Functional Protein Production in the TNT® SP6 High-Yield Protein Expression System

ABSTRACT The wheat germ cell-free TNT® SP6 High-Yield Protein Expression System produces high levels of functional protein. Functional protein yield can reach 100µg/ml in batch mode and 400–500µg/ml in dialysis mode. A method for the production of functional procaspase-3 and the mature protein, caspase-3, using the same vector with different modes of protein synthesis is described. Full-length procaspase-3 was produced (>70µg/ml) in batch mode, while functional processed caspase-3 was produced (~400–500µg/ml) in dialysis mode.

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We show functional procaspase-3 production and processing can be modulated by the mode of synthesis, batch or dialysis.

INTRODUCTION

Cell-free protein synthesis has emerged as a powerful alternative to cell-based protein expression systems for functional and structural proteomics. Wheat germ extract-based cell-free protein synthesis provides unique advantages over other cell-free lysates. These include room temperature incubations, time savings (~2 hours in batch mode), the ability to do high-throughput screening (e.g., screening of protein active site mutants; 1), flexibility to add auxiliary components, the ability to use modified tRNAs for labeling and site-specific incorporation of unnatural amino acids (2), expression of proteins toxic to cells and screening of protein folding and function (3,4). It is a popular alternative for *E. coli*-based cell-free systems because of its eukaryotic nature and its ability to produce soluble and functional proteins, as we have shown for 55 human proteins (8kDa to 151kDa; 5), and it complements rabbit reticulocyte lysate for applications that demand higher protein yield.

The TNT® SP6 High-Yield Protein Expression System[a,b] (Cat.# L3260) is an improved wheat germ coupled transcription/translation system that requires only the addition of purified plasmid DNA template containing the SP6 promoter and the protein-coding region for the protein of interest. This system eliminates the need for in vitro transcription and purification of the mRNA, while exceeding levels of expression in the Wheat Germ Extract Plus Expression System (6).

Wheat germ expression systems lend themselves to dialysis mode (5,7), thereby increasing the protein synthesis levels 4- to 7-fold over the batch mode. With the TNT® SP6 High-Yield System, the dialysis mode allows protein synthesis to continue for about 10 hours versus the batch mode in which protein synthesis increases linearly for about 2 hours.

We used the new TNT® SP6 High-Yield Protein Expression System to produce high protein levels that can be visualized on Coomassie® blue-stained SDS PAGE gels. We also examined the production of functional procaspase-3 and caspase-3 with the TNT® SP6 High-Yield System and show that functional procaspase-3 production and processing can be modulated by the mode of synthesis, batch or dialysis.

PROTEIN EXPRESSION, LABELING AND ANALYSIS

To inspect the level of protein production, we cloned the protein coding regions of firefly luciferase, Monster Green® Fluorescent protein and a synthetic *Renilla* luciferase, with or without the addition of an N-terminal HQ tag (a metal affinity tag) into pF3K WG (BYDV) Flexi® Vector (Cat.# L5681). Purified, supercoiled plasmid DNA templates were added directly to the TNT® SP6 High-Yield Extract without linearization. Labeled target proteins were produced in batch mode using the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System (Cat.# L5001), which incorporates the fluorophore BODIPY®-FL at the ε position of lysine residues. The labeled proteins were separated by SDS PAGE (Figure 1, Panel A) and detected using a laser-based fluorescent gel scanner. This fluorescent labeling eliminates the need to use radioactivity. Protein synthesized using the dialysis mode was identified by Coomasie® blue staining following gel elecrophoresis (Figure 1, Panel B). Since the limit of detection for Coomasie® Blue staining is 0.1–0.5µg of protein per band, the visible expression bands indicate that protein production levels exceeded 0.1µg/µl with the TNT® SP6 High-Yield System. Finally, we purified proteins with an N-terminal HQ tag using the one-step MagneHis™ Protein Purification System (Cat.# V8500) (Figure 1, Panel C). Using these methods, the general yields of target proteins can reach 100µg/ml in batch mode and ~400–500µg/ml in dialysis mode.

