Mammalian TAP-tagging technique – General protocols collection

Anne-Claude Gingras 2003/08/12

Content:

agingras@systemsbiology.org

1) Overview of the process

Derived from the system described by the Seraphin's laboratory, the Tandem Affinity purification system (TAP) is a two-step affinity purification scheme which generates protein complexes sufficiently clean to be analyzed by mass spectrometry.

I generated a series of vectors allowing to perform tandem affinity purification in mammalian cells. My vector backbone is the pcDNA3 vector from Invitrogen, and the TAP-tag moieties are derived from the S. pombe vectors of Dr. Kathy Gould, at Vanderbilt University. I have also developed different variations of the initial vectors, but haven't characterized them in depth.

The following is a collection of protocols which I have developed for my own research interest. I can in no way guarantee that they will work for you, but you are welcome to try.

Anatomy of an N-terminal TAP-tag vector

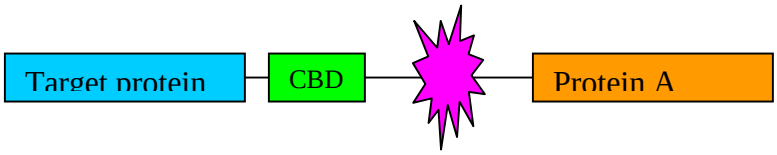
- Purify through ProteinA tag on IgG-sepharose
- Elute protein of interest using TEV protease
- Purify through CBD on calmodulin-sepharose (Ca++)
- Elute protein of interest through calcium chelation

2) Cloning notes

a. Overview of vectors available

i. pcDNA3-based C-terminal TAP-tag vectors

1. Calmodulin-binding domain, TEV cleavage site, protein A



= TEV protease cleavage site

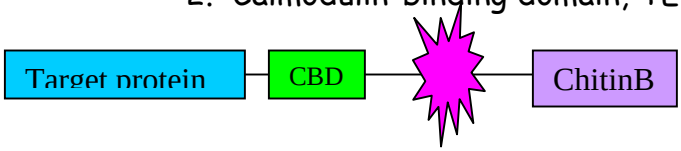
Polylinker: (HindIII, KpnI present in the fusion protein – DO NOT USE FOR CLONING)

```
                                    BamHI                                                                   EcoRI          EcoV
gac cca agc ttg gta ccg agc tcg gat cca cta gta acg gcc gcc agt gtg ctg gaa ttc tgc aga
D   P   S   L   V   P   S   C   D   P   L   V   T   A   A   S   V   L   E   F   C   R

                    NotI       XhoI      PmeI        AscI         PacI          start of CBD
tat cca tca cac tgg cgg ccg ctc gag agt tta aac ggc gcg cca tta att aac atg aag cga cga
Y   P   S   H   W   R   P   L   E   S   L   N   G   A   P   L   I   N   M   K   R   R

tgg aaa aag aat ttc
W   K   K   N   F
```

2. Calmodulin-binding domain, TEV cleavage site, chitin-binding



= TEV protease cleavage site

Polylinker: (HindIII, KpnI present in the fusion protein – DO NOT USE FOR CLONING)

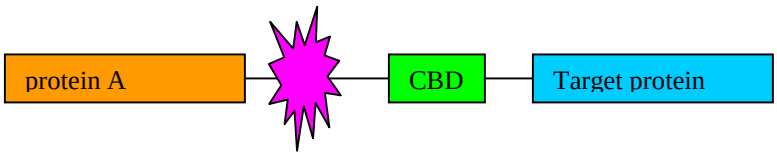
```
BamHI                                           EcoRI          EcoV
gac cca agc ttg gta ccg agc tcg gat cca cta gta acg gcc gcc agt gtg ctg gaa ttc tgc aga
D   P   S   L   V   P   S   C   D   P   L   V   T   A   A   S   V   L   E   F   C   R

                    NotI       XhoI      PmeI        AscI         PacI          start of CBD
tat cca tca cac tgg cgg ccg ctc gag agt tta aac ggc gcg cca tta att aac atg aag cga cga
Y   P   S   H   W   R   P   L   E   S   L   N   G   A   P   L   I   N   M   K   R   R

tgg aaa aag aat ttc
W   K   K   N   F
```

ii. pcDNA3-based N-terminal TAP-tag vectors

1. protein A, TEV cleavage site, Calmodulin Binding Domain)



= TEV protease cleavage site

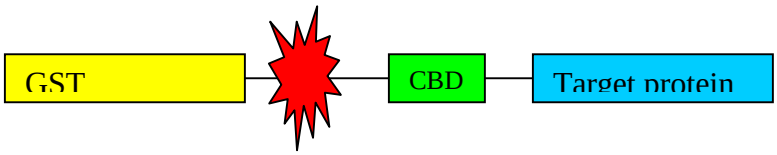
Polylinker: (Xho present in the TAP tag cassette – DO NOT USE FOR CLONING)

```
end of CBD                  PmeI       AscI       PacI      BamHI
ca tcc tcc ggg gca ctt gat agt tta aac ggc gcg cca tta att aag gat cca cta
S  S   S   G   A   L   D   S   L   N   G   A   P   L   I   K   D   P   L

                            EcoRI       EcoRV                NotI
gta acg gcc gcc agt gtg ctg gaa ttc tgc aga tat cca tca cac tgg cgg ccg ctc
V   T   A   A   S   V   L   E   F   C   R   Y   P   S   H   W   R   P   L

            XbaI    ApaI
gag cat gca tct aga ggg ccc
E   H   A   S   R   G   P
```

2. GST, Precission cleavage site, calmodulin binding domain



= Precission protease cleavage site

Polylinker: (only unique sites are shown):

```
end of CBD                  PmeI       AscI       PacI      BamHI
ca tcc tcc ggg gca ctt gat agt tta aac ggc gcg cca tta att aag gat cca cta
S  S   S   G   A   L   D   S   L   N   G   A   P   L   I   K   D   P   L

                            EcoRI       EcoRV                NotI      XhoI
gta acg gcc gcc agt gtg ctg gaa ttc tgc aga tat cca tca cac tgg cgg ccg ctc
V   S   A   A   S   V   L   E   F   C   R   Y   P   S   H   W   R   P   L

            XbaI    ApaI
gag cat gca tct aga ggg ccc
E   H   A   S   R   G   P
```

