InterPlay Mammalian TAP System

Instruction Manual

Catalog #240103 InterPlay N-terminal Mammalian TAP System
#240104 InterPlay C-terminal Mammalian TAP System
#240101 InterPlay N-terminal Mammalian TAP Vectors
#240102 InterPlay C-terminal Mammalian TAP Vectors
#240107 InterPlay TAP Purification Kit
#240099 InterPlay TAP Purification Buffer Kit

Revision D.0

For Research Use Only. Not for use in diagnostic procedures.

240101-12

Agilent Technologies

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Agilent. Agilent shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

ORDERING INFORMATION AND TECHNICAL SERVICES

Email

techservices@agilent.com

World Wide Web

www.genomics.agilent.com

Telephone

Location	Telephone
United States and Canada	800 227 9770
Austria	01 25125 6800
Benelux	02 404 92 22
Denmark	45 70 13 00 30
Finland	010 802 220
France	0810 446 446
Germany	0800 603 1000
Italy	800 012575
Netherlands	020 547 2600
Spain	901 11 68 90
Sweden	08 506 4 8960
Switzerland	0848 8035 60
UK/Ireland	0845 712 5292
All Other Countries	Please visit *www.agilent.com/genomics/contactus*

InterPlay Mammalian TAP System

CONTENTS

InterPlay Mammalian TAP System*

MATERIALS PROVIDED

Materials provided	Catalog #240103	Catalog #240104	Catalog #240101	Catalog #240102	Catalog #240107	Catalog #240099
TAP expression vectors						
pNTAP-A expression vector (1 µg/µl)	20 µg	—	20 µg	—	—	—
pNTAP-B expression vector (1 µg/µl)	20 µg	—	20 µg	—	—	—
pNTAP-C expression vector (1 µg/µl)	20 µg	—	20 µg	—	—	—
pCTAP-A expression vector (1 µg/µl)	—	20 µg	—	20 µg	—	—
pCTAP-B expression vector (1 µg/µl)	—	20 µg	—	20 µg	—	—
pCTAP-C expression vector (1 µg/µl)	—	20 µg	—	20 µg	—	—
TAP expression control vectors						
pNTAP-Mef2a expression control vector (1 µg/µl)	30 µg	30 µg	30 µg	30 µg	—	—
pCMV-Tag2-Mef2c expression control vector (1 µg/µl)	30 µg	30 µg	30 µg	30 µg	—	—
InterPlay TAP purification kit (#240107)[a]						
Lysis buffer	50 ml	50 ml	—	—	50 ml	50 ml
0.5 M EDTA	200 µl	200 µl	—	—	200 µl	200 µl
14.4 M β-mercaptoethanol	69 µl	69 µl	—	—	69 µl	69 µl
Streptavidin resin (#240105)[b]	1.25 ml	1.25 ml	—	—	1.25 ml	—
Streptavidin binding buffer (SBB)	25 ml	25 ml	—	—	25 ml	25 ml
Streptavidin elution buffer (SEB)	5 ml	5 ml	—	—	5 ml	5 ml
Streptavidin supernatant supplement	100 µl	100 µl	—	—	100 µl	100 µl
MS-Grade calmodulin resin (#240106)[b]	0.625 ml	0.625 ml	—	—	0.625 ml	—
Calmodulin binding buffer (CBB)	40 ml	40 ml	—	—	42 ml	40 ml
Calmodulin elution buffer (CEB)	2.5 ml	2.5 ml	—	—	2.5 ml	2.5 ml

[a] The InterPlay TAP purification kit provides reagents sufficient for 5 purifications (1×10^8 cells/purification).

[b] The resin volume listed applies to the settled resin only. The resin is supplied as a 50% slurry, bringing the total volume to twice the amount listed.

* Patent Pending

Revision D.0

STORAGE CONDITIONS

All vectors: Store at –20°C upon receipt.
All other components: Store at 4°C upon receipt. Do not freeze.

ADDITIONAL MATERIALS REQUIRED

Anti-Calmodulin binding protein epitope tag antibody (Upstate Catalog #07-482)
Anti-FLAG® M2 antibody (used to detect expression from the pCMV-Tag2-Mef2c control vector; Agilent catalog #200471 or 200472)
Mammalian cell transfection reagent
Media for cell growth and transfection
Microcon® YM-10 centrifugal filter unit (Millipore Catalog #42421)
Protease inhibitors [e.g., Protease inhibitor cocktail (Sigma Catalog #P8340), PMSF (Sigma Catalog #P7626), etc.]

NOTICE TO PURCHASER

For Research Use Only - Not for any clinical, therapeutic, or diagnostic use in humans or animals.
The purchase of the products containing the Streptavidin Binding Peptide Tag conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to resell, repackage, or sublicense) to use this product solely for research purposes. No other right or license is granted to the buyer whether expressly, by implication, by estoppel or otherwise. In particular, the purchase of this product does not include or carry any right or license to use, develop, or otherwise exploit this product commercially, and no rights are conveyed to the buyer to use this product or components of this product for any other purposes. This product is sold pursuant to an agreement with The General Hospital Corporation, and The General Hospital Corporation reserves all rights relating to this product, except as expressly set forth above. For information regarding obtaining a license for uses other than research purposes, please contact The General Hospital Corporation at (617) 726-8608.

INTRODUCTION

Identification of protein–protein interactions is at the core of understanding biological processes occurring in living cells. Traditionally, potential interacting proteins have been identified by genetic methods (two–hybrid screens) with subsequent verification of the interaction by co-immunoprecipitation. While this method has been successful for detection of two interacting proteins, it is of limited utility when more complex protein aggregates such as ribosomes, spliceosome complexes, or transcription complexes are investigated. To overcome this limitation, an alternative method was developed for purification of yeast protein complexes.[1, 2] This tandem affinity purification (TAP) method combines purification of a protein complex of interest using affinity purification tags with subsequent mass spectrometry identification of unknown protein complex components. The key feature of this technology is the use of two different affinity purification tags that are fused to at least one known component of the protein complex of interest by genetic methods. Performing two consecutive purification steps using affinity purification tags that have gentle washing and elution conditions allows for isolation without disruption of the targeted complex.

The Agilent Interplay TAP systems improve upon the original published protocol with two peptide tags that allow for isolation of exceptionally clean proteins without disrupting the targeted complex (see Figures 1 and 2). The SBP tag, a synthetic sequence isolated from a random peptide library, has a high affinity for the streptavidin resin provided (~2×10^{-9} M), and can be effectively eluted with biotin.[3, 4] The CBP tag, derived from a C-terminal fragment of muscle myocin light-chain kinase, has a high affinity for the calmodulin resin provided (~1×10^{-9} M) in the presence of calcium. Upon removal of calcium with a chelating agent, recovery of the tagged protein from the resin is achieved.[5–7] Both tags can be eluted from their respective resins with gentle washing and small molecule elution conditions thus increasing the amount and purity of the resulting purified protein complex. Protease digestion is not required to recover the interacting protein partners.

