

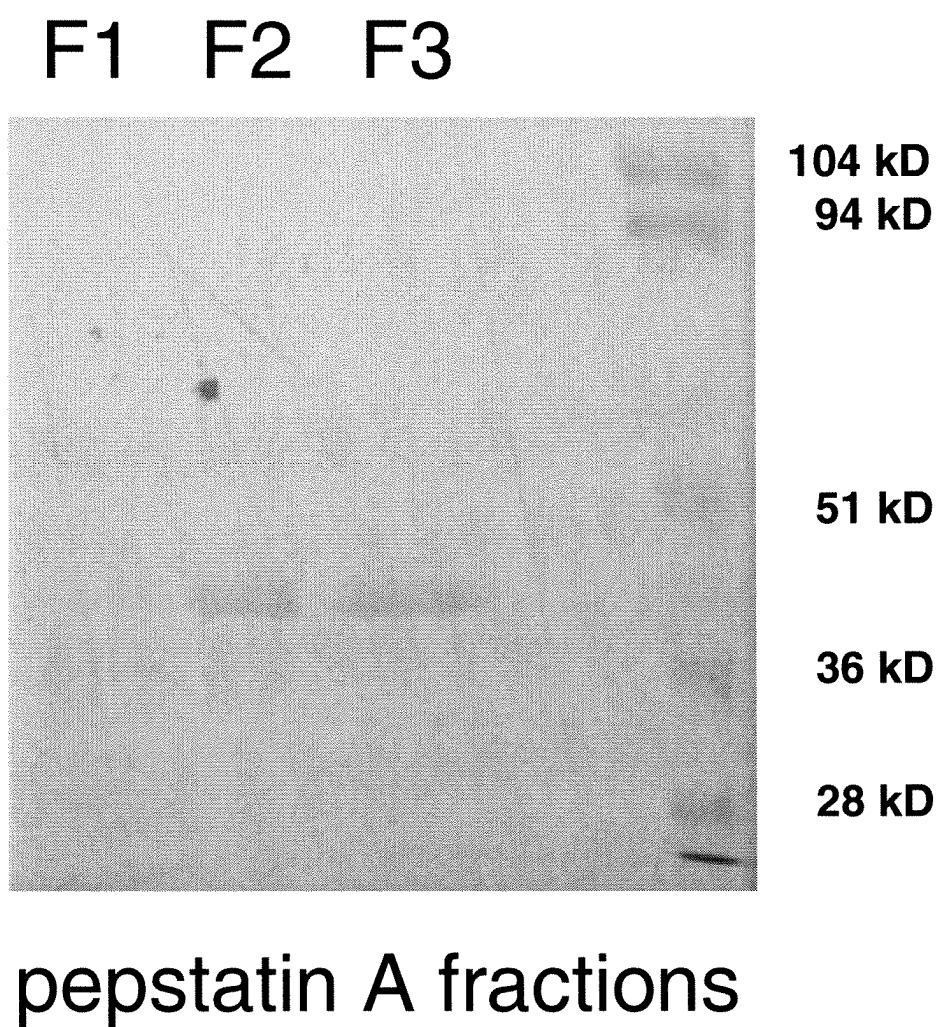


Figure 1

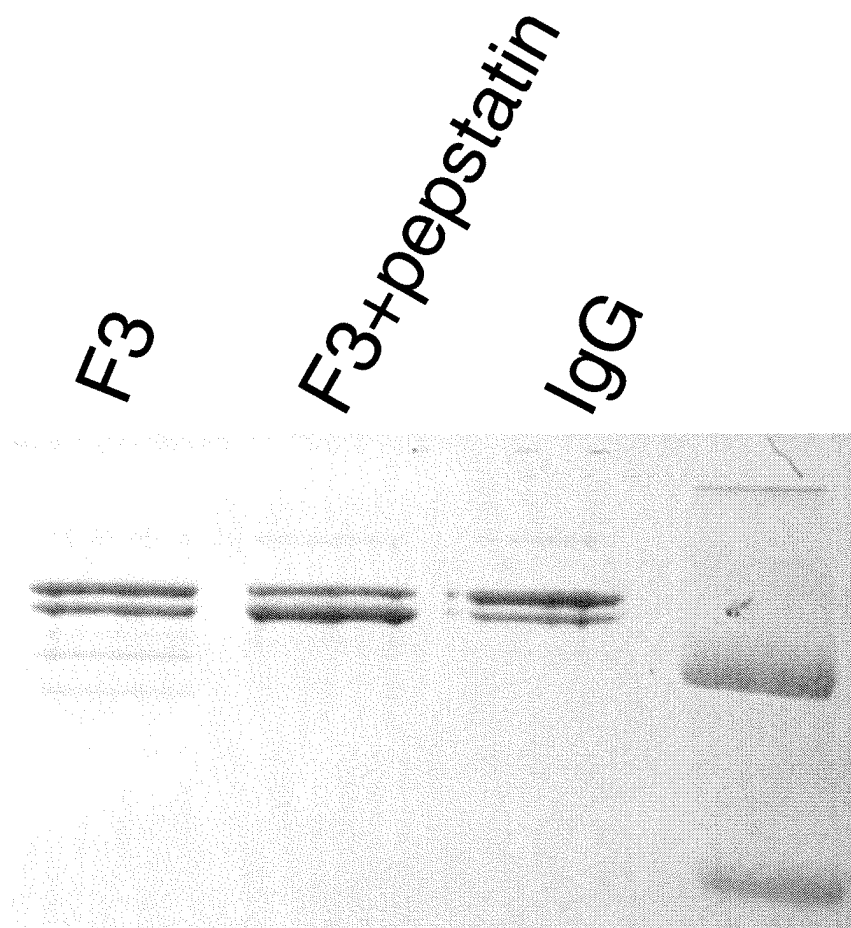
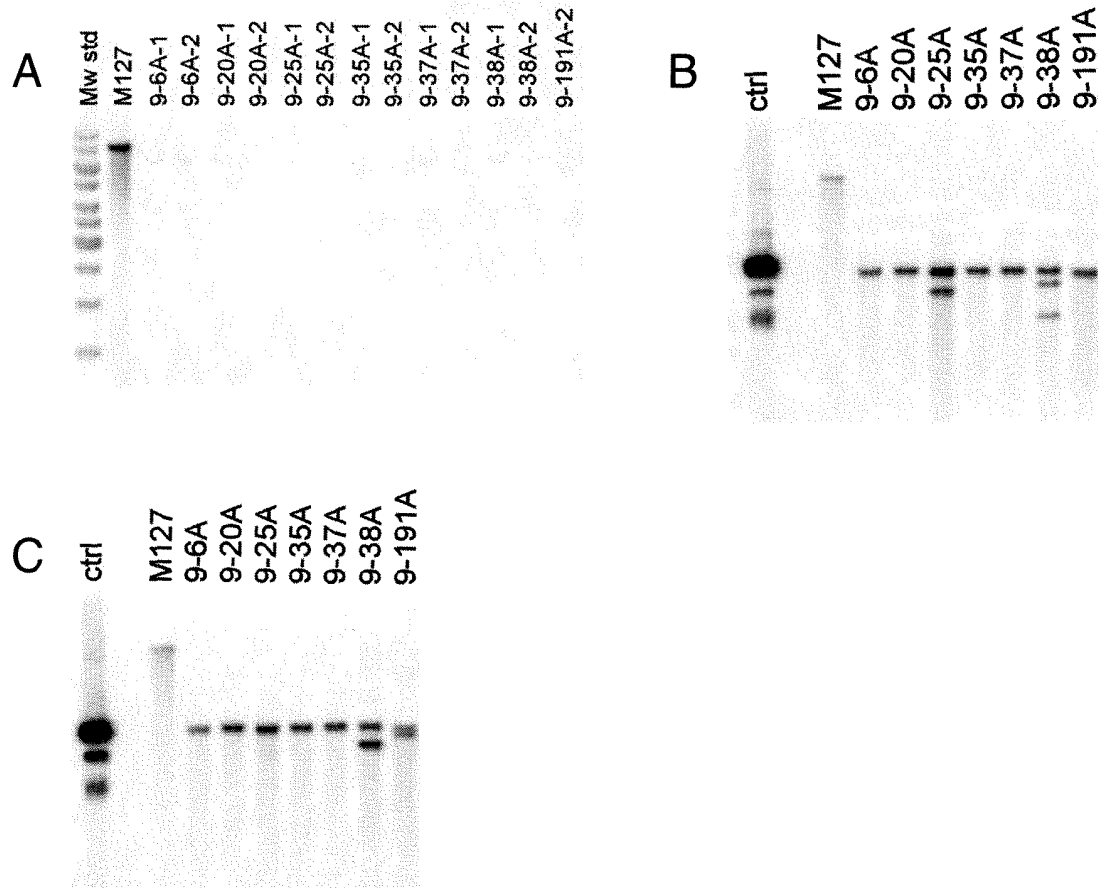


Figure 2



Generation of single protease deletion strains M181 and M195

M181 = repurified clone 9-20A-1 (i.e. originating from 9-20A)

M195 = repurified clone 9-35A-1 (i.e. originating from 9-35A)

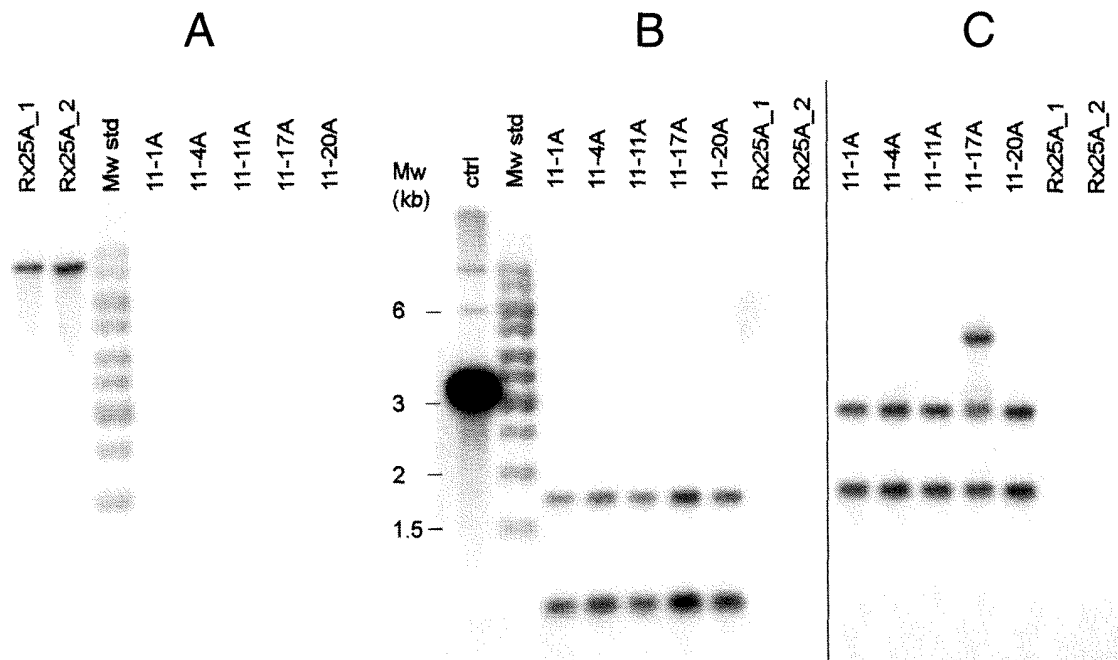
Expected signals:

A) *pep1* ORF: >8 kb from parent M127, no signal from transformants

B) *pep1* 5'flank: >8 kb from parent M127, 4 kb from transformants

C) *pep1* 3'flank: >8 kb from parent M127, 4.2 kb from transformants

Figure 3



Generation of rituximab, *pep1* deletion strain M182

M182 = 11-1A

M169, parent = Rx25A_1

Expected signals:

- A) *pep1* ORF: >8 kb from parent M169, no signal from transformants
- B) *bar*: 1.0 + 1.7 kb from transformants, 3.1 kb from pTTv41, nothing from M169
- C) *bar*: 1.8 + 2.8 kb from transformants, 3.1 kb from pTTv41, nothing from M169