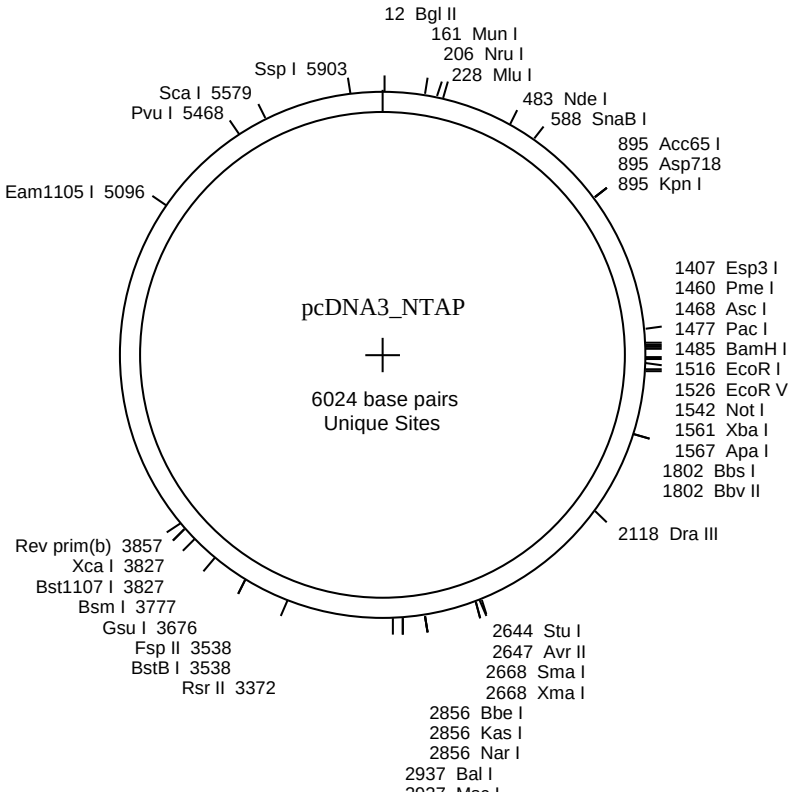
b. Detailed map of pcDNA3-ProtA-TEV-calmBP (alias pcDNA3-NTAP)

Amplified NTAP-tag cassette from ACG.

The NTAP-tag (2 Ig binding domains) from the pombe vector of Kathy Gould was amplified using a 5' primer introducing a Kpn I site, and a 3' primer, introducing sites for PmeI, AscI, PacI and BamHI. The PCR insert from below was digested with KpnI and BamHI and inserted in the KpnI and BamHI sites of pcDNA3. The insert was completely sequenced, with the exception of the portion shown in grey below, which was really blurry on the chromatograms. The portion from the original TAP-tag vector is shown in CAPITAL letters, and the portion added from ACG's cloning is in lower caps. Info. modified on 2002/09/12 acg.

ggtaccATGAAAGCTGATGCGCAACAAAATAACTTCAACAAAGATCAACAAAGCGCCTTCTATGAAATCTTGAACATGCC
TAACTTAAACGAAGCGCAACGTAACGGCTTCATTCAAAGTCTTAAAGACGACCCAAGCCAAAGCACTAACGTTTTAGGTG
AAGCTAAAAAATTAAACGAATCTCAAGCACCGAAAGCTGATAACAATTTCAACAAAGAACAACAAAATGCTTTCTATGAA
ATCTTGAATATGCCTAACTTAAACGAAGAACAACGCAATGGTTTCATCCAAAGCTTAAAAGATGACCCAAGCCAAAGTGC
TAACCTATTGTCAGAAGCTAAAAAGTTAAATGAATCTCAAGCACCGAAAGCGGATAACAAATTCAACAAAGAATCTAGTA
CCCCAACTACTGCTTCTGAAAATCTATATTTTCAAGGTGAACTAAAAACTGCTGCTTTGGCTCAACATGCGCTCGAGAAG
ATGAAGCGACGATGGAAAAAGAATTTCATAGCCGTCTCAGCAGCCAACCGCTTTAAGAAAATCTCATCCTCCGGGGCACT
TGAtagtttaaacggcgcgccattaattaaggatccactag

Map of the complete vector – Unique sites are shown



Full sequence of the pcDNA3-NTAP vector

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGTAT
CTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATT
GATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAA
CTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGT
AACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGT
ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTA
TGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAA
TGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACC
AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAG
GTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAG
GGAGACCCAAGCTTggtaccATGAAAGCTGATGCGCAACAAAATAACTTCAACAAAGATCAACAAAGCGCCTTCTATGAA
ATCTTGAACATGCCTAACTTAAACGAAGCGCAACGTAACGGCTTCATTCAAAGTCTTAAAGACGACCCAAGCCAAAGCAC
TAACGTTTTAGGTGAAGCTAAAAAATTAAACGAATCTCAAGCACCGAAAGCTGATAACAATTTCAACAAAGAACAACAAA
ATGCTTTCTATGAAATCTTGAATATGCCTAACTTAAACGAAGAACAACGCAATGGTTTCATCCAAAGCTTAAAAGATGAC
CCAAGCCAAAGTGCTAACCTATTGTCAGAAGCTAAAAAGTTAAATGAATCTCAAGCACCGAAAGCGGATAACAAATTCAA
CAAAGAATCTAGTACCCCAACTACTGCTTCTGAAAATCTATATTTTCAAGGTGAACTAAAAACTGCTGCTTTGGCTCAAC
ATGCGCTCGAGAAGATGAAGCGACGATGGAAAAAGAATTTCATAGCCGTCTCAGCAGCCAACCGCTTTAAGAAAATCTCA
TCCTCCGGGGCACTTGAtagtttaaacggcgccattaattaaggatccACTAGTAACGGCCGCCAGTGTGCTGGAATT
CTGCAGATATCCATCACACTGGCGGCCGCTCGAGCATGCATCTAGAGGGCCCTATTCTATAGTGTCACCTAAATGCTAGA
GCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCT
GGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTC
TGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCT
ATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGC
GGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCT
TTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGCATCCCTTTAGGGTTCCGATTTAGTGCTTTACGG
CACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTT
GACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTT
TTGATTTATAAGGGATTTTGGGGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTAA
TTCTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGGCAGGCAGAAGTATGCAAAGCATGCATCT
CAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGT
CAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGC
TGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTT
TGGAGGCCTAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGTATATCCATTTTCGGATCTGATCAAGAGACAGGATGAGGAT
CGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGG
GCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGAC
CGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCG
CAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCA
TCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTG
CCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATC
TGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTC
GTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCG
GCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTG
ACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTC
TGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCC
TTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGA
GTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATA
AAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACC
TCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACAT
ACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGC
CCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATT
GGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAA
GGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGA
ACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGT
CAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCC
GACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGT
ATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTA
TCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAG
CAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTG
GTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGT
AGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTAC
GGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGA
TCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTA
ATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTAC
GATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAG
CAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGT
TGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTC
ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCA
AAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCA
GCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTG
AGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAA
AAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAA
CCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAA
TGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTT
ATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTT
CCCCGAAAAGTGCCACCTGACGTC