We have validated the system by co-transfecting mammalian cells with vectors containing known interacting proteins. Since members of the myocin enhancing factor 2 (MEF2) family are known to interact, MEF2a and MEF2c are used to demonstrate tandem affinity purification. The pNTAP-Mef2a vector contains MEF2a, tagged at the N-terminus with SBP and CBP affinity tags. The pCMV-Tag2-Mef2c vector contains MEF2c with an N-terminal FLAG tag. When co-transfected, the expressed MEF2 proteins from each vector interact. Following transfection, cells are harvested and the proteins are purified using streptavidin resin followed by calmodulin resin (see Figure 2). Gentle washing and elution conditions allow the protein–protein interactions to remain intact. The purified protein complex is analyzed by SDS-PAGE and the MEF2c is detected by Western blotting using an antibody to the FLAG peptide, indicating that it interacted and co-purified with its partner, MEF2a. The proteins are further characterized by in-gel digestion with trypsin followed by mass spectrometry analysis, confirming interaction.

SBP tag	**MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPSGGCKLG**
CBP tag	**KRRWKKNFIAVSAANRFKKISSSGAL**

FIGURE 1 Amino acid sequences of the streptavidin (SBP) and calmodulin binding peptides (CBP).

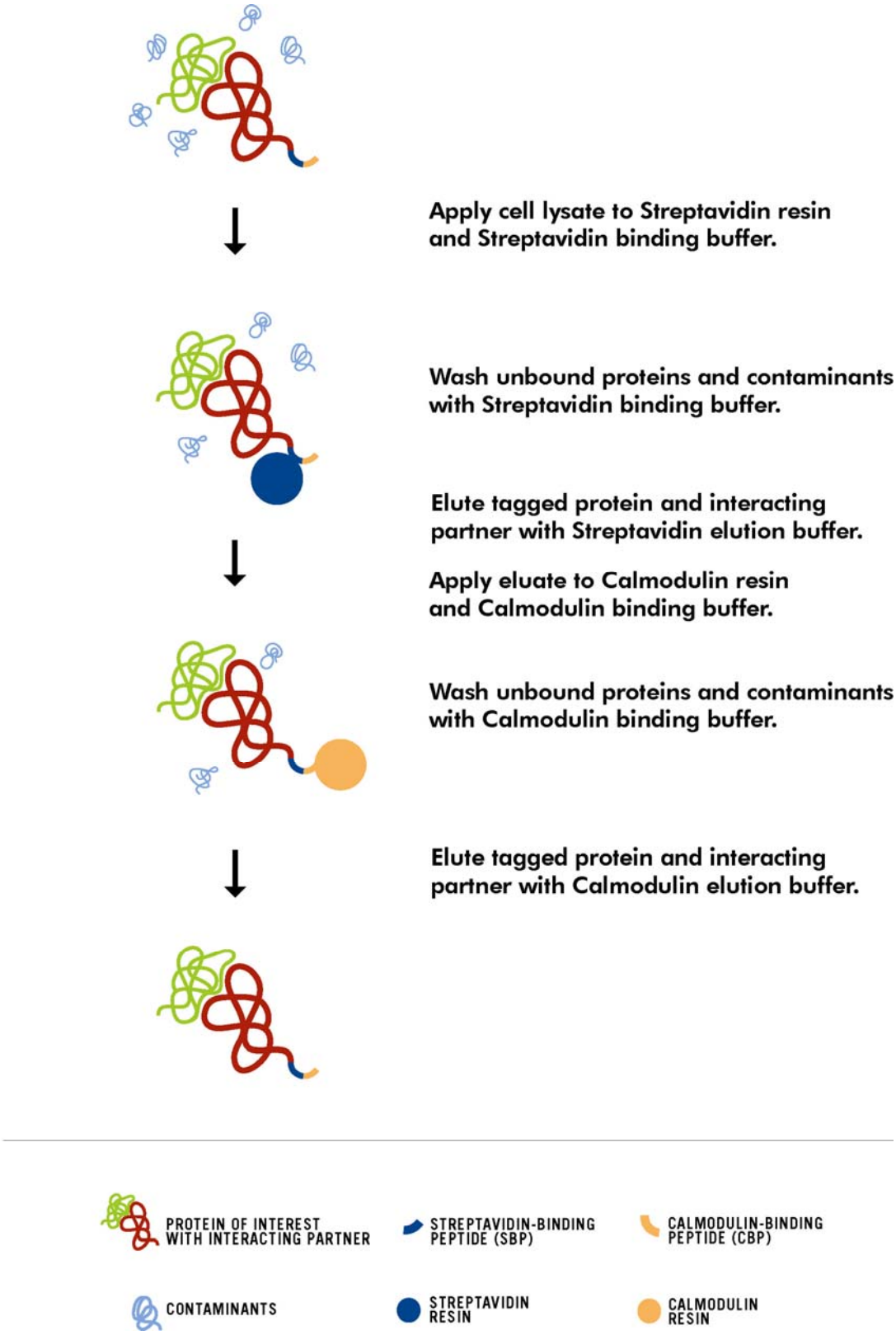


FIGURE 2 Tandem affinity purification of the tagged protein of interest and interacting proteins using streptavidin resin followed by calmodulin resin.

INTERPLAY TAP SYSTEM CONSIDERATIONS

For maximal protein-protein interactions, we strongly recommend using stable expression of the TAP-tagged protein, as opposed to transient expression. Stable transfection facilitates constant bait expression levels, while transient transfection allows dilution of the bait plasmid and its expression products over time. However, transient transfections can be performed to verify successful bait expression and to test antibody detection prior to expressing the TAP-tagged bait from stably-expressing cells for purification.

Ectopic expression of the TAP-tagged bait protein in the host cell results in competition of the TAP-tagged bait with the untagged endogenous protein. To ensure sufficient recovery of interacting proteins with the TAP-tagged bait, the TAP-tagged bait should be expressed at a level equal to or greater than the level of the endogenously expressed protein.

Although the TAP procedure results in extraordinary enrichment of the TAP-tagged bait and the associated proteins, some interactions (such as those with heat shock proteins) may be nonspecific. In order to confidently identify bait-specific interactions, performing a control purification with an unrelated bait is strongly recommended.

TAP-tagged bait proteins may behave differently depending on which terminus is fused to the TAP tag. Ideally, the best terminus for placement of the SBP and CBP tandem tags should be determined empirically. However, structure databases can provide valuable information about the accessibility of the respective termini (e.g., the NCBI structure database; *http://www.ncbi.nlm.nih.gov/Structure*). In general, tag placement at the N-terminus of the protein results in higher expression than placement at the C-terminus.

If the bait protein forms homodimers, there will be a tendency to preferentially purify tagged bait homodimers, thus potentially interfering with the purification of heterodimerizing target proteins. Certain modifications of the bait protein (mutations or truncations) may eliminate homodimerization, but this approach may also eliminate the formation of heterodimers.