CASE STUDY: PROCASPASE-3

Procaspase-3 is first expressed as a zymogen (proenzyme), and the mature form is a heterotetramer

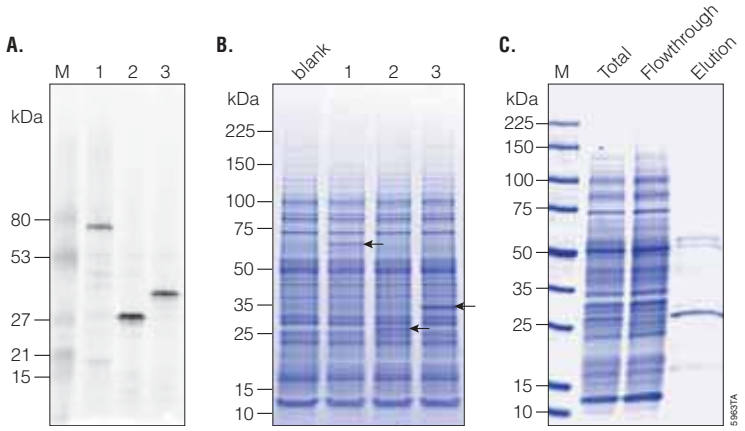

Figure 1. Protein synthesis and purification in TNT® SP6 High-Yield Protein Expression System. For all panels. each lane is equivalent to 1µl of reaction. All target protein contained an N-terminal HQ tag. All 4–20% Tris-Glycine gels in this figure and the rest of the article were from Invitrogen. Lane 1, firefly luciferase; lane 2, Monster Green® Fluorescent Protein phMGFP Vector; lane 3, synthetic *Renilla* luciferase. Arrows indicate expressed proteins. **Panel A.** Proteins were expressed in batch mode using 2µg of plasmid DNA and 1µl of FluoroTect™ $Green_{Lys}$ in a 50µl reaction. The reactions were incubated at 25°C for 2 hours. Synthesized proteins were detected on a Typhoon® 8600 scanner. Lane M, Fluorescent Molecular Weight Marker (Sigma). **Panel B.** Coomassie®-stained SDS PAGE. Proteins were expressed in dialysis mode using 8µg of plasmid and 60µl of TNT® SP6 High-Yield Extract in 100µl reactions. The reactions were incubated for 18 hours at 25°C in dialysis cups (MWCO 12,000 BioTec International, sold by Daiichi Pure Chemicals DBC Code 212956) and 2.5ml of dialysis buffer in a Uniplate (Whatman®). The dialysis buffer consisted of 12mM HEPES, 0.5mM spermidine, 5mM DTT, 80µM amino acids, 70mM KOAc, 1.7mM ATP, 0.6mM GTP, 0.6mM CTP, 6mM UTP, 20mM CP and 3.5mM $Mg(OAc)_2$. **Panel C.** Coomassie® Stained SDS PAGE. The N-terminal HQ-tagged Monster Green® Fluorescent Protein phMGFP Vector was purified by adding 150µl of MagneHis™ Binding/Wash Buffer, NaCl (500mM final concentration) and 30µl of MagneHis™ Magnetic Particles to 50µl of the reaction lysate. The mixture was incubated at 25°C for 5 minutes with periodic mixing to keep the particles from settling. Following incubations, the MagneHis™ Particles were captured on a magnetic stand and the supernatant was removed. The particles were washed with 150µl of MagneHis™ Binding/Wash Buffer, 500mM NaCl and 20mM imidazole. The particles were captured on a magnetic stand, and the supernatant was removed. The wash process was repeated a total of three times followed by a final wash with 100mM imidazole. The bound protein was eluted in 100µl of MagneHis™ Elution Buffer. Lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491).

