FPCount protocol - Full protocol

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Dec 05, 2021

1

dx.doi.org/10.17504/protocols.io.bztsp6ne

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FPCount is a complete protocol for fluorescent protein calibration, consisting of:

1. FP expression/purification using Thermo's HisPur Cobalt Resin.
2. FP concentration determination in a microplate reader.
3. FP fluorescence quantification in a microplate reader.

Results can be analysed with the corresponding R package, FPCountR.

Summary

DOI

dx.doi.org/10.17504/protocols.io.bztsp6ne

Eszter Csibra, Guy-Bart Stan 2021. FPCount protocol - Full protocol.
protocols.io
https://dx.doi.org/10.17504/protocols.io.bztsp6ne

fluorescent protein, calibration, plate reader

protocol ,

Nov 05, 2021

Dec 05, 2021

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External Manuals, Protocols and Guides:

- HisPur_Cobalt_Resin, 10mg/ml capacity (**RESIN centric: Basic method based on this)
- Batch and Spin Cup Methods (**SPIN CUP centric: Basic method based on this)
- HisPur™ Cobalt Resin
- Pierce™ Spin Columns - Snap Cap
- Amicon columns
- ThermoFisher Protein assay compatibility table
- microBCA assay / Protein by BCA in plates

Troubleshooting

- Protein expression yields low.
  - Lower temperature, increase incubation time, increase inducer/decrease inducer. Try different strain.
- Protein largely in insoluble fraction.
  - Lower temperature, decrease inducer. Try different strain.
  - Proteins can be refolded from insoluble fraction, but this isn't recommended.
  - Consider adding a solubilising fusion tag, such as maltose binding protein.
- Protein purification yields low.
  - concentrate existing elution fractions
  - (if that's not enough), use a larger fraction of the 50ml culture for purification, and pre-concentrate it before binding (remember to add 10mM imidazole)
  - increase binding time
  - increase number of elutions / elution time
  - if protein not binding column, adjust imidazole concentration down in Binding Buffer
  - if protein not eluting, check wash fractions
    - if protein washing off, do fewer washes or adjust imidazole concentration down
    - if protein just 'stuck' to column, adjust imidazole concentration up in EB or adding NaCl
  - consider moving His tag to opposite terminus or increasing the linker length between the tag and the protein.
- Protein in elutions not pure
  - increase washes and/or add 1M NaCl.

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- Expression
  - Luria Broth (LB), antibiotics, inducer
- Cell lysis
  - 1M Tris pH 7.5, filter-sterilised (f/s), or commercial
  - 2M NaCl (f/s)
  - ThermoFisher Pierce Protease Inhibitor Tablets (EDTA-free) or equivalent (often abbreviated to 'pi')
  - lysozyme (eg ThermoFisher 89833 or equivalent), stored as powder, or as 50ug/ml (500X) stock aliquoted in -20oC
  - DNase I (MP Biomedicals 219006210, bovine pancrease, lyophilised) or equivalent
  - Sonicator (eg Q125 QSonica)
- Purification
  - 1M imidazole (f/s)
  - HisPur Cobalt resin
  - spin columns
  - Amicon spin-columns for buffer exchange (10K or appropriate cutoff)
- Verification
  - SDS-PAGE reagents or premade gels (eg Protean PreCast Gels)
  - SDS-PAGE running buffer
  - 2X Laemmli's buffer (2X LB)
  - Color pre-stained protein marker or equivalent
  - Coomassie blue stain or commercial variant (eg InstantBlue, Sigma ISB1L-1L)
- Quantifications
  - 96-well plates, uvclear for A280 assays (eg. UV-transparent clear microplates, Greiner, 655801)
  - 96-well plate used in your assays (eg. Corning 3370 or Greiner 655090)
  - Plate reader
  - BSA standards (eg from ThermoFisher 23209; or Thermo MicroBCA kit comes with a set)
  - MicroBCA kit (ThermoFisher 23235), or equivalent
  - Multipipette (optional); P200 multichannel

f/s - filter sterilised

Expression

1 [Day 1]

Overnight culture set-up:

- 50ml LB
- 50ul cam
- 50ul arabinose **0.02%**
- glycerol stock scraping of BL21/pS381_ara_His-FP transformant (or equivalent)
- 30oC 250rpm
- overnight expression...

Harvesting and Washing

2 [Day 2]

Citation: Eszter Csibra, Guy-Bart Stan FPCount protocol - Full protocol https://dx.doi.org/10.17504/protocols.io.bztsp6ne

Buffers:

- **Wash buffer = T50N150**
- 50 mM Tris-Cl pH 7.5, 150 mM NaCl
- Doesn't need protease inhibitors
- Can be substituted by T50N300
- **Resuspension buffer = T50N300+pi**
- 50 mM Tris-Cl pH 7.5, 300 mM NaCl
- protease inhibitors (pi; 1 tablet/10ml)
- filter sterilise as pi makes things cloudy/doesn't go into solution well
- **Lysis Buffer = T50N300+pi**
- lysozyme 1X (100ug/ml)
- DNase I (1000U/ml)
- CaCl2 stock
- MgCl2 stock
- 2X Laemmli's buffer (2xLB)
- **Binding Buffer (BB) = T50N300+pi+10imid**
- T50N300+pi, 10 mM imidazole
- **Elution Buffer (EB) = T50N300+pi+150imid**
- T50N300+pi, 150 mM imidazole
- **Dilution Buffer = T5N15+pi**
- For protein assays
- **How much buffer will I need?**
- T50N150 - maybe 35ml/FP
- T50N300 + pi (f/s) - make master stock > 10ml per FP.
- T50N300 + pi + lysozyme = 5ml per FP
- T50N300 + pi + 10mM imid = 4ml per FP
- T50N300 + pi + 150mM imid = 1ml per FP
- T5N15 + pi = {5ml for BSA + 8ml for each FP}
- microBCA working reagent (WR) = 12ml per plate (plate = 3 FPs) OR {3ml for BSA + 3ml per FP}
- total pi tablets required = (18ml per FP + 5ml BSA) = </= 23ml per FP = </= 3 pi per FP

Procedure:

- Prechill 1x 50ml falcon tube per FP on ice, 15min
- Prechill 1-2x 50ml falcon tubes per FP on ice - for sonication (choose 1x aliquot if using 20 OD cells, or 2x for 40 OD cells)
- Prechill big centrifuge, 4oC
- Remove culture from incubator; for some FPs it will be clear by eye if expression levels are good.
- Transfer to falcon on ice; cool for 20min
- From now on cultures and protein should be kept on ice and spun at 4oC unless otherwise stated.
- Take OD (use 100ul of culture 1:10 in LB)
- Expect maybe 4-6 OD/ml.
- Calculate volume or fraction of total required for 20 or 40 OD worth of cells. (20 OD = 1x 20OD/2ml aliquot for sonication; 40 OD = 2x 20OD/2ml aliquot for sonication)

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- Expect fraction to be 0.1-0.2 meaning we're only using 10-20% of the culture for this purification even if we get through the whole 40 OD (which would require loading 7* 600ul on the spin columns). So expecting vol to be 5-10ml.

Example OD calculation:

A	B	OD of 1:10	OD of neat	total OD	fraction that is 40 OD	ml for 40
					40/total OD	40/total * 50
1	mCherry	0.418	4.18	209	0.19	9.57
2	eg1	0.3	3	150	0.267	13.3
3	eg2	0.6	6	300	0.133	6.67

- Add 20 or 40 OD to the prechilled tubes set aside for aliquotted cultures.
- (Original cultures can stay on ice or be stored in fridge.)
- Spin 3,220xg, 10min, 4oC
- Resuspend in 5ml WASH buffer w pipetboy/5ml stripette; Add 30ml more WASH buffer or so
- Spin 3,220xg, 10min, 4oC
- Resuspend in 2ml (for 20 OD) or 4ml (for 40 OD) Lysis Buffer.
- Lysis Buffer = T50N300 + pi + lysozyme. (No DTT for His tag purifs.)
- eg. for 5 purifications, Take 22 ml of T50N300+pi and add 44ul lysozyme.
- If using 40 OD, split cells into 2x falcons of 20OD/2ml each.

Lysis

3 *Prep for next stage: pre-chill the microfuge to 4oC.*

Lysis by Sonication

- Stand falcon in small plastic beaker full of ice.
- Sonicate: **50% amplitude, 10s on/off, 2min**.
- NB. 2min means 2min of sonication. as we're doing 10s on/off, this takes 4min.
- Solution should go from turbid to clear.
- If doing 40 OD, you will have two falcons.
- If you have many FPs, after 6 falcons you have enough sample to fill the microfuge for the next stage - it's worth starting the DNase step (30min) then the spin (30min) before the other samples are sonicated.

DNase I treatment

Essential if using A280 assay later. **Note that DNase I = 31 kDa meaning similar in size to FPs in a way that would affect estimates in Gel1 (though not Gel2 as it shouldn't bind the column), and is sensitive to vortexing.**

- Prepare DNase I stock: 1000 U/ml DNase I in ddH2O

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- To lysates in T50N300, add:
- DNase I to 50 U/ml final (20X dilution)
- CaCl2 to 5mM final (13mM ideal for DNase I, <5mM recommended with His resins)
- MgCl2 to 50mM final
- Mix thoroughly
- Reaction: 30min at 4oC

Fractionation

4 *Prep for next stage: prep eppies and cool them for after the spin*

[Day 2]

- Spin out insoluble fraction.
- Split 2ml lysates into 4x 0.5ml prechilled eppies.
- Spin in prechilled microfuge - 30min 16Kg 4oC.
- Result: 4x 0.5ml of soluble lysate (if using 20 OD) or 8x 0.5ml soluble lysate (if using 40 OD)
- Transfer SOLUBLE fractions to new tubes - can mix so that you have fewer eppies.
- Keep ONE INSOLUBLE PELLET for each FP. Add 500ul 1XLB and resuspend. Need this for Gel1.

