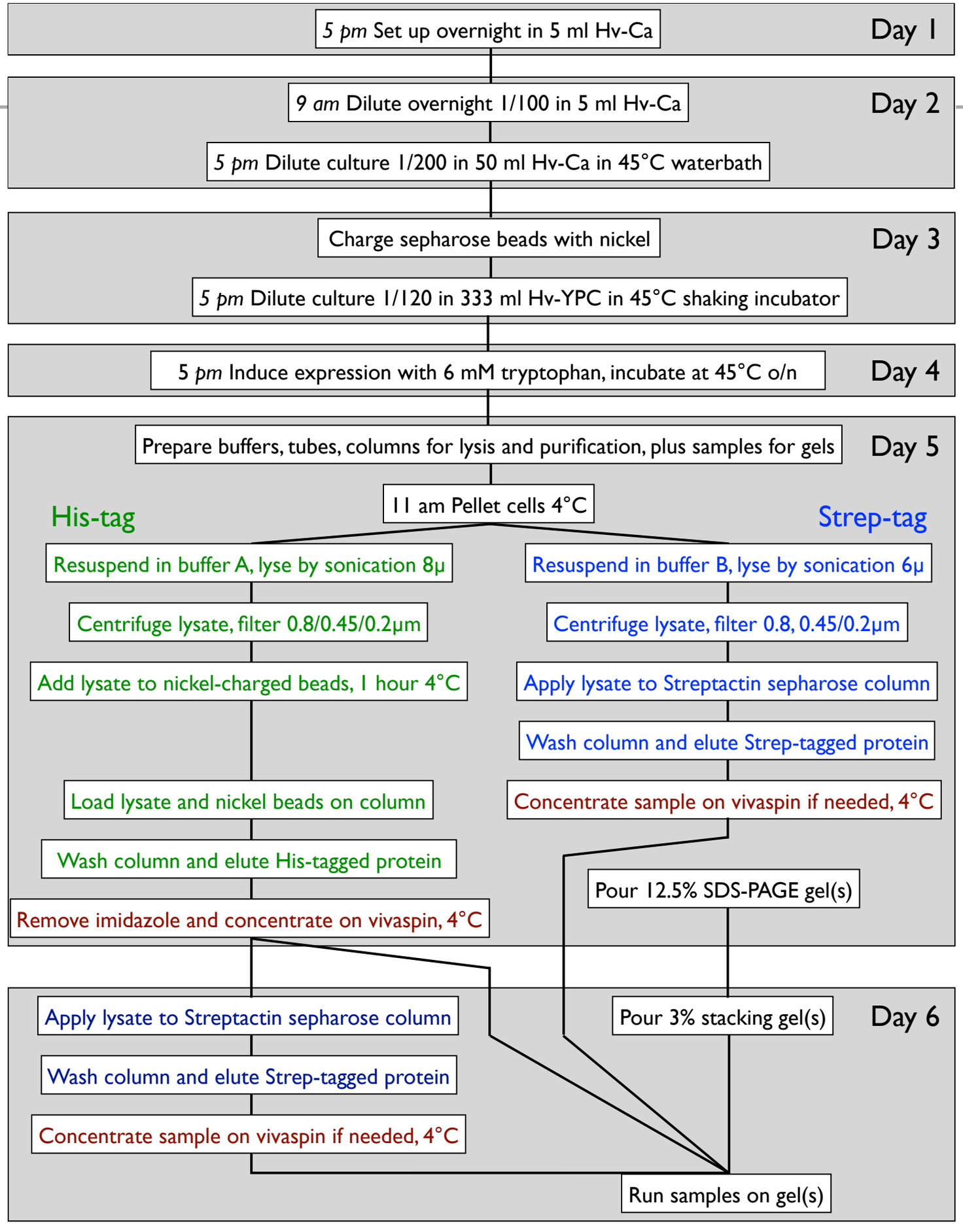
PULLDOWNS: PROTEIN PREP

MAGIC BOOK

FLOWCHART



Allers et al (2010); Haque et al (2020); Karan et al (2024)

DAY 1

- Set up 5 ml O/N of strain with over-expression plasmid in Hv-YPC (if native expression) or Hv-Ca+ (if over-expressing) using ~1-2 colonies, grow at 45°C on rotator.