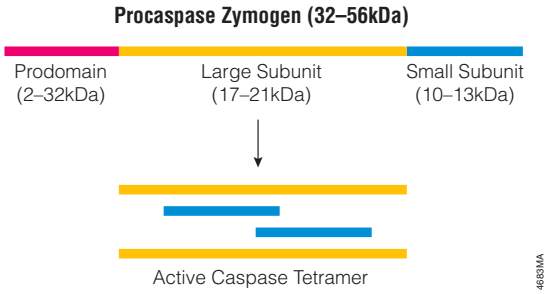

Figure 2. Activation of caspases. Caspase zymogens are cleaved between the large and small subunits, and the prodomains are removed. The active site is formed by a heterodimer that contains one large and one small subunit. Two heterodimers associate to form the fully active tetramer.

The TNT® SP6 High-Yield Protein Expression System produced more full-length procaspase-3 in batch mode using a plasmid DNA template than we previously demonstrated with our RNA-based Wheat Germ Extract Plus.

of small and large subunits generated following internal cleavage (Figure 2; 8). Caspases involved in apoptosis are generally categorized into two different classes: initiator caspases, and effector caspases.

Elucidating the activation mechanisms of caspases is crucial for delineating events that lead to apoptosis. Initiator caspases like caspase-8 usually exist as monomers before activation. They are activated through oligomerization followed by autoprocessing (9). For the effector proteases like caspase-3, the zymogens are believed to be homodimers with low protease activity before activation. Once the proenzymes are partially processed by initiator caspases between the large and small domains, the activated intermediate will undergo autocatalytic processing and remove the prodomain from the large subunit to yield the mature enzyme (10). Both pathways are linear, and fully activated (mature) caspases will not efficiently act on their pro-forms (9–11).

The TNT® Quick Coupled Transcription/Translation Systems (Cat.# L1170 for T7 promoter and L2080 for SP6 promoter) have been used extensively in studying the activation mechanism of procaspase-3, where ^{35}S labeled methionine was incorporated into the zymogen and used for detection of the activation patterns (10). To evaluate the feasibility of performing similar experiments with the TNT® SP6 High-Yield Protein Expression System, we cloned procaspase-3 into the pF3K WG(BYDV) Flexi® Vector with and without an N-terminal HQ tag. The proteins were expressed in the TNT® SP6 High-Yield System and labeled with the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System.

FULL-LENGTH PROCASPASE-3 PRODUCTION

The TNT® SP6 High-Yield Protein Expression System produced more full-length procaspase-3 in batch mode using a plasmid DNA template than we previously demonstrated with the RNA-based Wheat Germ Extract Plus (Cat.# L3250; 1). Compared to TNT® Quick Coupled Transcription/Translation Systems, the TNT® SP6 High-Yield System produced at least tenfold more functional protein for activation analysis (data not shown). The zymogen produced can be activated by caspase-8 (Figure 3, Panels A and B). Using the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System did not significantly affect the activity of caspase-3 (Figure 3, Panel B) or its activation by caspase-8; therefore, downstream activation studies of procaspase-3 can be achieved without the use of radioactivity.

Full-length procaspase-3 can be produced in *E. coli*, but careful manipulation of induction conditions are needed due to endogenous activation of the zymogen (12). We also observed the production of caspase-3 in different processed forms with the pF1A T7 Flexi® Vector (Cat.# C8441) and pFN6K (HQ) Flexi® Vector (Cat.# C8521) in the Single Step (KRX) Competent Cells (Cat.# L3001; Figure 4).