DESCRIPTION OF THE VECTORS

In each of the TAP expression vectors, eukaryotic expression is driven by the human cytomegalovirus (CMV) immediate early promoter which directs constitutive expression of cloned inserts in a wide variety of mammalian cell lines.

Each vector contains the neomycin/kanamycin-resistance gene under control of the prokaryotic β-lactamase promoter to provide kanamycin resistance in bacteria and the SV40 early promoter to provide G418 resistance in mammalian cells.

pNTAP Expression Vectors

The pNTAP expression vector is used to clone a gene of interest with the SBP and CBP affinity tags adjacent to the 5´ end of the gene. The pNTAP vector is available in three different reading frames to simplify subcloning. These reading frames, designated as A, B, and C, differ only by one or two bases. Thus, each pNTAP vector has a reading frame that will allow cloning a gene of interest so that it is fused correctly with the affinity tags. The multiple cloning site (MCS) of the pNTAP vectors allows for a variety of cloning strategies, resulting in fusion of the TAP tags to the N-terminus of the protein of interest. A Kozak consensus sequence provides optimal expression of the fusion protein.[8] (See Figure 3.)

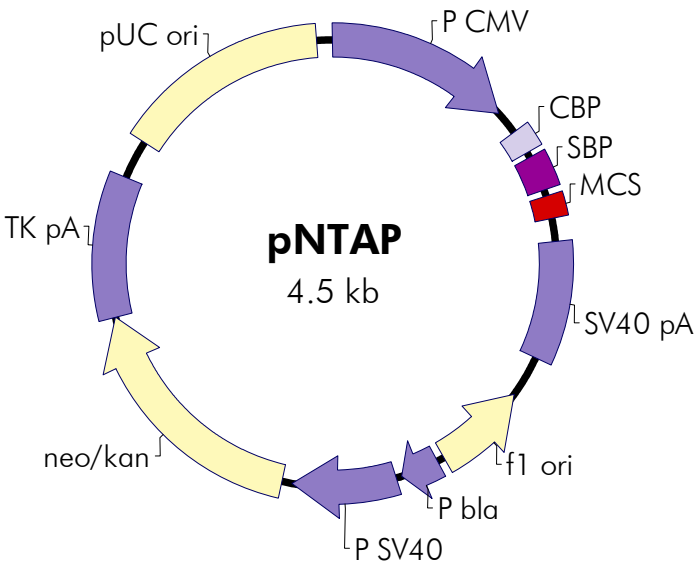
pCTAP Expression Vectors

The pCTAP expression vector is used to clone a gene of interest with the SBP and CBP affinity tags adjacent to the 3´ end of the gene. The pCTAP vector is available in three different reading frames to simplify subcloning. These reading frames, designated as A, B, and C, differ only by one or two bases. Thus, each pCTAP vector has a reading frame that will allow cloning a gene of interest so that it is fused correctly with the affinity tags. The multiple cloning site (MCS) of the pCTAP vectors allows for a variety of cloning strategies, resulting in fusion of the TAP tags to the C-terminus of the protein of interest. (See Figure 4.)

pNTAP-Mef2a and pCMV-Tag2-Mef2c Expression Control Vectors

The pNTAP-Mef2a control vector contains the MEF2a ORF tagged at the N-terminus with SBP and CBP tags. The pCMV-Tag2-Mef2c control vector contains the MEF2c ORF tagged at the N-terminus with the FLAG tag. (See Figure 5.) When co-transfected, expressed MEF2 proteins from each vector interact and are co-purified via tandem affinity purification using the streptavidin and calmodulin resins. The purified MEF2c protein is detected by Western blotting using an antibody to the FLAG peptide, indicating that it interacted and co-purified with its partner, MEF2a. The control proteins can be further characterized by mass spectrometry analysis, validating interaction.

pNTAP Vector Map



pNTAP Multiple Cloning Site Region
(sequence shown 889–1051)

SBP tag (3′ end)

```
P   S   G   G   C   K   L   G
CCC TCC GGC GGC TGC AAG CTG GGC * GCC CGG GCG GAT CCC CCG GGC TGC AGG AAT TC...
                                  Srf I     BamH I          Pst I     EcoR I

   EcoR V    Hind III                Acc I/Sal I  Xho I        Apa I
...G ATA TCA AGC TTA TCG ATA CCG TCG ACC TCG AGG GGG GCC CCG GTA CCT...
```

T7 promoter

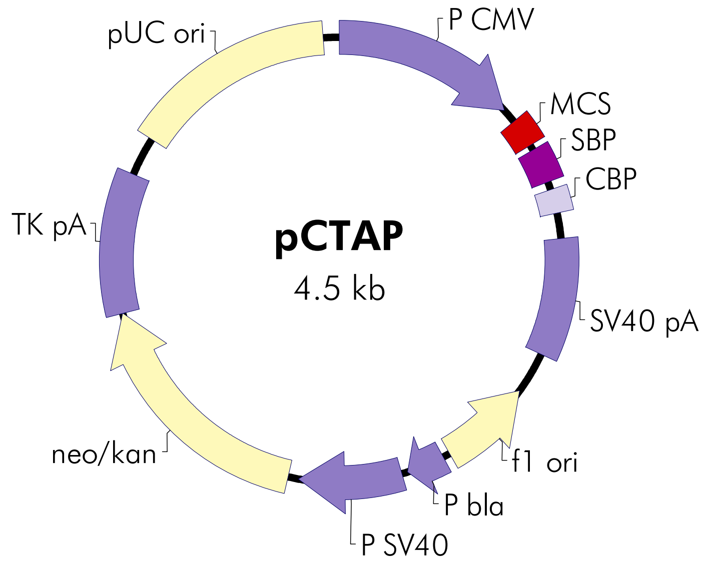
```
...TAATTAATTAAGGTACCAGGTAAGTGTACCCAATTCGCCCTATAGTGAGTCGTATTAC
   MULTIPLE STOP CODONS
```

* In pNTAP-A, no bases inserted; in pNTAP-B, T inserted; in pNTAP-C, TT inserted

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5′ AATTAACCCTCACTAAAGGG 3′]	620–639
calmodulin binding peptide (CBP)	682–759
streptavidin binding peptide (SBP)	778–912
multiple cloning site	913–986
T7 promoter and T7 primer binding site [5′ GTAATACGACTCACTATAGGGC 3′]	1030–1051
SV40 polyA signal	1063–1446
f1 (–) origin of ss-DNA replication	1584–1890
bla promoter	1915–2039
SV40 promoter	2059–2397
neomycin/kanamycin resistance ORF	2432–3223
HSV-thymidine kinase (TK) polyA signal	3224–3682
pUC origin	3811–4478

FIGURE 3 Circular map of the pNTAP A–C expression vectors. The positions listed in the table above correspond to pNTAP-A. See Table I for a complete list of feature positions for the pNTAP A–C vectors.

pCTAP Vector Map



pCTAP Multiple Cloning Site Region
(sequence shown 620–797)

Sac I, BstX I, Sac II, Not I, Srf I

T3 promoter

A ATT AAC CCT CAC TAA AGG GAA CAA AAG CTG GAG CTC CAC CGC GGT GGC GGC CGC TCT AGC...

