Expression and Purification of His-tagged 3C Protease (H6H3C)

pH6H3C is a Kanamycin (Kan) resistant T7-driven expression vector used for expression of His-tagged 3C protease. We saw slightly better expression levels when using an *E. coli* strain that compensates for rare codons and therefore use Rosetta(DE3) cells which are Chloramphenicol (Cap) resistant. The protein is expressed in inclusion bodies. Unless otherwise noted [Kan]=30ug/mL and [Cap]=34ug/mL.

Expression in Shaker Flasks:

1. Transform pH6H3C into Rosetta(DE3) cells (EMD4Biosciences) and plate on Kan/Cap plates.
2. Inoculate starter culture of LB (10mL per liter of culture that will be grown) with Kan/Cap and a colony from plate. Incubate O/N at 37°C with shaking at ~250rpm.
3. Inoculate LB with overnight culture (1:100 dilution) and antibiotics. Incubate at 37°C with shaking.
4. Grow cells until an OD_{600} of 0.6-0.8 is reached.
5. Induce cells with 0.5 mM IPTG and incubate with shaking at 37°C for another 3 hours.
6. Harvest cells by centrifugation (6K rpm, 10 min) and freeze cell pellet at -80°C.
7. Check the expression by SDS-PAGE before proceeding with purification. Protein has a molecular weight of ~22 kDa. See Figure 1.

Expression in Benchtop fermentor:
This typically yields 8-10g wet cell pellet per liter induction.

1. Prepare the standard rich medium used for recombinant *E. coli* fermentation from the New Brunswick Scientific document: “Fundamentals of Fermentation.”
2. Inoculate LB (50mL per liter that will be grown) containing Kan/Cap with a small clump of colonies from plate. Incubate O/N at 37°C with shaking.
3. On the day of your growth add any sterile filtered components to your rich medium. Use [Kan] =100ug/mL and [Cap]=34ug/mL.
4. Seed the rich medium at OD_{600}~0.1-0.2. This is ~20-30 mL of overnight starter per liter of culture.
5. Use a DO setpoint=30 and a DO cascade where agitation and air are regulated sequentially.
6. Grow cells at 37°C until an OD_{600} of 2.0-2.5 is reached.
7. Induce cells with 0.5 mM IPTG and incubate at 37°C for another 3 hours.
8. Harvest cells by centrifugation (6K rpm, 10 min) and freeze cell pellet at -80°C.
9. Check the expression by SDS-PAGE before proceeding with purification. See Figure 1.

Amino Acid Sequence of H6H3C:

M G S S H H H H H H G G G L V P R G P N T E F A L S L L R K N I M T I T T S K G E F T
G L G I H D R V C V I P T H A Q P G D D V L V N G Q K I R V K D K Y K L V D P E N I N
L E L T V L T L D R N E K F R D I R G F I S E D L E G V D A T L V V H S N N F T N T I L
E V G P V T M A G L I N L S S T P T N R M I R Y D Y A T K T G Q C G G V L C A T G K I
F G I H V G G N G R Q G F S A Q L K K Q Y F V E K Q

Molecular Weight: 21818.9 Da
Extinction Coefficient (280nm): 5480 $M^{-1}cm^{-1}$
pI: 8.78