c. Design of oligonucleotides

In theory, all the sites in the polylinker from the maps included in this document can be used. In practice, I have been using mostly *PmeI* and *PacI*, for cloning in the NTAP or CTAP vectors. In a few cases, I already had primers around to add *EcoRI* and *XhoI* sites for use in the CTAP vector, so I used these instead without any problem.

i. Cloning into the *PmeI* and *PacI* sites.

I make most of my constructs this way because both *PmeI* and *PacI* are rare cutters (8 bases in the recognition sequence), and are thus rarely present in the inserts I'm trying to clone. They are also the sites that generate the smaller additional sequences from the vector. Finally, they both can work in the New England Biolabs buffer 4. *PmeI* is a blunt cutter, though, so the *PmeI*/*PacI* is a sticky/blunt cloning. I have made other vectors with different tags which have the same polylinker, so that the inserts can be shuttled from a vector to another.

1. Cloning into the pcDNA3-NTAP

Below is a portion of the polylinker for pcDNA3-NTAP, including the *PmeI* and *PacI* sites. The reading frame of the N-terminal fusion is indicated below the DNA sequence. It is critical that the reading frame is kept from the PmeI site for N-terminal fusions. For N-terminal fusions, the natural stop codon of your protein is part of the clone. The PacI site follows immediately:

Polylinker:

```
end of CBD                  PmeI       AscI        PacI      BamHI
ca tcc tcc ggg gca ctt gat agt tta aac ggc gcg cca tta att aag gat cca cta
S  S   S   G   A   L   D   S   L   N   G   A   P   L   I   K   D   P   L
```

Desired PCR product to be cloned:

```
gtttaaacATGxxx xxx xxx xxx xxx xxx xxxTGAttaattaa
PmeI   Start  protein of interest    Stop PacI
```

yellow:	PmeI restriction site – frame between restriction site and ATG is critical
green:	original initiation codon from your protein of interest (essential for CTAP fusions)
red:	anti-parallel to the original stop codon from your protein of interest (essential for NTAP fusions)
magenta:	*PacI* restriction site

Oligos design: (If you follow the guidelines below, the same 5' oligo can be used for cloning in the NTAP and in the CTAP vectors; the 3' oligo will need to be different)

5'Primer (5'PmeI):

```
gatcgtttaaacATGNNNNNNNNNNNNNNNNN
```

grey:	a few added nucleotides to facilitate digestion by PmeI
yellow:	PmeI restriction site – frame between restriction site and ATG is critical
green:	original initiation codon from your protein of interest (essential for CTAP fusions)
blue:	first 15-20 nucleotides (after ATG) from your protein of interest

3'Primer (3' STOP PacI):
gatcttaattaaTCANNNNNNNNNNNNNNNNN

grey:	a few added nucleotides to facilitate digestion by *PacI*
magenta:	*PacI* restriction site
red:	anti-parallel to the original stop codon from your protein of interest (essential for NTAP fusions)
dark blue:	anti-parallel to the last 15-20 nucleotides (before stop) from your protein of interest

2. Cloning into the pcDNA3-CTAP

Below is a portion of the polylinker for pcDNA3-CTAP, including the *PmeI* and *PacI* sites. The reading frame of the C-terminal fusion is indicated below the DNA sequence. It is critical that the reading frame is kept from the PacI site for C-terminal fusions, and that the stop codon is NOT included in the PCR primer.

Polylinker:

```
                  NotI      XhoI     PmeI       AscI        PacI        start of CBD
tat cca tca cac tgg cgg ccg ctc gag agt tta aac ggc gcg cca tta att aac atg aag cga cga
Y   P   S   H   W   R   P   L   E   S   L   N   G   A   P   L   I   N   M   K   R   R
```

Desired PCR product to be cloned:

```
gtttaaacATG xxx xxx xxx xxx xxx xxx xxx xxx tta att aa
PmeI    Start  protein of interest/NOStop   PacI
```

Oligos design:
You can use the same 5' primer as you used for the N-terminal fusions. The 3'primer is designed in the same way as for the NTAp primer, minus the stop codon. Make sure the frame stays as shown on the schema above.

3'Primer (3' NOSTOP PacI):

gatcttaattaaNNNNNNNNNNNNNNNNN

grey:	a few added nucleotides to facilitate digestion by *PacI*
magenta:	*PacI* restriction site
dark blue:	anti-parallel to the last 15-20 nucleotides (before stop) from your protein of interest

d. Design of sequencing primers

Mini-preps (QIAGEN) of the constructs are tested for the presence of inserts. Positive minipreps are sequenced with the appropriate primers:

i. Constructs in NTAP:
   1. Sp6 (commercial primer)
   2. calm.FWD (5'-CATAGCCGTCTCAGCAGCCAACCG)

ii. Constructs in CTAP:
   1. T7 (commercial primer)
   2. calm.REV (5'-CGGTTGGCTGCTGAGACGGCTATG)

3) Expression

a. Cell lines

I have been using both 293T and 293 cells for the transfections – each cell line being used for a different purpose, but they are grown and transfected in the same manner. You may want to use a cell line which is more physiological for your purposes, but I would suggest that you also use the HEK293 or 293T cells in transient transfections to detect whether your construct can be expressed prior to establishing stable cell lines. In all cases, you should start with a known source of cells – this is especially important when making stable lines: I try using a similar (and low) passage after reception of the cells from the ATCC for all my experiments.