DAY 2

- *9 am:* When OD 1.0, dilute into 5 ml Hv-YPC/Hv-Ca+ cultures, grow at 45°C on rotator. A total of 3 dilutions, to allow for growth rate: 1/250 (20 µl), 1/100 (50 µl), 1/50 (100 µl), 1/25 (200 µl).

- *5 pm:* When OD 0.1 (after ~8 hours), dilute into 50 ml YPC/Ca+ broth in trigene-free flasks. A total of 3 dilutions, to allow for growth rate: 1/100 (0.5 ml), 1/200 (0.25 ml), 1/500 (0.1 ml).

- Incubate for 24h in 45°C waterbath, with shaking 150 rpm until OD 0.5.

DAY 3

- *5 pm:* Dilute YPC/Ca+ culture (OD = 0.5) into 333 ml YPC broth. 1 or 2 dilutions, to allow for growth rate: 1/120 (2.8ml), or 1/60 (5.4ml).
- Incubate overnight (~16 hours) in 45°C incubator, with shaking 150 rpm until OD 0.5. Maximum of 8 flasks with 333 ml YPC broth (2.66 litres), fits in 6 centrifuge bottles for SLA3000.
- If purifying via His-tag charge beads with nickel:
- Aliquot 0.5 ml IMAC Sepharose 6 Fast Flow per sample to 15ml Falcon tube. Vortex first to resuspend, maximum of 1 ml if purifying from 2.66 litres (8 flasks).
- Spin 1 min at 4,000 rpm, wash 2x with ~5 ml dH_2O.
- Equilibrate with 1 ml of 0.2 M $NiSO_4$ for 30 minutes at 4°C, on rotator in cold room.
- Meanwhile prepare 25 ml buffer A (20 mM imidazole) and 5 ml buffer A (500 mM imidazole), no PMSF.
- Wash 2x with ~5 ml dH_2O, then wash 1x with 1 ml buffer A + 500 mM imidazole. Then wash 3x with 5 ml buffer A (20 mM imidazole).
- Resuspend in 0.5 (or 1) ml buffer A per sample, store overnight at 4°C.

DAY 4 – OPTIONAL

- *Induce expression if required:* When OD 0.5, add 0.4 g of powdered Trp (~6 mM Trp) to 333 ml flask (1.2 g to 1 L). Incubate at 45°C o/n.

DAY 5

- Prepare solutions:
- **5 M NaCl:** 146.1 g for 500 ml (in H_2O)
- **1 M HEPES pH 7.5:** 60 g for 250 ml (in H_2O), pH equilibrated to 7.5 with NaOH, store at 4°C
- **1 M Imidazole:** 1.36 g for 20 ml (in H_2O) or 3.4 g for 50 ml (can be kept a few weeks at 4°C)
- **100 mM PMSF:** 35 mg in 2 ml 100% ethanol (prepare fresh, toxic) 
- ***10X SigmaFAST:*** 1 tablet for 10 ml (in H_2O) (can be kept a few weeks at 4°C

DAY 5

- Spin the cells for 10 min at 6000 rpm at 4°C, use SLA3000 rotor.
- Resuspend cell pellets in 7 ml in required buffer (A/B + protease inhibitor), transfer to 50 ml Falcon tube. Combine resuspended cells, then aliquot to maximum 15 ml / Falcon tube.
- Lyse cells by sonication on ice. Pulse for 3x for 30 sec at 8 µ (becomes clear).
- Transfer to 15 ml round-bottom tube, spin for 30 min at 20000 rpm at 4°C. May need to repeat centrifugation step if lysate is viscous or cloudy.
- Combine the supernatants into 50 ml (or 15 ml) Falcon tube on ice. Syringe filter sequentially through 0.8 µm, 0.45 µm and 0.2 µm filters into Falcon tube on ice. Take 37.5 µl sample *Lysate sample*
- *His purification:* Add equilibrated beads to cell lysate in Falcon tube, rotate gently for 1 hour at 4°C. Use 0.5 ml per sample, maximum of 1 ml if purifying from 2.66 litres (8 flasks).
- Meanwhile resuspend the cell pellet in 200 µl buffer A and take a 37.5 µl sample. *Pellet sample*