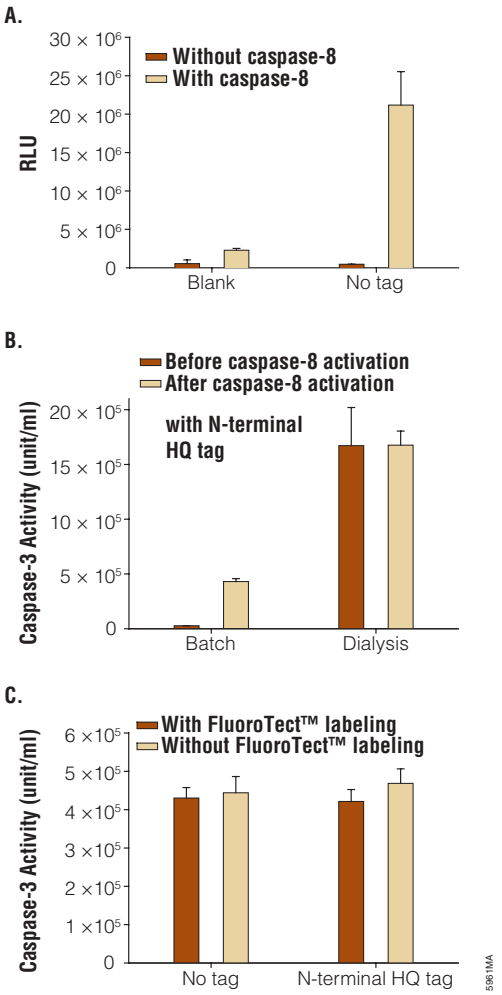

Figure 3. Caspase-3 activity assay. Caspase-3 activity was measured with Caspase-Glo® 3/7 Assay (Cat.# G8091). Procaspase-3 was activated by treating 10µl of lysate with 100U (1µl) caspase-8 (BioMol) for 1 hour at 37°C. Mock-treated, matched lysates received 1µl of 10mM HEPES (pH 7.5) and were incubated for 1 hour at 37°C. After treatment or mock treatment with caspase 8, lysates were diluted to 1:20,000 in 10mM HEPES (pH 7.5). Caspase-Glo® 3/7 Assay measurements were made at steady state (approximately 20 minutes after the reagent was added). Results are presented as active caspase activity (unit/ml) calculated from a standard curve (r^2 = 0.999) using recombinant human caspase-3 (BioMol SE-169, diluted 1:20,000 from 200U/ml in Wheat Germ Extract Plus lacking input RNA) that had a specific activity of 5,982.9 units/µg (BioMol defines one activity unit as producing 1pmol/minute of DNA product using Ac-DEVD-pNA at 200µM at 30°C). **Panel A.** Activation of procaspase-3 with caspase-8 detected by Caspase-Glo® 3/7 Assay. The relative light units (RLU) data show specificity of the assay towards caspase-3 rather than caspase-8. "Blank" is a reaction without added DNA template. **Panel B.** Activities of N-terminal HQ-tagged caspase-3 expression in batch vs. dialysis modes, with or without caspase-8 treatment. Estimated yield from activity is 78 ± 6 and 444 ± 58µg/ml of cell-free reaction. **Panel C.** Incorporation of FluoroTect™ $Green_{Lys}$ does not hamper caspase-3 activity or activation by caspase-8, either with or without an N-terminal HQ tag when produced in batch mode.