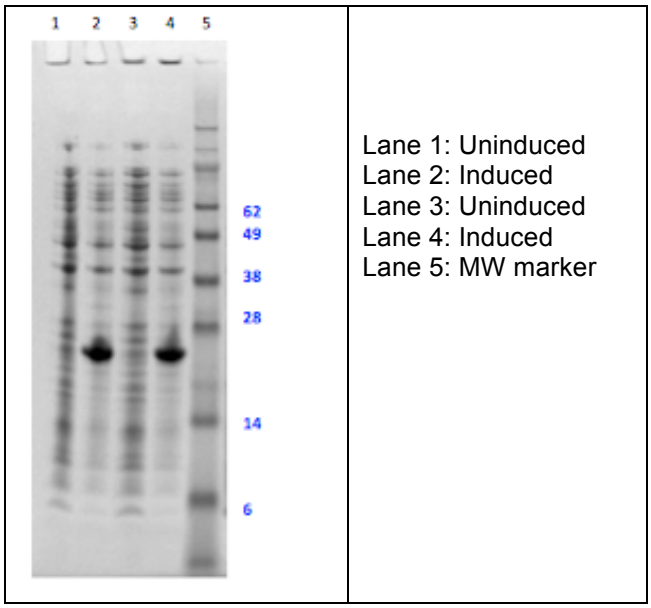

Figure 1. SDS-PAGE of expression of H6H3C protease in Rosetta (DE3) cells shows a strong induced protein band.

IMAC Purification under denaturing conditions:
We found that if you use cobalt affinity resin the protease develops a brown color following dialysis, but it does not seem to affect activity. Using nickel affinity resin does not have this effect.

All urea solutions must be made fresh, just prior to use. Do not use old urea solutions or leave your protein in high concentrations of urea overnight as it can carbamylate your protein. Steps 5-11 should be completed in a single day. *Note: We saw less ppt in the dialysis/refolding step when we did an acid elution vs. imidazole.*

Lysis Buffer (250 mL)	Elution Buffer (at least 50 mL)	Dialysis Buffer (4 L)
100 mM NaH_2PO_4	50 mM Sodium Acetate	20 mM MES
10 mM Tris-Cl	6 M Urea	1 mM EDTA
6 M Urea	pH 5.0	1 mM DTT
pH 8.0		10 % Glycerol (v/v)
		pH 6.5

1. Resuspend cells in 50 mM Tris-Cl pH 8.0 (9ml per gram weight cell pellet).
2. Add lysozyme to resuspension (1.5mg lysozyme per gram weight).
3. Sonicate for 10 minutes on ice (cycle 5sec on/off to avoid heating solution).
4. Centrifuge at 22000 rpm for 20 minutes at 4°C. Discard supernatant. The pellet should be large and opaque. At this point, you can stop and freeze pellet at 80°C until ready to process or move on to step 5.
5. Resuspend pellet in lysis buffer (5mL per gram weight of initial cell pellet). Homogenize solution thoroughly to break up any clumps.
6. Centrifuge at 22000 rpm for 25 minutes at 4°C to pellet cellular debris.
7. Meanwhile, equilibrate IMAC resin in Lysis buffer. You will need ~1ml of beads per 6mL supernatant.
8. Carefully decant supernatant into the container to be used for rocking. (The pellet tends to be very loose and jellylike so you may have to decant and do a second spin down of supernatant.). Add pre-equilibrated IMAC Resin to supernatant and rock on rotary shaker at 4°C for 1 hour.
9. Pour resin slurry into column and wash with 10 column volumes of Lysis buffer.
10. Elute the protein with at least five column volumes of elution buffer. Check fractions on SDS-PAGE. Pool fractions containing H6H3C and make ~100x (mol/mol) with DTT.
11. Dialyze pooled samples in 3,500 MWCO dialysis tubing at 4°C against 2L of the Dialysis buffer for 1-2 hours. Swap into fresh 2L of Dialysis buffer and dialyze overnight at 4°C.
12. If protease precipitates during dialysis, it can be reclaimed. Centrifuge the solution to separate the soluble protein solution from the precipitate. Resolubilize precipitate in minimal amount of elution buffer. Add DTT to 1 mM. Redialyze the protein solution in 4L of the Dialysis buffer. This step often yields a more concentrated protease solution that is still fully active.
13. Yield is usually ~25 mg/L induction and protease is ~95% pure. If greater purity is desired continue with polishing step (see next section). Otherwise, aliquot protease and store at -80°C.

Optional Final Polishing step (this is usually not necessary and rarely done anymore):
All steps carried out on an AKTA-FPLC at 4°C.