Soluble fraction

Prep for next step: Change temp on microfuge to 21oC, and open lid to let it warm up. If incubations are done at RT to speed up kinetics, they should be spun at ~RT too. Proteins should be protected by the protease inhibitors.

Gel1: Verification of Expression/Fractions

5 **[Day 2]**

Sample prep for gels:

- boil samples in SDS/DTT to denature proteins.
- soluble fractions: 10ul + 10ul 2XLB -> boil -> load 10ul.

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- insoluble fractions: boil whole 500ul eppie (once), use 5ul of sample in 1X LB to load.
- Boiled samples no longer need to be kept at 4oC.

Gel prep for gels:

- Prep SDSPAGE running buffer
- Prepare 12% gel / prepare precast gel
- Fill up both reservoirs with running buffer.
- Wash out wells with pastette.
- Load samples in order below…

Gel loading:

- M = colour prestained protein markers, 5ul
- soluble fractions: load 10ul.
- insoluble fractions: load 5ul.

Gel1 example:

- M
- FP1 sol
- FP1 insol
- FP2 sol
- FP2 insol

Run gel:

- Run gel at 10mA (0.01A) per gel until dye front gets to the green seal about 1cm from bottom
- Wash 10’ dH2O (get rid of SDS).
- Stain overnight: 25ml Instant Blue.
- **[Day 3]**: Rinse gel twice in dH2O
- Wash 20’ in water with slow agitation.
- Image: camera using lightbox /Typhoon imager.

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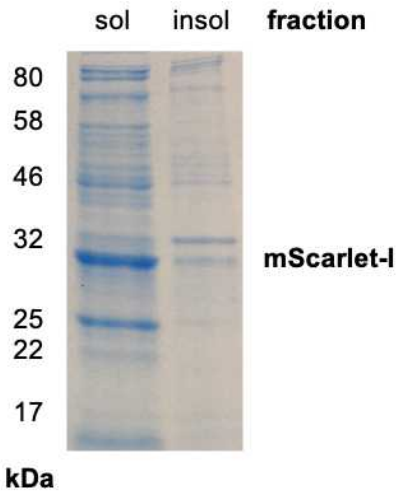

Gel1: mScarlet expresses well and is found in the soluble fraction

Protein abundance guesstimation:
My observation from running SDS-PAGE gels with 5ul protein markers beside BSA standards is that the apparent intensities of most bands in the markers correspond to the intensity you get from BSA standards loaded at 250-500ng protein lane, after staining with Instant Blue. So it's possible to make rough guesses at the FP concentration per OD just by looking at the markers. However, this isn't generally necessary, and more information can be gotten from comparing the relative intensity of the band corresponding to your FP to the other bands in a particular lane. If the FP is clearly visible in the induced vs uninduced lanes, there's some reasonable expression, and if it is mainly in the soluble fraction that is good news. Further, if it is one of the strongest bands in the soluble fraction that's all you need to know - your FP is expressing at very high levels (it is one of the most abundant proteins per cell) without causing toxicity, and most of it is soluble so it should be easy to purify.

Affinity Purification

6 There are a number of ways to purify His-tagged proteins. I opted for the HisPur Cobalt resin for a few reasons. Resins are available in agarose bead and magnetic bead format - magnetic beads are attractive for high throughput and automated protocols, but their binding capacities are 100-1000fold lower per volume than agarose resins. While I don't need grams of protein, I did require ug-mg yields, so it had to be agarose. The most popular resin is the Nickel resin (NiNTA) but I opted for Cobalt because, while its capacity is lower this is not a problem at the scales I require, but its specificity is higher ensuring purer protein.

Back of the envelope calculation to illustrate:

- HisPur Cobalt resin.
- Capacity of the resin: 10mg/ml (lower than NiNTA but higher specificity).
- spin columns
- I use Pierce Spin Columns - Snap Cap. The standard/smallest one has a max volume of 300ul 50% resin, which means a total yield of 3mg protein can be obtained from Cobalt

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resins, or 20mg protein max from NiNTA. This is typically sufficient: assuming all protein ends up in one quant assay, we're talking a starting conc for the highest conc in the plate would be (E1 neat: 3mg/100ul = 3000ug/100ul = 30ug/ul; E1/10 =) 3ug/ul. If I get a conc of 500ng/ul here I'm happy (6x less). Minimum is probably 100ng/ul (30x less).

Nickel and Cobalt resins tolerate most common reagents except EDTA (so there's no EDTA in the lysis buffer). DTT is disputed - manual for HisPur says to avoid it completely. DTT use (1mM) was tested and found to make not much difference to purification yields. Meaning DTT 1mM shouldn't negatively affect the columns. Subsequently however I didn't use it because it interferes w microBCA assays, and FPs seem happy enough without (but many of them only have a single Cys anyway).

[Day 2 contd]

Resin

- Take the HisCobalt resin out of the fridge.
- Shake and gently vortex resin stock bottle to make sure it is well mixed.
- Take 600ul 50% resin -> add it to an assembled spin column (max. volume 600ul).
- Spin 1' 1000g to remove storage buffer
- Discard flowthrough (fth).