Figure 4

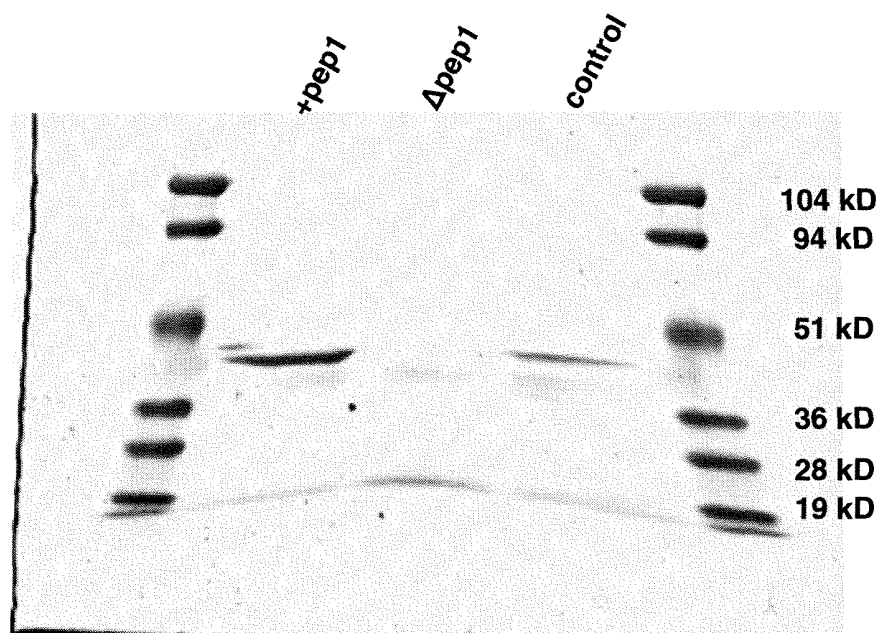


Figure 5

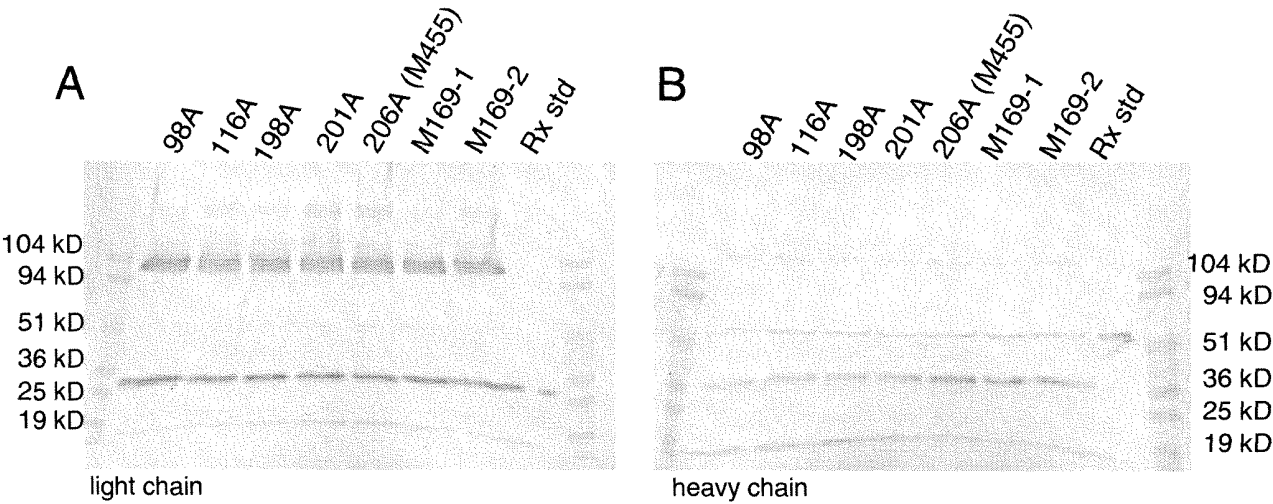


Figure 6

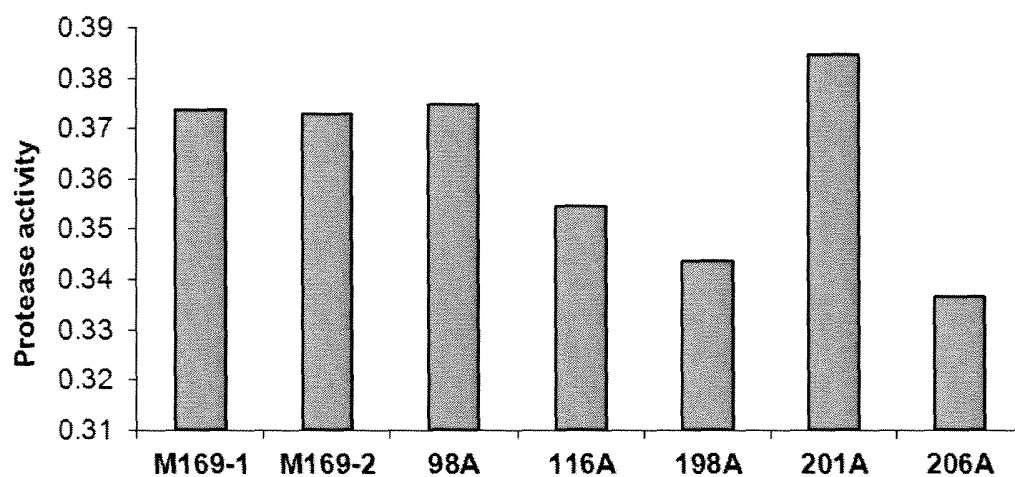


Figure 7

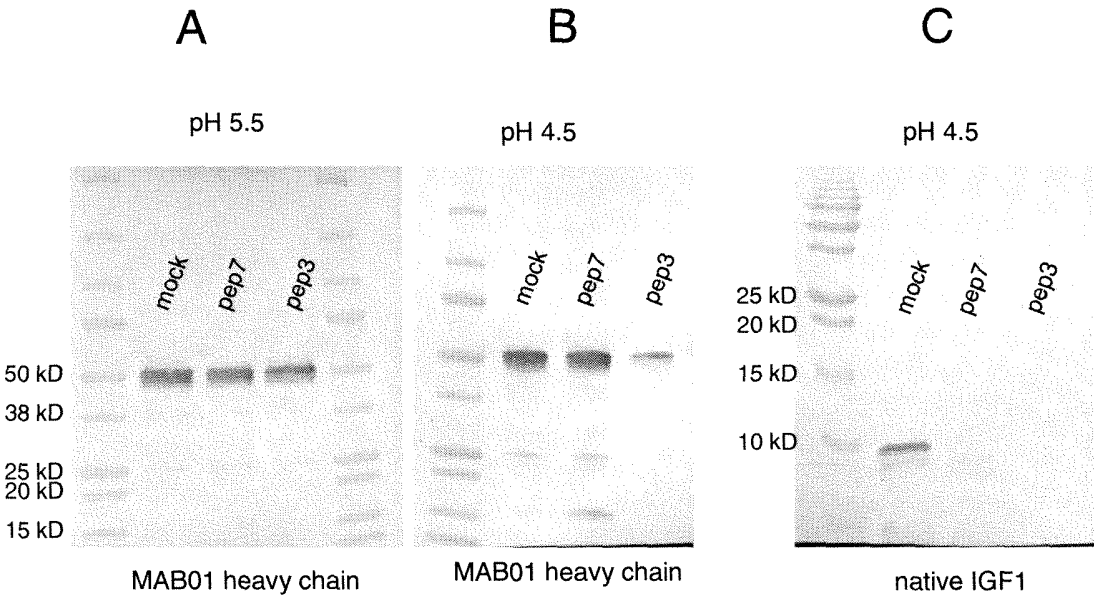


Figure 8

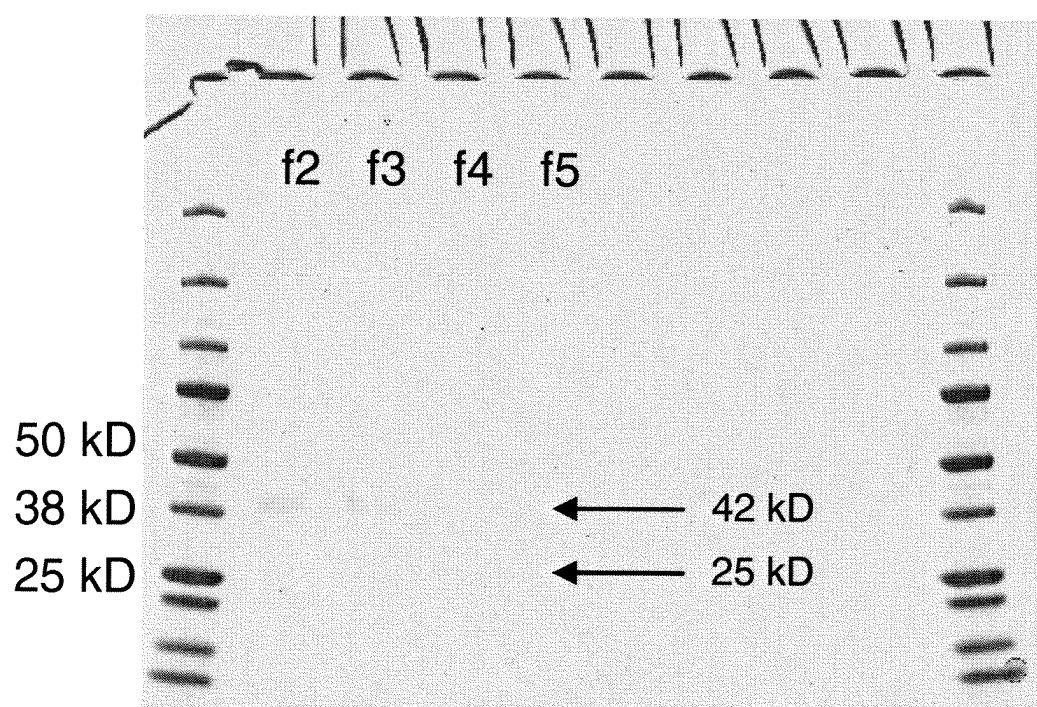


Figure 9

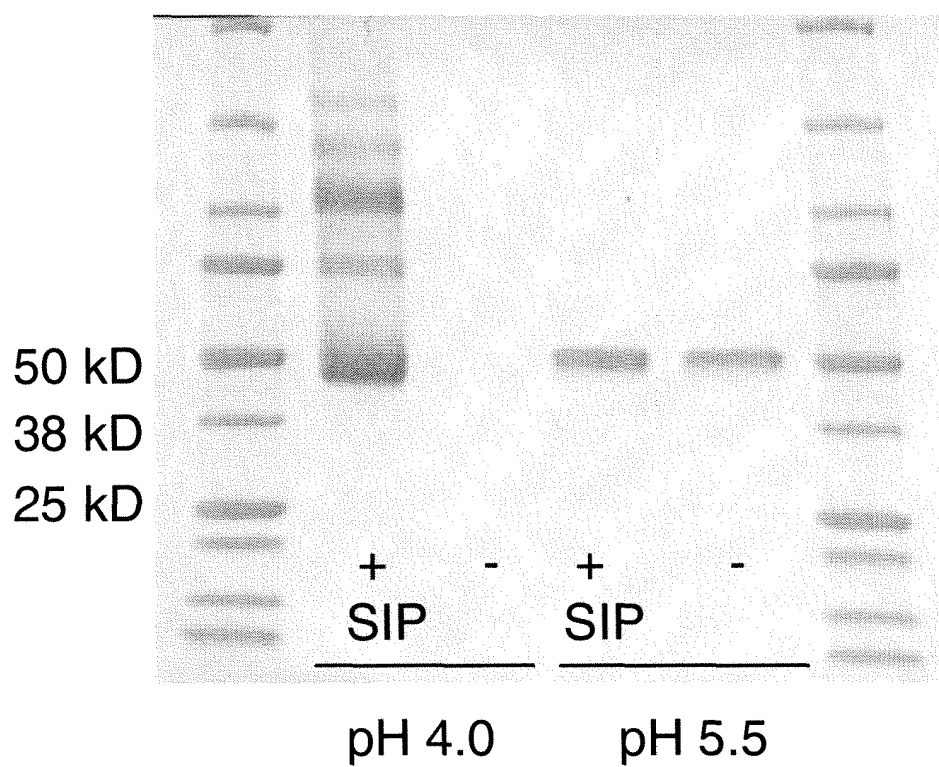


Figure 10

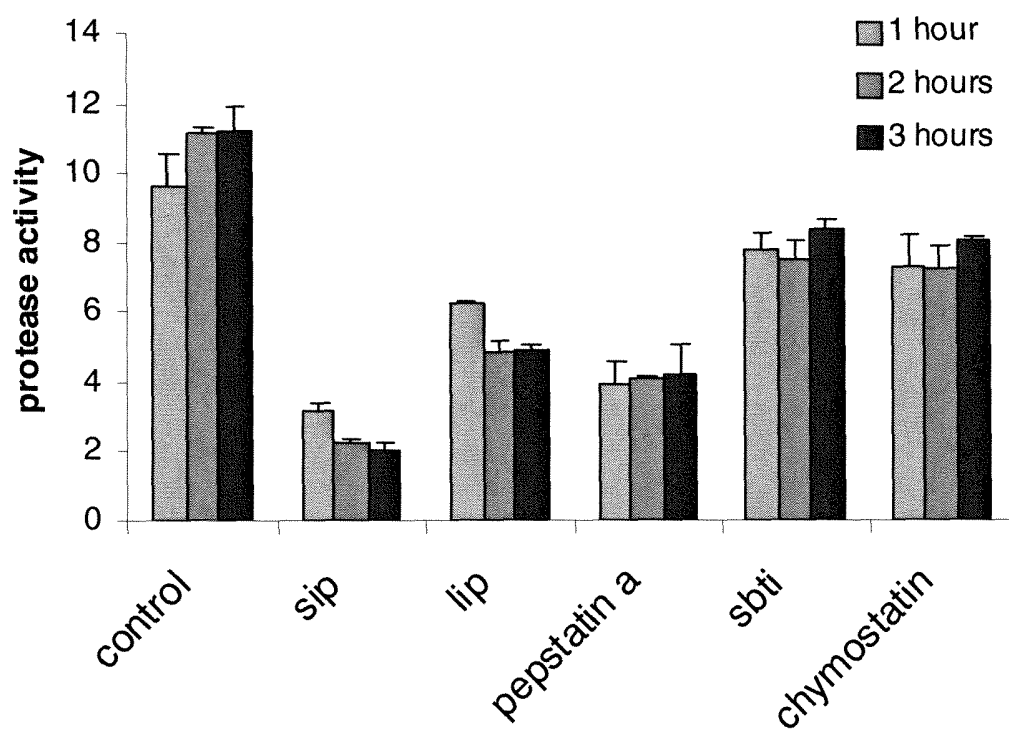


Figure 11

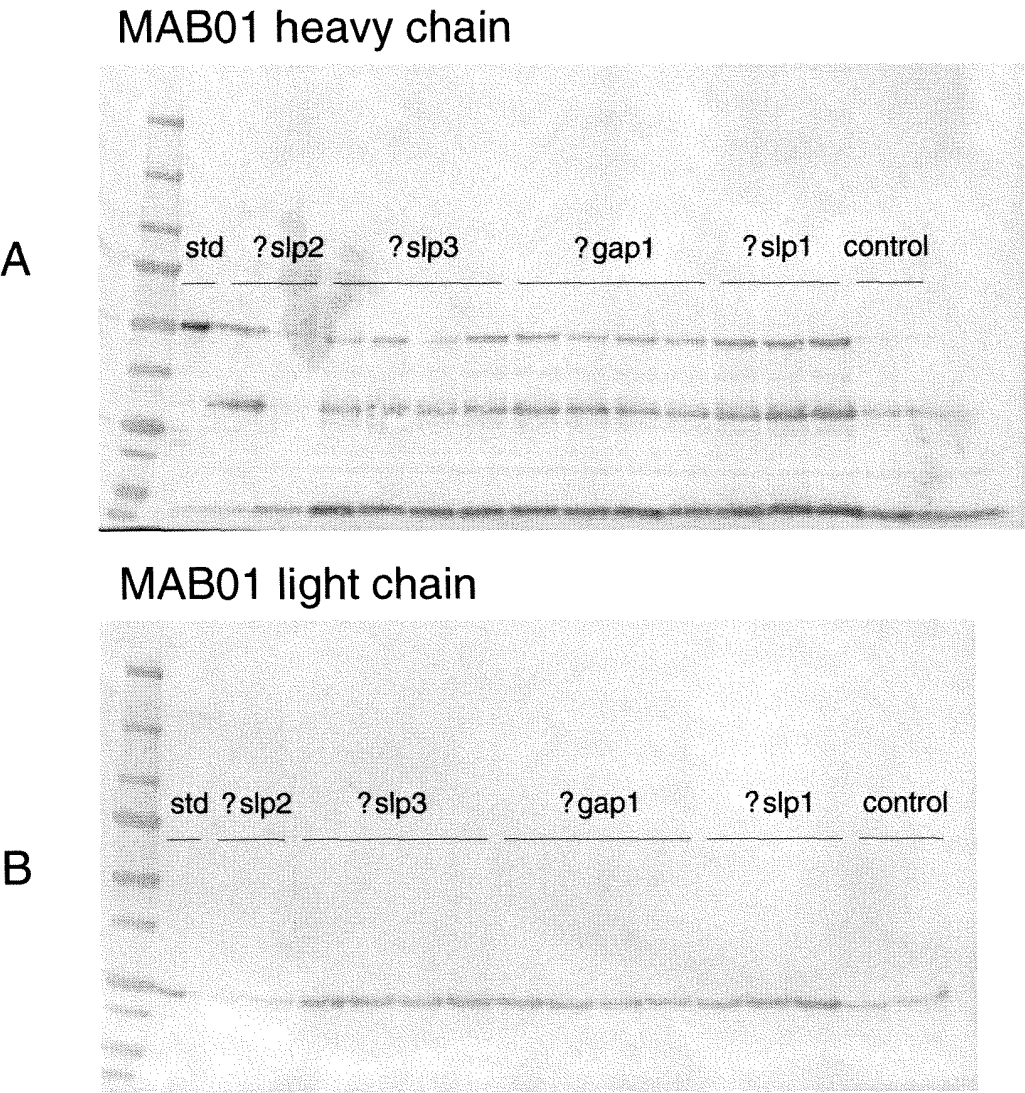


Figure 12

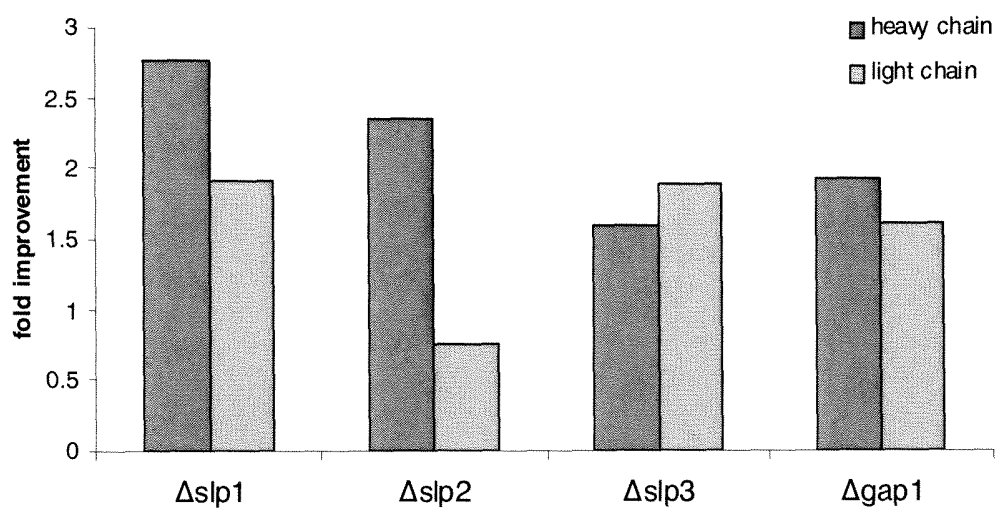


