**Reference Publications**

Iterative Synthetically Phosphorylated Isomers (iSPI) described in:

* Gassaway, Brandon M; Li, Jiaming; Rad, Ramin; Mintseris, Julian; Mohler, Kyle; Levy, Tyler; Aguiar, Mike; Beausoleil, Sean; Paulo, Joao A; Rinehart, Jesse; Huttlin, Edward L.; Gygi, Steven P. *A Multi-purpose, Regenerable, Proteome-scale, Human Phosphoserine Resource for Phosphoproteomics*. Nature Methods 2022 *in press*.

rEcoli XpS and pSerOTS-C1\* (V70) described in:

* Mohler, Kyle; Moen, Jack; Rogulina, Svetlana; Rinehart, Jesse. Principles for Systematic *Optimization of an Orthogonal Translation System with Enhanced Biological Tolerance*. BioRxiv 2021, 2021.05.20.444985; https://doi.org/10.1101/2021.05.20.444985

**Relevant Reagents Available through Addgene**

* rEcoli XpS ***Addgene #: 192872***

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* pSerOTS-C1\* (V70) ***Addgene #: 188537***
* iSPI\_pSer\_Full\_library ***Addgene #: 188536***
* iSPI\_pSer\_Subpool#1 ***Addgene #: 188526***
* iSPI\_pSer\_Subpool#2 ***Addgene #:*** *1****88527***
* iSPI\_pSer\_Subpool#3 ***Addgene #: 188528***
* iSPI\_pSer\_Subpool#4 ***Addgene #: 188529***
* iSPI\_pSer\_Subpool#5 ***Addgene #: 188530***

* iSPI\_pSer\_Subpool#6 ***Addgene #: 188531***

* iSPI\_pSer\_Subpool#7 ***Addgene #: 188532***

* iSPI\_pSer\_Subpool#8 ***Addgene #: 188533***

* iSPI\_pSer\_Subpool#9 ***Addgene #: 188534***

* iSPI\_pSer\_Subpool#10 ***Addgene #: 188535***

**Overview of Experimental Methods**

1. Preparation and transformation of electrocompetent rEcoli XpS cells with pSerOTS-C1\*
2. Preparation and transformation of electrocompetent (rEcoli XpS + pSerOTS-C1\*) with iSPI libraries
3. Expression and Purification of iSPI library proteins

*Technical Note:* This protocol describes making any one library (e.g., iSPI\_pSer\_Subpool#1) in its phosphorylated-serine form. A serine containing library could also be generated by substituting the SupD plasmid (Addgene #: 68307) for the pSerOTS-C1\* plasmid (Addgene #: 188537).

***I.* Preparation and transformation of electrocompetent rEcoli XpS cells with pSerOTS-C1\***

*Two days before electrocompetent rEcoli XpS cell preparation*

1. Streak rEcoli XpS onto LB agar plate (no antibiotic) and grow overnight at 37°C to obtain single colonies.

 rEcoli XpS Genotype: *C321 mutS+, λ-, Δ(ybhB-bioAB)::zeoR, ΔprfA, ΔserB*

*One day before electrocompetent rEcoli XpS cell preparation*

1. Inoculate 5 mL of LB (no antibiotic) with 3-4 colonies from the rEcoli XpS plate and grow overnight at 37°C, 250 rpm.

*Day of electrocompetent rEcoli XpS cell preparation*

1. Inoculate 20 mL of LB (no antibiotic) with 200 µL (1:100 dilution) of rEcoli XpS overnight culture.

1. Grow at 37°C, 250 rpm and monitor O.D.600nm until the culture reaches an O.D. ~0.4-0.5.
2. Place culture on ice and pellet cells by centrifugation at 8,000 rpm for 90 sec. Decant supernatant.

\*\*\*Remaining steps performed on ice\*\*\*

1. Wash cells using 20 mL of ice-cold, sterile 10% glycerol by gently pipetting of shaking the culture tube.
2. Pellet cells by centrifugation at 8,000 rpm for 90 sec. Decant and repeat wash step (two washes total).
3. After the second wash, resuspend pelleted cells in ~500 µL of 10% glycerol and transfer to a 2 mL microcentrifuge tube.
4. Pellet cells by centrifugation at 8,000 rpm for 60 sec and carefully remove supernatant using a pipet.
5. Resuspend cells in 100 µL sterile, ice-cold 10% glycerol (this is enough for two 50 µL transformations).
6. Move 50 µL of cell suspension to a pre-chilled 1.5 mL microcentrifuge tube (50 µL per transformation).