i. 293T (ATCC CRL-11268):

I only use the 293T cells for transient transfections and mostly for testing expression and/or purification on small scale. The yield in protein is significantly higher in 293Ts, so you need less DNA/cells to obtain the same amount of proteins for your experiments. I would recommend to start the small scale tests using 293Ts, especially if you don't know how well your own (test) protein will behave (see below for suggested amounts for transfection).

ii. HEK293 (CRL-1573):

I use the 293 for making all my stable cell lines. When possible to generate, stable cell lines are my preferred choice for the real purification experiments. They express more moderate amount of protein, and are less likely to produce artifacts due to overexpression, such as mislocalization, and association with unnatural partners. They also are less likely to be misfolded and coated with chaperones. When stable cell lines cannot be obtained (for example when the protein is toxic), I still use 293 (and NOT 293T) in transient transfections to generate sample for large scale purification and mass spectrometry. In that case, I transfect 2 – 4 x 150 mm plates and split them to obtain 8 – 16 x 150 mm plates. I usually harvest at 48-60 hours, but this can be modulated depending of your protein of interest.

iii. General growth of the cells:

The cells are kept in DMEM high glucose supplemented with 5% FBS and 5% serum supreme (Biowhittaker) and Penicillin/Streptomycin. Alternatively, they can also be grown in DMEM high glucose supplemented with 10% FBS and Pen/Strep. The Serum Supreme is simply fortified calf serum and is much cheaper than FBS (40$ versus 200$ for a 500ml bottle, so the costs are significantly reduced in the first recipe). The cells can be trypsinized – they detach quite quickly and should not be incubated in trypsin for too long. For routine growth, I split the cells 1 → 4 to 1 → 8 (they do not like being too sparse).

b. Transfection - transient

I have been using the lipofectamine PLUS system from Invitrogen. Regular lipofectamine, lipofectamine 2000, and a range of cationic lipids from Invitrogen, Qiagen or other companies can also be used. HEK293 and 293T cells can also be easily transfected by calcium phosphate or electroporation. The bottom line is: if you have a system which works well for your lab, go ahead and use it.

The transfection procedure is essentially the one described in the Invitrogen protocol, but below are the quantities I've been using when transfecting in 6 well plates. The 6 well plate format is perfect for just testing your expression (i.e. to make sure your protein can be expressed). You can transfect miniprep DNA in the 6 well if it is of really good quality. The spin procedure from Qiagen works quite well. In general the DNA concentration is just over 100ng/ul following the purification, so you only need 8-10ul for the transfection (per well). If you want to troubleshoot your purification on a small scale (with transient transfection), I recommend transfecting at least 1 x 100mm plate per construct (of course you'll need maxiprep DNA for this). If you are establishing stable cell lines (see next section), you actually don't need to transfect that many cells – I routinely transfect one 6 well or one 6 cm plate, and have plenty.

Each well of a 6-well plate of HEK293 or HEK293T cells (60-90% confluent) is transiently transfected as follows:

- 1µg DNA (~ 8 µl miniprep DNA)
- 2.5µl PLUS reagent from Invitrogen
- 100µl Opti-MEM from Invitrogen, no phenol, no antibiotic added, pre-warmed (37°C)

MIX the PLUS and the Opti-MEM first. Add the DNA, MIX well and incubate at room temp. 15-30 minutes

- 2.5µl lipofectamine from Invitrogen
- 100µl Opti-MEM

MIX and combine with DNA mixture and incubate 15-30 minutes at room temp.

Rinse the cells 1x with Opti-MEM. Add 1ml Opti-MEM per well, and the combined DNA/PLUS/lipofectamine mixture. Gently rotate the plate to distribute the media and incubate for 3 hours at 37°C.

Stop by adding 1.2ml of DMEM containing 20% FBS (Do not remove transfection mixture). Incubate overnight (12-18hours).

The next day, split each well ~1:3 to 1:6 (e.g. 1 well into one or two x 6 cm plates). Grow for an additional 30-50 hours. Rinse the plates 2x with PBS, scrape and lyse. Lysis can be performed by freeze-thaw, or in TAP-tag lysis buffer (see protocol for the purification of TAP-tagged proteins).

Analyze the expression by running 25-50µg extract on SDS-PAGE, followed by western blotting. If no antibody is available to the tagged protein, the expression of the protein A

moiety can be easily detected using standard rabbit sera (1:2000) as a primary, and anti-rabbit-HRP as a secondary antibody.

If you are doing the transfection in a 100 mm plate, you can use the same procedure, but increase the amount. You can try the following as a starting point:

- 8 ug DNA
- 25 ul PLUS
- 1.2 ml Opti-MEM to mix with PLUS and DNA
- 25 ul lipofectamine
- 1.2 ml Opti-MEM to mix with lipofectamine
- 5 ml Opti-MEM on the cells
- 7.5 ml of DMEM + 20% FCS to stop the reaction

c. Transfection - stable

Once the DNA has been sequenced and has showed expression in transient transfection, cells of interest are stably transfected. You need to decide whether you will want to obtain a pool of stably transfected cells, or individual clones of transfected cells. If you are working with only a few targets, you should consider establishing individual clones because the expression has the tendency to be more stable in a clone than in a pool (where often cells who do not express or express little of the protein of interest grow faster than the high expressers and tend to take over the culture after some time). In addition, you can know the expression of your protein of interest in all the cells from a clone, whether you can only detect the average from a pool. If working with clones, however, you may want to combine 3 – 4 decent* expressers for each experiment to prevent artifacts due to clonal variation. (* I never choose the clones which express HUGE amounts of proteins, by fear that artifact might arise. Instead, I'm trying to obtain clones which express in the same range as the endogenous protein).