DAY 5 – HIS COLUMN PURIFICATION: BUFFER A

Buffer A	50ml	37.5ml	25ml	15ml	
20 mM HEPES pH 7.5	1ml	750µl	500µl	300µl	of 1 M
2M NaCl	20ml	15ml	10ml	6ml	of 5M
20mM imidazole	1ml	750µl	500µl	300µl	of 1M
1mM PMSF	500µl	375µl	250µl	150µl	of 100mM
H_2O	27.5ml	20.6ml	13.75ml	8.25ml	

+ protease inhibitor: Add 10X SigmaFAST protease inhibitor immediately before use

20 mM HEPES pH 7.5	1ml	750µl	500µl	300µl	of 1 M
2M NaCl	20ml	15ml	10ml	6ml	of 5M
20mM imidazole	1ml	750µl	500µl	300µl	of 1M
1X Sigma*FAST*	5ml	3.75ml	2.5ml	1.5ml	of 10X
H_2O	23ml	17.25ml	11.5ml	6.9ml	

DAY 5 – HIS COLUMN PURIFICATION 1/2

- To make 5 ml buffer A with different concentrations of imidazole: 100µl 1M HEPES, 2ml 5M NaC, 50µl 100mM PMSF +

	Imidazole conc. mM							
	50	**100**	150	**200**	250	300	400	**500**
1M imidazole	0.25ml	**0.5ml**	0.75ml	1ml	1.25ml	1.5ml	2ml	2.5ml
H_2O	2.6ml	**2.35ml**	2.1ml	1.85ml	1.6ml	1.35ml	0.85ml	0.35ml

- Prepare on ice:
  - Buffer A (20 mM imidazole)
    - 7 ml (+ protease inhibitor) per 333 ml flask of culture (for resuspending pellet)
    - 15 ml per column for washing
  - Elution buffers (buffer A + 100, 200, 500 mM imidazole), 5 ml per column
  - Eppendorfs with 12.5 µl 4x loading dye, need 8 per pulldown. Lysate, pellet, FT, Wash, E100 E200, E500, Viva
  - 4 ml round-bottom tubes for sample collection, need 3 per pulldown. E100, E200, E500
  - Bio-Rad Poly-Prep gravity column and two 30 ml universals for collection.

DAY 5 – HIS COLUMN PURIFICATION 2/2

His gravity column

- In cold room load beads on Bio-Rad Poly-Prep gravity column, collect flow-through in universal. If using 1 ml beads, load onto 2 gravity columns (0.5 ml beads per column maximum).
- Load the flow-through on the column again (use to rinse out beads) and collect flow-through. *FT sample (37.5 µl)*
- Wash the column with 5 ml of buffer A, collecting flow-through in a fresh 30 ml universal. *Wash sample (37.5 µl)*
- Wash again with 10 ml of buffer A.
- Elute with 1 ml of buffer A + 100 mM imidazole, collect elution in 4 ml round-bottom tube. *E100 sample (37.5 µl).* Repeat (2 ml total)
- Elute with 1 ml of buffer A + 200 mM imidazole, collect elution in 4 ml round-bottom tube. *E200 sample (37.5 µl).* Repeat (2 ml total)
- Elute with 1 ml of buffer A + 500 mM imidazole, collect elution in 4 ml round-bottom tube. *E500 sample (37.5 µl).* Repeat (2 ml total). Note: elution with 500 mM imidazole is normally unnecessary and requires extensive dialysis/filtration to remove imidazole before purification on Strep column.
- Add to 37.5 µl *samples*: 12.5 µl loading dye 4x + 0.5 µl DTT 1 M, boil 10 min at 94°C and store at 4°C. Repeat with 37.5 µl *samples* below.

DAY 5 – VIVASPIN (CHOOSE CORRECT MWCO)

- Wash Vivaspin 20 with 5 ml dH_2O, spin 5 min at 4000 rpm in swing-bucket at 4°C.
- Remove retentate and filtrate, then wash with 5 ml buffer B, spin again.
- Combine elutions (15 ml max), load on Vivaspin.
- Spin in 10 min intervals at 4000 rpm (3000 x *g*) in swing-bucket at 4°C until 1-2 ml left.

Optional: Use pressure-fuge to halve spin times. Fit pressure head, pressurise to 5 bar, spin 15 min at 4000 rpm in swing-bucket at 4°C.