Figure 13

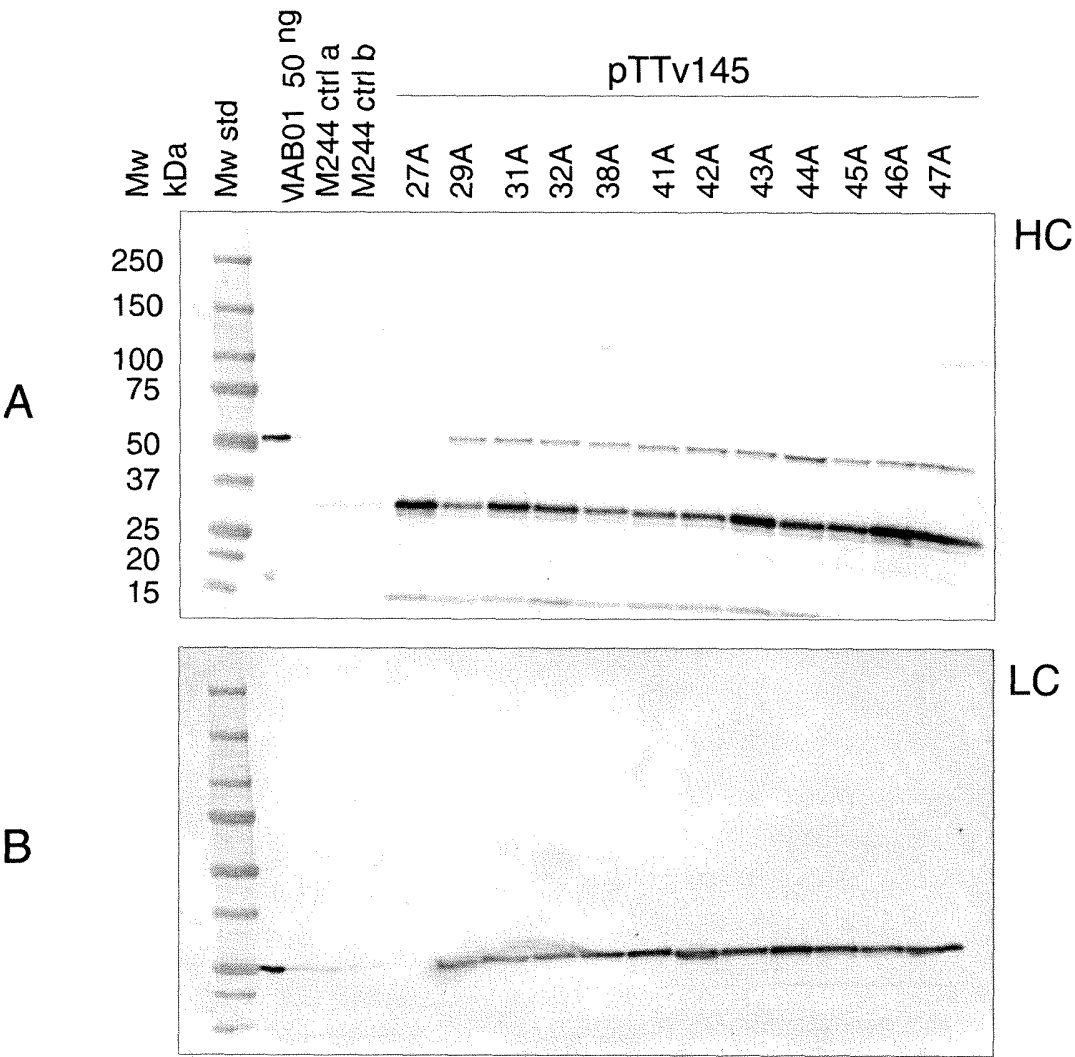


Figure 14

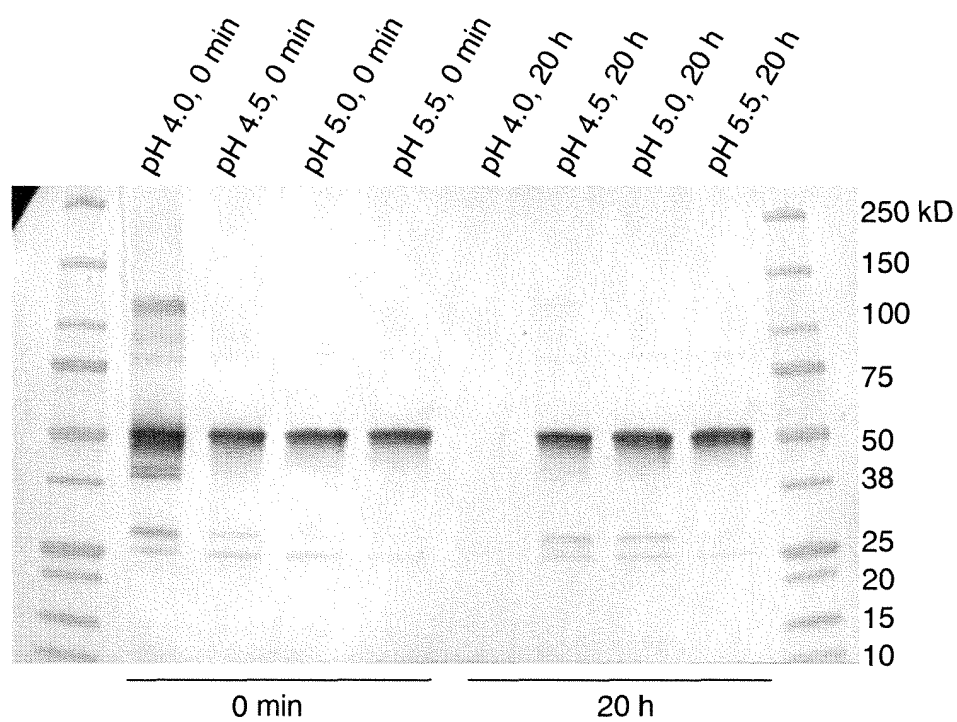


Figure 15

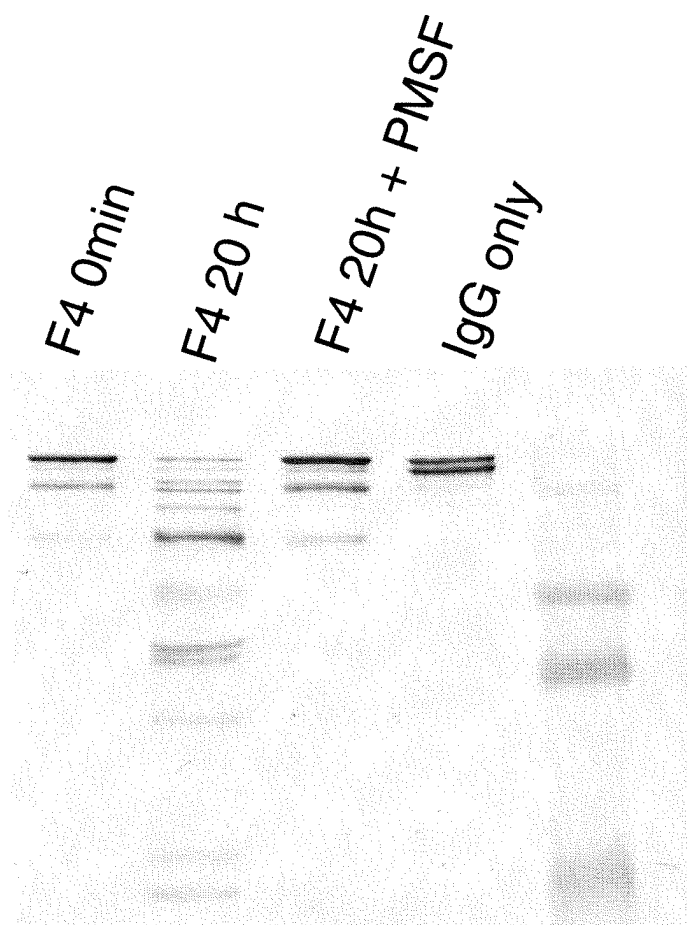


Figure 16

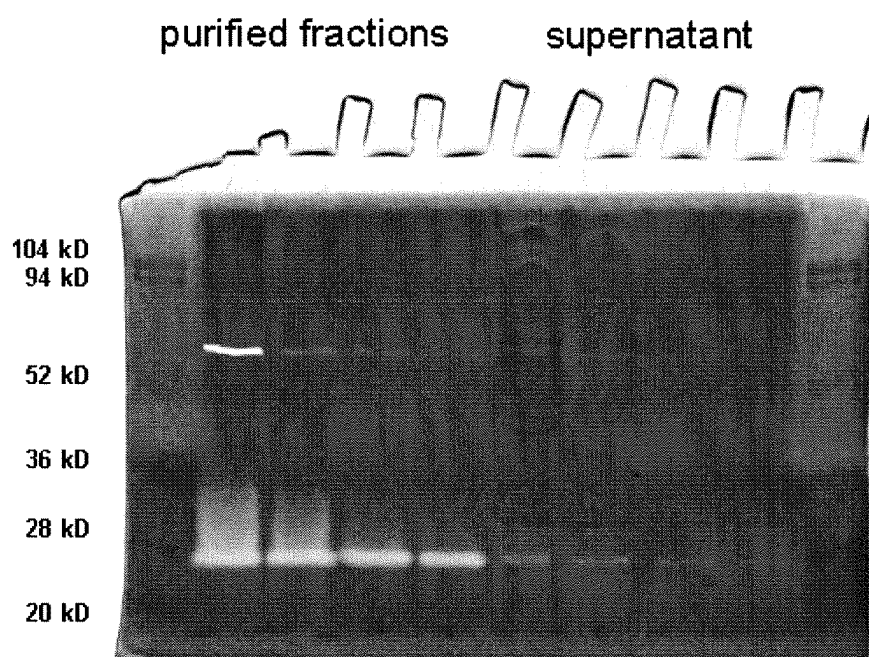


Figure 17

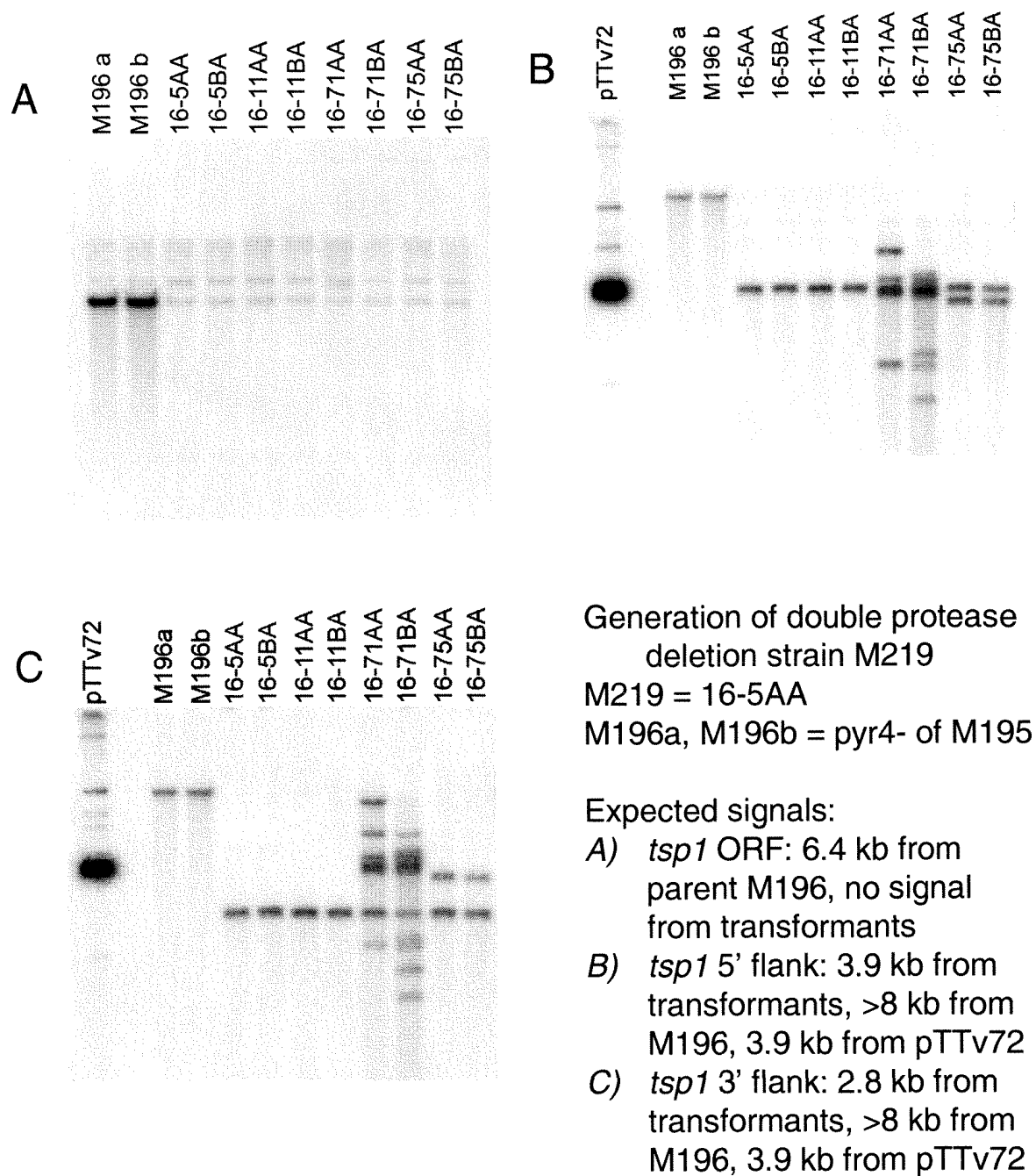
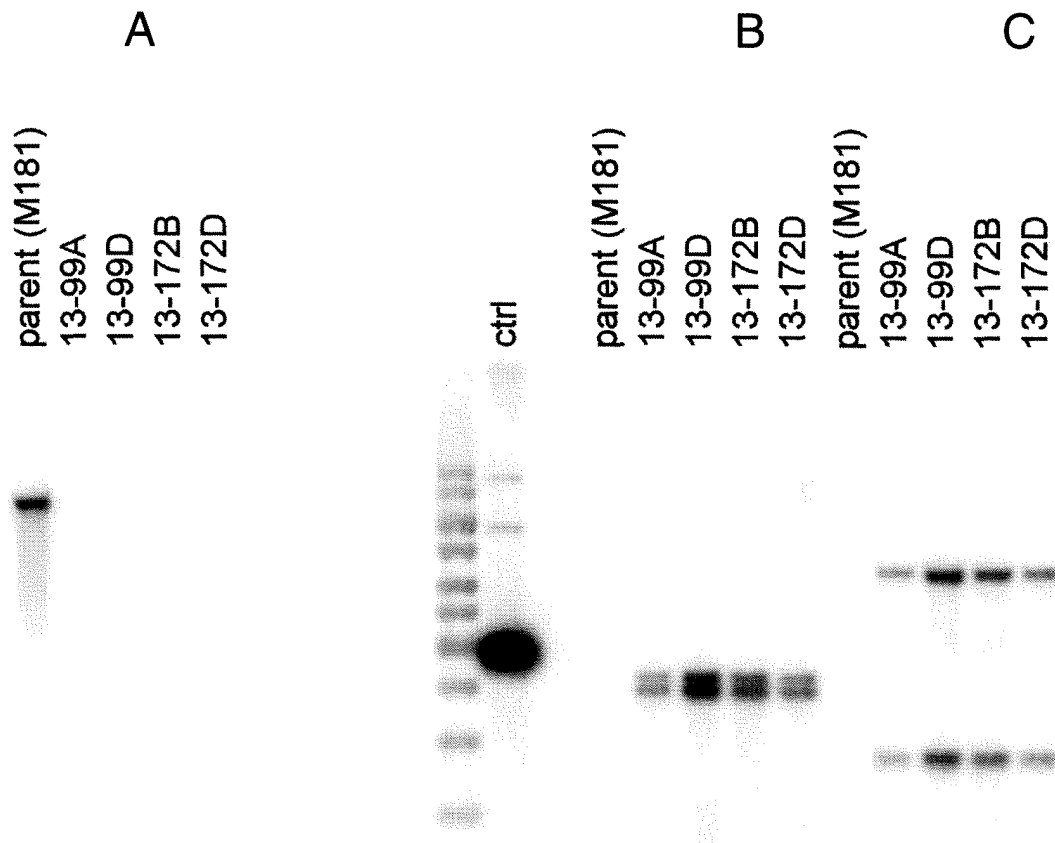


Figure 18



Generation of *pep1tsp2* deletion strain M194 (for MAB01 transformations)

M194 = 13-172D

M181 = parent

Expected signals:

A) *tsp1* ORF: kb from parent M181, no signal from transformants

B) *bar*: 1.4 + 2.5 kb from transformants, 2.9 kb from pTTv42, nothing from M181

C) *bar*: 1.9 + 3.2 kb from transformants, 2.9 kb from pTTv42, nothing from M181

Figure 19

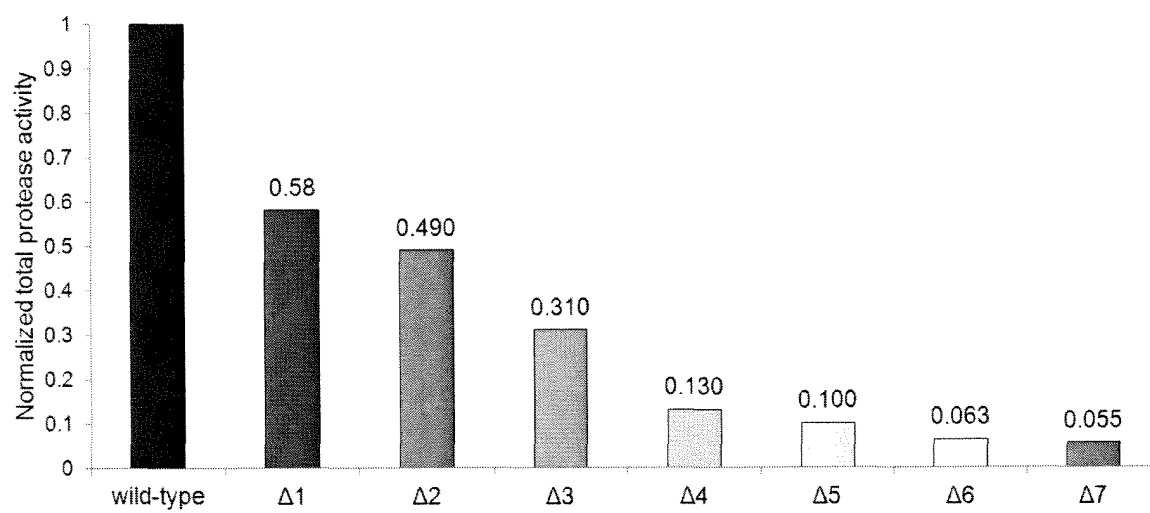


Figure 20

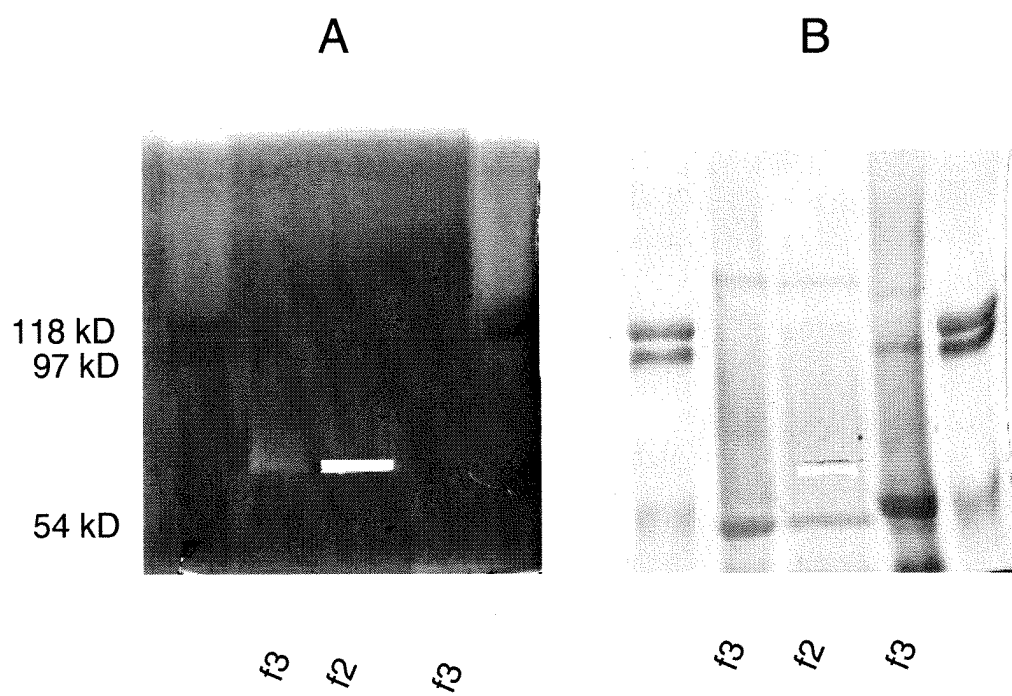


Figure 21

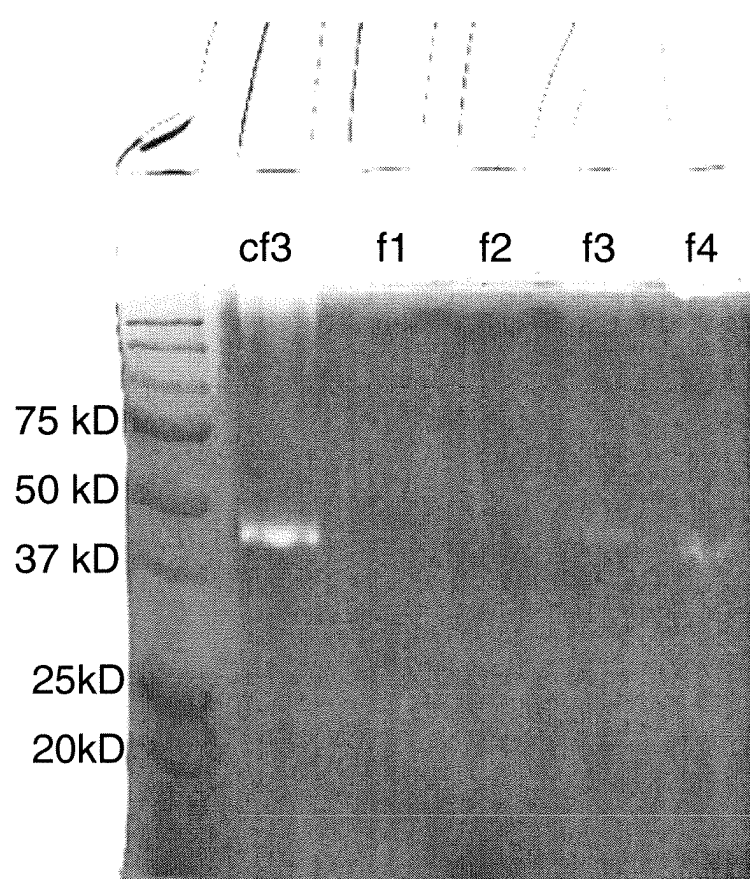


Figure 22

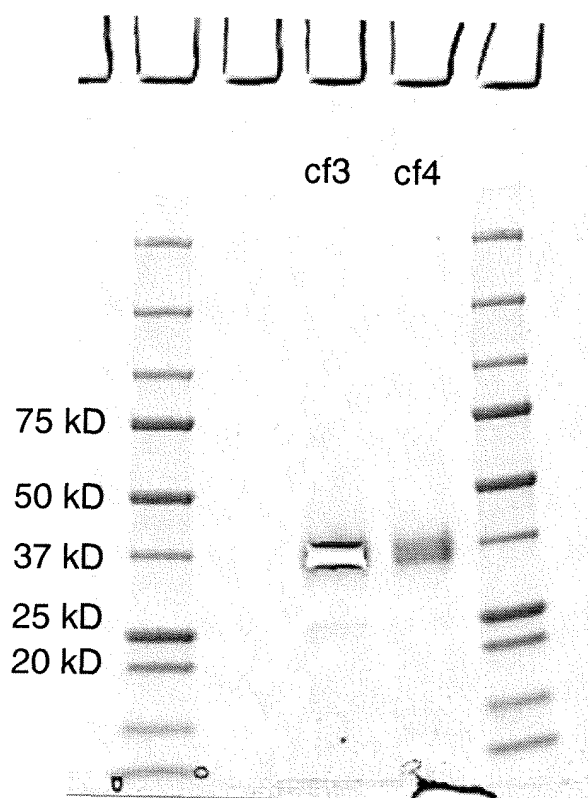


Figure 23

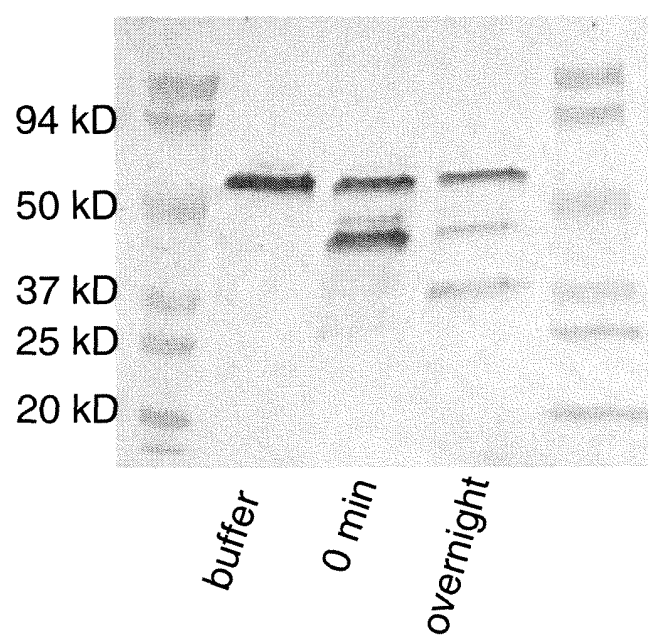


Figure 24

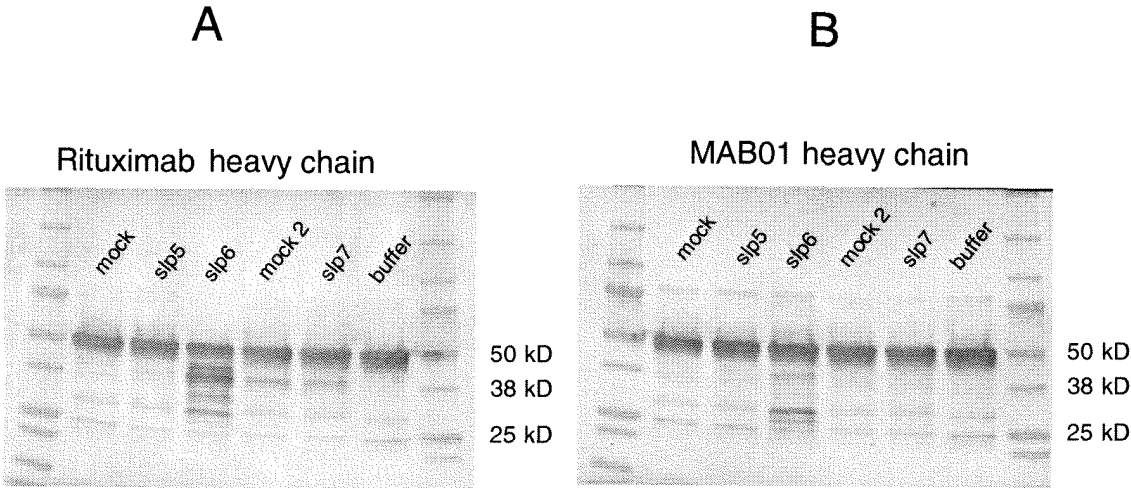


Figure 25

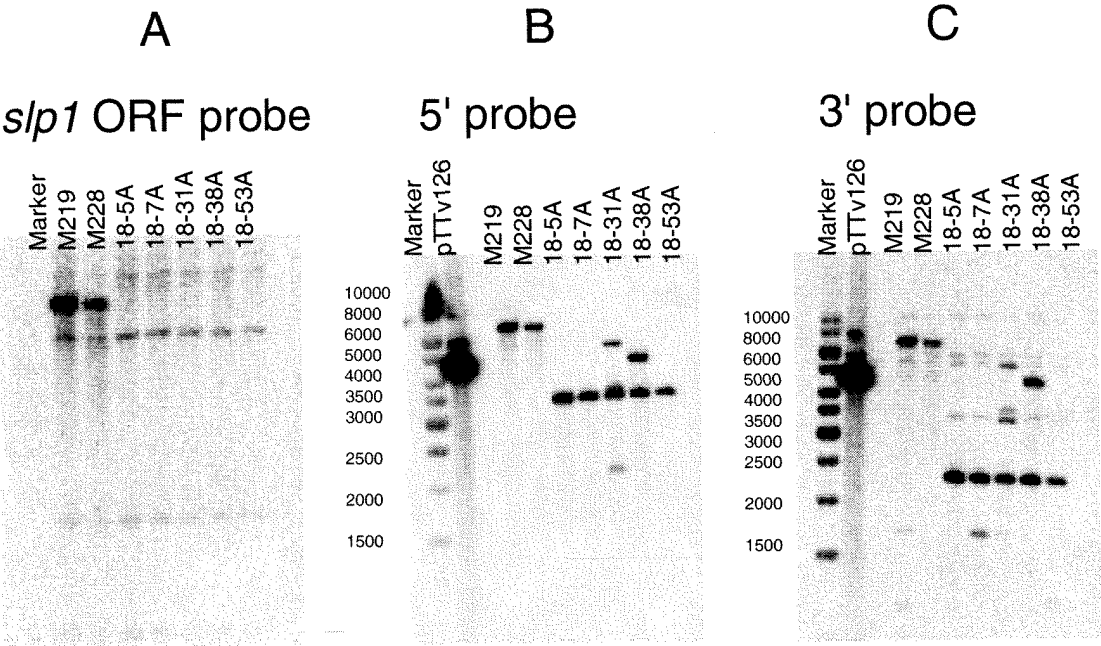


Figure 26

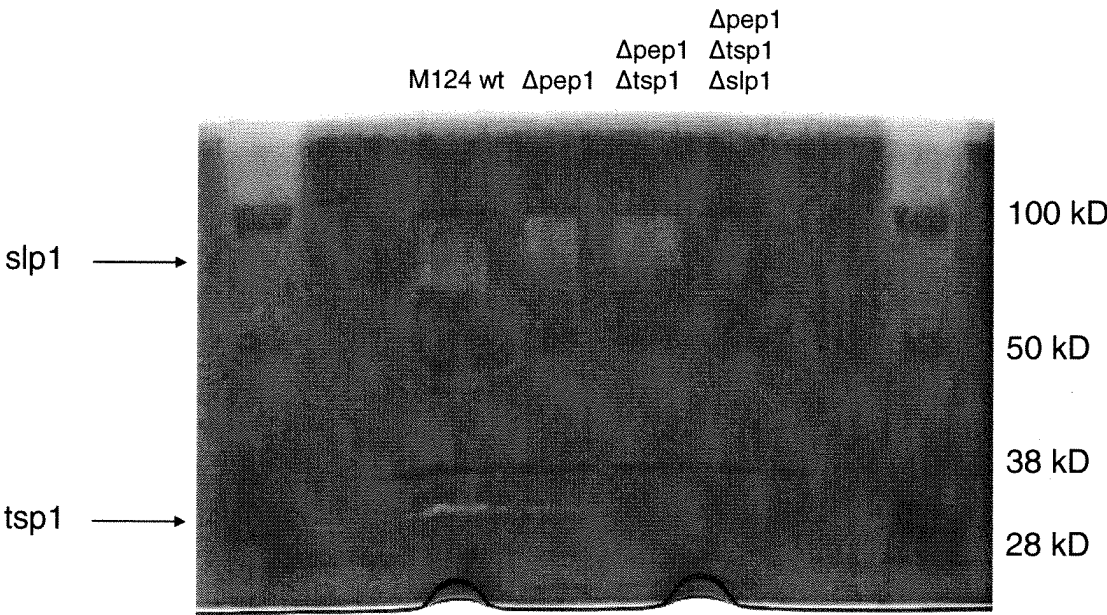


Figure 27

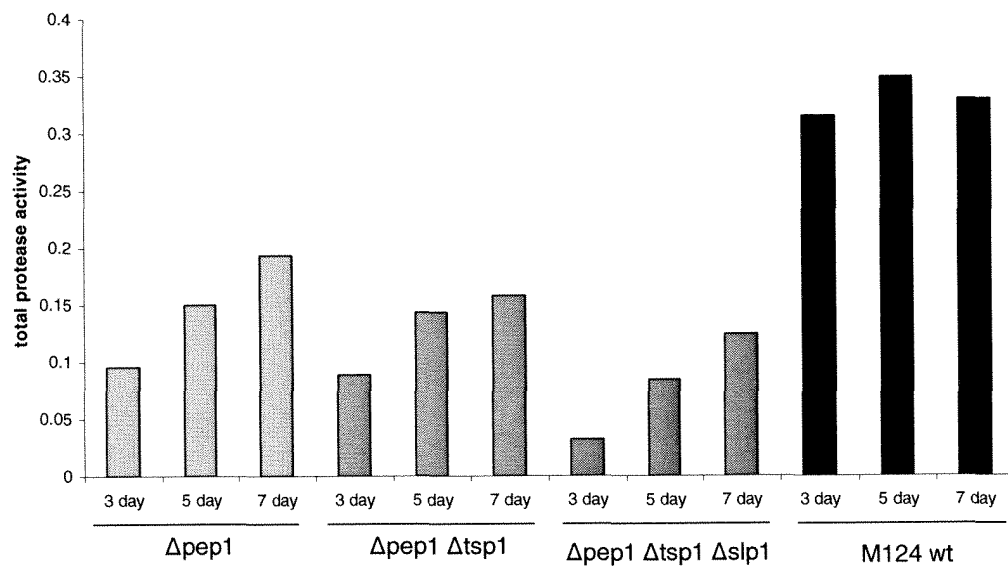


Figure 28

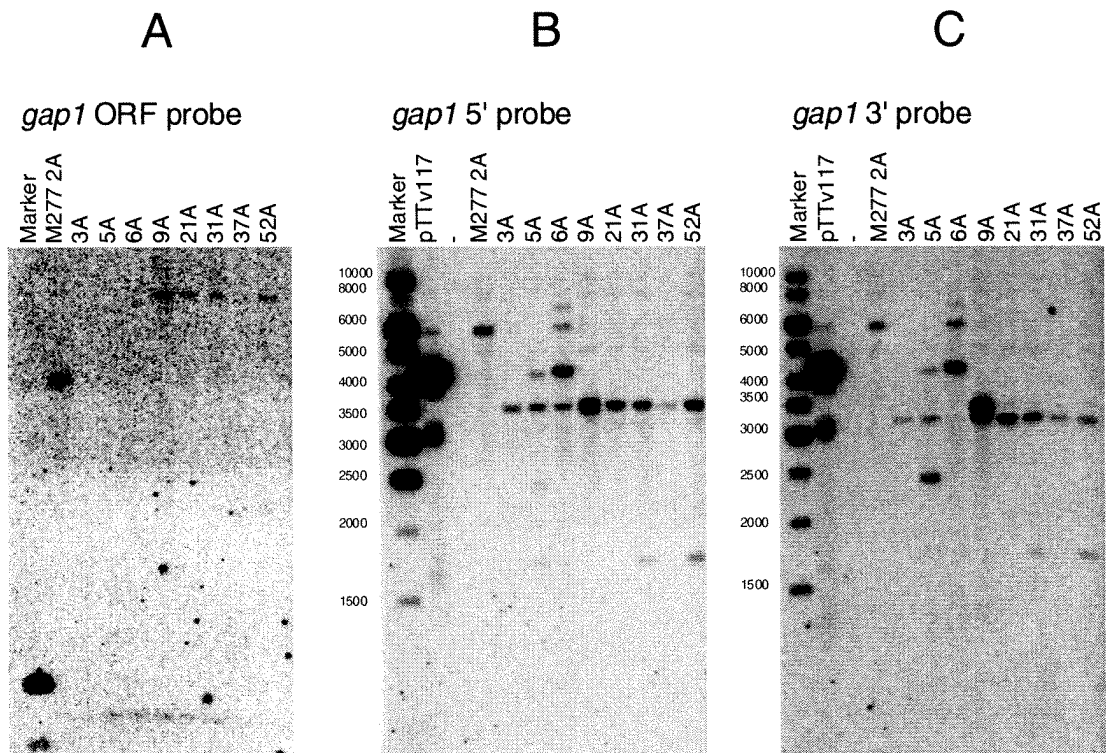


Figure 29

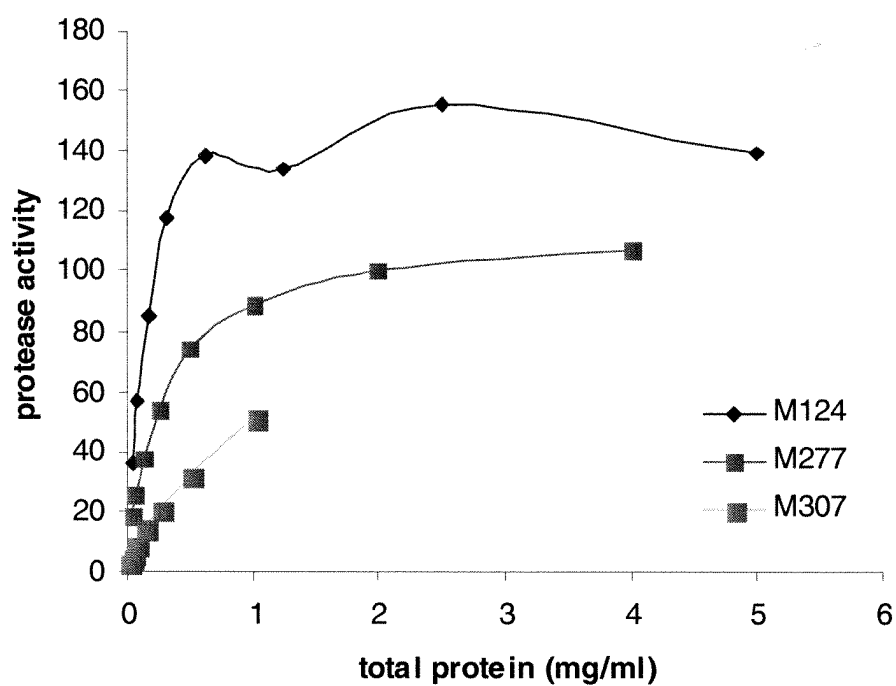


Figure 30

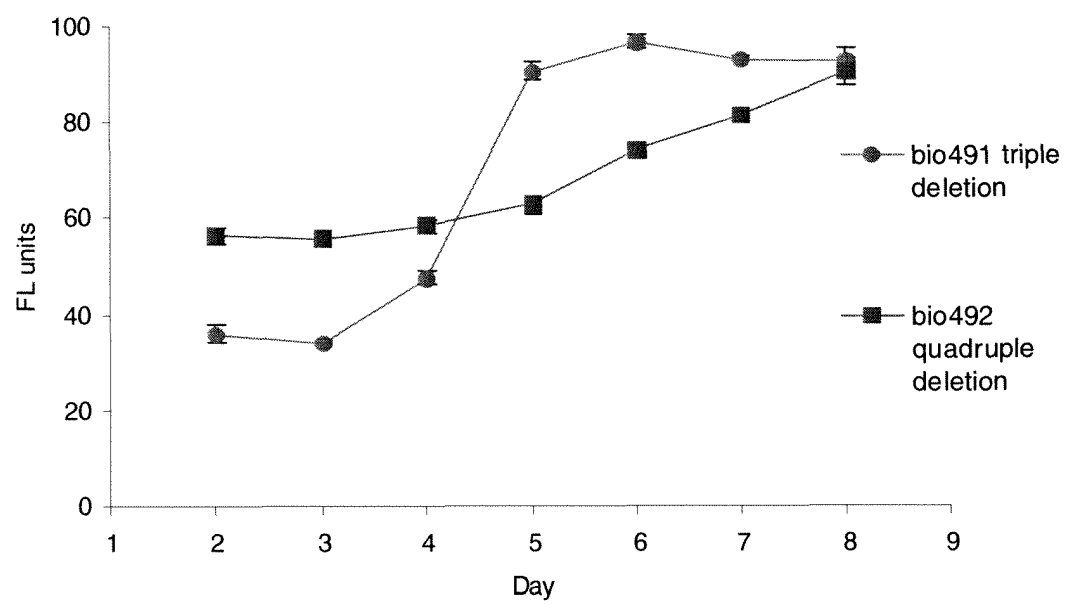
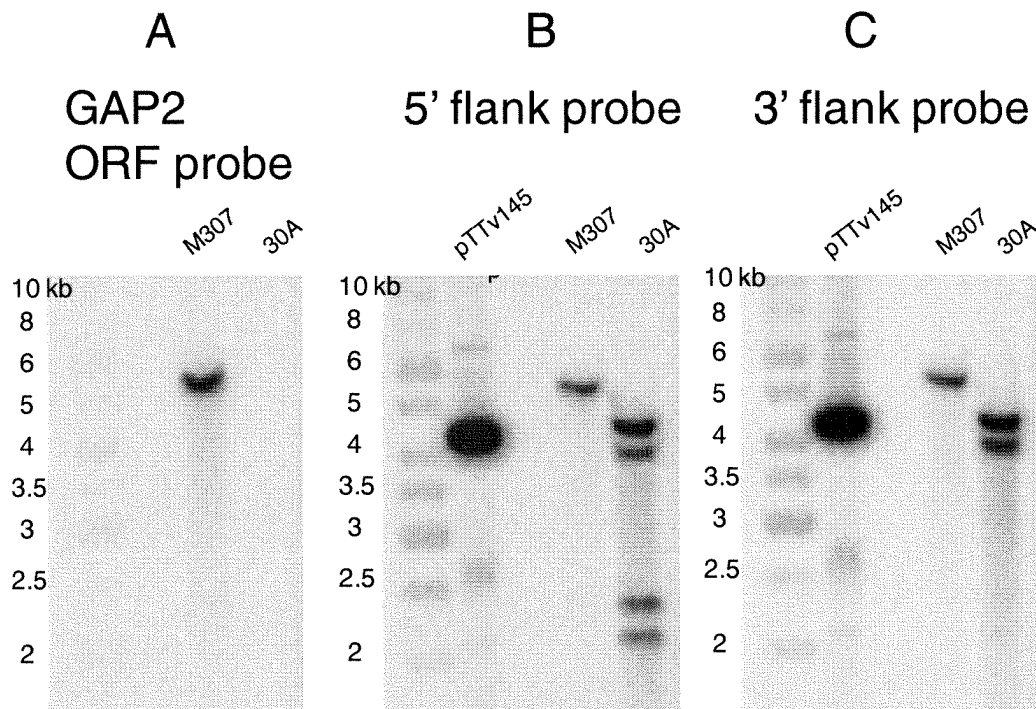