Use only low-passage cells for this procedure. Transfect as above (or using any transfection protocol adapted to the cell line of interest). For 293 cells, cells are transfected (6cm plate) when they are ~80% confluent. The next day, they are split into 6 x 10cm plates, using serial 2- or 3-fold dilutions (so the final dilution will be ~1:6, 1:12, 1:24, and so forth). If you are only trying to obtain pools, perform only 3 serial dilutions. The cells are returned at 37°C for 24 hours. The next day, the medium is changed for DMEM containing 1g/L G418. The cells are incubated in this medium for about 12-15 days, with occasional re-feeding. At the end of this period, the plates on which colonies are well defined/separated from their neighbors will be selected for picking clones. The other plates will be trypsinized and transferred into 150mm plates for use as "pools". For picking the clones, the position of the clones is first marked at the bottom of the plate. The plate is then rinsed with 5ml PBS. Sterile 3mm cloning disks (Sigma) are soaked in trypsin-EDTA, and carefully placed on each colony, using sterile forceps. HEK293 detach really fast (2-5 minutes), so proceed quickly. The disks are then transferred into the wells of a 24 or 48-well plate already containing medium. Clones and pools are grown in selection media and

passaged as necessary. As soon as possible, they are screened for expression, and frozen away. Often, pools lose their expression quite fast, so clones are better for a more permanent line.

e. Reagents needed

DMEM high glucose, Fetal bovine serum, trypsin-EDTA, Sterile PBS, G418 sulfate, Penicillin-streptomycin can be purchased through Mediatech Cellgro, Biowhittaker, Invitrogen, etc.

All the TC plastics are purchased from TPP (plates), BD-Falcon (plates, pipettes), or Fisher (pipettes) – equivalent material can also be used.

Chemiluminescence detection reagents and films can be obtained from Amersham, and similar products can also be obtained through other suppliers.

Product description	Supplier	catalog number	unit size/price
Serum Supreme	Biowhittaker	14-492F	42$/500ml
Opti-MEM no phenol	Invitrogen	11058021	24$/500ml
Lipofectamine	Invitrogen	18324012	210$/1ml
PLUS reagent	Invitrogen	11514015	110$/0.85ml
Sterile 3mm cloning disks	Bel-Art / Fisher	37847 0001	18$/100
Rabbit normal serum	ICN	2941149	24.85$/100ml
Donkey anti-rabbit HRP	Amersham	NA934	158$/1ml
HEK293	ATCC	CRL-1573	175$/EA
293T	ATCC	CRL-11268	283$/EA

Purification of TAP-tagged (protein A/calmodulin) proteins for direct analysis by LC-MS
ACG 2003/02/18

This procedure has been adapted from the protocol of Dr. Séraphin. I have optimized it for the purification of CYTOSOLIC MAMMALIAN proteins. The lysis and purifications conditions will need to be optimized for the purification of nuclear or membrane-associated proteins. The final washes + elutions are performed in a buffer allowing for the direct analysis of trypsin-digested proteins by LC-MS.

Troubleshooting notes. It is really important, especially when performing the TAP-tagging expression/purification for the first time, or with a new protein, to take aliquots for analysis of the purification procedures at all the steps indicated in red. Steps which are not yielding an efficient recovery (see section II for expected recoveries) should be troubleshooted individually (see troubleshooting notes, section III).

The following protocol is aimed at small-scale purification and at purification of larger amounts for mass spectrometry, and approximation for the quantities to used are indicated (yellow highlight for the large scale; green highlight for the small scale).

PART I: PURIFICATION OF TAP-TAGGED PROTEINS

1. Extract preparation:

Wash cells 3x in ice-cold PBS. Lyse cells in lysis buffer for 30 minutes (see appended recipe), and harvest by scraping (you can also scrape in PBS, and then lyse). For each 150mm plate, I use ~0.5 to 1ml lysis buffer. Perform 2 freeze-thaw cycles to improve protein recovery (I alternate between liquid nitrogen and 37 degrees water bath – nerver let the temperature go over 4 degrees in the tubes). Spin down the debris for 10 minutes at maximum speed in a microfuge. (If larger volumes are used, the spins can be performed in a SS34 rotor at 10000rpms for 15 minutes, or in a clinical centrifuge at 2650g for at least 30 minutes.) Recover the cleared supernatant and monitor the protein concentration (I usually recover 5-10mg protein / 150mm plate of 90% confluent 293 cells). KEEP AN ALIQUOT FOR ANALYSIS (A – read section II for suggested quantities).

2. Binding to IgG sepharose:

NOTES: All spin steps involving beads are performed at very low speeds (e.g. 1500 rpm in a microfuge for 1-2 minutes). Removal of the liquid is performed slowly, preferably with a transfer pipette, or a pipetman. All spins and incubations are performed at 4°C, and all the buffers are pre-chilled on ice.

Wash the appropriate amount of IgG beads 3x with lysis buffer. Remove excess liquid. Add the cleared lysate to the washed beads. Incubate with gentle rocking (or end-over-end rocking) at 4°C for 2 – 4 hours (make sure the slurry is well resuspended). The quantity of

beads used is ~1-5 µl packed beads / mg extract, with the following exceptions: 1) When working with smaller quantities of lysate, I never use less than 15µl packed beads, to make it easier to see at the bottom of the tube; 2) Even when working with large amounts of cells (10-20 plates, or 50-200mg extract), I never use more than 75 - 100 µl packed beads (the capacity of the beads is >2mg protein A/ml packed gel, i.e. >200 µg protein A/100 µl packed beads). When working with large volumes of lysate, I incubate for longer periods (4 hours).

3. Cleavage with tobacco etch virus protease (TEV):

Spin down the IgG beads. Recover supernatant (USE FOR ANALYSIS - B). Wash the beads 3x with 1ml lysis buffer, and 3x with TEV buffer (see appended recipe). KEEP AN ALIQUOT OF THE BEADS PRE-CLEAVAGE FOR ANALYSIS (C). Drain liquid after the last wash. Resuspend beads in TEV buffer containing TEV protease. The quantity of TEV buffer to add is important. For extract from 5 to 10 plates bound to ~100µl packed protein A beads, I currently use ~100 units TEV in 300 µl TEV buffer. In preliminary experiments, I have used 5-10 units of TEV in 15-30 µl buffer for 5-10 µl packed IgG beads /mg protein – you may have to increase the volume for the slurry to stay resuspended. Incubate the slurry (again with gentle agitation) for at least 4 hours at 4°C (In most case, more is NOT detrimental – if the cleavage is inefficient, try incubating overnight). Make sure there is enough liquid so that the slurry stays resuspended.