- Wash with 10 ml buffer B, pipetting to wash protein from filter. Spin again 15-30 min.
- Wash up to twice more (≤3X total) with 10 ml buffer B to remove imidazole.

Optional: Use diafiltration cup to continuously remove imidazole.

- Once sample has been concentrated to 2 ml, insert diafiltration cup and fill with 10 ml buffer B. Spin 15-30 min at 4000 rpm in swing-bucket at 4°C until diafiltration cup is empty.
- Remove retentate (1-2 ml), washing protein from filter, transfer to eppendorf on ice. *Viva sample (37.5 µl)*

DAY 5 – STREP COLUMN PURIFICATION: BUFFER B

Buffer B	50ml	37.5ml	25ml	15ml	
20 mM HEPES pH 7.5	1ml	750µl	500µl	300µl	of 1 M
2M NaCl	20ml	15ml	10ml	6ml	of 5M
1mM PMSF	500µl	375µl	250µl	150µl	of 100mM
H_2O	28.5ml	21.4ml	14.25ml	8.55ml	

+ protease inhibitor: Add 10X SigmaFAST protease inhibitor immediately before use

20 mM HEPES pH 7.5	1ml	750µl	500µl	300µl	of 1 M
2M NaCl	20ml	15ml	10ml	6ml	of 5M
1X Sigma*FAST*	5ml	3.75ml	2.5ml	1.5ml	of 10X
H_2O	24ml	18ml	12ml	7.2ml	

- **Elution Buffer (5 mM D-desthiobiotin):** To 15 ml Buffer B add 0.016 g D-desthiobiotin

DAY 5 – STREP COLUMN PURIFICATION FROM CELL LYSATE 1/2

- Prepare on ice:
  - Buffer B
    - 7 ml (+ protease inhibitor) per 333 ml flask of culture (for resuspending pellet)
    - 7 ml per column for washing
    - Elution buffers (buffer B + 5mM D-desthiobiotin), 3 ml per column
    - Eppendorfs with 12.5 µl 4x loading dye, need 8 per pulldown. Lysate, pellet, FT, Wash, E1, E2, E3, Viva
    - 2 ml reppendorfs for sample collection, need 3 per pulldown. E1, E2, E3
    - Bio-Rad Poly-Prep gravity column and two 30 ml universals for collection.
- In cold room, aliquot 1 ml IBA *Strep*-Tactin Sepharose to each Bio-Rad Poly-Prep gravity column. Vortex gently first to resuspend, use 1 ml each in two columns if purifying from 2.66 litres (8 flasks).
- Equilibrate *Strep*-Tactin Sepharose with 2 ml buffer B, repeat and cap column. 2x 2 column bed volumes (CV).

DAY 5 – STREP COLUMN PURIFICATION FROM CELL LYSATE 2/2

Strep gravity column

- Add cell lysate to equilibrated *Strep*-Tactin Sepharose on Poly-Prep gravity column, collect flow-through in universal. Apply up to 20 CV cell lysate to column, i.e. 20 ml cell lysate per 1 ml *Strep*-Tactin per column.

- Load the flow-through on the column again and collect flow-through. Repeat once more if the lysate flows through quickly. *FT sample (37.5 µl)*

- Wash the column with 1 ml of buffer B, collecting flow-through in a fresh 30 ml universal. *Wash sample (37.5 µl)*. Wash four times more with 1 ml of buffer B (total of five washes). 5x 1 CV wash total.

- Elute with 0.8 ml of elution buffer (B + 5 mM desthiobiotin), collect in 2 ml eppendorf. *E1 sample (37.5 µl)*
  Then elute with 1.4 ml of elution buffer, collect in 2 ml eppendorf. *E2 sample (37.5 µl)*
  Then elute with 0.8 ml of elution buffer, collect in 2 ml eppendorf. *E3 sample (37.5 µl).*
  3 CV elution total (0.8 CV + 1.4 CV + 0.8 CV), most of protein will be in E1 or E2.

- Add to 37.5 µl *samples*: 12.5 µl loading dye 4x + 0.5 µl DTT 1 M, boil 10 min at 94°C and store at 4°C. Repeat with 37.5 µl *samples* below.