Resin equilibration

- Resin equilibration rounds:
- **cap**
- add 300ul Binding Buffer (BB; T50N300, pi, 10mM imidazole - no lysozyme)
- flick to mix
- **uncap**
- spin 1' 1000g
- discard fth

- Repeat 3X. **I frequently incubate third equilibration 15' RT.**

Equilibrations	Tick when done
1	
2	
3	

Binding

– – – **Remember to add 10mM imidazole to samples before adding to column!** – – –

- Binding rounds:
- **cap**

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- add 600ul FP lysate (in T50N300, pi, lysozyme, 10mM imidazole)
- flick to mix
- **Bind at RT for 15min-90min**
- flick to mix every 5-10min
- **uncap**
- 1’ 1000g

Keep Flowthrough from at least one Binding Round for gel2: transfer flowthrough (after spin) from standard elution tube to a 1.5ml eppy.

- Repeat 1X-7X.

Binding can be done with just one sample of 600ul, or the full 40 OD cells (4ml lysate ~ 6+ sets of 600ul). (Though column can saturate by the 5th round of loading.)

Binding rounds	Tick when done
1	
2	
3	
4	
5	
6	
7	

Washing

Prep for next stage: prep EB if not done. Put EB at RT if not done.

- Washing rounds:
- cap
- add 300ul-600ul BB
- flick to mix
- uncap
- 1’ 1000g
- discard fth

- Repeat 5X.

Standard recommended washing is with 5 column volumes BB, as 5X with 1 col vol (300ul), but when doing a high number of binding rounds, I like to increase this to 10 col vols (eg. 5X 600ul). Can add 1M NaCl to increase stringency.

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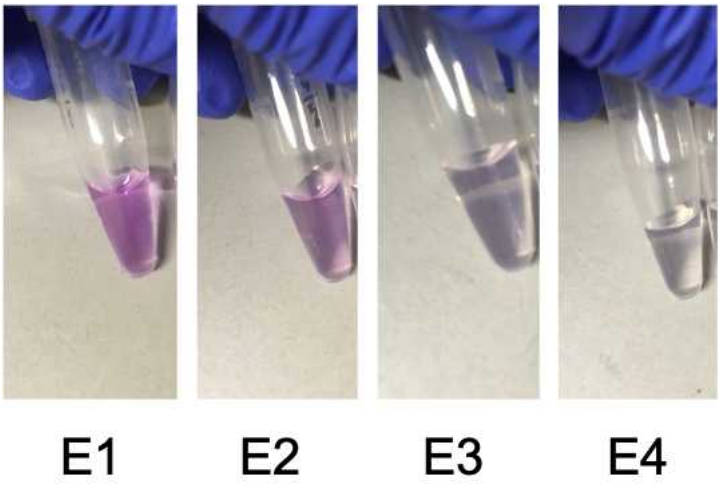
Washing rounds	Tick when done
1	
2	
3	
4	
5	

Elutions

- Elution rounds:
- cap
- add 300ul Elution Buffer (EB; T50N300, pi, 150mM imidazole)
- flick to mix
- **Elution incubation, 5' RT**
- uncap and **Transfer to Elution eppy (1.5ml)**
- 1' 1000g
- Elutions to fridge.

- Repeat 3X.

Elutions	Tick when done
1	
2	
3	



Elutions of mScarlet

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Gel2: Verification of Purification

7

[Day 3]

Check purification: lysate vs flowthrough vs elution(s). Optional - run beside BSA standards.

- Sample prep for gels: boil samples in SDS/DTT to denature proteins.
- Typical sample: 10ul + 10ul 2XLB -> boil -> load 10ul.
- Diluted sample, eg 1:2 dilution: 10ul sample + 10ul T50N300 -> take 10ul + 10ul 2X LB -> boil -> load 10ul
- Gel prep for gels
- AS FOR GEL1.
- Gel loading
- M = colour prestained protein markers, 5ul
- samples: load 10ul.
- Gel2 example
- M
- Pick your favourite FP [eg mScarlet] and run all fractions:
- sol 1/2 (1:2 dilution: 10ul sol fraction + 10ul T50N300 -> take 10ul + 10ul 2XLB -> load 10ul)
- fth1 1/2
- fth5 1/2
- E1 1/8
- E2 1/8
- E3 1/8
- For others, just run E1. [Run E1 neat if you only loaded protein once, run 1/8 if loaded several and expect high levels, run 1/2 if loaded several and expect low levels.]
- mch E1 1/8
- snap E1 1/8
- fast E1 1/8
- mrfp E1 1/8
- Run/Wash/Stain overnight AS FOR GEL1.
- **[Day 4]**: Rinse/Wash/Image AS FOR GEL1.