4. Binding to calmodulin-sepharose:

Use about the same amount of calmodulin-sepharose beads as used for the IgG sepharose (you can usually get away with half as much). Wash the calmodulin sepharose beads 3x with calmodulin binding buffer (see recipe below).

Spin down the IgG beads post-cleavage. KEEP AN ALIQUOT OF SUPERNATANT FOR ANALYSIS (D). ALSO, WASH THE BEADS 3XWITH TEV BUFFER, AND USE FOR ANALYSIS (E). Transfer the supernatant to a fresh tube. Add 1 volume of calmodulin binding buffer. Invert to mix. Spin down the IgG beads again. Transfer this wash to a fresh tube. Repeat twice (if you started with 300 ul TEV buffer, you should now have 1200 ul combined mixture). Add 1/250 volume (~5 ul) 1M CaCl2, mix by inversion. Spin again to remove traces of IgG sepharose and transfer the supernatant to the tube containing the calmodulin-sepharose. Incubate 90 minutes with gentle agitation at 4°C.

5. Elution from calmodulin-sepharose:

Spin down the calmodulin beads. Remove the supernatant (KEEP FOR ANALYSIS – F). At this point (and especially with the large scale), I transfer the beads (using calmodulin binding buffer) to a clean Bio-spin column to perform the subsequent washes and elution steps. These steps can also all be performed in eppendorf tubes, with spinning (especially for small scale). Wash the beads 3x with 10 – 20 volumes calmodulin-***binding*** buffer. (If using the Bio-spin, resuspend the slurry in buffer, let drip the buffer through, pushing the liquid out gently with a rubber bulb if the buffer does not drip by itself). Wash 2x with 10

– 20 volumes calmodulin ***rinsing*** buffer. (KEEP AN ALIQUOT OF THE BEADS PRE-ELUTION FOR ANALYSIS – G). Drain the beads well (push the remaining droplets with a rubber bulb). Transfer the Bio-spin column + beads into clean tubes (2ml screw-cap tubes work well). Resuspend beads in one volume of calmodulin elution buffer (e.g. 100µl for 100µl packed beads), incubate a few minutes, lift the Bio-spin to let drip, and push the remaining droplets out with a rubber bulb. Add another volume (100µl) calmodulin elution buffer to the Bio-spin column and let drip in the same eppendorf tube (push the remaining droplets out with a rubber bulb). Transfer the Bio-spin column to a new tube and repeat these steps 3 more times. ANALYSE A FRACTION OF EACH OF THESE ELUTIONS (H, I). ALSO ANALYSE THE BEADS POST-ELUTION (J). Freeze the final eluates until the analysis is performed. NOTE: If performing a small scale (test) experiment, Bio-spin columns are not suggested. Simply spin down beads and recover the supernatant for each elution.

PART II: ANALYSIS OF THE PURIFICATION PROCEDURE.

The expressed protein is detected by Western blot using antibodies specific to the expressed protein. If antigen-specific antibodies are not available, expression can also be detected through the presence of the protein A (using whole rabbit sera as a primary antibody, and HRP-coupled anti-rabbit as a secondary antibody) and calmodulin tags (using biotinylated calmodulin, followed by HRP-coupled avidin – I have never performed this latter detection myself). In all cases, chemiluminescent detection is performed and the amounts of protein in each fraction are approximated. By loading each fraction, it is really easy to see which steps require further optimization. The following table is an indication of how much sample I have loaded for each of these fractions in test experiments (starting material = 500µg). The proportions are decreased when starting with more material. The gels are loaded in the order A to J. If an assessment of the complexity of the samples is needed, the final eluates can also be analyzed by silver staining of the SDS-PAGE gel.

ID	fraction name	function	amount loaded
A	total lysate	efficiency of binding to IgG beads	25µg = 1/20
B	lysate post IgG beads	efficiency of binding to IgG beads	25µg = 1/20
C	IgG beads pre-TEV	efficiency of binding to IgG beads; TEV cleavage efficiency	1/20
D	TEV eluate	TEV cleavage efficiency	1/10
E	IgG beads post-TEV	TEV cleavage efficiency; efficiency of binding to calmodulin beads	1/10
F	supernatant post-calmodulin (unbound)	efficiency of binding to calmodulin beads	1/10
G	calmodulin beads pre-elution	efficiency of binding to calmodulin beads efficiency of elution from calm. beads	1/10
HI	elutions 1, 2	efficiency of elution from calm. beads	1/5
J	calm. beads post elution	efficiency of elution from calm. beads	1/5

PART III: TROUBLESHOOTING NOTES

You should seriously consider troubleshooting individual steps is the recovery is significantly less than the values indicated below:

1) binding to IgG sepharose > 80%
2) TEV cleavage > 50%
3) binding to the calmodulin sepharose > 80%
4) elution with EGTA > 50%
5) total recovery > 30%

Things to try…

1) binding to IgG sepharose
    a. Make sure you wash you cells well prior to lysis
    b. Try different batch / brand IgG sepharose
    c. Make sure there are no mutations in 2 Ig domains of tag
2) TEV cleavage
    a. If cleavage is incomplete: Increase amount of TEV, time of incubation, temperature of incubation; try a different lot or source of TEV
    b. If cleavage occurs, but cleaved protein is not recovered: maybe your protein sticks to the beads; try reducing IgG amount, and/or adding more detergent; determine whether your protein sticks to sepharose
3) Binding to calmodulin sepharose
    a. Make sure you have added calcium and that there is no excess of EDTA

b. Try a different brand / lot of the calmodulin-sepharose

4) Elution with EGTA
   a. Decrease the amount of calmodulin-sepharose you are using – your protein might stick to it
   b. Incubate for longer periods, at room temp.; incubate with higher concentrations of EGTA; perform repeated elutions
   c. Try adding some Rapigest (detergent which is compatible with mass spectrometry after cleavage – sold by Waters)

PART IV: REAGENTS AND RECIPES

Product description	Supplier	catalog number	unit size/price
IgG sepharose 6 fast flow	Amersham	17-0969-01	10ml / 243$
calmodulin-sepharose 4B	Amersham	17-0529-01	10ml / 212$
recombinant TEV	Invitrogen	10127017	1000U / 86$
Micro Bio-spin columns	Bio-rad	732-6204	100 / 80$
Protease inhibitor cocktail	Sigma	P8340	5ml / 126$