STOP

BamH I, Pst I, EcoR I, EcoR V, Hind III, Acc I/Sal I

...CCG GGC GGA TCC CCC GGG CTG CAG GAA TTC GAT ATC AAG CTT ATC GAT ACC GTC GAC *...

Xho I

SBP tag (5´ end)

M D E K T T G W R G G H

...CTC GAG GGA AGC GGT AGC GGT ACC ATG GAC GAG AAG ACC ACC GGC TGG CGG GGC GGC CAC

* In pCTAP-A, no bases inserted; in pCTAP-B, A inserted; in pCTAP-C, AA inserted

Note ***The presence of a stop codon (TAG) in-frame with the SBP and CBP affinity tags must be considered when inserting genes into the MCS of the pCTAP-C vector. Do not use the Sac I, BstX I, Sac II, or Not I sites for cloning unless the cloning strategy removes the stop codon by double-digestion using one of these upstream sites plus a site downstream of the stop codon.***

Feature	Nucleotide Position
CMV promoter	1–602
T3 promoter and T3 primer binding site [5´ AATTAACCCTCACTAAAGGG 3´]	620–639
multiple cloning site	651–743
streptavidin binding peptide (SBP)	762–896
calmodulin binding peptide (CBP)	903–980
T7 promoter and T7 primer binding site [5´ GTAATACGACTCACTATAGGGC 3´]	1032–1053
SV40 polyA signal	1065–1448
f1 (–) origin of ss-DNA replication	1586–1892
bla promoter	1917–2041
SV40 promoter	2061–2399
neomycin/kanamycin resistance ORF	2434–3225
HSV-thymidine kinase (TK) polyA signal	3226–3684
pUC origin	3813–4480

FIGURE 4 Circular map of the pCTAP A–C expression vectors. The positions listed in the table above correspond to pCTAP-A. See Table I for a complete list of feature positions for the pCTAP A–C vectors.

TABLE I

Locations of Features for the pNTAP-A–C and pCTAP-A–C Vectors

Feature	pNTAP-A	pNTAP-B	pNTAP-C	pCTAP-A	pCTAP-B	pCTAP-C
P CMV	1–602	1–602	1–602	1–602	1–602	1–602
CBP	682–759	682–759	682–759	903–980	904–981	905–982
SBP	778–912	778–912	778–912	762–896	763–897	764–898
MCS	913–986	914–987	915–988	651–743	651–744	651–745
SV40 pA	1063–1446	1064–1447	1065–1448	1065–1448	1066–1449	1067–1450
f1 origin	1584–1890	1585–1891	1586–1892	1586–1892	1587–1893	1588–1894
P *bla*	1915–2039	1916–2040	1917–2041	1917–2041	1918–2042	1919–2043
P SV40	2059–2397	2060–2398	2061–2399	2061–2399	2062–2400	2063–2401
neo/kan	2432–3223	2433–3224	2434–3225	2434–3225	2435–3226	2436–3227
TK pA	3224–3682	3225–3683	3226–3684	3226–3684	3227–3685	3228–3686
pUC origin	3811–4478	3812–4479	3813–4480	3813–4480	3814–4481	3815–4482

Control Vector Maps

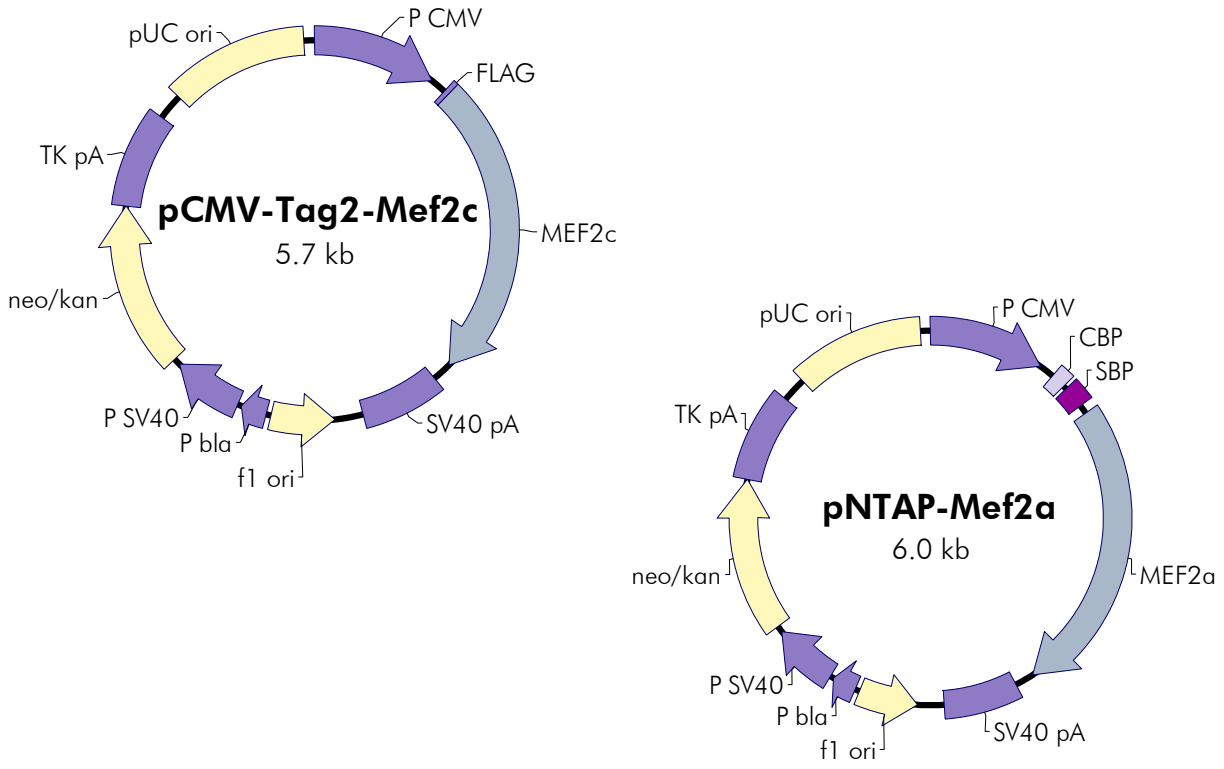


FIGURE 5 Circular maps of the control vectors.

PREPARING THE VECTOR

Preparing the pNTAP/pCTAP Expression Vector

- When cloning into the pCTAP vector, design the insert to contain a Kozak sequence. A complete Kozak sequence includes CC$\frac{A}{G}$CC**ATG**G, although CC**ATG**G, or the core **ATG**, is sufficient.