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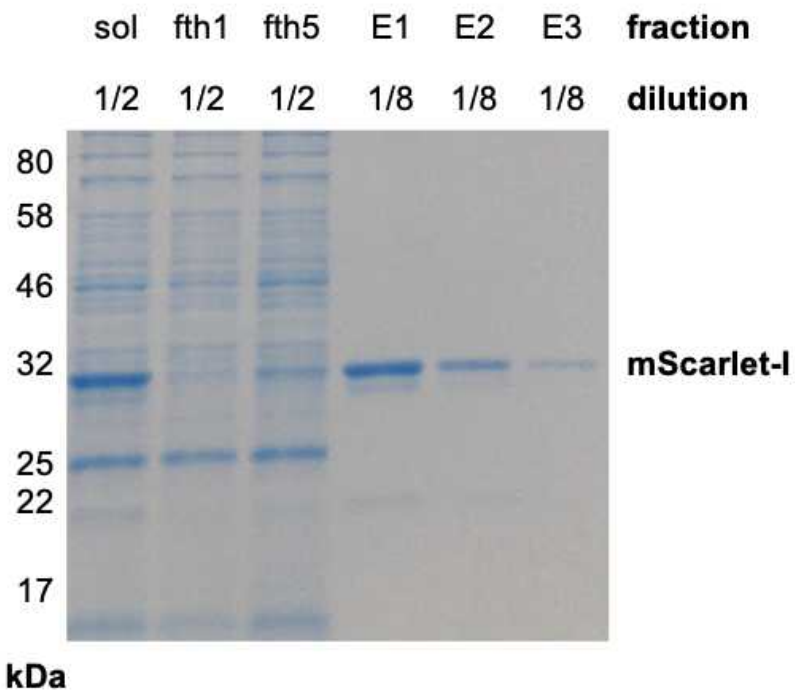

purified mScarlet - high yield and high purity

Quantification by eye

Can make guesstimates of Neat E1 concns within 30min of staining usually. Assume most markers about 500ng/ul. Guesstimate E1 band - X ng/ul. Work out conc of E1 neat, using effective volume loaded (5ul if loaded 10ul of a sample:LB mixture) and dilution used.

Calculation example:

FP / Fraction loaded	Est. mass (ng/lane)	Vol loaded (ul)	Est. conc (ng/ul)	Diln	Est. conc of neat (ng/ul)
mCherry E1	500	5	100	1/8	800

Protein concentration (not always optional)

8 **I do a buffer exchange every time (to T5N15-+pi) because it is essential for microBCA and A280 assay accuracy. If microBCA assays are used, and BSA standards are being used, it is best to treat the BSA in parallel with the FPs to make sure the buffers are identical. However, if only ECmax assays are going to be done, technically this step is optional. T5N15 + pi should be made fresh. There is no data on pi stability so in practise I never use >1day old buffer. Filter sterilise to get rid of cloudy precipitate (doesn't disappear even when dissolved at 37oC.**

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According to the manual, this is normal, but it may interfere with assays.)

Minimum concentrations for FP elutions in the next steps are > 100 ng/ul, as the highest conc to be measured in will be Elution/10, and 10 ng/ul is still within range of the protein assays. It usually makes sense to merge all elutions before starting.

Notes before starting:

1. As E1>>E2>>E3, combining all three and concentrating back to the original volume of E1, 300ul, is unlikely to make a big difference in the conc of E1 pre-concentration to conc of E123 post-concentration. However, it should increase. But if this is further concentrated to 100ul then at least a 3X increase in concentration should result.

2. Amicons claim 100 mM imidazole is the maximum compatibility of the columns. The elutions contain 150 mM. So technically the first step should be to DILUTE the elution 2-fold, but in practice it works fine as is.

3. Amicons are not compatible with >0.1% Triton X100 and Triton can interfere w A280 absorbance in protein assays. If sonication was used, no problem. If Triton was used, typically this means there's 0.1% in the lysis buffer. If the bound resin was washed with 10 column volumes of Triton-free buffer, perhaps Triton shouldn't be an issue, but this hasn't been tested.

4. Amicon concentration can be taken as an opportunity for buffer exchange, eg. dilute out imidazole and protease inhibitors and buffer exchange from T50N300 to T5N15. But obviously to compare different proteins they should be treated the same. Note: for the experiments in the paper, I (i) merged and concentrated E1+E2+E3 (900ul) to 50ul, (ii) buffer exchanged (3x450ul) into T5N15 and made up to 100ul, (iii) split the FPs into 2x50ul and buffer exchanged (3x450ul) either into T5N15 again, or T5N15+pi. Step (ii) was required to remove imidazole, and step (iii) to add pi back to one half of the sample, because I wanted to compare T5N15 and T5N15+pi in the following assays. In practise, it makes sense to do only one buffer exchange, straight into your buffer of choice.