*all other chemicals are molecular biology grade (or equivalent) from Sigma

Lysis buffer:

final concentration	product	stock concentration	vol. for 100ml 1x
10 %	glycerol	100 %	10 ml
50 mM	Hepes-KOH pH 8.0	1 M	5 ml
100 mM	KCl	2 M	5 ml
2 mM	EDTA	0.5 M	0.4 ml
0.1 %	NP-40	10 %	1 ml
2 mM	DTT	1 M	0.2 ml
1 x	Sigma prot inhibitor	500 x	0.2 ml
10 mM	NaF	1 M	1 ml
0.25 mM	NaOVO3	100 mM	0.25 ml
5 nM	okadaic acid	10µM	25µl
5 nM	calyculin A	10µM	25µl
50 mM	ß-glycerolphosphate	1 M	5 ml
	H2O		to 100ml

Aliquot and freeze.

TEV buffer:

final concentration	product	stock concentration	vol. for 100ml 1x
10 mM	Hepes-KOH pH 8.0	1M	1 ml
150 mM	NaCl	5 M	3 ml
0.1 %	NP-40	10 %	1 ml
0.5 mM	EDTA	0.5 M	100 µl
1 mM	DTT	1 M	100µl
	H2O		to 100µl

Aprotinin, leupeptin and PMSF do not inhibit TEV, and can therefore be used in this buffer if endogenous protease activity is suspected. Phosphatase inhibitors okadaic acid, calyculin A, ß-glycerolphosphate and NaOVO3 can also bee used to prevent phosphatase activity (they should not inhibit TEV or binding to calmodulin).

Calmodulin-binding buffer:

final concentration	product	stock concentration	vol. for 100ml 1x
10 mM	ß-mercaptoethanol	14 M	69.7 µl
10 mM	Hepes-KOH pH 8.0	1 M	1 ml
150 mM	NaCl	5 M	3 ml
1 mM	MgOAc	1 M	100µl
1 mM	imidazole	1 M	100µl
0.1 %	NP-40	10 %	1 ml
2 mM	CaCl2	1 M	100 µl
	H2O		100 ml

Protease inhibitors can be used in this buffer if endogenous protease activity is suspected. Phosphatase inhibitors okadaic acid, calyculin A, ß-glycerolphosphate and NaOVO3 can also be used to prevent phosphatase activity

Calmodulin-rinsing buffer for direct analysis by LC-MS:

final concentration	product	stock concentration	vol. for 100ml 1x
50 mM	Amm.bicarb. pH 8.0	1 M	5 ml
75 mM	NaCl	5 M	1.5 ml
1 mM	MgOAc	1 M	100µl
1 mM	imidazole	1 M	100µl
2 mM	CaCl2	1 M	100 µl
	H2O		100 ml

Calmodulin-elution buffer for direct analysis by LC-MS:

final concentration	product	stock concentration	vol. for 100ml 1x
50 mM	Amm.bicarb. pH 8.0	1 M	5 ml
25 mM	EGTA	0.5 M	5 ml
	H2O		100 ml

The ammonium bicarbonate is volatile, and will be evaporated in the speed vac, following the tryptic digest. The EGTA will not be evaporated, and there will also be traces of NaCl, MgOAc, imidazole and CaCl2. After the tryptic digest, dry the sample by speed-vac. Resuspend in 200 ul water, and speed-vac to remove traces of Ammonium bicarbonate. Resuspend in buffer A and load onto the C18 reverse-phase column used for MS. Wash extensively offline, with 5% acetonitrile in buffer A. Analyse by LC-MS. If necessary, load onto a large capacity C18 reverse-phase column first, then elute in acetonitrile, and lyophilize prior to running on the LC-MS. Alternatively, a strong cation-exchange step can be performed. Samples eluted in NH4OH:MeOH can be lyophilized and loaded on the LC-MS reverse-phase column.

6) Appendix

a. molecular biology techniques

The following are the techniques I've been using most often for the cloning into the pcDNA3-NTAP / CTAP vectors. Any similar technique should also work, but if you do experience cloning problems with your technique, you may want to give a shot at the following.

i. PCR

I get my oligos from IDT DNA. I generally get the smallest – 25 nmole - synthesis scale, desalted, but not purified. I resuspend all the oligos at 500 pmoles/ul for the stock (which I keep at -20 degrees), and I make a working dilution of 5 pmoles/ul. The DNA is used at 5-10 pmoles/ul for plasmid DNA (1:20 dilution of a QIAGEN miniprep works fine).

I try using the highest fidelity enzymes available. Pfu Ultra or Pfu Turbo (Stratagene) work well for most templates, but in some cases, I have had to use Herculase (Roche), of Taq+ Precision (Stratagene). I do not use regular Taq for cloning. I have been using the dNTPs from Stratagene (25 mM each). The following reaction is with Pfu Turbo. I use filter tips to pipet from the stock enzymes and dNTPs. Make sure you keep the enzyme at -20 degrees until needed.

As for the temperature and the number of cycles, this will depend upon your template/ primers. The denaturation/ annealing time will depend of the type of machine you use (here = single block), and the elongation time will also depend on the length of your template. I usually start with the following set up (here, with 26 cycles; you can go to 30 cycles if more product is needed):

Denaturation:	94 degrees – 3 minutes
First 8 cycles:	94 degrees – 30 seconds
	48 degrees – 30 seconds
	72 degrees – 1 minute per kb of template to be amplified
Next 18 cycles:	94 degrees – 30 seconds
	58 degrees – 30 seconds
	72 degrees – 1 minute per kb of template to be amplified
Final elongation:	72 degrees – 10 minutes
Keep at:	4 degrees

<u>Setting up the reaction:</u>

For a 50ul final reaction volume:

1 ul DNA (5 pmoles/ul)
1 ul primer 5' (5 pmoles/ul)

1 ul primer 3' (5 pmoles/ul)
0.2 ul dNTPs (25mM each)
0.4 ul Pfu Turbo
5 ul 10 x cloned Pfu buffer
41.4 ul water

The last 4 ingredients can be pre-mixed and distributed (47 ul) to tubes of wells containing the DNA and the primers.