DAY 5 – STREP COLUMN PURIFICATION AFTER HIS COLUMN 1/2

- Prepare on ice:
  - Buffer B
    - 7 ml per column for washing
  - Elution buffers (buffer B + 5mM D-desthiobiotin), 3 ml per column
  - Eppendorfs with 12.5 µl 4x loading dye, need 6 per pulldown. FT, Wash, E100 E200, E500, Viva
  - 2 ml reppendorfs for sample collection, need 3 per pulldown. E1, E2, E3
  - Bio-Rad Poly-Prep gravity column and two 30 ml universals for collection.
- In cold room, aliquot 0.5 ml IBA *Strep*-Tactin Sepharose to each Bio-Rad Poly-Prep gravity column. Vortex gently first to resuspend.
- Equilibrate *Strep*-Tactin Sepharose with 2 ml buffer B, repeat and cap column. 2x 2 column bed volumes (CV).

DAY 5 – STREP COLUMN PURIFICATION AFTER HIS COLUMN 2/2

Strep gravity column

- Add protein to equilibrated *Strep*-Tactin Sepharose on Poly-Prep gravity column, collect flow-through in universal. Apply up to 10 CV cell lysate to column, i.e. ≤5 ml cell lysate per 0.5 ml *Strep*-Tactin.

- Load the flow-through on the column again and collect flow-through. Repeat. *FT sample (37.5 µl)*

- Wash the column with 0.5 ml of buffer B, collecting flow-through in a fresh 30 ml universal. *Wash sample (37.5 µl).* Wash four times more with 0.5 ml of buffer B (total of five washes). 5x 1 CV wash total.

- Elute with 0.4 ml of elution buffer (B + 5 mM desthiobiotin), collect in 2 ml eppendorf. *E1 sample (37.5 µl)*
  Then elute with 0.7 ml of elution buffer, collect in 2 ml eppendorf. *E2 sample (37.5 µl)*
  Then elute with 0.4 ml of elution buffer, collect in 2 ml eppendorf. *E3 sample (37.5 µl).*
  3 CV elution total (0.8 CV + 1.4 CV + 0.8 CV), most of protein will be in E1 or E2.

- Add to 37.5 µl *samples*: 12.5 µl loading dye 4x + 0.5 µl DTT 1 M, boil 10 min at 94°C and store at 4°C. Repeat with 37.5 µl *samples* below.

DAY 6 – STORAGE AND RUNNING THE GEL

- For short-term, store elutions at 4°C overnight. If possible, first reduce imidazole concentration to ≤100 mM using Vivaspin.
- For long-term, store at -80°C: add 1/8 final volume of 80% glycerol 6% SW and mix. Alternatively, if concentrating protein by Vivaspin, diafiltrate with buffer B + 10% glycerol. Aliquot 100 µl samples into 1.5 ml eppendorfs, snap-freeze on dry ice / ethanol.

Run samples on a gel

- Prepare 12.5% SDS-PAGE gels (SDS PAGE GEL). Pour ~6 ml 12.5% gel to 1st line, overlay with 200 µl isopropanol, leave to set.
- Rinse out isopropanol from SDS-PAGE gel thoroughly with dH_2O, dry with tissue. Pour ~2 ml 3% stack to top, insert comb (12 or 15 well), leave to set.
- Boil samples 10 min at 94°C. Load 10-15 µl sample, plus 5 µl page ruler protein ladder (not boiled).
- see SDS PAGE GEL for further details

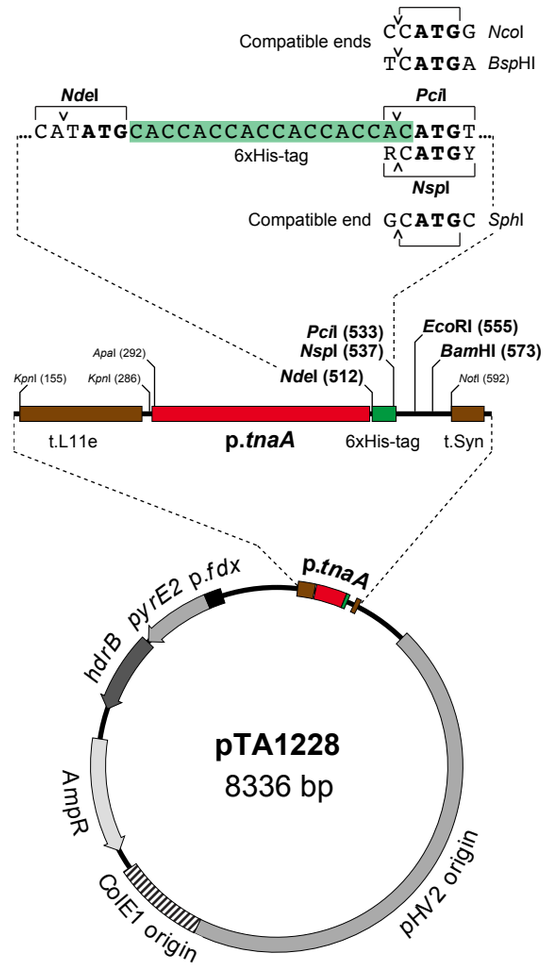
PLASMIDS FOR PROTEIN OVER-EXPRESSION (1)