- In-frame stop codons are provided in each of the vectors. The N-terminal fusion vector pNTAP contains a multiple stop codon cassette that introduces a stop codon downstream of the insert in all three possible reading frames. The C-terminal fusion vector pCTAP contains in-frame stop codons immediately downstream of the epitope tags.

- If the insert DNA is phosphorylated, dephosphorylate the digested vectors with CIAP prior to ligation. If more than one restriction enzyme is used, the background can be reduced further by gel purification of the vector.

- After purification and ethanol precipitation of the vector DNA, resuspend in a volume of TE buffer (see *Preparation of Media and Reagents*) that will allow the concentration of the plasmid DNA to be the same as the concentration of the insert DNA (~0.1 µg/µl).

- The following are restriction enzymes with compatible ends to cloning sites in the TAP vectors:

Enzyme	Compatible enzymes
BamH I	*Bcl* I, *Bgl* II, *Bst* I, *Mob* I, *Sau3*A I, *Xho* II
*Eco*R I	*Mun* I, *Mfe* I
*Eco*R V	Blunt ends
Not I	*Eae* I, *Eag* I, *Gdi* II, *Xma* III
Pst I	*Hgi*A I[a], *Nsi* I
Sal I	*Ava* I[a], *Pae*R71, *Xho* I
Srf I	Blunt ends
Xho I	*Ava* I[a], *Pae*R71, *Sal* I

[a] A subset of the sites apply.

Ligating the Insert

The ideal insert-to-vector molar ratio of DNA is variable; however, a reasonable starting point is 2:1 insert-to-vector. The ratio is calculated using the following equation:

$$X\ \mu\text{g of insert required} = \frac{(\text{Number of base pairs of insert})\,(\,0.1\,\mu\text{g of TAP vector})}{\sim 4.5\,\text{kb of TAP vector}}$$

where *X* is the quantity of insert (in micrograms) required for a 1:1 insert-to-vector molar ratio. Multiply *X* by 2 to get the quantity of insert required for a 2:1 ratio.

1. Prepare three control and two experimental 10-µl ligation reactions by adding the following components to separate sterile 1.5-ml microcentrifuge tubes:

Ligation reaction components	Control			Experimental	
	1[a]	2[b]	3[c]	4[d]	5[d]
Prepared TAP vector (0.1 µg/µl)	1.0 µl	1.0 µl	0.0 µl	1.0 µl	1.0 µl
Prepared insert (0.1 µg/µl)	0.0 µl	0.0 µl	1.0 µl	*Y* µl	*Y* µl
rATP [10 mM (pH 7.0)]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
Ligase buffer (10×)[e]	1.0 µl	1.0 µl	1.0 µl	1.0 µl	1.0 µl
T4 DNA ligase (4 U/µl)	0.5 µl	0.0 µl	0.5 µl	0.5 µl	0.5 µl
Double-distilled (ddH_2O) to 10 µl	6.5 µl	7.0 µl	6.5 µl	*Z* µl	*Z* µl

[a] This control tests for the effectiveness of the digestion and the CIAP treatment. Expect a low number of transformant colonies if the digestion and CIAP treatment are effective.

[b] This control indicates whether the plasmid is cleaved completely or whether residual uncut plasmid remains. Expect an absence of transformant colonies if the digestion is complete.

[c] This control verifies that the insert is not contaminated with the original plasmid. Expect an absence of transformant colonies if the insert is pure.

[d] These experimental ligation reactions vary the insert-to-vector ratio. Expect a majority of the transformant colonies to represent recombinants.

[e] See *Preparation of Media and Reagents*.

2. Incubate the reactions for 2 hours at room temperature or overnight at 4°C. For blunt-end ligation, reduce the rATP to 5 mM and incubate the reactions overnight at 12–14°C.

Transformation

Transform competent bacteria with 1–2 µl of the ligation reaction, and plate the transformed bacteria on LB-kanamycin agar plates (see *Preparation of Media and Reagents*). Please see reference 9 for a protocol for producing and transforming competent cells. (Alternatively, ready-to-use Agilent competent cells with transformation efficiencies ≥5 × 10^9 cfu/µg are also available.)

Verification of Insert Size, Orientation, and Percentage

Examine DNA from individual colonies to identify plasmids containing inserts of the correct size and orientation by PCR directly from the colony or by restriction analysis. If desired, calculate the percentage of vectors with inserts after analysis of a sufficient number of colonies.

PCR Amplification of DNA from Individual Colonies

The presence and size of a DNA insert in a vector may be determined by PCR amplification of DNA from individual colonies.

1. Prepare a PCR amplification reaction containing the following components:

   4.0 µl of 10× *Taq* DNA polymerase buffer
   0.4 µl of dNTP mix (25 mM each dNTP)
   0.8 µl of 10 µM 5′ primer (see table below for sequence)
   0.8 µl of 10 µM 3′ primer (see table below for sequence)
   0.4 µl of 10% (v/v) Tween® 20
   2.0 U of *Taq2000* DNA polymerase
   dH_2O to a final volume of 40 µl

Primers for Insert Amplification from the pNTAP Vector

Primer	Nucleotide sequence (5′ to 3′)
5′ primer (T3)	5′ AATTAACCCTCACTAAAGGG 3′
3′ primer (T7)	5′ GTAATACGACTCACTATAGGGC 3′

Primers for Insert Amplification from the pCTAP Vector

Primer	Nucleotide sequence (5′ to 3′)
5′ primer	5′ GGTCTATATAAGCAGAGCTGGT 3′
3′ primer (T7)	5′ GTAATACGACTCACTATAGGGC 3′

2. Touch the transformed colonies with a sterile toothpick and streak onto antibiotic-containing patch plates for future reference. Incubate the patch plates at 37°C overnight. Immediately following the streak onto plates, swirl this same toothpick into a separate PCR reaction tube.

3. Gently mix each reaction, overlay each reaction with 30 µl of mineral oil and perform PCR using the following cycling parameters:

Cycling Conditions for RoboCycler Temperature Cyclers and Single-Block Thermal Cyclers

Number of cycles	Temperature	Length of time
1 cycle	94°C	3 minutes
30 cycles[a]	94°C	30 seconds
	55°C	30 seconds
	72°C	1 minute
1 cycle	72°C	5 minutes

[a] This segment must run for at least 30 cycles.

4. Analyze the PCR products using standard 1% (w/v) agarose gel electrophoresis. Because the PCR/sequencing primers are located on both sides of the MCS, the expected size of the PCR product in the pNTAP vector is 432 bp plus the size of the insert. The expected size of the PCR product in the pCTAP vector is 498 bp plus the size of the insert.

   Note *Additional information can be obtained by further restriction analysis of the PCR products if a restriction site was included in the insert.*

5. Following identification of colonies containing the correct insert, return to the patch plates made in step 2 above and pick a portion of each of the positive colonies into aliquots of LB–kanamycin broth (see *Preparation of Media and Reagents*). Grow overnight at 37°C with shaking.

6. The next morning, purify the plasmid DNA from the liquid cultures. The PCR amplification primers should be used in separate reactions to determine the nucleotide sequence of the insert. It is important to sequence the vector insert prior to transfection to confirm the insert sequence and cloning success.

