow the cells containing the plasmids in liquid culture overnight, and then make glycerol stocks using 500 uL of cell culture and 500 uL of sterile 50% glycerol solution. Streak cells on agar plates containing appropriate antibiotics from the glycerol stocks. Start cultures from the plates to express your proteins of interest. You will get much more reproducible phosphoserine protein expression following this method.
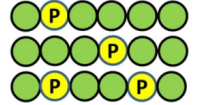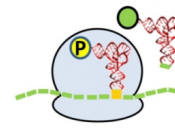
Phosphoprotein expression

Note: Start by using strains from glycerol stocks. Streak cells on agar plates containing appropriate antibiotics from the glycerol stocks. Start cultures from the plates to express your proteins of interest. You will get much more reproducible phosphoserine protein expression following this method.

Plasmids supplied
1. B40 OTS - pKD-SepRS-EFSep-5xtRNASep (Kanamycin 25 ug/mL)
2. pCRT7 NT Topo tetR/pLtetO Amp-WT sfGFP (Ampicillin 100 ug/mL) – this can be the starting point for the *user defined phosphoprotein plasmid.* Replace the sfGFP with gene of interest (GOI). Add TAG amber codon(s) at the position(s) in GOI where phosphoserine incorporation is desired.

To clone new genes for your proteins of interest into the pCRT7 NT vector provided, use 5'KpnI- 3' HindIII restriction sites.

See (*3*) for information on the tetR/pLtetO promoter system used for inducible expression in the pCRT7 NT vector.

The phosphoproteins (or sfGFP) expressed via the pCRT7 pLtetO/tetR plasmid is induced with 100 ng/mL anhydrotetracycline (*3*).

The SepOTS on the B40 OTS plasmid is induced with 1 mM IPTG. This gene system is a combination of genes/plasmids described in (*4*). Phosphoserine incorporation at amber codons *REQUIRES* SepOTS induction.

Alternatives to the pCRT7 NT vector. The pGEX-6P-1 plasmid from GE healthcare is compatible with EcAR7+B40 OTS plasmid and has been validated in the Rinehart lab for GST-Tagged phosphoprotein expression. All other plasmids must be experimentally validated by the user to be EcAR7+B40 OTS compatible.

Starting point for phosphoprotein expression: Note, this is still protein over-expression in *E. coli* and most proteins need optimization for the best overexpression conditions. One rule of thumb is to try our starting point conditions (below) compared to the conditions that you know work for the non-phosphoserine version of your protein of interest (30 deg. Temp for EcAR7 and OTS induction suggestion still apply).

Stock Solutions:
- IPTG stock- 500 mM made fresh
- ATC- 0.2 mg/mL (50% Ethanol/50% H20)
- Phosphoserine (Sigma) prepared as 125 mM solution, pH 6.8
- 20% glucose
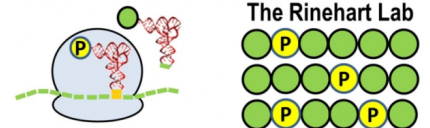
1) Pick 5-20 colonies of transformed EcAR7 + B40 OTS + pCRT7 pLtetO/tetR from freshly streaked plate and grown in LB media with appropriate antibiotics and 0.08% glucose overnight to confluency

2) Dilute precultures into desired amount of media (LB media with appropriate antibiotics, 2 mM Phosphoserine, and 0.08% glucose) for protein expression to OD_{600} of ~ 0.15 and grow at 30 °C ,230 rpm to an OD_{600} 0.8

3) Added freshly prepared solutions to a final concentration of: 1 mM IPTG, 100 ng/mL anhydrotetracycline (ATC) for induction. SepOTS induction reduces growth rate (see ref (1)) and IPTG should be added with ATC at mid-log (~OD_{600} 0.8) to aid in phosphoprotein overexpression.

4) Express for ~ 20 hrs at temperature ranging between 20°C - 30 °C ,230 rpm (depends on the stability and expression of your protein of interest). * The sfGFP provided is expressed at 30 °C for 20 hrs.

References: (please cite our work).

1. Heinemann, I. U., Rovner, A. J., Aerni, H. R., Rogulina, S., Cheng, L., Olds, W., Fischer, J. T., Soll, D., Isaacs, F. J., and Rinehart, J. (2012) Enhanced phosphoserine insertion during Escherichia coli protein synthesis via partial UAG codon reassignment and release factor 1 deletion, FEBS Lett 586, 3716-3722.

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3. Lutz, R., and Bujard, H. (1997) Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements, Nucleic Acids Res 25, 1203-1210.

4. Park, H. S., Hohn, M. J., Umehara, T., Guo, L. T., Osborne, E. M., Benner, J., Noren, C. J., Rinehart, J., and Söll, D. (2011) Expanding the genetic code of Escherichia coli with phosphoserine, Science 333, 1151-1154.

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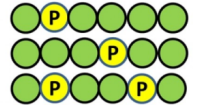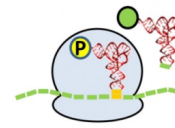
Plasmid maps and sequences

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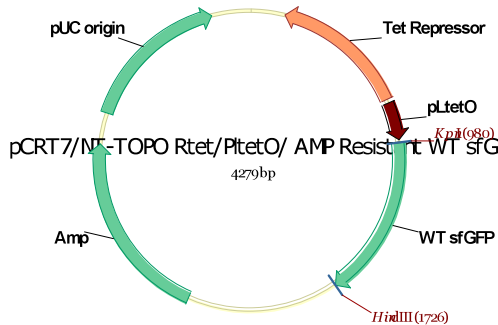
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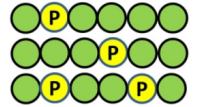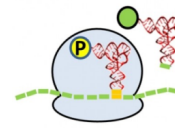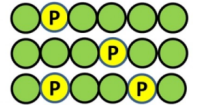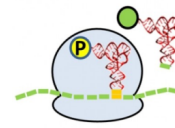
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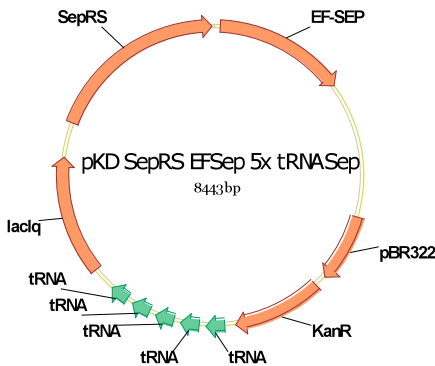
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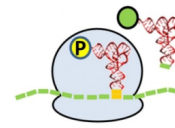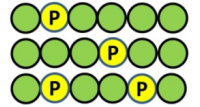
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