Figure 31

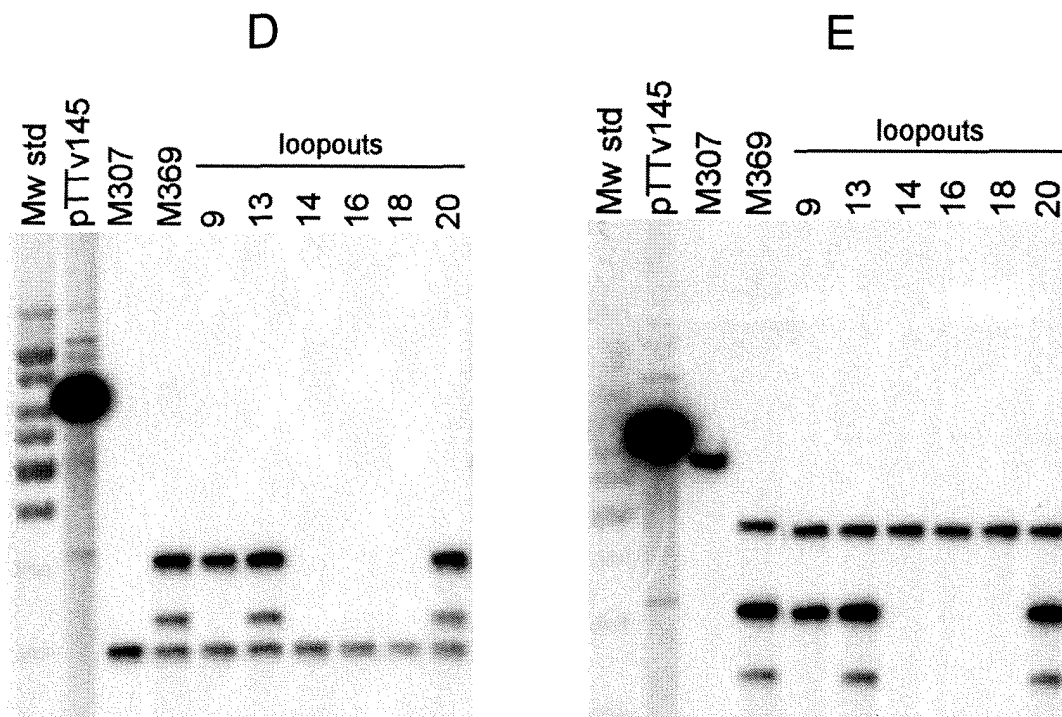


Generation of quintuple protease deletion strain M369, clone 7-30A = M369

Expected signals:

- A, gap2 ORF: 4.9 kb from parent (M307), no signals from transformant
- B, gap2 5'flank: 4.9 kb from parent, 2.3 kb from transformant, 2.3 kb from plasmid control pTTv145
- C, gap2 3'flank: 4.9 kb from parent, 3.8 kb from transformants, 3.8 kb from plasmid control pTTv145

Figure 32



Generation of pyr4⁻ from the quintuple protease deletion strain M369, resulting strain M381 (clone 14)

M307 = 4-fold del strain, M369 = 5-fold del strain

Expected signals:

- D) gap2 5'flank: 1.5 kb from all strains, 4.1 kb from plasmid control pTTv145

- E) gap2 3'flank: 3.6 kb from M307, 2.7 kb from M369 + loopout clones, 3.8 kb from plasmid control pTTv145

Figure 32 Continued

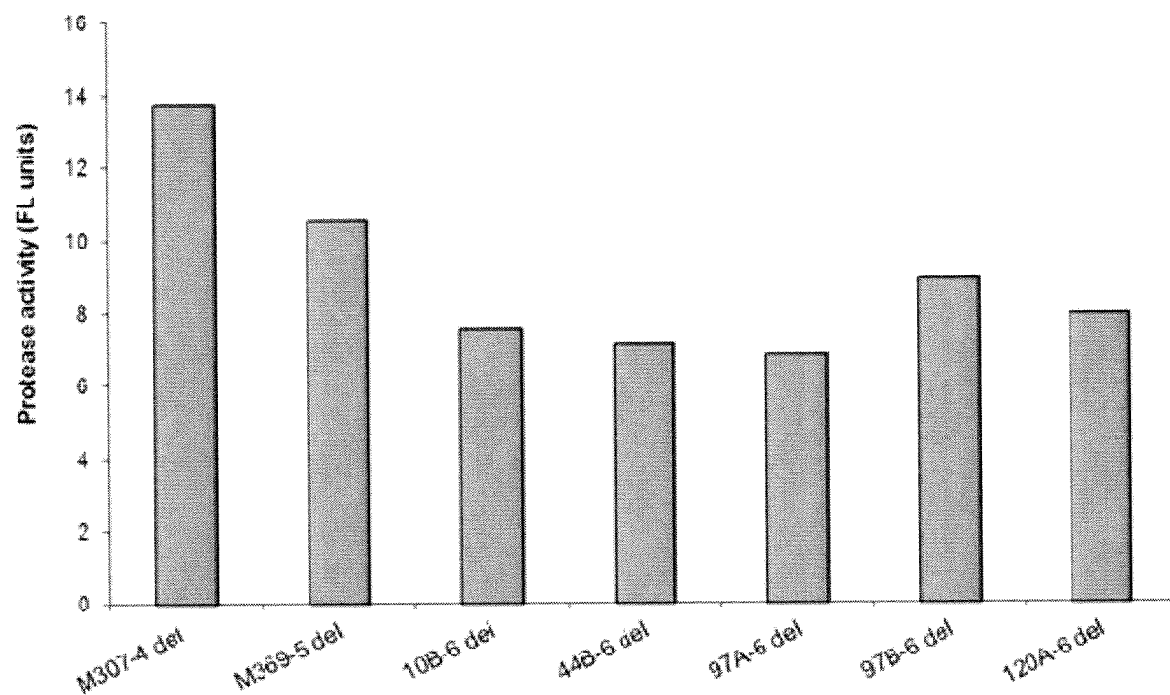
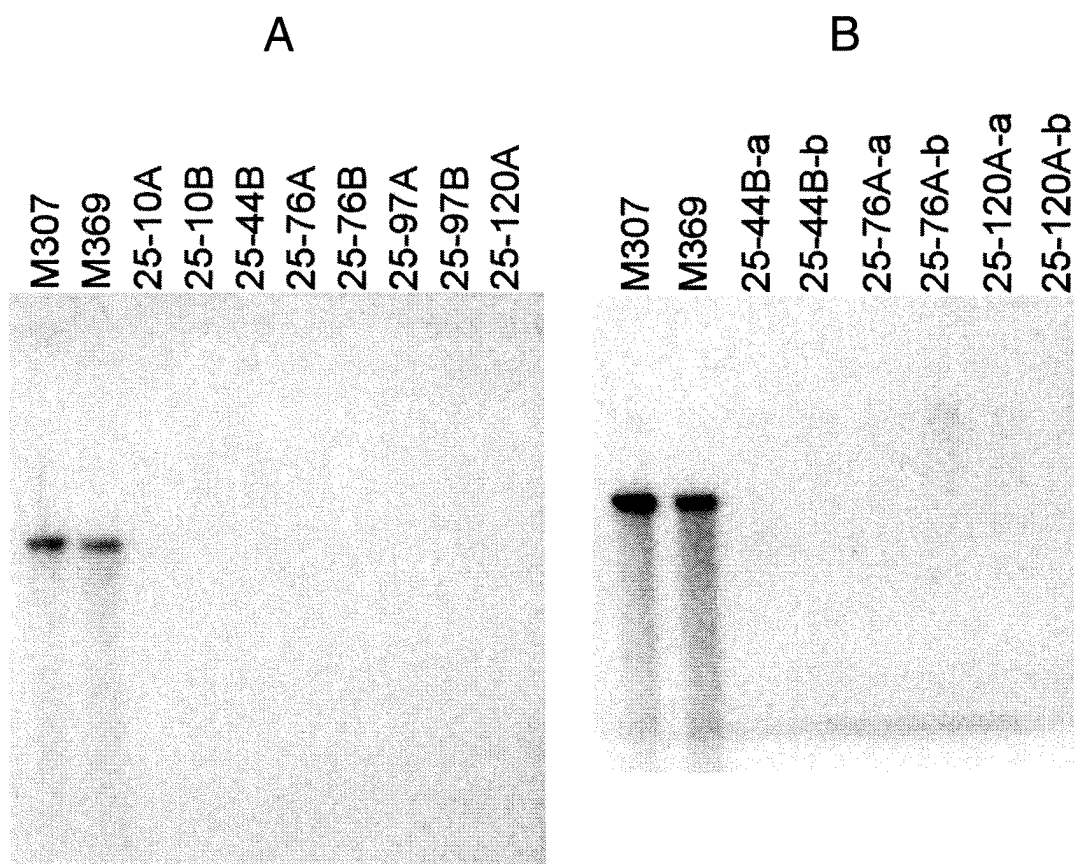


Figure 33



Generation of 6-fold protease deletion strains M396 and M400

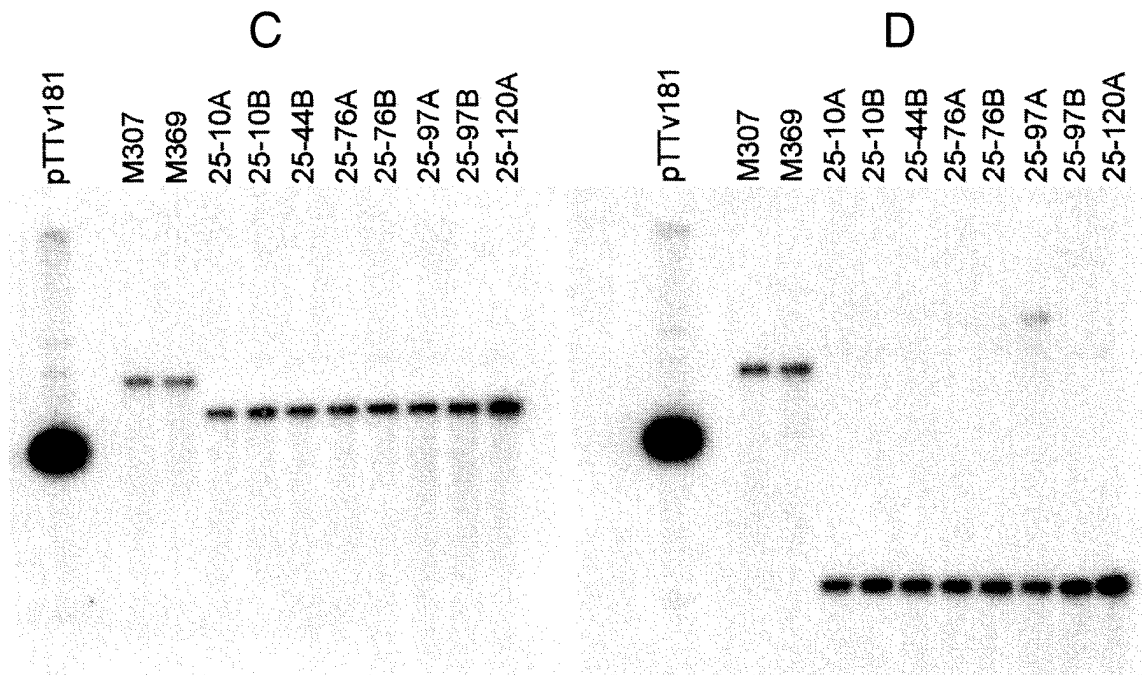
M396 = 25-120A

M400 = 25-120A-a, i.e. repurified clone from M396

Expected signals:

- Aa & Ab) pep4 ORF: 6.3 kb from M307 and M369, no signals from transformants

Figure 34

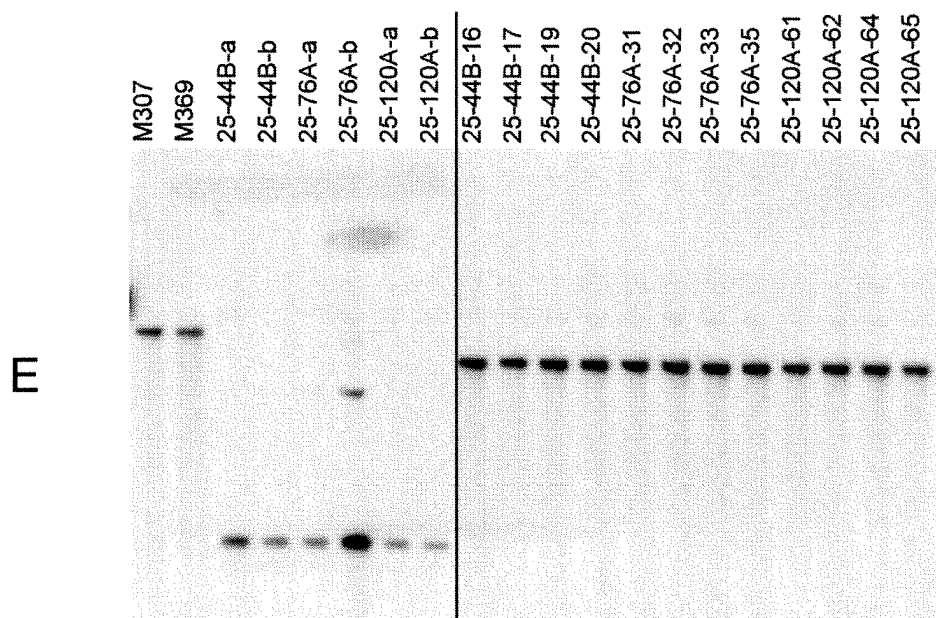


Generation of 6-fold protease deletion strains M396 and M400
M396 = 25-120A

Expected signals:

- B) pep4 5' flank: 6.3 kb from M307 and M369, 4.8 kb from transformants, 4.0 kb from pTTv181
- C) pep4 3' flank: 6.3 kb from M307 and M369, 2.1 kb from transformants, 4.0 kb from pTTv181

Figure 34 Continued



Generation of *pyr4*- from 6-fold protease deletion strains M396
 M396 = 25-120A (> repurified clone 25-120A-a = M400)

Expected signals:

- D) *pep4* 3' flank: 6.3 kb from M307 and M369, 2.1 kb from repurified transformants, 4.9 kb from the loopout clones