MAMMALIAN CELL TRANSFECTION

After the nucleotide sequence of the gene insert is confirmed, prepare DNA of appropriate amount and purity for the mammalian cell transfection procedure.

Transfecting the Experimental and Control Vectors

The efficiency of transfection will vary depending on DNA quality and the host cell line used. Choose a transfection method that will give the highest transfection efficiency for the chosen cell line. Protocols for transfection of mammalian cell lines can be found in Sambrook, *et al*. (1989).[10] The amount of TAP vector required for expression of the gene of interest may vary depending upon the expression level of the gene, the transfection efficiency, and the host cell line used. Varying the amount of vector may be required to achieve optimal gene expression. In most cases, expression of the genes should be detectable 24–72 hours after transfection. The length of time is dependent upon mRNA and protein stability. A time course is recommended to identify the optimal time to detect gene expression. Perform the appropriate assays to determine protein expression levels (e.g., Western blotting).

Protein purification from cells that stably express the TAP-tagged bait protein is strongly recommended. Select for TAP-tagged bait plasmid-containing transfectants using the G418 antibiotic. Mammalian cell lines are not equally sensitive to the G418 antibiotic–the minimal lethal concentration can range from 100 µg/ml to 1 mg/ml. The antibiotic concentration to be used for selection must be determined for each cell line before beginning the experiment. Consult the available literature on the sensitivity of your cell line to G418. If no sensitivity information is available, perform a kill-curve by growing cultures in a multiwell plate using a range of antibiotic concentrations in the individual wells. The optimal concentration is the lowest one that kills all of the cells within 10–14 days. Rapidly dividing cells may be killed more readily since theG418 antibiotic appears to act mainly on dividing cells.

When transfecting COS-7 cells, grow the cells in DMEM + 10% FBS and antibiotics in a T-175 flask overnight to a confluency of 50–60%. Perform a lipid-based transfection and incubate the cells at 37°C for 48 hours. Wash the cells with PBS three times and remove the PBS from the cells. Add 5 ml of ice cold PBS to each flask containing approximately 1×10^7 cells each and transfer the cell suspension(s) to a 15 ml conical tube. Centrifuge the cells for 10 minutes at 1500 × g. Remove the PBS before purifying the protein complexes.

Transfecting the Control Vectors

Note *When performing the TAP purification protocol using COS-7 cells co-transfected with the control vectors, it is necessary to process cells from 10 T-175 flasks (containing 1 × 10^7 cells each) in order to obtain sufficient amounts of protein for mass spectrometry analysis. A single flask (containing 1 × 10^7 cells) yields sufficient protein for Western blot analysis.*

Co-transfect mammalian cells with 30 µg of each control vector per T-175 flask using the optimal transfection method and conditions for the host cell line used. Include a no-DNA transfection control. The positive control reactions should show expression of the control genes 48 hours after transfection.

Detecting Gene Expression

Approximately 48 hours post-transfection, harvest the cells and assay for protein expression by performing a Western blot. When detecting expression from the pCMV-Tag2-Mef2c control vector, the MEF2c protein is detected using an antibody to the FLAG peptide at a 1:500 dilution.

Lysis buffer

Prepare the lysis buffer by adding protease inhibitors (see guidelines below), then keep the prepared buffer at 4°C. Prepare only the amount of lysis buffer required for one day of experimental work.

For example, for each milliliter of lysis buffer, add 10 µl of a protease inhibitor cocktail (Sigma Catalog #P8340) and 10 µl of 100 mM of PMSF (Sigma Catalog #P7626). The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.

Streptavidin binding buffer (SBB)

Prepare the SBB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of SBB. Mix the buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation. However, the prepared buffer may be stored at 4°C for up to two weeks.

Note *To further prevent protein degradation, 100 µl of protease inhibitor cocktail and 100 µl of 100 mM of PMSF per 10 ml of prepared SBB may be added. The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.*

Streptavidin elution buffer (SEB)

The SEB must be prepared and kept at 4°C and must be protected from light. Prepare only the amount of buffer required for one day of experimental work. Prepare the SEB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of SEB. Mix the buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation.

Note *To further prevent protein degradation, 100 µl of protease inhibitor cocktail and 100 µl of 100 mM of PMSF per 10 ml of prepared SEB may be added. The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.*

Calmodulin binding buffer (CBB)

Prepare the CBB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of CBB. Mix the buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation. However, the prepared buffer may be stored at 4°C for up to two weeks.

Note *To further prevent protein degradation, 100 µl of protease inhibitor cocktail and 100 µl of 100 mM of PMSF per 10 ml of prepared CBB may be added. The PMSF must be added to the buffer immediately before use. Mix the buffer gently by inverting the tube.*

Calmodulin elution buffer (CEB)

Prepare the CEB by adding 7 µl of 14.4 M β-mercaptoethanol (provided) to 10 ml of CEB. Mix buffer gently by inverting the tube. Best results are obtained when the buffer is used within 24 hours of preparation. However, the prepared buffer may be stored at 4°C for up to two weeks.

Note *Do not add protease inhibitor to the CEB. The protease inhibitor proteins will interfere with subsequent analysis of the TAP-purified proteins.*

TAP PROTOCOL

Notes *Perform all protein purification steps at 4°C to prevent the interacting proteins from dissociating.*

The quantities of the reagents given are recommended for processing 1 × 10^7 cells or 1 × 10^8 cells.

It is recommended to use non-stick tubes during the purification procedure to reduce sample loss due to absorption to the tube walls. This is especially relevant during the calmodulin-resin binding and elution steps when protein concentrations can be relatively low.

When performing the TAP purification protocol using HeLa cells, for example, it is recommended to process cells from at least 10 T-175 flasks (containing approximately 1 x 10^7 cells each) in order to obtain sufficient amounts of protein for mass spectrometry analysis. A single T-175 flask yields sufficient protein for Western blot analysis.

When processing multiple flasks, cell preparations may be scaled-up, pooled, and processed concurrently.