Steps:

- Situation: E1 is estimated at 100ng/ul
- Plan:
- 1) merge E1, E2, E3 (=300x3 = 900ul) and concentrate to 50ul
- 2) buffer exchange and concentrate to < 100ul
- 3) resuspend to 100-300ul
- Use Amicon Ultra 10K columns
- 500ul capacity (500ul -> 15ul possible)
- **spin at 14Kg 10' to concentrate**
- **spin at 1Kg 1' to recover**

- Step1. Concentration.
- Add E1 (300ul) to 10K amicon column

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- 5’ 14Kg spin at 21oC
- expect it to go down to 100ul
- discard flowthrough
- Add E2 (300ul)
- 5’ 14Kg spin at 21oC
- expect it to go down to 100ul
- discard flowthrough
- Add E3 (300ul)
- 10’ 14Kg spin at 21oC
- expect it to go down to 50ul (needs to be <100ul)
- Recover result (needs to be <50ul)
- turn column over into fresh eppy
- 1’ 1000g
- Take sample, measure volume precisely, dilute back to exactly 50ul (for single buffer exchange) or 100ul if needs to be split into two buffer exchanges (to compare buffers).

- Step2. Buffer exchange into T5N15 -/+ protease inhibitors
- Assuming we’re starting from 50ul protein:
- Add E123 (50ul) to (fresh) 10K amicon column
- 10’ 14Kg spin at 21oC
- expect it to go down to 50ul
- discard flowthrough
- Add 450ul buffer (1)
- 10’ 14Kg spin at 21oC
- expect it to go down to 50ul
- discard flowthrough
- Add 450ul buffer (2)
- 10’ 14Kg spin at 21oC
- expect it to go down to 50ul
- discard flowthrough
- Add 450ul buffer (3)
- 10’ 14Kg spin at 21oC
- expect it to go down to 50ul
- discard flowthrough
- Recover result (needs to be <50ul)
- turn column over into fresh eppy
- 1’ 1000g
- Take sample, measure volume precisely, dilute back to 100ul+. 100ul is enough for one quantification, 200ul+ will allow for repeats.

FP Calibration in Plate Readers Protocol

9 The idea behind this protocol is to make most efficient use of protein. Therefore, ideally, one 100ul aliquot of the merged/concentrated Elution (E123), is all that is needed for all the fluorescence and concentration assays, and these can be done consecutively on a single dilution series. Currently an ‘exhaustive’ workflow includes 1 fluorescence assay and 3 protein

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assays. As this is the ‘exhaustive’ protocol, I will list a workflow to do all three.

Summary:

- You will need 100ul elution of each FP to be calibrated; plus 100ul of 2mg/ml BSA; plus the microBCA kit.
- Access to all plate readers to be calibrated for a clear few hours is ideal, but the time required depends entirely on ambition: the number of instruments, channels and FPs.
- Prep dilutions as 225ul/well in a uvclear plate -> absorbance scans.
- Transfer 200ul to clear plates (the same type of plate as you use for bacterial assays) -> fluorescence quants.
- Transfer 150ul to new clear plate -> microBCA.

Quantification of FP Concentration (part1)

10 There are three protein concentration assays (‘protein assays’) in this workflow.

1. microBCA assay
2. A280 assay
3. ECmax assay

As A280 and ECmax are simply absorbance assays, they need to be done before a microBCA, if multiple concentration measurements are going to be done. In addition, the A280 assay requires a different sort of plastic - uvclear plates. These absorb far less in the UV range and allow us to pick up protein signals at A280 that wouldn’t be possible with standard polystyrene. Therefore, this requires an extra step that I decided to do before the fluorescence assay. While in principle it shouldn’t matter, transferring samples between plates is not loss-free, and A280 assays are not typically very sensitive. Therefore, using as much as possible for the A280 is ideal - I went for 225ul, as 2x225ul can reliably be pipetted from a 500ul dilution series.

Therefore plates to scan in order are:

- 225ul uvclear - a280/ecmax
- 200ul clear - fluor
- 150ul clear - microbca

–

[Day 2 contd]

Prepare dilutions of BSA in parallel with FPs to allow for parallel treatment of BSA/FPs and microBCA standardisation later. Run scans in plate reader for FP quantification.

11 **1. Make Protein Dilutions in Eppies**

In order to validate the protein quantitation assays, I dilute each FP using serial dilutions and measure the concentration and fluorescence of each. In order to maximise both the dilution

Citation: Eszter Csibra, Guy-Bart Stan FPCount protocol - Full protocol https://dx.doi.org/10.17504/protocols.io.bztsp6ne

series and the number of FPs quantifiable per plate, I typically arrange FPs in 96-well plates in pairs of rows (AB, CD, EF, GH) - each FP is therefore measured as a duplicate. This leaves the columns for the dilution series itself, with 11 used for protein dilutions, and one for the buffer. (NB: In theory, arranging FPs in columns, with 7 dilutions plus one buffer for each FP, would allow for 6 proteins to be measured at the same time.)

If the microBCA assay is to be used, you need one BSA dilution per plate, or two if the two FPs to be measured are in different buffers (each FP/buffer combination needs a corresponding BSA/buffer dilution).

I recommend preparing dilution series in 1.5ml eppies: they are easier to handle (and see into) than wells of a deep well plate, important for avoiding errors.