1. Add 1µL [20-50 ng/µL] of pSerOTS-C1\* plasmid to the 50 µL rEcoli XpS cell suspension.
2. Mix the DNA:cell suspension by gently pipetting and transfer to a pre-chilled electroporation cuvette (0.1 cm electrode gap). Electroporate using the following parameters: 1800 V, 200 Ohm, 25 mF
3. Immediately remove the cuvette and pipet ~600-800 µL of LB into the cuvette to dilute and transfer the transformed cells to a fresh 1.5 mL tube for recovery and outgrowth prior to plating. Gently mix tube while incubating for 60 min. at 37°C.
4. After recovery, transfer 100-200 µL from transformed outgrowth to an LB+KAN [50 µg/mL] agar plate and streak for single colonies. Grow overnight at 37°C.
5. Inoculate 5 mL of LB+KAN with 6-8 colonies from the transformation plate and grow overnight at 37°C, 250 rpm.
6. Using the overnight culture, make a glycerol stock and store at -80ºC.

(continue with library transformation protocol, if desired)

***II.* Preparation and transformation of (rEcoli XpS + pSerOTS-C1\*) cells with iSPI libraries**

*Two days before electrocompetent (rEcoli XpS + pSerOTS-C1\*)* *cell preparation*

1. Streak (rEcoli XpS + pSerOTS-C1\*) cells onto LB+KAN [50 µg/mL] agar plate and grow overnight at 37°C to obtain single colonies.

*One day before electrocompetent (rEcoli XpS + pSerOTS-C1\*)* *cell preparation:*

1. Inoculate 5 mL of LB+KAN with 3-4 colonies from (rEcoli XpS + pSerOTS-C1\*) plate and grow overnight at 37°C, 250 rpm.

*Day of electrocompetent (rEcoli XpS + pSerOTS-C1\*)* *cell preparation*

1. Inoculate 20 mL of LB+KAN with 200 µL (1:100 dilution) of (rEcoli XpS + pSerOTS-C1\*) overnight culture.

1. Grow at 37°C, 250 rpm and monitor O.D.600nm until the culture reaches an O.D. ~0.4-0.5.
2. Place culture on ice and pellet cells by centrifugation at 8,000 rpm for 90 sec. Decant supernatant.

\*\*\*Remaining steps performed on ice\*\*\*

1. Wash cells using 20 mL of ice-cold, sterile 10% glycerol by gently pipetting of shaking the culture tube.
2. Pellet cells by centrifugation at 8,000 rpm for 90 sec. Decant and repeat wash step (two washes total).
3. After the second wash, resuspend pelleted cells in ~500 µL of 10% glycerol and transfer to a 2 mL microcentrifuge tube.
4. Pellet cells by centrifugation at 8,000 rpm for 60 sec and carefully remove supernatant using a pipet.
5. Resuspend cells in 100 µL sterile, ice-cold 10% glycerol (this is enough for two 50 µL transformations).
6. Move 50 µL of cell suspension to a pre-chilled 1.5 mL microcentrifuge tube (50 µL per transformation).

1. Add 1µL [100 ng/µL] of desired iSPI library to the 50 µL (rEcoli XpS + pSerOTS-C1\*) cell suspension.
2. Mix the DNA:cell suspension by gently pipetting and transfer to a pre-chilled electroporation cuvette (0.1 cm electrode gap). Electroporate using the following parameters: 1800 V, 200 Ohm, 25 mF
3. Immediately remove the cuvette and pipet 600 µL of SOC recovery media into the cuvette to dilute and transfer the transformed cells to a 15 mL culture tube for recovery and outgrowth prior to plating. Repeat cuvette wash with an additional 600 µL of SOC and pool with previous fraction. Mix tube while incubating for 60 min. at 37°C, 250 rpm.
4. After recovery, check transformation efficiency by plating serial dilutions (generally, 100 µL of 10^-3 and 10^-4 dilutions are sufficient to calculate C.F.U./mL) on LB+KAN+AMP [50 µg/mL + 100 µg/mL] agar plates. **Library coverage should ideally be >10,000,000 C.F.U.**
5. Inoculate 20-50 mL LB+KAN+AMP with 1 mL of transformed outgrowth culture and grow overnight at 37°C, 250 rpm. (continue with library expression and purification protocol)

*Technical Note: This protocol can also be used for transformation of DNA library into cloning strain to re-generate a stock of the plasmid library. We recommend using a commercial strain of super competent bacterial cells designed for plasmid propagation. Also note that each re-transformation and preparation of the plasmid library may result in a loss of library diversity from the original preparation.*

***III.* Expression and Purification of iSPI library proteins**

*Technical Note:* This protocol describes Ni-NTA and Amylose purification with optional protease cleavage to remove the MBP tag. We have also used Ni-NTA purification only followed by digestion and IMAC phosphopeptide enrichment to make tryptic peptide library samples (e.g., Gassaway Nature Methods).