Preparing the Protein Extracts

Step #	Protocol step	For processing 1 × 10^7 cells	For processing 1 × 10^8 cells
1	Resuspend the cells in lysis buffer in a polypropylene centrifuge tube. Subject the cells to three successive rounds of freeze-thawing by incubating the cells on dry ice for 10 minutes or in a –80°C freezer for 20 minutes to freeze the cells, followed by incubating the cells for 10 minutes in cold water to thaw the cells.	1 ml lysis buffer	10 ml lysis buffer
2	Centrifuge the cells at 16,000 × g for 10 minutes to pellet the cell debris. Collect the supernatant.	—	—
3	Transfer a small aliquot of the supernatant to a microcentrifuge tube. Store the aliquot at –20°C for later Western blot analysis (load 5 µl per lane).	—	—
4	To each milliliter of cell lysate, add 0.5 M EDTA and 14.4 M β-mercaptoethanol (provided).	4 µl 0.5 M EDTA 0.7 µl 14.4 M β-ME	40 µl 0.5 M EDTA 7 µl 14.4 M β-ME

Preparing the Streptavidin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
5	Centrifuge the 50% streptavidin resin slurry at 1500 × g for 5 minutes to collect the resin. Discard the supernatant to remove the ethanol storage buffer. Resuspend the resin in 1 ml of SBB. When processing multiple preps, the resin can be pooled and washed in 1 ml of SBB. Repeat this wash step.	50 µl 50% streptavidin resin slurry (25 µl resin) 1 ml SBB/wash	500 µl 50% streptavidin resin slurry (250 µl resin) 1 ml SBB/wash
6	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant and resuspend the resin in SBB.	25 µl SBB	250 µl SBB

Purifying the Protein Complexes Using Streptavidin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
7	Add the washed streptavidin resin (50% slurry) to the lysate from step 4.	50 µl washed resin (50% slurry)	500 µl washed resin (50% slurry)
8	Rotate the tube at 4°C for 2 hours to allow the tagged proteins to bind to the resin.	—	—
9	Collect the resin by centrifugation at 1500 × g for 5 minutes. Remove and freeze the supernatant for possible analysis later. Resuspend the resin in 1 ml of SBB by rotating the tube at 4°C for 5 minutes. Repeat this wash step.	1 ml SBB/wash	1 ml SBB/wash
10	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant. Add SEB to the resin.	100 µl SEB	1 ml SEB
11	Rotate the tube at 4°C for 30 minutes to elute the protein complexes.	—	—
12	Collect the resin by centrifugation at 1500 × g for 5 minutes. Carefully transfer the supernatant to a fresh tube (the supernatant contains the eluted proteins).	—	—
13	Transfer an aliquot of the cell supernatant to a microcentrifuge tube. Store the aliquot at –20°C for later Western Blot analysis (load 10 µl per lane).	—	—
14	Add the streptavidin supernatant supplement to the supernatant. Then, add CBB to the supplemented supernatant.	2 µl streptavidin supernatant supplement 400 µl CBB	20 µl streptavidin supernatant supplement 4 ml CBB

Preparing the Calmodulin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
15	Centrifuge the 50% calmodulin resin slurry at 1500 × g for 5 minutes to collect the resin. Discard the supernatant to remove the ethanol storage buffer. Resuspend the resin in 1 ml of CBB. When processing multiple preps, the resin can be pooled and washed in 1 ml CBB. Repeat this wash step.	25 µl 50% calmodulin resin slurry (12.5 µl resin) 1 ml CBB/wash	250 µl 50% calmodulin resin slurry (125 µl resin) 1 ml CBB/wash
16	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant and resuspend the resin in CBB.	12.5 µl CBB	125 µl CBB

Purifying the Protein Complexes Using Calmodulin Resin

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
17	Add the washed calmodulin resin (50% slurry) to the eluted proteins from step 14.	25 µl washed resin (50% slurry)	250 µl washed resin (50% slurry)
18	Rotate the tube at 4°C for 2 hours to allow the protein complexes to bind to the resin.	—	—
19	Centrifuge the resin at 1500 × g for 5 minutes. Remove and freeze the supernatant for possible analysis later. Resuspend the resin in 1 ml of CBB by rotating the tube at 4°C for 5 minutes. Repeat this wash step.	1 ml CBB/wash	1 ml CBB/wash
20	Collect the resin by centrifugation at 1500 × g for 5 minutes. Discard the supernatant. Proceed to steps 21a or 21b to separate the protein complexes from the calmodulin resin, depending on the downstream methods of analysis chosen. If the purified protein is required to be in native configuration for subsequent applications, such as determination of enzymatic activity, elute the purified protein from the calmodulin resin as described in step 21a. If the protein is analyzed using other methods, such as mass spectroscopy or immunoblotting, proceed to the boiling method in step 21b.	—	—

Step #	Protocol step	For processing 1×10^7 cells	For processing 1×10^8 cells
21a	**Elution method:** To separate the protein complexes from the resin by elution, add CEB to the collected calmodulin resin. Rotate the tube at 4°C for 30 minutes to elute the protein complexes. Collect the resin by centrifugation at 1500 × g for 5 minutes. Carefully transfer the supernatant to a fresh tube. The supernatant contains the Tandem Affinity Purified protein complexes. Addition of 10% glycerol may help to prevent loss of enzyme activity due to freeze–thaw cycles. The supernatant may be stored at –20°C for future processing.	50 µl CEB	500 µl CEB
21b	**Boiling method:** Resuspend the resin in an equal volume of 1× loading buffer suitable for the separation system used in the subsequent analysis. Boil the resuspended resin for 5 minutes. Carefully transfer the supernatant to a fresh tube. The supernatant contains the Tandem Affinity Purified protein complexes. The supernatant may be stored at –20°C for future processing. It may be necessary to reduce the volume of the supernatant in order to load a sufficient amount on a gel, for analysis by mass spectroscopy. A protocol for concentrating the supernatant is given in the *Appendix*. **Note** *It is essential to use the MS-grade calmodulin resin provided with this procedure.*	—	—

DETECTION OF PURIFIED PROTEINS

TAP-purified proteins may be analyzed by Western blotting and mass spectrometry.

Western Blotting

To detect the purified proteins, resolve the protein preparation by SDS-PAGE. Experimental proteins may be detected using protein-specific antibodies, or by detection with the anti-calmodulin binding protein epitope tag antibody (Upstate Catalog #07-482). The anti-FLAG antibody used at a 1:500 dilution will detect the MEF2c protein (53 kDa), expressed from the pCMV-Tag2-Mef2c control vector.

Notes *Anti-FLAG M2 antibody (Agilent Catalog #200471 or 200472) is available for detecting FLAG-tagged proteins.*

Some TAP-tagged baits may not efficiently elute from the calmodulin resin. In this case, boil the beads for 5 minutes in 1× loading buffer before loading onto the gel.

Mass Spectrometry

To characterize the TAP-purified proteins, concentrate samples by TCA precipitation and resolve the interacting proteins by SDS-PAGE. Perform in-gel digestion on individual protein bands with trypsin and characterize the corresponding peptide fingerprints by mass spectrometry.

APPENDIX: CONCENTRATING THE TAP PROTEIN COMPLEX-CONTAINING SUPERNATANT

Sample concentration using Millipore Amicon® Microcon® devices (specifically, the Microcon® YM-10 centrifugal filter unit) is recommended to achieve maximum retention of purified proteins. Consult the manufacturer's product specifications and guidelines prior to use.

Unit Pre-treatment

In order to increase recovery, pre-treat the plastic device before use in order to block available binding sites.

1. Insert the sample reservoirs into vials.

2. Pipet 0.5 ml of a passivation solution (see *Preparation of Media and Reagents* for a list of suggested passivation solutions) into each sample reservoir. Cap and soak for at least 2 hours (or overnight) at room temperature.