Figure 34 Continued

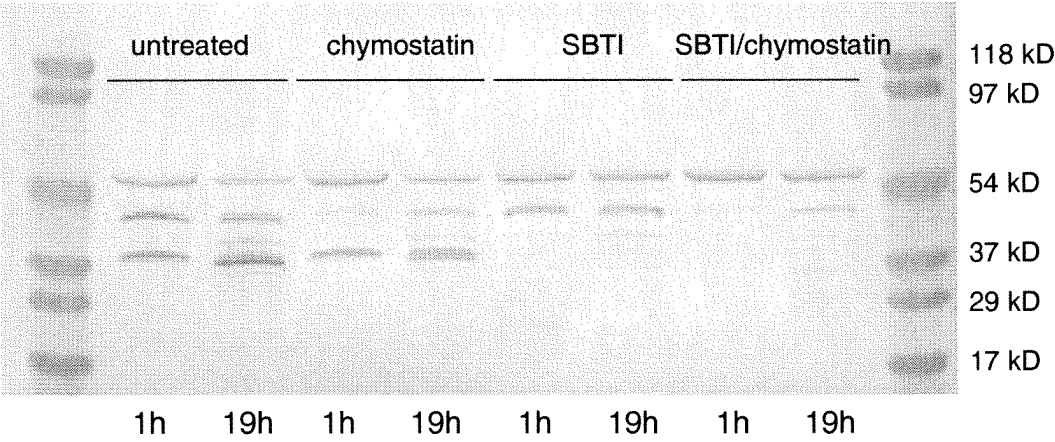


Figure 35

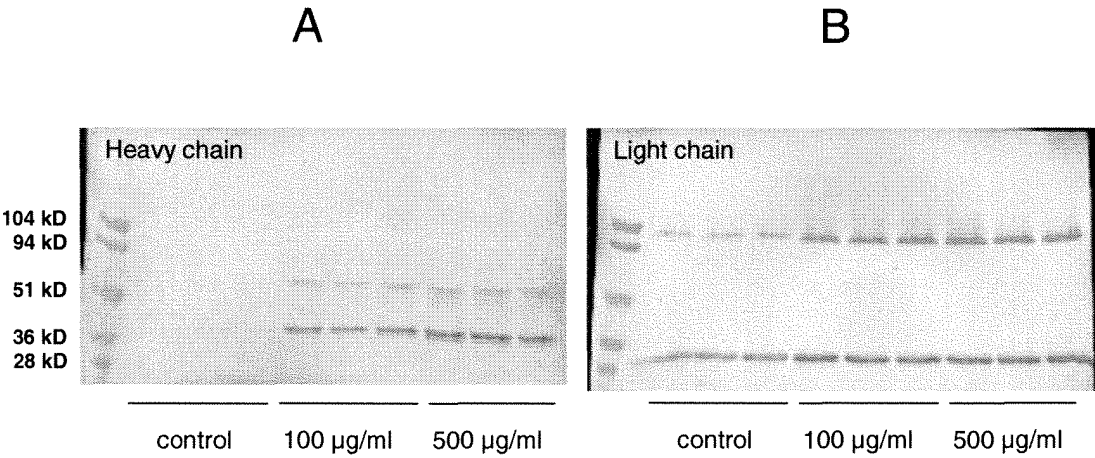


Figure 36

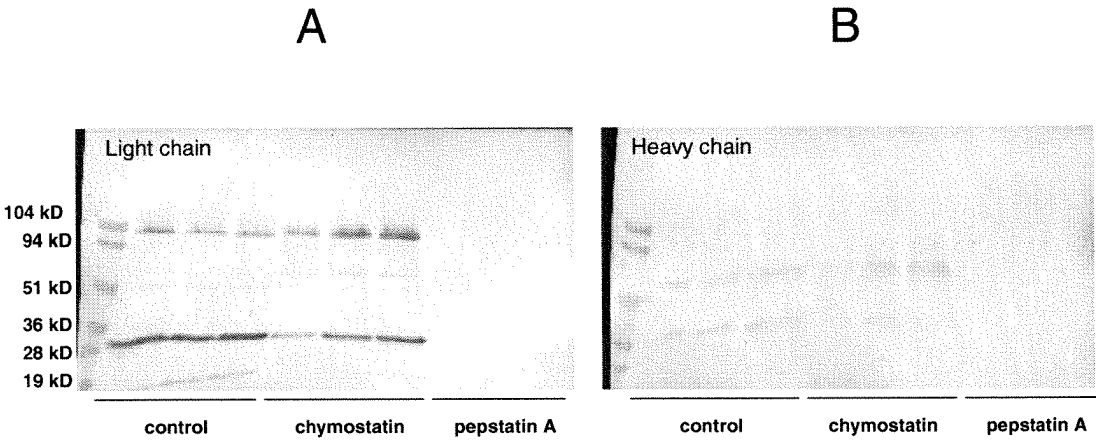


Figure 37

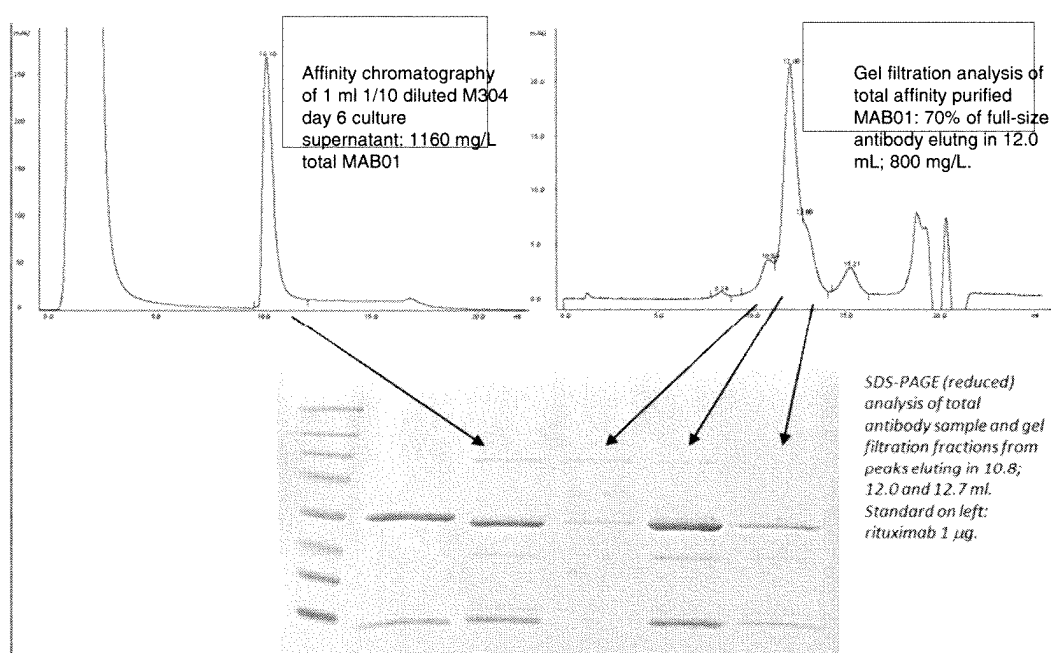
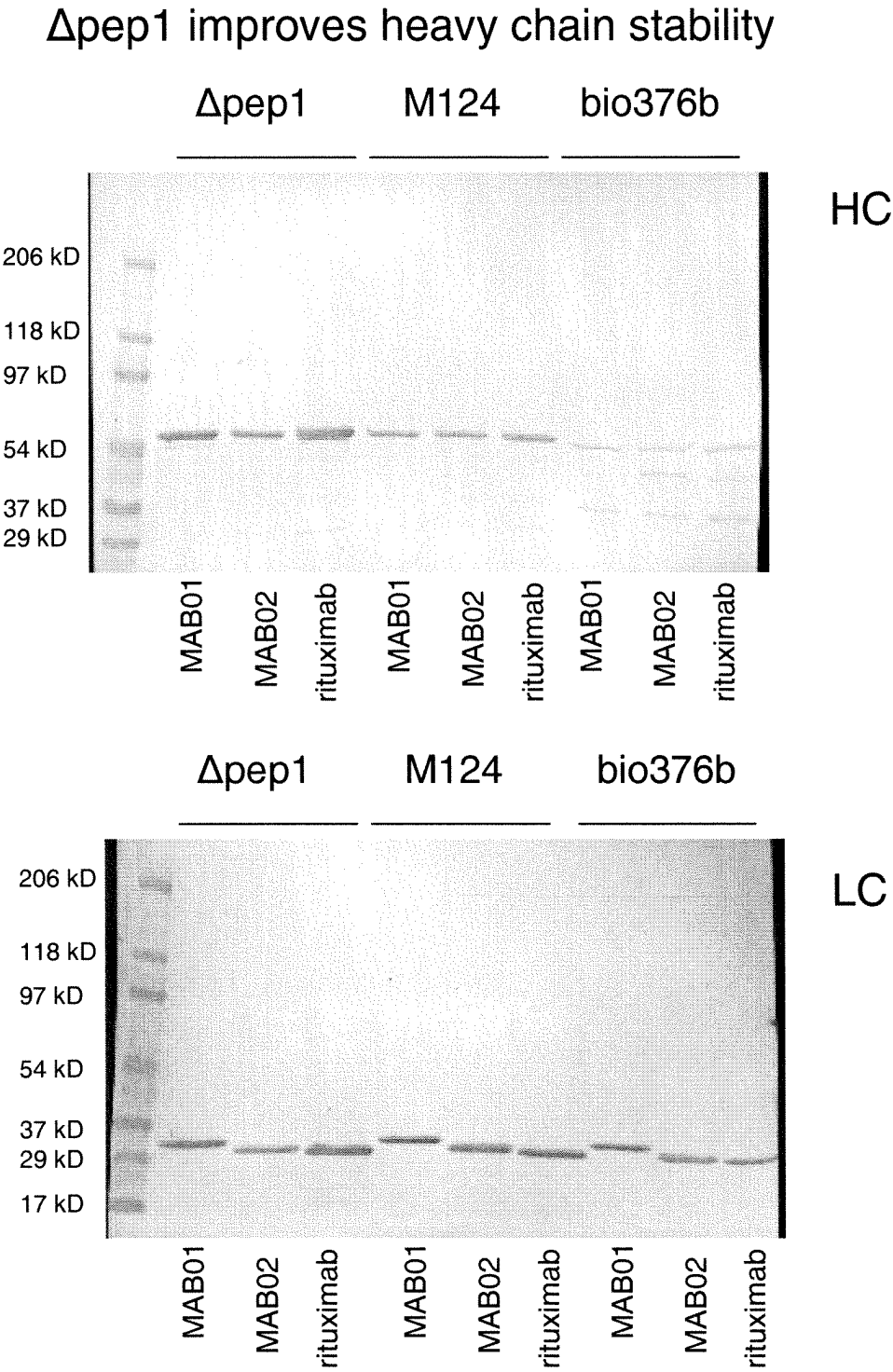


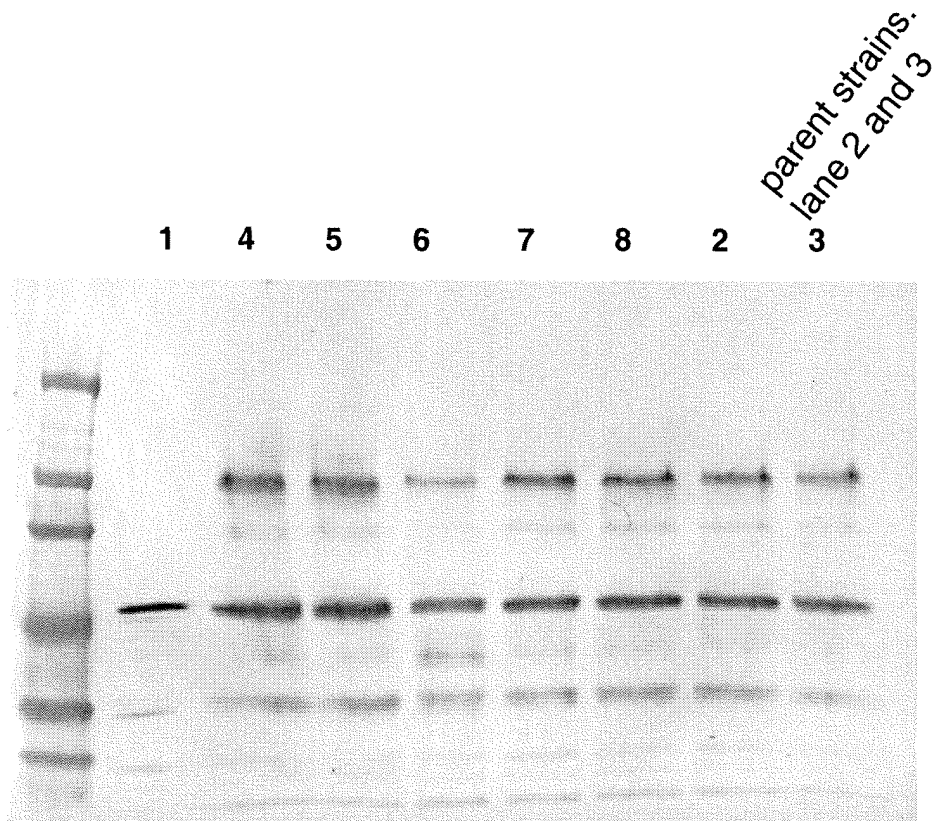
Figure 38



2 mg/ml large shake flask supernatant (Δ pep1 and M124); 2 mg/ml fed batch fermentation supernatant; 18 hour incubation at 37°C at pH 5.5

Figure 39

Δ tsp1 improves the rituximab heavy chain production levels on day 5.

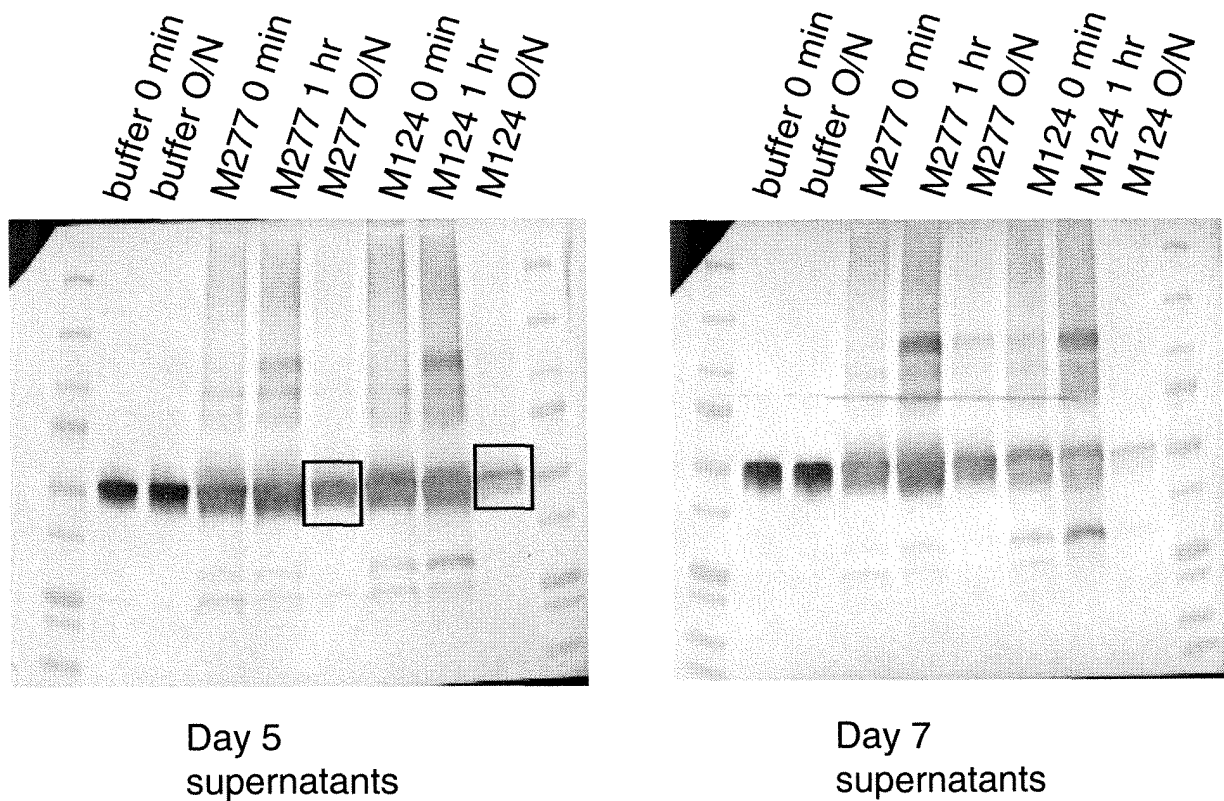


Lanes: 1) 50 ng Rx control, 2 & 3) parental strain Rx 25A Δ tsp1 4) 12-2A, 5) 12-16A, 6) 12-19A, 7) 12-20A, 8) 12-34A

transformants in lane 4 and 5, clearly show more heavy chain than in lane 2 and 3.

Figure 40

MAB01 heavy chain degradation is reduced in triple deletion strain supernatant M277



2.5-fold more heavy chain on day 5

4-fold more heavy chain on day 7

Supernatants diluted to 6 mg/ml, spiked with MAB01 0.05 $\mu\text{g}/\mu\text{l}$, and incubated at 37°C. M277 is $\Delta\text{pep1 } \Delta\text{tsp1 } \Delta\text{slp1}$; M124 is full protease containing control strain.