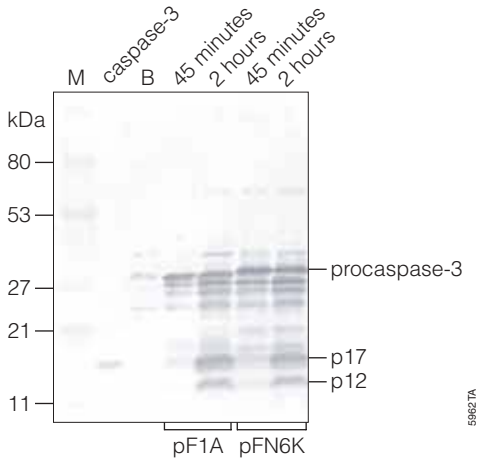

Figure 4. Caspase-3 expression in *E. coli* KRX strain. Procaspase-3 (GenBank® Accession #U26943) coding region was cloned into pF1A Flexi® Vector then transferred to pFN6K Flexi® Vector. The resulting plasmids were transformed into the Single Step (KRX) Competent Cells (Cat.# L3001) that provided rhamnose-inducible expression (13). A single colony was used to inoculate 10ml LB medium start culture with proper antibiotics and grown at 37°C overnight. One milliliter of start culture was added to 50ml of LB medium with proper antibiotics and grown to an $O.D._{600}$ ~0.6–0.8. The cells were induced with 0.2% (w/v) final concentration of L-rhamnose (Sigma) and grown at 37°C. One milliliter cultures were collected before induction (lane B) as well as 45 minutes and 2 hours post induction; we also loaded 50 units of caspase-3 (BioMol) as a control. Cells were spun down and resuspended with 100µl of 1X FastBreak™ Cell Lysis Reagent (Cat.# V8571) with protease inhibitor cocktail (Roche). A total of 2.5µl of the cell lysate was loaded in each lane. Caspase-3 enzyme (BioMol) was used as standard, and the primary antibody was rabbit anti caspase-3 polyclonal antibody (BioMol, used at a 1:1,000X dilution). This antibody reacted more strongly with the large subunit of caspase-3 (p17) than the small subunit (p12). Goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate was used at 1:200X dilution. The TMB Stabilized Substrate for Horseradish Peroxidase (Cat.# W4121) was used for final detection.

PROCASPASE-3 PROCESSING BY CASPASE-8

Processing of procaspases has been studied in the TNT® Quick Coupled Transcription/Translation Systems with ^{35}S-methionine labeling (8,9). We synthesized procaspase-3 with the TNT® SP6 High-Yield Protein Expression System and labeled the protein with the FluoroTect™ System. The activation patterns were confirmed by Western analysis (Figure 5, Panel A). We were able to tentatively assign the processing sites in procaspase-3 (Figure 5, Panel B), which agreed with those reported in the literature (10).

Procaspase-3 processing is a linear process, in which mature caspase-3 cannot process its zymogen (9,10). We have generated data supporting this mechanism (Figure 5, Panel C). However, we also observed limited but detectable processing of procaspase-3 by its mature enzyme with longer incubation times (Figure 5, Panel D).