Typical BSA dilution series:

dilution	BSA (ul)	Buffer (ul)	Vol left (ul)	Conc (ng/ul)
1	100 of stock (2mg/ml)	900	500	200.0000000
2	500 of prev dilution	500	"	100.0000000
3	"	"	"	50.0000000
4	"	"	"	25.0000000
5	"	"	"	12.5000000
6	"	"	"	6.2500000
7	"	"	"	3.1250000
8	"	"	"	1.5625000
9	"	"	"	0.7812500
10	"	"	"	0.3906250
11	"	"	"	0.1953125

Typical FP dilution series:

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dilution	FP (ul)	Buffer (ul)	Vol left (ul)
1	100 of elution	900	500
2	500 of prev dilution	500	"
3	"	"	"
4	"	"	"
5	"	"	"
6	"	"	"
7	"	"	"
8	"	"	"
9	"	"	"
10	"	"	"
11	"	"	"

12 2. Fill uvclear 96-well plate

Typical arrangements, depending on 0/1/2 BSA dilutions to be used:

UVClear Plate: ***225ul per well.***

0 BSA, 4 FPs

A	B	C	1	2	3	4	5	6	7	8	9	10	11	12
FP1	row1	A	dilution1 (neat)	dilution2 (1:2)	d3	d4	d5	d6	d7	d8	d9	d10	d11	buffer
	row2	B	dilution1 (neat)	dilution2 (1:2)	d3	d4	d5	d6	d7	d8	d9	d10	d11	buffer
FP2		C												
		D												
FP3		E												
		F												
FP4		G												
		H												

1 BSA, 3 FPs

Citation: Eszter Csibra, Guy-Bart Stan FPCount protocol - Full protocol https://dx.doi.org/10.17504/protocols.io.bztsp6ne

A	B	C	1	2	3	4	5	6	7	8	9	10	11	12
BSA	row1	A	dilution1 (neat)	dilution2 (1:2)	d3	d4	d5	d6	d7	d8	d9	d10	d11	buffer
	row2	B	dilution1 (neat)	dilution2 (1:2)	d3	d4	d5	d6	d7	d8	d9	d10	d11	buffer
FP1		C												
		D												
FP2		E												
		F												
FP3		G												
		H												

2 BSA, 2 FPs

A	B	C	1	2	3	4	5	6	7	8	9	10	11	12
BSA1	row1	A	dilution1 (neat)	dilution2 (1:2)	d3	d4	d5	d6	d7	d8	d9	d10	d11	buffer
	row2	B	dilution1 (neat)	dilution2 (1:2)	d3	d4	d5	d6	d7	d8	d9	d10	d11	buffer
FP1		C												
		D												
BSA2		E												
		F												
FP2		G												
		H												

Steps:

- Distribute 225ul buffer into column 12 with multipipette (Dispensing mode; 5ml; 225ul * 8; Asp 4, Disp 5).
- Add protein dilutions to plate with single channel manual pipette. Pipette tip use can be minimised if dispensing from the lowest concentration protein (dilution11) first.

13 3. Measure absorbance in UVClear plate - A280 and ECmax assays

Prewarm plate reader to temp used for cell growth curve assays to make sure the calibrations are valid for those temperatures - eg. 30oC.

Plate reader runs with UVClear plate:

A	instrument	plate	lid	scan	time taken
1	instrument1	uvclear	none	A200-1000	2 min

Citation: Eszter Csibra, Guy-Bart Stan FPCount protocol - Full protocol https://dx.doi.org/10.17504/protocols.io.bztsp6ne

Only one scan is needed here. That is an absorbance scan between 200nm and 1000nm. On the Spark this is the maximum range you can do, and you don't need more anyway. But the UV end is important for A280 measurements, the middle for ECmax, and the top for path length quantification. It also encompasses the A562nm baseline quantification for the microBCA assay.

Other runs can also be done optionally. Scans with a seal can be done if evaporation is a worry (in practice with 225ul it shouldn't be), but be aware that seals can distort absorbance readings particularly at A280 so it's not recommended. I sometimes run an extra, shorter, scan of 400nm-800nm as this lets me visualise the FP peaks by eye and verify easily that there is a decent amount of FP in the purification(s). (BFP in particular is not visible by eye, so the elution colour doesn't tell you how much of it you have purified!)

Quantification of FP Fluorescence

14 The purpose of this step is to quantify the fluorescence of each FP dilution **in the same plate that the E. coli growth curves will be carried out in**.

[Day 3 contd]

Measure fluorescence in clear plate

- Transfer 200ul into clear plate

Plate reader runs with Clear plate (200ul):

A	instrument	plate	lid	scan	time taken
1	instrument in which the microBCA assay will be done	clear	none or seal	A200-1000	2 min
2	instrument1	clear	seal	FP-dependent fluorescence channel1	10min
3	instrument1	clear	seal	FP-dependent fluorescence channel2	10min
4	instrument2	clear	seal	FP-dependent fluorescence channel1	10min
5	instrument2	clear	seal	FP-dependent fluorescence channel2	10min

I usually run another absorbance scan here with or without a seal (without is 'nicer' but

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evaporation should be taken into account for longer incubation times above 25oC). Alternatively, if the ECmax assay is the only protein assay to be used, the uvclear plate step can be skipped in favour of running an absorbance scan here.