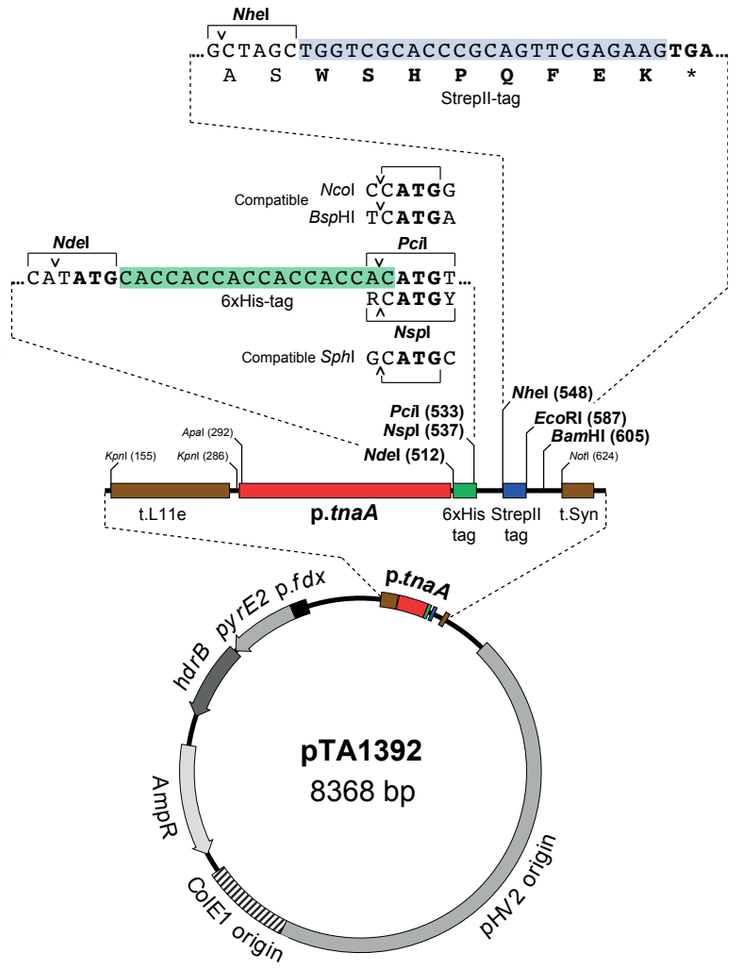
N-TERMINAL HIS TAG

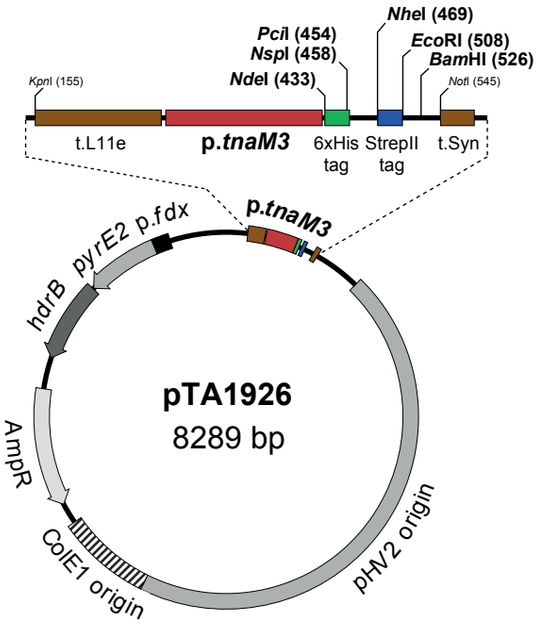
- NdeI site in plasmid is directly upstream of 6xHis tag, for cloning without His-tag use PCR to incorporate NdeI site at 5′ end of gene
- For cloning with 6xHis-tag, insert gene at PciI or NspI site in plasmid, in-frame with 3' end of His tag:
  - If second codon starts with T, G, or A, cut plasmid with PciI and incorporate PciI (A/CATGT), NcoI (C/CATGG) or BspHI (T/CATGA) site at 5′ end of gene, respectively
  - If second codon starts with C, cut plasmid with NspI site and incorporate SphI (GCATG/C) site at 5′ end of gene
- Incorporate EcoRI/BamHI/NotI site at 3′ end of gene