**Cell Lysis and Lysate Preparation**

*Lysis Buffer (1x):*

50 mM Na2HPO4

300 mM NaCl

10 mM Imidazole

1 mM MgCl2

Add 1 large protease inhibitor (EDTA free)

Add 1x PhosStop Solution

1 mg/ml lysozyme

125 units/mL Benzonase

pH to 8.0 with HCl

*Cell Lysate Preparation Protocol:*

1. Add 2 mL of lysis buffer per gram of wet cell pellet and resuspend by vortexing.
2. Incubate lysis mixture in 37°C water bath for 25 minutes.
3. Briefly vortex the lysis mixture (~2 sec) to break up cells, then sonicate at 40% power with 10 seconds on, 20 seconds off for 1 minute of total on time.
4. Distribute lysate into 30 mL centrifuge bottle and spin for 25 minutes at 40,000 *xg* at 4°C. Carefully decant supernatant to a new 30 mL bottle and spun again for 25 minutes. Transfer clarified lysate to a 50 mL falcon tube.

**Ni-NTA Resin (6xHis Tag) Purification Protocol**

*Ni-NTA Wash Buffer (1x): Ni-NTA Elution Buffer (1x):*

50 mM Na2HPO4 50 mM Na2HPO4

300 mM NaCl 300 mM NaCl

50 mM NaF 250 mM Imidazole

1 mM NaVO3 pH to 8.0 with HCl

10 mM Imidazole

pH to 8.0 with HCl

*Ni-NTA Resin Purification Protocol:*

1. During the lysate clarification, prepare a gravity column by adding 3-4 mL of Ni-NTA resin (50% slurry) and equilibrating with 10 mL of lysis buffer.
2. Pass clarified lysate through the column twice (if time allows).
3. Wash the column with 100-150 mL of wash buffer.
4. Elute bound protein with 10 mL of elution buffer.
5. Proceed to Amylose Purification Protocol or concentrate and perform buffer exchange into application specific buffer (e.g., storage buffer or trypsin digestion buffer).

**Amylose Resin (MBP Tag) Purification Protocol**

*Amylose Dilution Buffer (2x): Amylose Equilibration/Wash Buffer (1x):*

40 mM Tris-HCl, pH 7.4 20 mM Tris-HCl, pH 7.4

2 mM EDTA 1 mM EDTA

200 mM NaCl

*Amylose Elution Buffer (1x):*

20 mM Tris-HCl, pH 7.4

1 mM EDTA

200 mM NaCl

10 mM Maltose

*Amylose Resin Purification Protocol:*

1. Add 10 mL of amylose dilution buffer to 10mL Ni-NTA Elution Fraction and mix thoroughly by inverting.
2. Prepare a gravity column by adding 3 mL of Amylose Resin (bed volume) and equilibrating with 25 mL of amylose equilibration/wash buffer (Note: this can be setup and equilibrated in advance).
3. Apply diluted Ni-NTA elution to column and pass through twice (if time allows).
4. Wash the column with 100 mL amylose wash buffer.
5. Elute bound protein using 10-15 mL amylose elution buffer.
6. Concentrate samples down to 500 µL using an Amicon Ultra centrifugal filter (Note: select filter with kD cutoff no larger than half the kD of recombinant protein).
7. If you do not wish to remove MBP, perform buffer exchange using suitable storage buffer for your recombinant protein and add glycerol to final concentration of 40-50%. Store at -80°C. If removing MBP tag using PreScission Protease, continue below.

**MBP Tag Removal (Protease Cleavage) Protocol**

*PreScission Protease Cleavage Buffer (1x):*

50 mM Tris-HCl, pH 7.4

150 mM NaCl

1 mM EDTA

1 mM DTT

*PreScission Protease Cleavage Protocol:*

1. Perform 3 rounds of buffer exchange using PreScission Protease buffer. Ensure that the final volume of protease buffer is 500 µL.
2. Add 20µL of PreScission Protease and incubate overnight with mixing at 4°C.
3. Following cleavage, remove protease and cleaved MBP using centrifugal filter with appropriate kD cutoff (if possible). Alternatively, you can remove protease using GST resin and MBP using Amylose resin depending on the desired purity of your sample.
4. Perform buffer exchange using suitable storage buffer for your recombinant protein (without glycerol).
5. Add glycerol to final concentration of 40-50% and store at -80°C
6. Analyze collected fractions by SDS-PAGE with Coomassie stain. Load BSA as standard curve.

**Example Analysis of Purification Steps by SDS-PAGE**