1. Pooled samples were further purified by using a 1 mL Resource S (Pharmacia Biotech) cation exchange column.
2. Column was equilibrated with 2 CV of Buffer A (20mM MES pH 6.0) prior to sample injection, and washed with an additional 5 CV of Buffer A post-injection.
3. Protein was eluted on a 0-100% gradient of Buffer B (20 mM MES pH 6.0 with 0.5 M NaCl) with a total elution volume of 20 CV. The elution gradient was then held out at a 100% Buffer B concentration for an additional 5 CV to elute any additional protein.
4. Fractions of peaks were checked on SDS-Page for presence and purity of protein.
5. Protease may be stored at -80°C in 10% glycerol.

Activity Assay of H6H3C:
To verify activity of the refolded protease, we monitor cleavage of a test substrate (HisMBP-RPA70AB) as a function of time. The expected cleavage products are His-MBP tag (~44 kDa) and RPA70AB protein (~27 kDa). The assay is conducted at a 50:1 molar ratio (substrate:enzyme) in a buffer containing 50mM Tris, 150mM NaCl, 10mM EDTA, pH8.2. Figure 2 below shows the complete cleavage of the substrate by the enzyme after 15min at both room temperature and at 4°C.

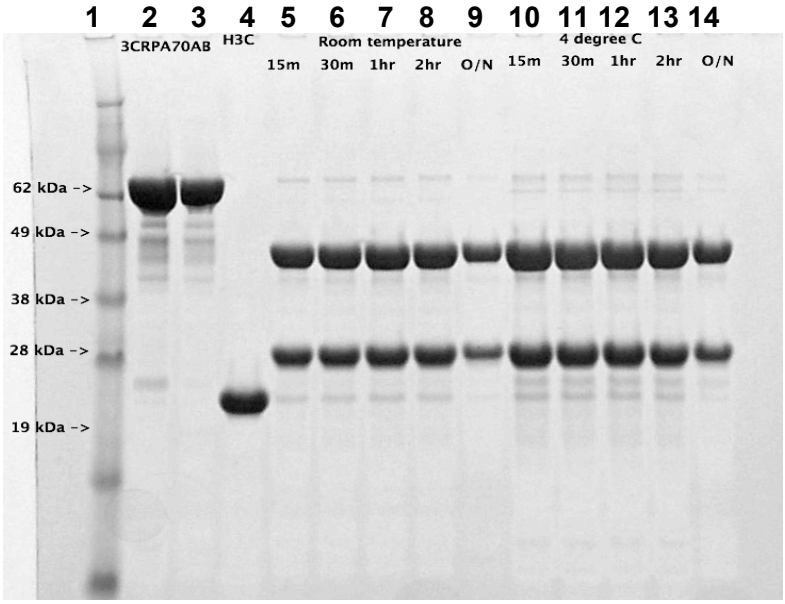

Figure 2: Activity test of H6H3C at room temperature and 4°C with samples taken at various time points.

Lane 1: MW marker
Lanes 2&3 : Uncut substrate
Lane 4 : H6H3C
Lanes 5-9 : Room temperature incubation
(15 min, 30min, 1hr, 2 hr, overnight)
Lanes 10-14 : 4°C incubation
(15 min, 30min, 1hr, 2 hr, overnight)

EMD4Biosciences (formerly Novagen) markets His-tagged 3C protease and their User Protocol TB420 details some general information on the protease and factors that affect activity:

Component	**Relative HRV 3C Protease activity (%)**
0.8 M NaCl	150
0.2 M NaCl	110
0.75 mM Leupeptin	50
8 mM PMSF	50
> 1 mM TLCK	50
10% glycerol	114
100 mM $ZnCl_2$	50
2 M Urea	0
1 M Urea	40
1 M Guanidine	0
1 mM DTT	100
50 mM EDTA	100
50 mM EGTA	100
0.1% Triton™ X-100	> 100
0.1% Tween™ 20	> 100
0.1% Nonidet™ P-40	> 100
1% Triton X-100	100
1% Tween 20	100
1% Nonidet P-40	100