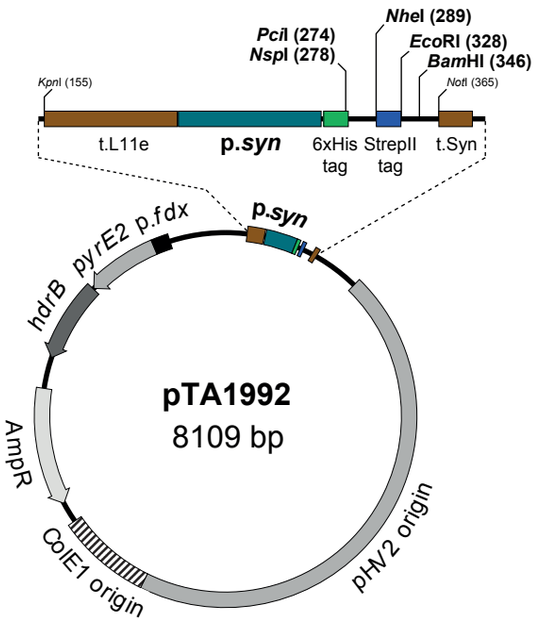
C-TERMINAL STREPII TAG

- EcoRI and BamHI sites are downstream of StrepII tag, for cloning without Strep-tag use EcoRI or BamHI site at 3′ end of gene, after stop codon
- For cloning with StrepII tag, replace stop codon of gene with NheI site (G/CTAGC), insert at NheI site of plasmid