Figure 41

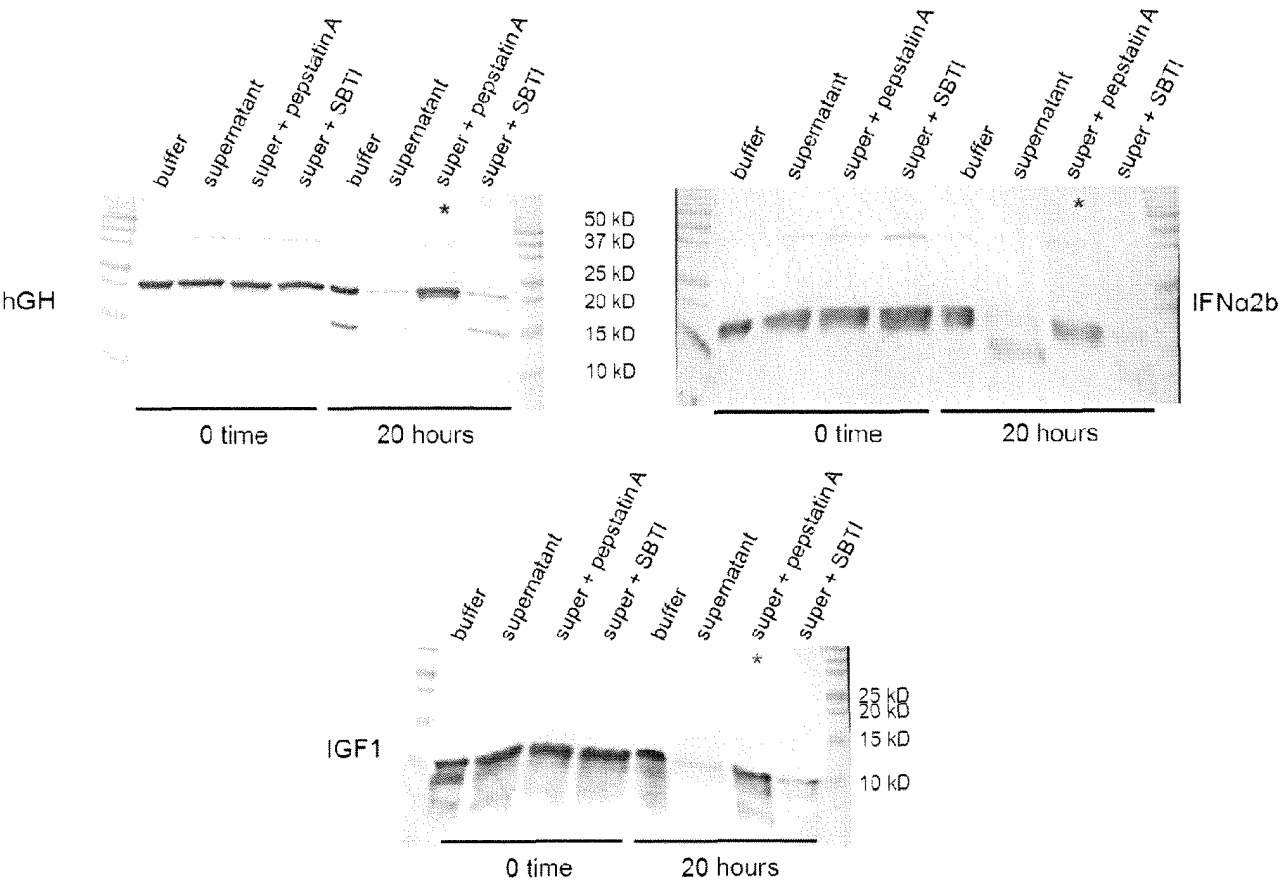


Figure 42

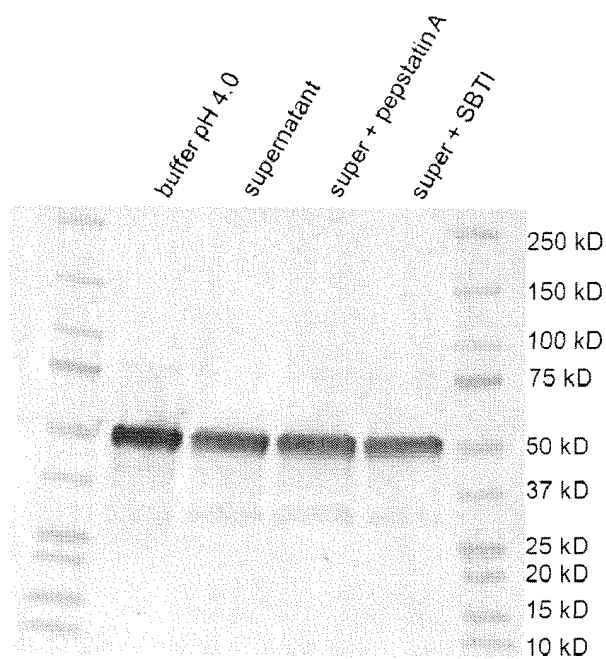


Figure 43

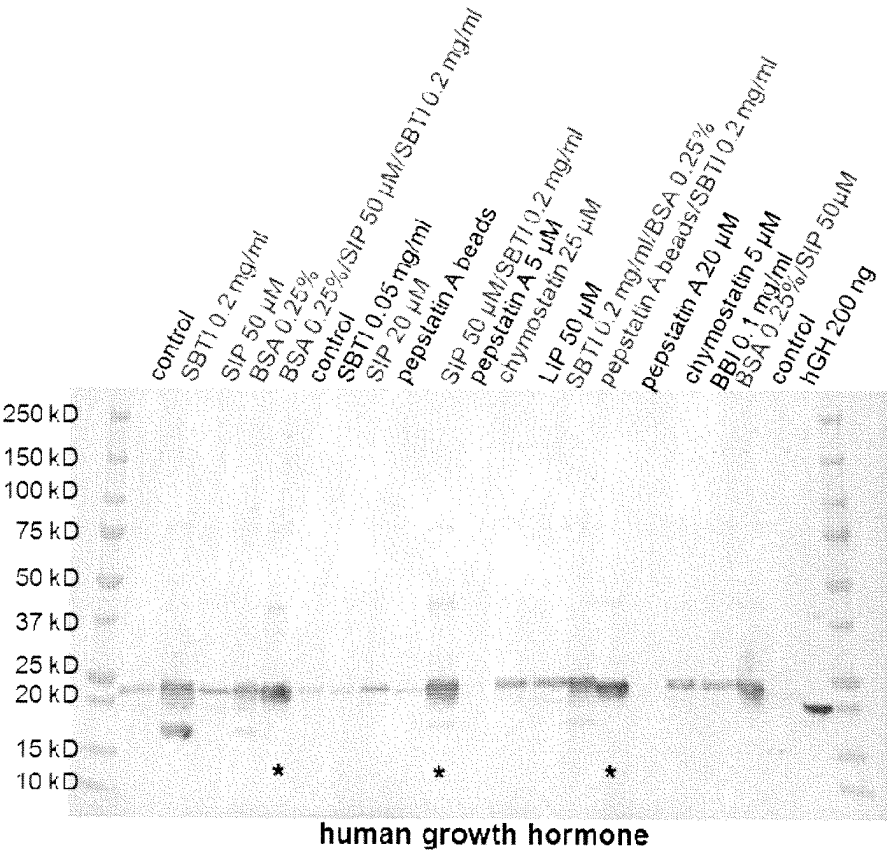


Figure 44

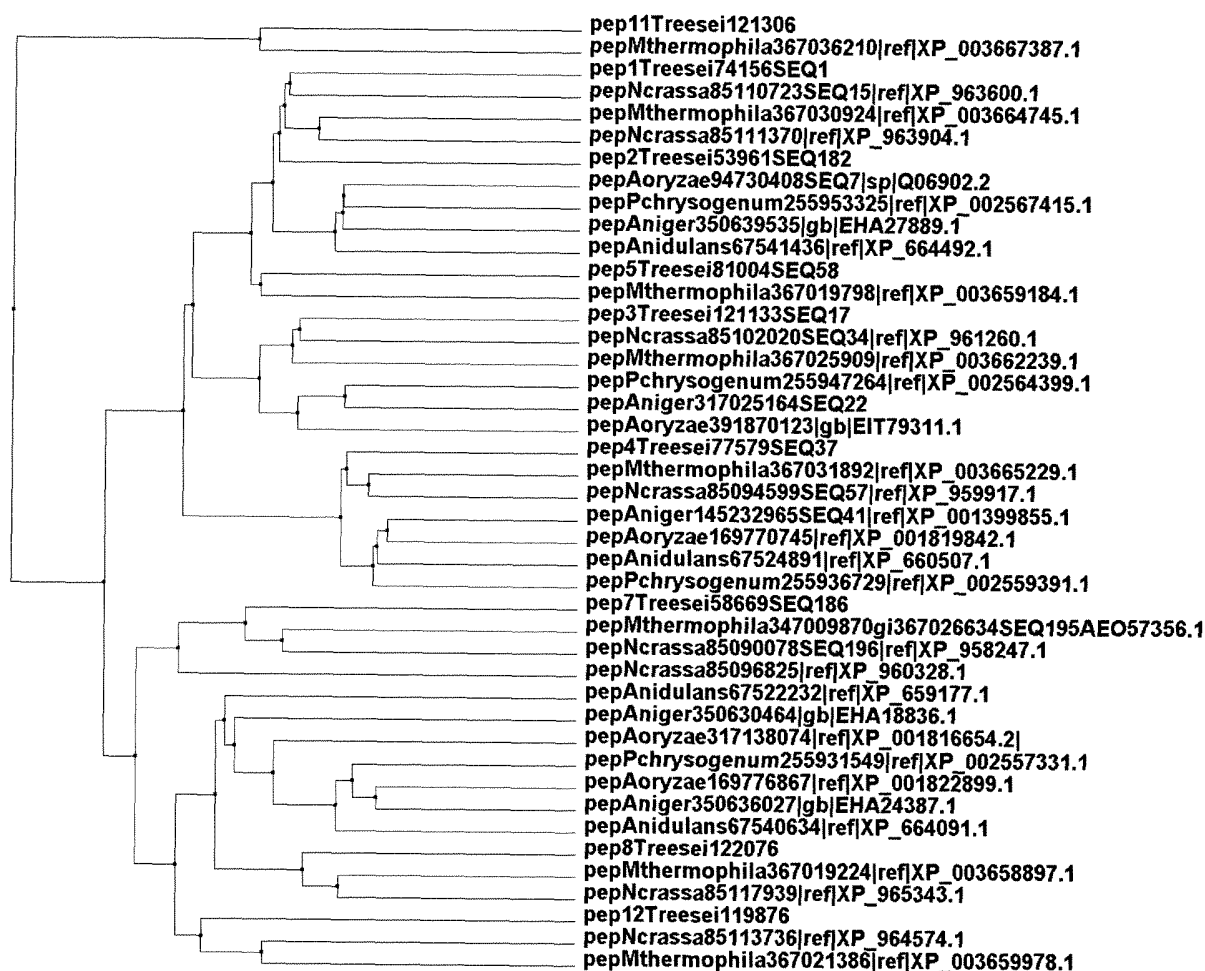


Figure 45

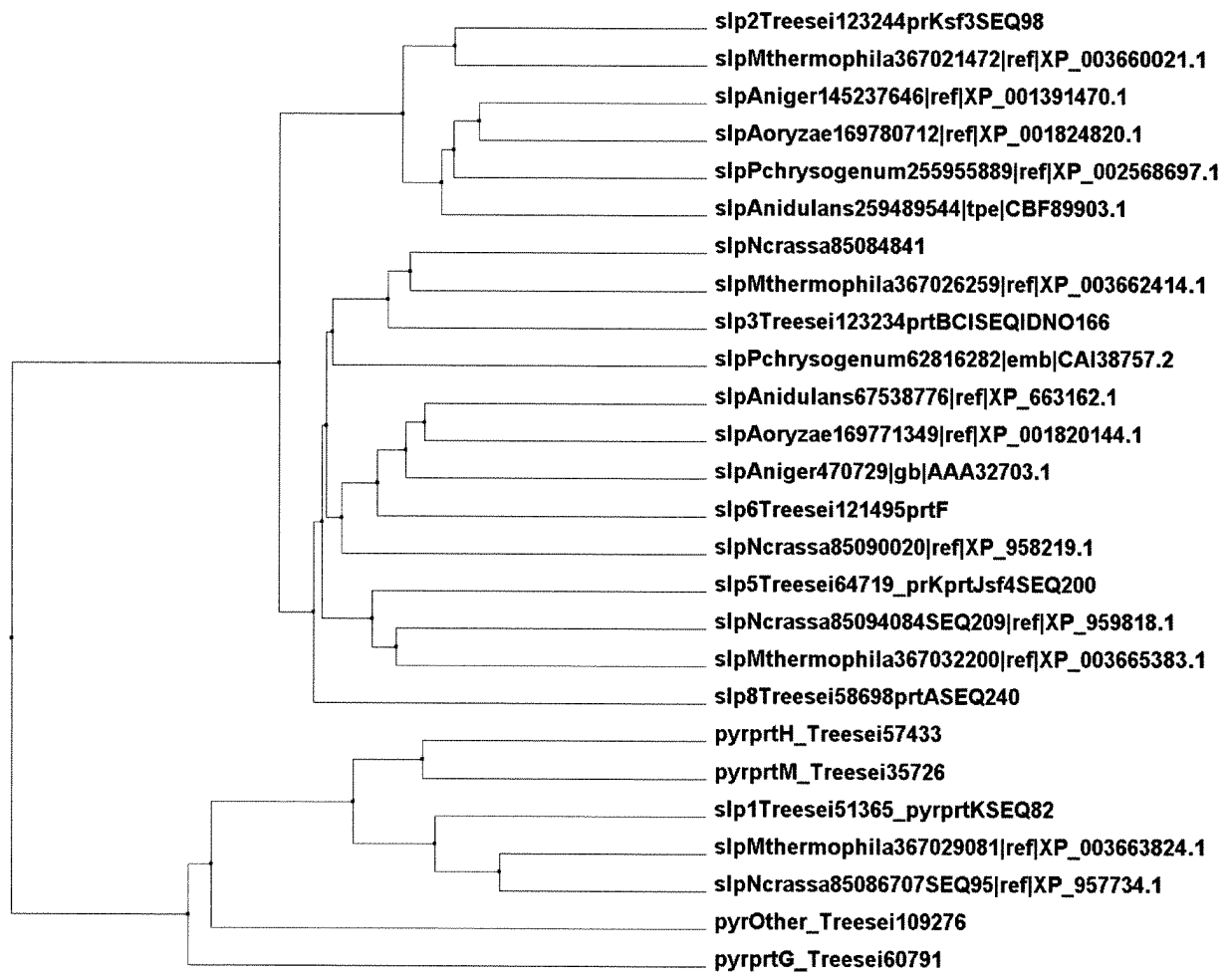
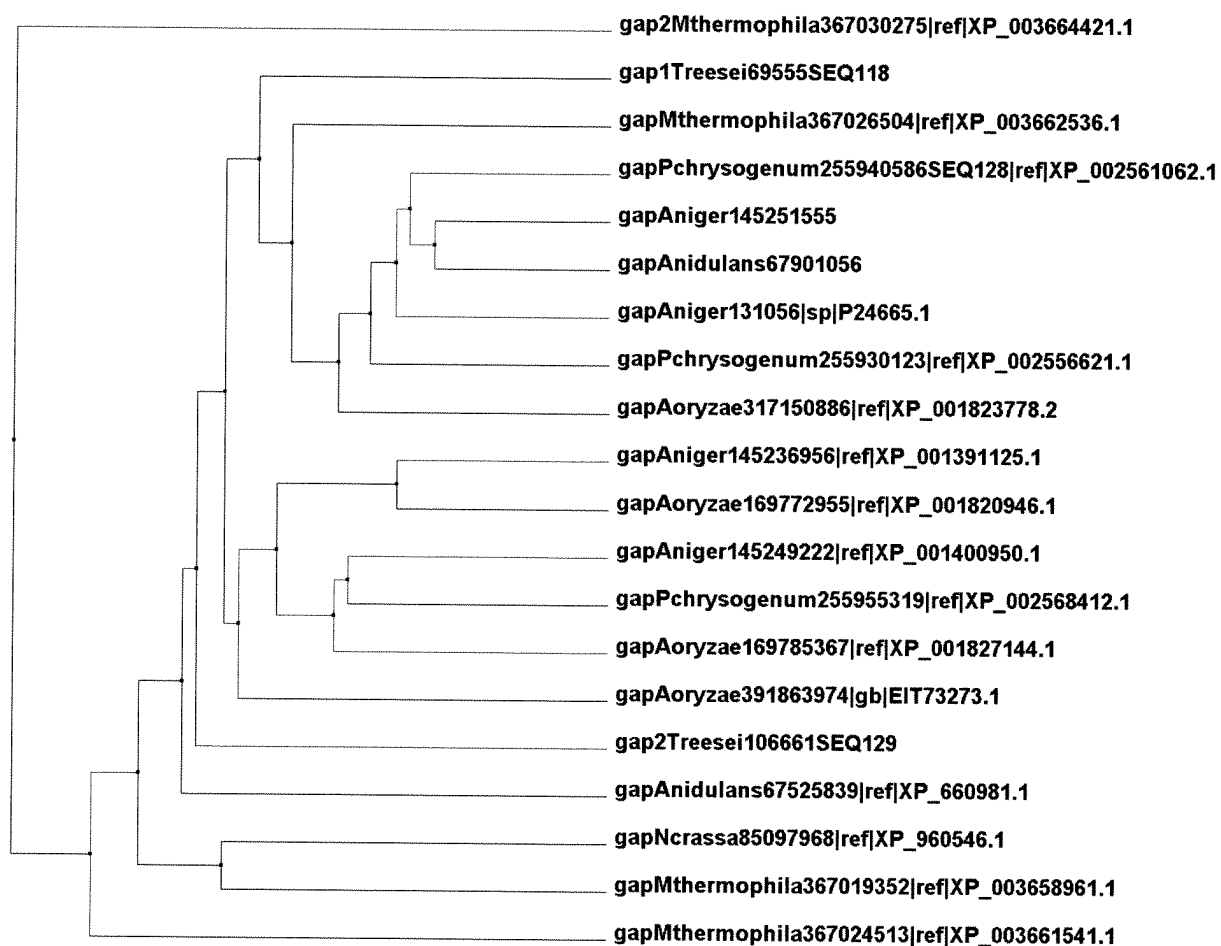


Figure 46

**Figure 47**

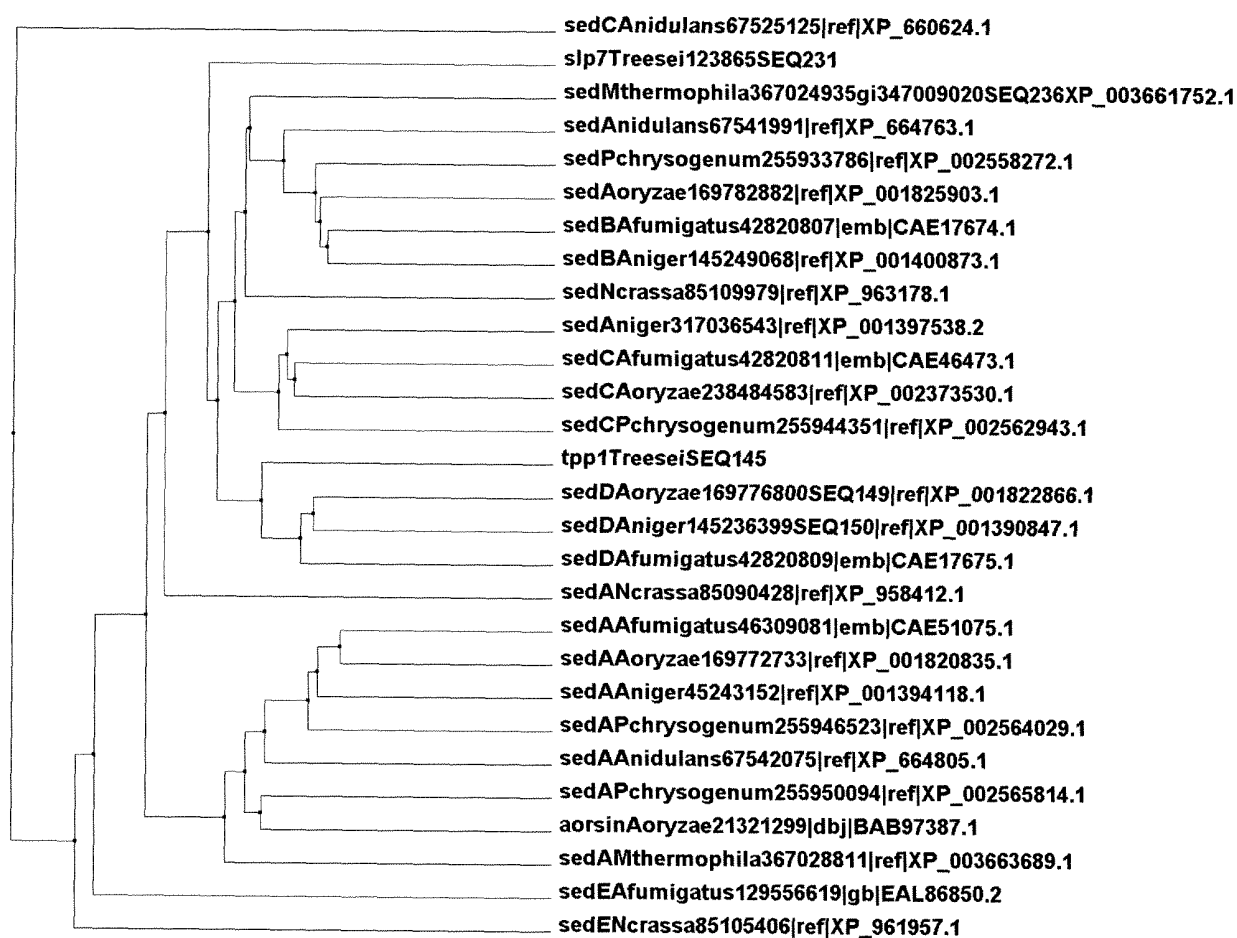
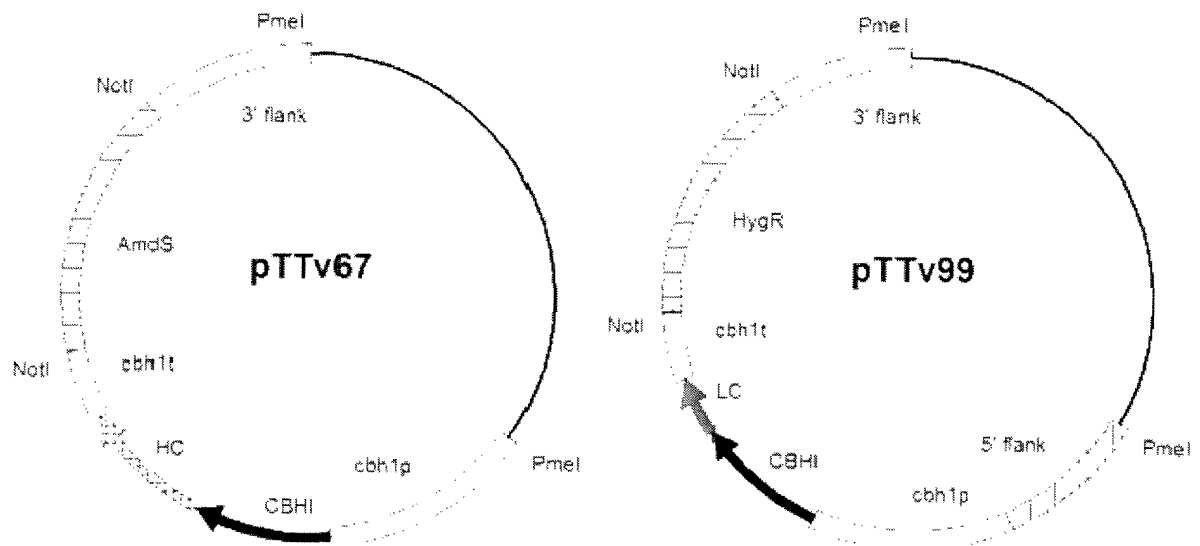
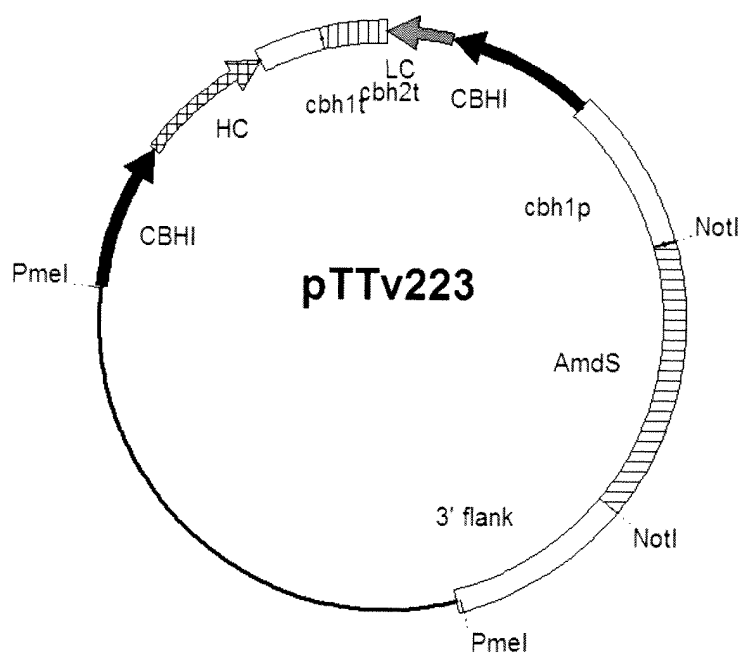
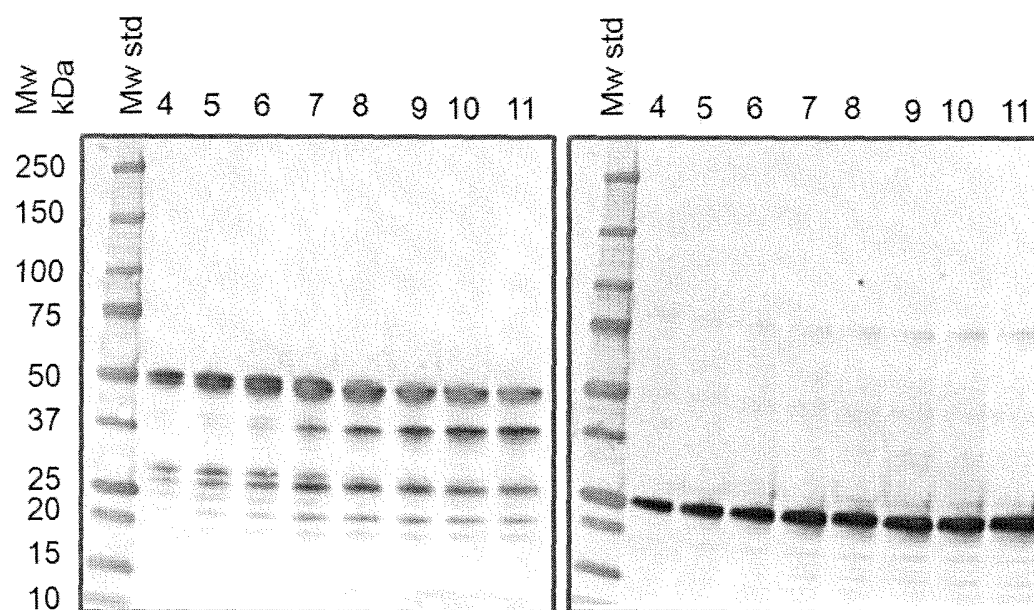
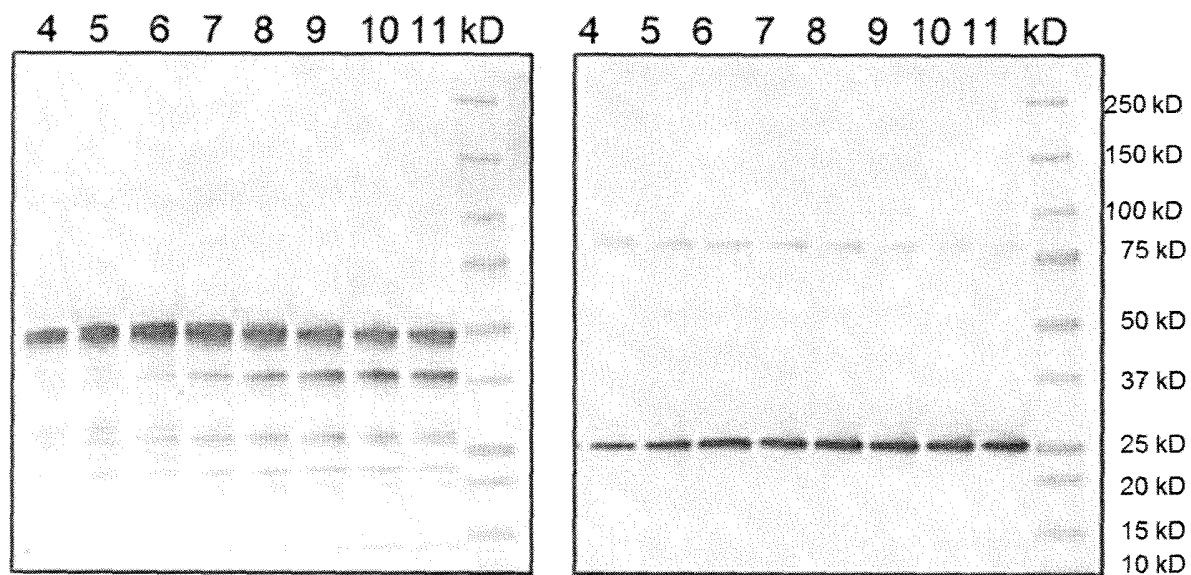