3. Uncap and rinse all devices thoroughly with distilled water.

4. To remove any residual solution, add 0.5 ml of distilled water to each device, close the cap, and spin at 1,000 × *g*. Repeat.

5. To eliminate the remaining water, invert the sample reservoir in the vial, and then spin once more at 1,000 × *g* for 3 minutes.

6. If not using the devices immediately, add 100 µl of distilled water to the assembled sample reservoirs. Cap and store at 4°C.

Protein Concentration

Note *Use non-stick tubes during the concentration procedure to reduce loss of protein to absorption to the tube walls.*

1. Place the Microcon sample reservoir into a non-stick vial. Pipet the CBP-purified protein sample (i.e., the supernatant) into the sample reservoir. The maximum sample reservoir volume capacity is 0.5 ml. Avoid touching the membrane with the pipet tip. Close the cap.

2. Place the assembly into a microcentrifuge. Centrifuge the assembly at 14,000 × *g* for 50 minutes at 4°C or for 35 minutes at room temperature.

   Note *The centrifugation speed and duration given here are recommended for the Microcon YM-10 centrifugal filter unit and should be adjusted when using other devices.*

3. Following centrifugation, remove the assembly from the microcentrifuge and carefully remove the sample reservoir from the vial. Retain the sample reservoir.

4. Invert the sample reservoir and place the reservoir in a fresh non-stick vial. Close the cap.

5. Place the assembly into a microcentrifuge. Spin the assembly at 1000 × *g* for 3 minutes to transfer the concentrate to the vial.

6. Remove the assembly from the microcentrifuge and discard the sample reservoir. Retain the flow-through in the vial; this contains the concentrated proteins.

TROUBLESHOOTING

Observation	Suggestion
Low transformation efficiency or low colony number	Perform a control transformation using a supercoiled vector to verify transformation efficiency of the competent cells.
	During transformation, grow cells under non-selective conditions for 1 hour to allow for expression of kanamycin gene prior to plating on selective medium.
The transfection efficiency is low	DNA quality is poor. Reisolate DNA using a method that removes endotoxins.
	Use a transfection protocol that has been optimized for the cell line used.
	Verify the DNA concentration.
The incidence of cell toxicity is high	DNA quality is poor. Reisolate DNA using a method that removes endotoxins.
	Transfection reagents may be toxic to the cell line. Use a different method of transfection.
	Expression of the exogenous gene(s) may affect cell viability.
	When using cells expressing the SV40 large T antigen, the amount of SV40 origin-containing vector in the transfection reaction may need to be decreased by up to ten-fold.
The TAP-tagged MEF2a protein is present following the TAP-purification, but the MEF2c is not detected	Interacting protein partners may dissociate at temperatures above 4°C. Perform all TAP purification steps at 4°C.
	The biotin in the SEB buffer is photosensitive. Protect the SEB buffer from light at all times.
All expected proteins not detected following TAP purification	Verify that the cell lysate contains the expected proteins before proceeding with the TAP purification. Protein expression must be detected by Western blotting using appropriate antibodies, following transfection and before performing the TAP purification.
	Determine if the proteins are present in the wash buffers to be discarded.
	Increase the salt concentration in the elution buffer(s). The salt concentration of each elution buffer provided is 150 mM NaCl.
Western analysis does not detect tagged protein(s)	Insert is cloned out of frame. Sequence to ensure correct reading frame. Reclone if insert is out of frame.
	Transfer of proteins is poor. Repeat transfer and optimize time of transfer, current and gel concentration and/or use molecular weight markers that cover the range to be transferred.
	Membrane preparation is inadequate. Ensure proper membrane hydration.
	Primary or secondary antibody concentration is too low. Titrate antibody conjugates for optimum concentrations.
	Protein has degraded during storage of the membrane. Use fresh blots.
	Poor isolation of tagged protein. Use a different cell lysis procedure.
	Proteolytic cleavage. Include protease inhibitors in lysis buffer.
The membrane produces excessive background	Insufficient blocking solution may have been used or the membrane was not thoroughly washed. Check the concentration of the blocking solution and/or wash thoroughly.
	Too much protein was loaded on gel. Load less protein on gel.
	Contamination by fingerprints and/or keratin has occurred. Use fresh membranes. Avoid touching the membrane. Use gloves and blunt forceps.
	The concentration of the anti-FLAG or secondary antibody is too high. Check the concentration of the antibodies and dilute if necessary.
	Do not add protease inhibitors to the CEB buffer.

(table continues on the next page)

(*table continues from the previous page*)

Absence of peaks in mass spectrum	An insufficient amount of protein was used. Mass spectrometry requires at least 50 ng of protein. An adequate number of flasks must be processed to purify detectable amounts of protein. When characterizing the control purification products, 1×10^8 cells must be processed.
Too many peaks in the mass spectrum	The sample may be contaminated with keratin or other exogenous proteins. Wear gloves throughout the procedure and take care to use implements (razor blades, spatulas and glass plates) that have been freshly cleaned with ethanol.

PREPARATION OF MEDIA AND REAGENTS

TE Buffer

10 mM Tris-HCl (pH 7.5)
1 mM EDTA

10× Ligase Buffer

500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)

Note *rATP is added separately in the ligation reaction*

LB Agar (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB Broth (per Liter)

10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust pH to 7.0 with 5 N NaOH
Autoclave

LB–Kanamycin Agar (per Liter)

Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB–Kanamycin Broth (per Liter)

Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml filter-sterilized kanamycin

Suggested Passivation Solutions

1% IgG in PBS
1% BSA in PBS
5% Tween® 20 in distilled water
1% powdered milk in distilled water
5% PEG compound in distilled water
5% Triton® X in distilled water
5% SDS in distilled water

REFERENCES

1. Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E. et al. (2001) *Methods* 24:218–229.
2. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. et al. (1999) *Nat Biotechnol* 17:1030–1032.
3. Wilson, D. S., Keefe, A. D. and Szostak, J. W. (2001) *Proc Natl Acad Sci U S A* 98:3750–3755.
4. Keefe, A. D., Wilson, D. S., Seelig, B. and Szostak, J. W. (2001) *Protein Expr Purif* 23:440–6.
5. Stofko-Hahn, R. E., Carr, D. W. and Scott, J. D. (1992) *FEBS Lett* 302(3):274-8.
6. Carr, D. W., Stofko-Hahn, R. E., Fraser, I. D., Bishop, S. M., Acott, T. S. et al. (1991) *J Biol Chem* 266(22):14188-92.
7. Means, A. R., Bagchi, I. C., Van Berkum, M. F. and Rassmussen, C. D. (1991). In *Cellular Calcium: A Practical Approach*,J. G. McCormack and P. H. Cobbold (Eds.). IRL Press, Oxford, England.
8. Kozak, M. (1991) *J Biol Chem* 266(30):19867-70.
9. Hanahan, D. (1983) *J Mol Biol* 166(4):557-80.
10. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

ENDNOTES

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.