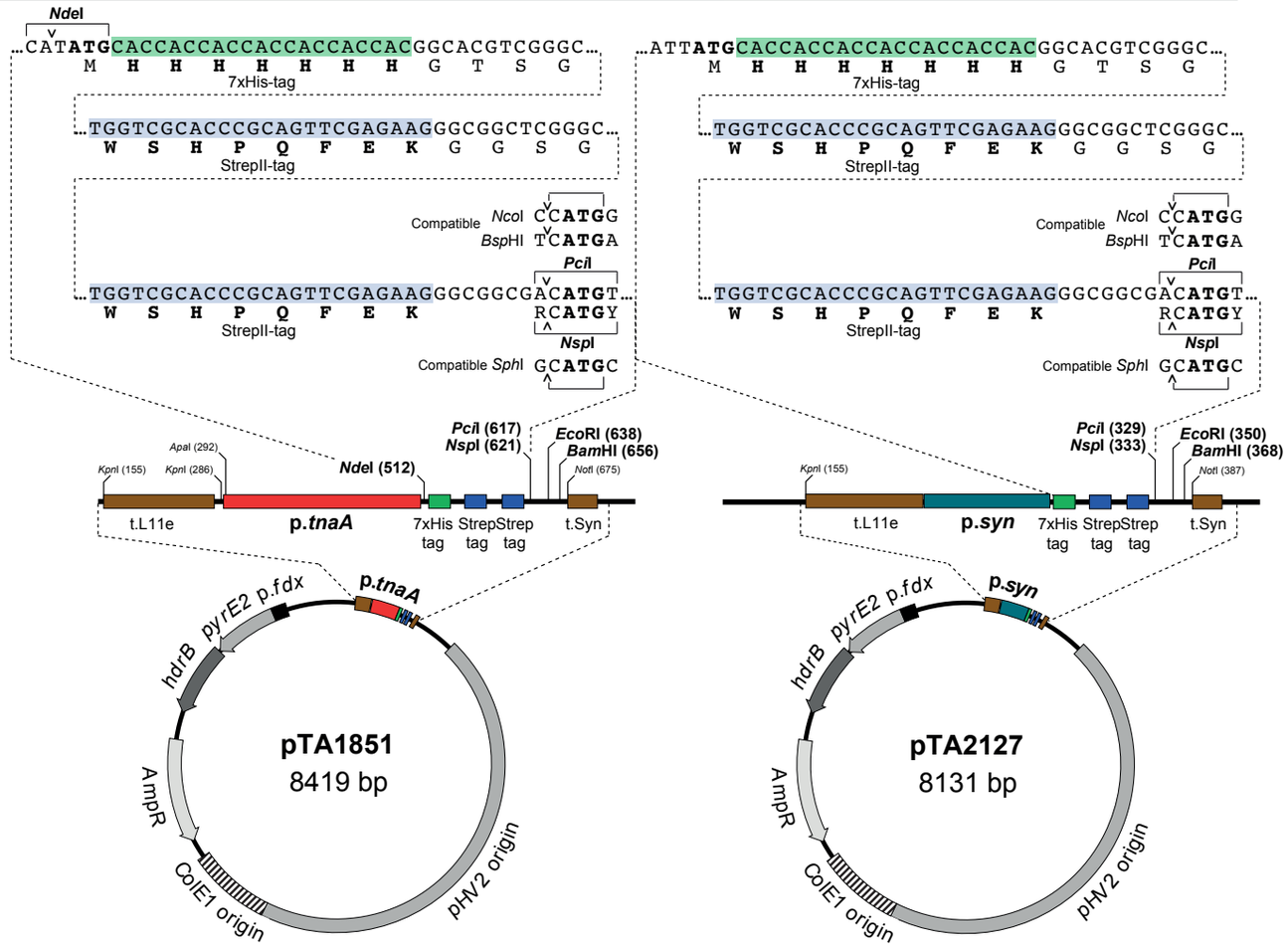




PLASMIDS FOR PROTEIN OVER-EXPRESSION (2)

N-TERMINAL TANDEM 7XHIS-2XSTREPII TAG

- NdeI site in plasmid is upstream of N-terminal tandem 7xHis-2xStrepII tag, for cloning without any tag incorporate NdeI site at 5′ end of gene.
- For cloning with 7xHis-2xStrepII tag, insert gene at PciI or NspI site in plasmid, in-frame with 3' end of His tag:
  - If second codon starts with T, G, or A, cut plasmid with PciI and incorporate PciI (A/CATGT), NcoI (C/CATGG) or BspHI (T/CATGA) site at 5′ end of gene, respectively.
  - If second codon starts with C, cut plasmid with NspI site and incorporate SphI (GCATG/C) site at 5′ end of gene.
- Incorporate EcoRI/BamHI/NotI (or compatible) site at 3′ end of gene.



Plasmid	Relevant properties
pTA1228	pTA963 (not shown) and pTA1228 have N-terminal His tag only. Both have p.tnaA tryptophan-inducible promoter. pTA1228 has better cloning sites (NspI)
pTA1392	pTA1392 has an N-terminal His tag and a C-terminal StrepII tag, which works reasonably well. It also has the p.tnaA tryptophan-inducible promoter
pTA1403	pTA1403 (not shown) is like pTA1392 but the tags are the other way round: N-terminal StrepII tag and C-terminal His tag. This doesn't seem to work well
pTA1926	pTA1926 is like pTA1392 but has 50% strength p.tnaM3 promoter, which has 50% basal and induced activity for "toxic" proteins. The tags are like pTA1392
pTA1992	pTA1992 is like pTA1392 but has the strong constitutive p.syn promoter for "harmless" proteins. The tags are like pTA1392
pTA1851	pTA1851 has N-terminal tandem tag: 7xHis 2x StrepII, separated by linkers. This normally works best. It has the p.tnaA tryptophan-inducible promoter
pTA2127	pTA2127 is like pTA1851 but has the strong constitutive p.syn promoter for "harmless" proteins. The tandem 7xHis tag 2x StrepII tag is like pTA1851

Allers et al (2010); Haque et al (2020); Karan et al (2024)