Figure 48

**Figure 49A****Figure 49B**

**Figure 50A****Figure 50B**

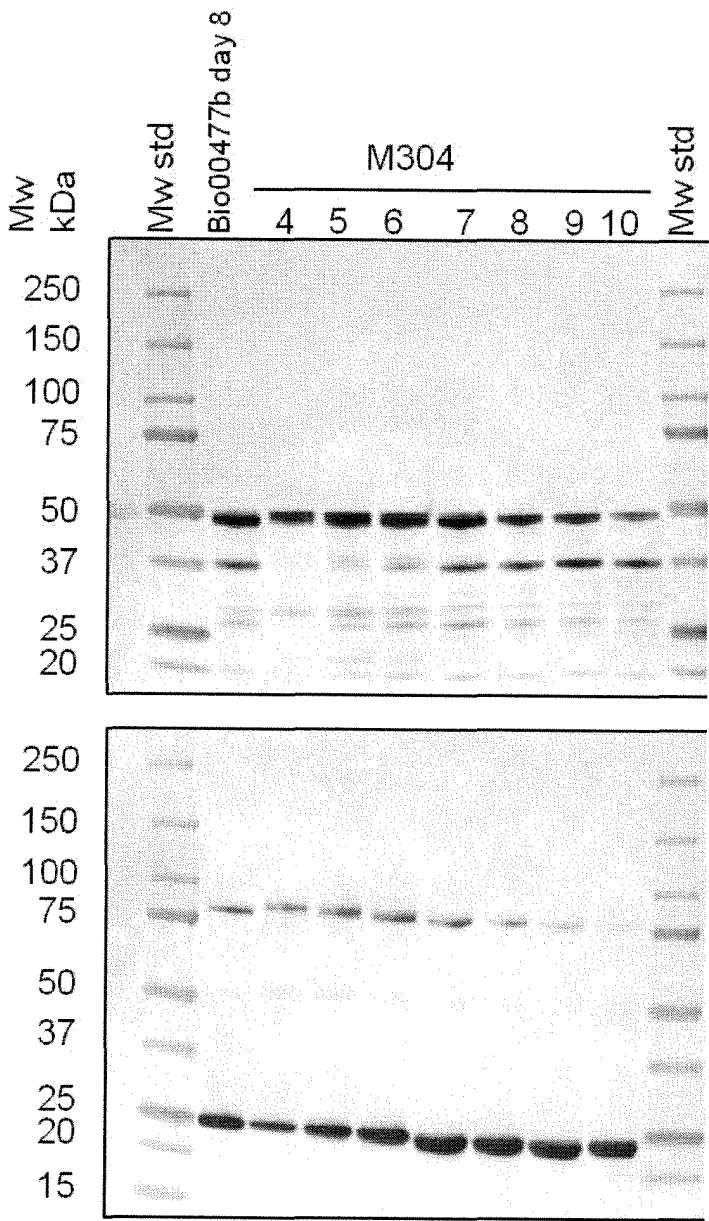
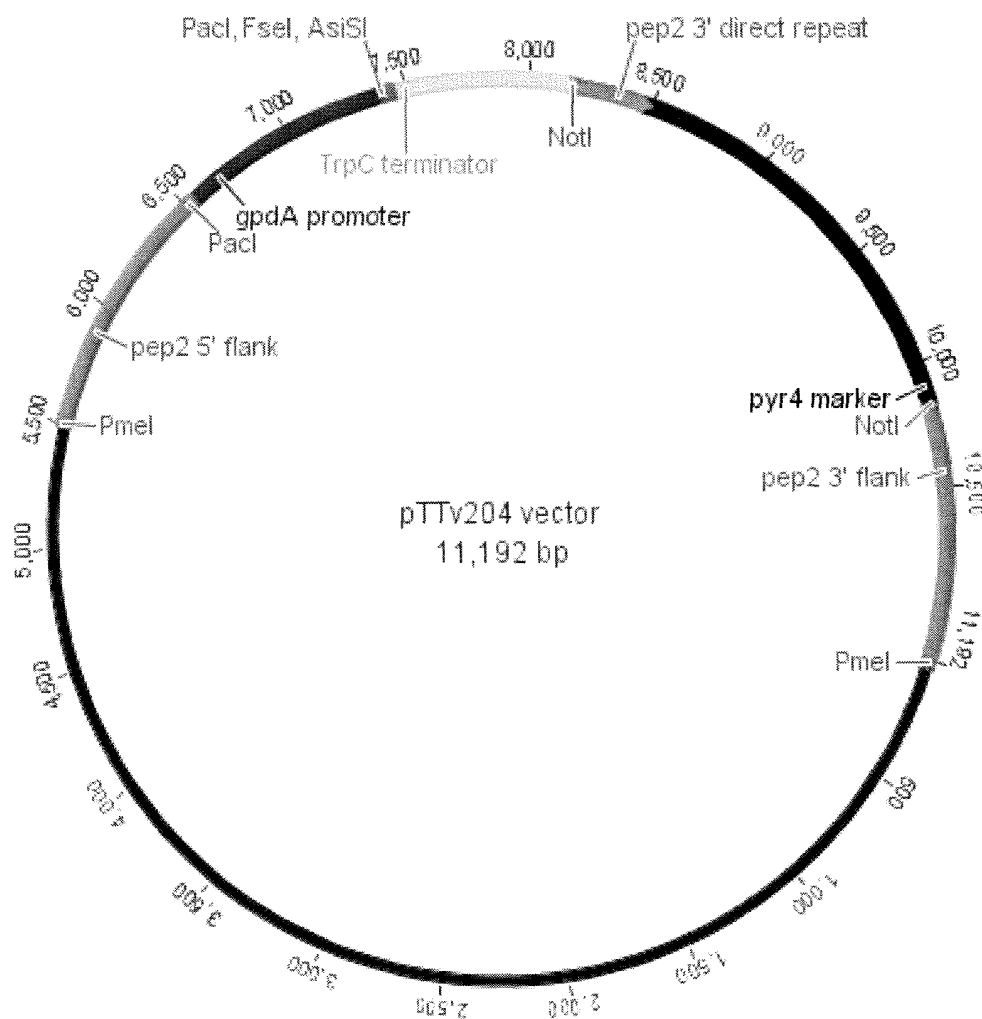


Figure 51

**Figure 52**

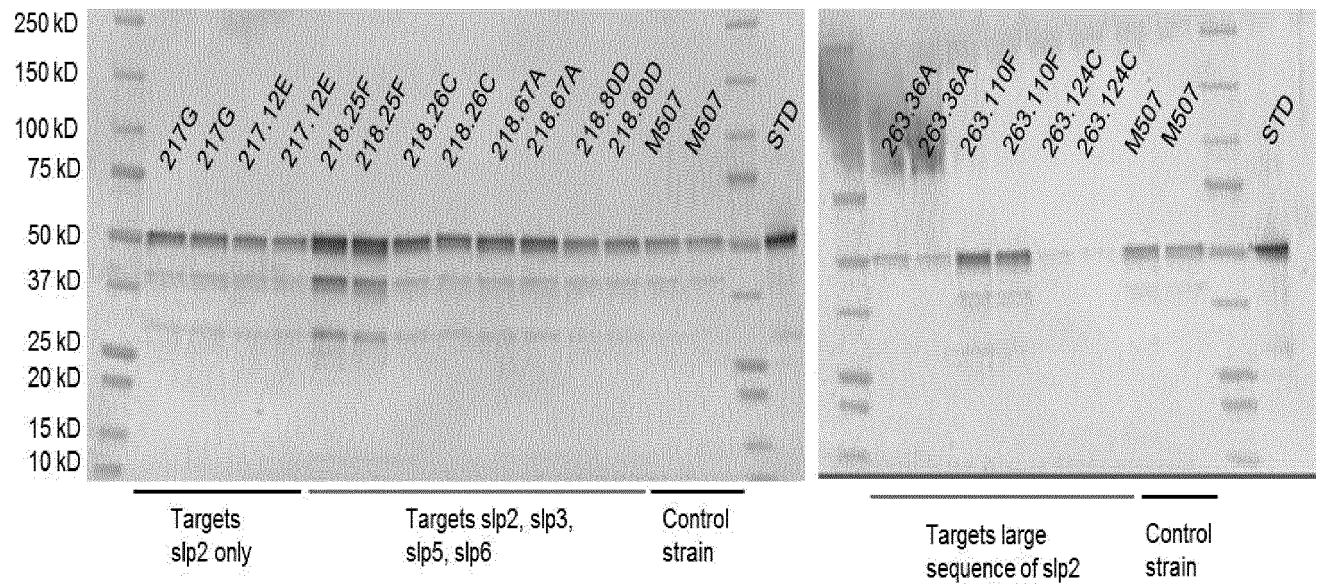
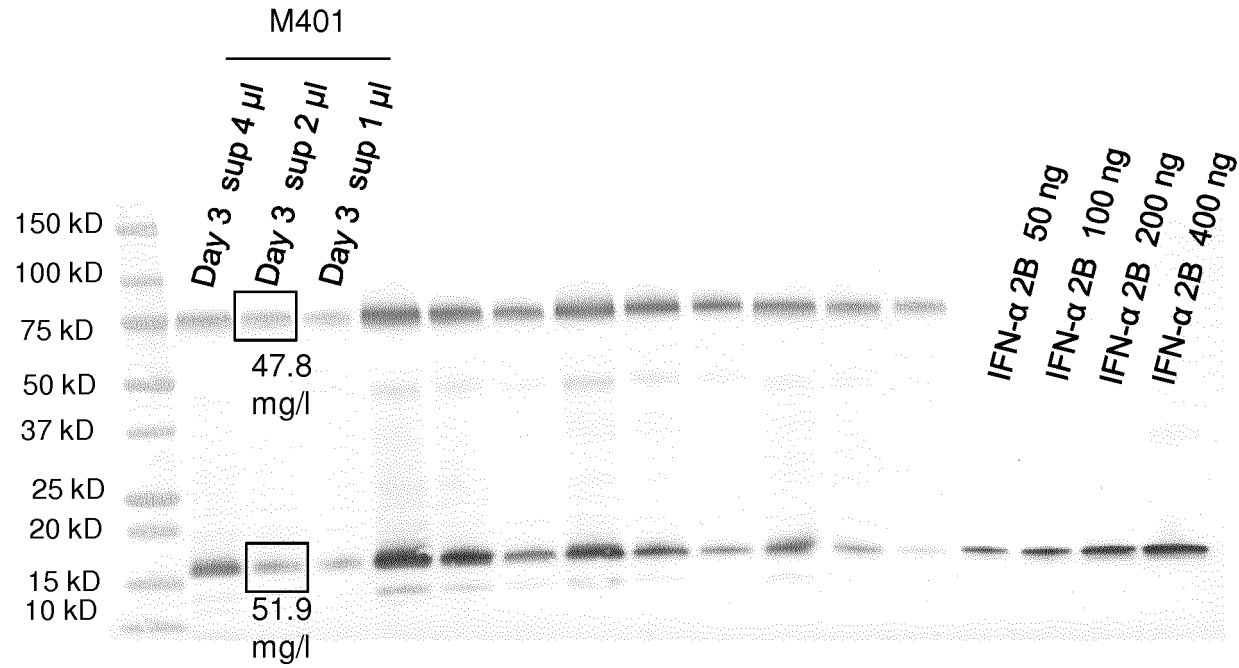


Figure 53.

A.



B.

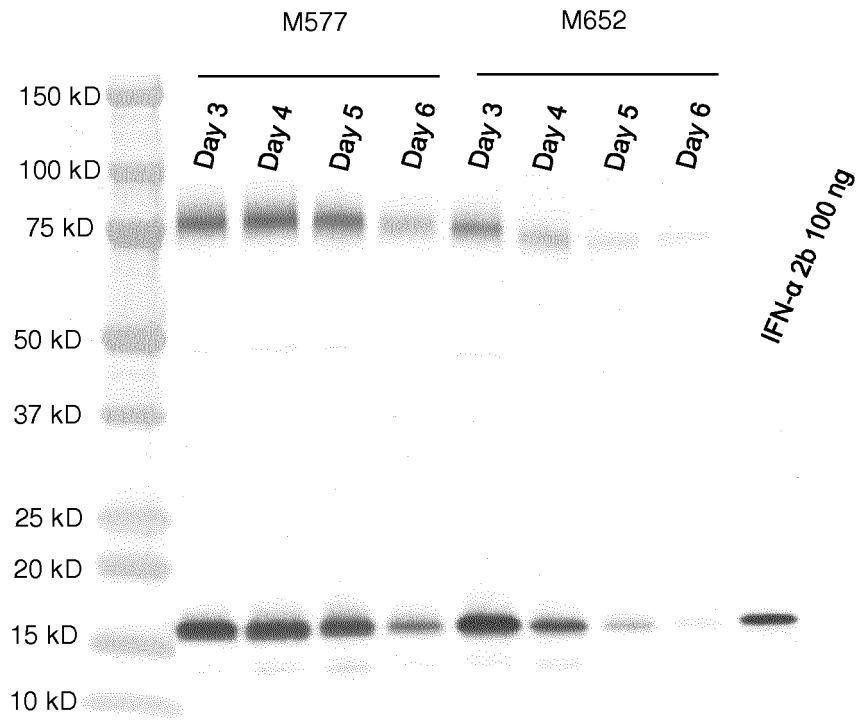


Figure 54

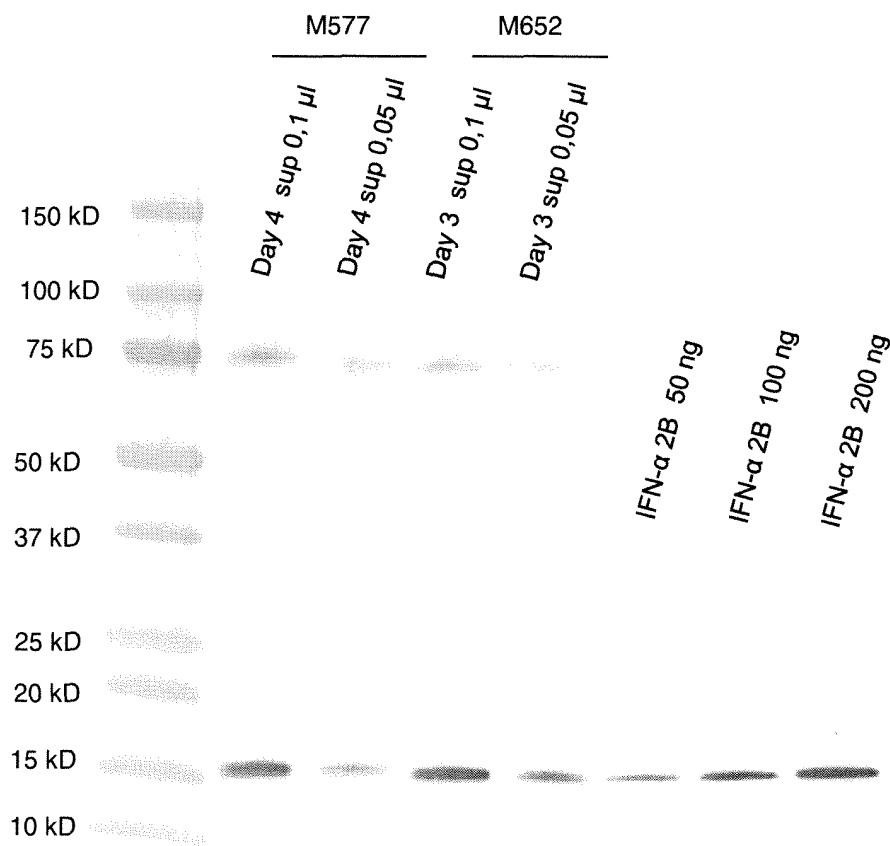


Figure 55