Fluorescence data should be gathered on a range of gains (I use 40-120), for one (or more) channels, as required. For eg. while mScarlet is best measured at emission channel 595/35, it can be useful to measure it in 620/20 as well, in cases where the 595/35 channel would pick up too much crosstalk from another FP (such as YFP).

How I name channels (filter sets):
Channels are named as a combination of the excitation, then emission filter, which are given names to indicate the region of the visible spectrum they cover, followed by numbers. eg. the standard GFP filter set is 'green1green2', which excites at wavelength 485nm (bandwidth 20nm) and measures emission at wavelength 535nm (bandwidth 25nm).

Quantification of FP Concentration (part 2)

15 **[Day 3 contd]**

The third FP concentration assay is the microBCA. Note that as the microBCA assay requires the addition of a reagent to your FP that cannot be easily separated again, this step needs to be the last in the workflow - after quantification of protein via A280/ECmax and after fluorescence assays.

Why microBCA is ideal:
1. It has low protein to protein variation (11%, much lower than Coomassie-based assays).
2. Its sensitivity of microplate version is linear at 2-40 ng/ul. (The test tube version of the microBCA protocol is apparently more sensitive but for that you'd need (a) 1ml of protein, (b) to incubate the plate at 60oC which polystyrene plates can't handle.)
3. It is optimised for microplates / high-throughput measurements.
4. As a colourimetric reaction, it can be verified (sanity check) by eye.
5. It includes its own BSA standards in ampule format.
6. It is tolerant to detergents (<5% Triton), if that's ever a requirement.

Watch out, though - microBCA is sensitive to buffer:
- High conc TBS (T50N300) leads to an inhibition of reaction.
- 1X PBS leads to a background reaction (observed).
- Protease inhibitors also have some background (because two of the inhibitors are actually peptides!).
- DTT and reagents that chelate copper are not tolerated.
- Specific single amino acids will also produce color in the BCA assay (cysteine, tyrosine, and tryptophan).

16 **3. MicroBCA assay**

3A. Prepare reagents

Citation: Eszter Csibra, Guy-Bart Stan FPCount protocol - Full protocol https://dx.doi.org/10.17504/protocols.io.bztsp6ne

The MicroBCA kit makes this step super simple. Just mix the three reagents, these are stable at RT for at least a few hours and don't need protecting from light. I make them up ahead of time while the fluorescence scans are running.

- Working reagent (WR) to make up: #columns(incl buffer) * #rows * 150ul = WR needed.
- I typically only do the assay for the first 8 dilutions + buffer, as this is beyond the sensitivity of the assay in all cases.
- So a full plate would need: 9 * 8 * 150ul = 10.8ml. Round up to 12ml.
- WR components need to be mixed in the ratio 25A:24B:1C.
- Calculation: (Total volume)/50 = 1 part. eg. 12ml/50 = 240ul.
- A = 25*part = 6ml
- B = 24*part = 5.76ml
- C = 1*part = 240ul
- Vortex.
- Distribute into in new clear plate with multipipette (Dispensing mode; 5ml; 150ul * 18; Asp 4, Disp 5).

17 3B. Prepare samples

Add 150ul from most recent plate (200ul clear plate or 180 black plate) into corresponding wells - use multichannel and mix carefully by pipetting up/down.

18 3C. Reaction and Measurement (in plate reader)

Both incubation and measurement is done in a plate reader. This ensures an even distribution of heat compared to ventilated incubators and ensures that the cooling to 25oC step is done properly - I can't control the room temperature or measure the temperature of the plate contents if I cool a plate by putting it on the bench.

- assay, 37oC 2h
- how to program:
- temp 37oC, 'await temp before continuing', unselect 'stop temp control after this step'
- "wait" step, 2h
- cool to RT, 25oC 10'
- how to program:
- temp 25oC, 'await temp before continuing', unselect 'stop temp control after this step'
- "wait" step, 10'
- measurement at A562nm
- how to program:
- Absorbance measurement, 562 nm, rest of settings on defaults.

This takes about 2h30 as the cooling takes time. NB. If the cooling takes >30min, eg. in the summer in a poorly air-conditioned lab, it is best to switch off the 'await temp before

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continuing' step.

Protein storage

19 Proteins should be stored in the fridge protected from the light, which testing indicated kept proteins stable and active for up to 4 weeks. Long term storage and re-testing post freeze-thaw has not been tested.

Analysis - Calibration of Plate Reader

20 The aim of this protocol is that for a given FP, we can relate the number of FP molecules to the 'relative' fluorescence units observed in a given instrument, with a given filter set, and gain. The previous steps described how to purify FPs to produce calibrants and how to run the assays. The output data from such assays must now be analysed to obtain calibrations.

To assist with this process, we have developed an R package called FPCountR. The full details are on the GitHub page. In brief, functions in this package are provided for each analytical step. parser() functions parse plate reader data into idealised formats. get_concentration() functions calculate protein concentration from absorbance data. The generate_cfs() function obtains conversion factors using concentration and fluorescence data. Finally, process_plate() and calc_percell() functions extract data from microbial growth curves in units of molecules, and molecules/cell.

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