After the PCR is finished, analyze 5 ul (1/10 of the reaction) on a 1 to 1.2 % TAE-agarose gel containing EtBr. The products should be easy to detect – make sure the size is correct.

ii) cloning the PCR product into pcDNA3-NTAP or pcDNA3-CTAP.

PCR cleanup and DNA precipitation
You should have about 45 ul of the PCR reaction left. Transfer it to a 1.5 ml microfuge tube and add 150 ul of water. Add 200 ul of phenol:chloroform:isoamylalcool (25:24:1) and vortex for 1 minute. Centrifuge at max speed for 5 minutes. Transfer the upper, aqueous phase to a new 1.5 ml microfuge tube (make sure you do not take any of the organic solvent phase or the interface). Add 1/10 volume of 3 M NaOAc pH 6.0 (20 ul), 0.5 ul glycogene (MBI Fermentas) and 3 volumes (600 ul) 100% EtOH. Mix by inversion and centrifuge at max speed for 30 minutes at 4 degrees. Gently aspirate off the supernatant, making sure not to disturb the pellet (which should be visible at the bottom of the tube). Add 100 ul of 70% EtOH to wash the pellet, centrifuge at max speed for 5 minutes. Remove the supernatant with a P200. Spin 1 minute, and remove traces of the supernatant with a P20. Air dry.

Digestion of the inserts
You can resuspend the dry DNA pellet directly in the digestion buffer containing the enzymes. The following is for the PmeI/PacI cloning.

For a 20 ul reaction volume:
2 ul 10 x restriction buffer (here New England Biolabs buffer 4)
0.5 ul PmeI (10000 U/ml)
0.5 ul PacI (10000 U/ml)
0.2 ul BSA (NEB)
16.8 ul water

All these ingredients are pre-mixed and use to resuspend the DNA pellet. The digests are incubated at 37 degrees for 2 – 4 hours.

Digestion of the vectors

In parallel, the vector is also digested with the same enzymes. Here is a typical reaction:

For a 50 ul reaction volume:
5 ul vector DNA at 1 ug/ul
5 ul 10 x restriction buffer (here New England Biolabs buffer 4)
1 ul PmeI (10000 U/ml)
1 ul PacI (10000 U/ml)
0.5 ul BSA (NEB)
37.5 ul water

The digests are incubated at 37 degrees for 2 – 4 hours.

Dephosphorylation of the vector

To prevent self-ligation of the vector (which occurs if the vector gets digested by only one of the enzymes), the vector is dephosphorylated with Calf Intestine Phosphatase (CIP; NEB). Simply add 1 ul of the CIP to the 50 ul vector digestion, mix well, spin briefly, and incubate at 37 degrees for one more hour. DO NOT DEPHOSPHORYLATE THE INSERTS!

Gel purification

Prepare a clean 1 – 1.2% agarose-TAE gel containing EtBr, using wells big enough to contain the reactions (about 25 ul for the insert), and enough wells to leave at least one empty lane between each loaded lane (to prevent cross-contamination). Add loading buffer to the digested samples, and load the entire reaction on the prepared gel. Let the gel migrate sufficiently (the xylene cyanol should be at least 2 cm into the gel, unless very small fragments are to be purified.

Bring the gel to the dark room, and transfer to a few layers of Saran Wrap. Use an hand-heald "long UV" lamp (which causes less damage to the DNA) to detect the position of the bands. Using CLEAN disposable scalpel blades (change blade in between samples) to isolate the gel fragments containing the DNA of interest, and transfer into clean 1.5 ml tubes. After all the bands have been cut, take a picture of the gel to make sure the size of the bands is OK.

Extraction of the DNA from the gel

I've been using the Geneclean Turbo kit from Qbiogene lately, but the standard Geneclean kit, as well as equivalent products from QIAGEN or Invitrogen also work fine. You should follow the manufacturer's instructions closely.

Ligation

You can follow any standard protocol for the ligation, or even use the new "quick ligation kits" available from NEB or MBI Fermentas. Here is basically how I do it (the respective amounts of vector and insert are from an average experiment: I do not measure these amounts anymore).

For a 5 ul reaction volume:
3 ul insert (10% of the eluate from the Geneclean Turbo prep)
1 ul vector (3% of the eluate from the Geneclean Turbo prep)
0.5 ul 10 x ligase buffer (NEB)
0.5 ul ligase (NEB)

Mix and incubate at room temperature 4 hours to overnight.

Transformation

I usually purchase chemically competent cells. XL10 Gold (Strategene) work really well and one tube (100 ul) allows to perform up to 15 transformations. For each transformation, I use 0.2 – 0.5 ul of the ligation, and 7.5 – 15 ul of the competent cells containing the amount of 2-mercaptoethanol suggested by the manufacturer. The cells / DNA are incubated on ice for 30 minutes, heat-shocked at 42 degrees for 30 seconds, and incubated on ice for 1 minute. 200 ul of medium (SOC, LB, Circlegrow, etc. without antibiotics) is added, and the cells are incubated at 37 degrees for 40 minutes. The entire reaction is then plated onto LB + 100 ug / ml ampicillin, and the plates are incubated overnight at 37 degrees.

Minipreps

Individual colonies (2 – 4 per construct) are picked from the plate and transferred into 5 ml of Circlegrow (Qbiogene) + 100 ug/ ml ampicillin (LB, 2YT or any similar medium can also be used). The cells are grown overnight, with agitation, at 37 degrees. I usually perform minipreps on 1.5 ml bacterial culture, using the QIAGEN spin procedure, which gives DNA of really high quality. You can just follow the manufacturer's instructions. I then test the constructs for the presence of inserts by performing a double digest with the enzymes used for cloning.

For a 5 ul reaction
4.15 ul DNA
0.5 ul 10 x restriction buffer (here NEB buffer 4)
0.05 ul BSA (NEB)
0.15 ul PmeI
0.15 ul PacI

The last 4 ingredients can be pre-mixed and added to the DNA to be analyzed. The digest can be incubated for 1 hour at 37 degrees, and analyzed on a 1 – 1.2%

agarose-TAE gel containing EtBr. Negative minipreps are discarded. One or two positives minipreps are further analyzed by sequencing – see section 2) d.