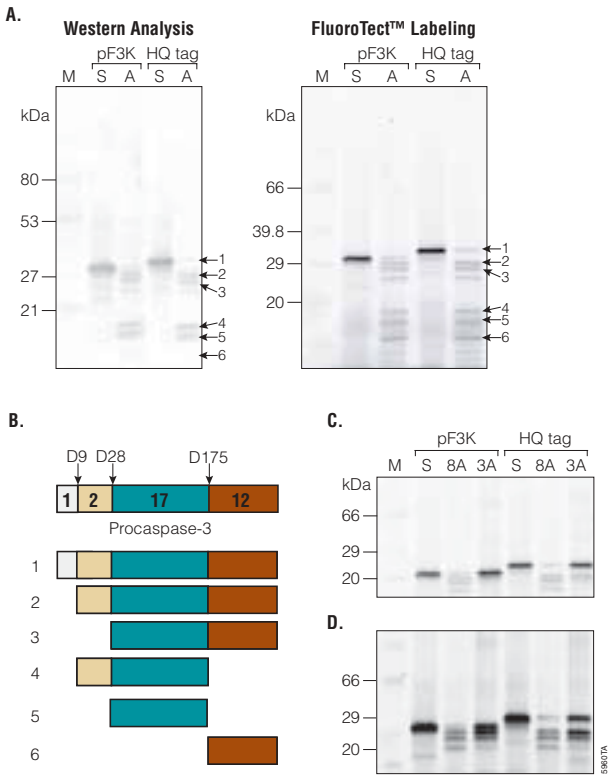

Figure 5. Detection of procaspase-3 processing in batch mode with FluoroTect™ Green$_{Lys}$ labeling. Procaspase-3 with or without an N-terminal HQ tag (in pF3K WG (BYDV) Flexi® Vector) was synthesized in batch mode with FluoroTect™ Green$_{Lys}$ labeling. Procaspase-3 was activated (**Panel A**) by treating 10µl of lysate with 100U (1µl) caspase-8 or caspase-3 (BioMol) for 1 hour at 37°C. Mock-treated, matched lysate supernatants (S) received 1µl of 10mM HEPES (pH 7.5) and were incubated for 1 hour at 37°C. Each lane is equivalent to 1µl of the original reaction. **Panel A.** Activation patterns of procaspase-3 are shown as detected by fluorescent labeling or Western analysis. Lanes S, supernatant without caspase-8 treatment; lanes A, supernatant after caspase-8 treatment. Lane M for fluorescent labeling was the Fluorescent Molecular Weight Marker, (Sigma); lane M for Western analysis was ProSieve® color protein markers (Cambrex). Western analysis was performed as described in Figure 4. **Panel B.** Tentative assignments of the cleaved bands at residues D9, D28 (for removal for prodomain) and D175 (for initial activation) are shown. Numbers inside the bars are rough estimations of fragment molecular weight (kDa); p17 is the protein fragment that is approximately between D28 and D175, and p12 is the carboxy-terminal fragment of the protein. **Panel C.** Gels show extensive processing of procaspase-3 by caspase-8 (lanes 8A) and the lack of processing of procaspase-3 by caspase-3 (lanes 3A) for 1 hour at 37°C. **Panel D.** Limited processing of procaspase-3 by its own mature form (lanes 3A) was observed if the reactions from Panel C were incubated for another 24 hours at 4°C. Lanes M for Panels C and D, Fluorescent Molecular Weight Marker (Sigma).

We were able to tentatively assign the processing sites in procaspase-3 (Figure 5, Panel B), which agreed with those reported in the literature (10).

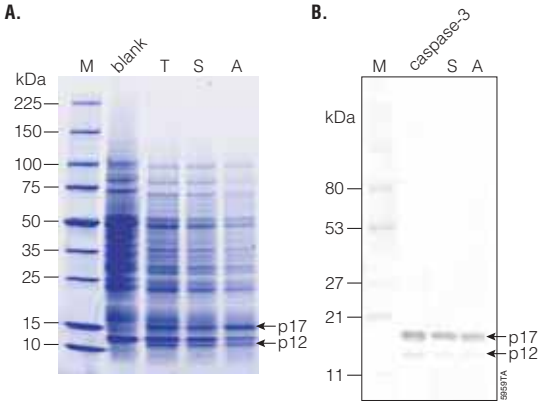

Figure 6. Mature caspase-3 production in dialysis mode. Panel A. Coomassie® blue-stained SDS PAGE. Each lane is equivalent to 1µl of reaction lysate. The blank was performed without any DNA template; lane T is total; lanes S contain the soluble fraction; lane A contains the protein activated by caspase-8 treatment. Arrows indicate the p17 and p12 subunits. Lane M, Broad Range Protein Molecular Weight Markers (Cat.# V8491). **Panel B.** Western analysis of the synthesized protein. 100 units of caspase-3 standard (BioMol) and 0.1µl of each reaction was loaded. Detection was performed as described for Figure 5. Arrows indicate large and small subunits of caspase-3. Lane M, ProSieve® Color Protein markers (Cambrex).

MATURE CASPASE-3 PRODUCTION IN DIALYSIS MODE

Although procaspase-3 has low basal activity before activation, possibly due to structural constraints around the active site (10), concentration-dependent self-activation of procaspase-3 (>0.2mg/ml) has been reported (13). In dialysis mode, which produces greater than 0.4mg/ml of protein, mature caspase-3 from the same constructs can be made as a possible consequence of concentration-dependent activation of the zymogen; however, we cannot exclude other factors that might lead to Procaspase-3 activation. The small subunit (12kDa) was visible with Coomassie® blue staining (Figure 6, Panel A) and was further verified by Western blot analysis (Figure 6, Panel B). The activity of caspase-3 is already at its full capacity before caspase-8 activation (Figure 3, Panel B). These results suggest that the amount and nature of protein synthesis can be modulated with different modes of experimental setups using the TNT® SP6 High-Yield Protein Expression System, depending on the property of target protein and the functional state of interest.

CONCLUSIONS

We demonstrated that the TNT® SP6 High-Yield Protein Expression System synthesizes enough protein in dialysis mode that the target protein can be identified on a Coomassie®-stained gel. The proteins can be purified by Ni-affinity resins if an HQ tag is present. Further, functional protein production can be achieved with this system. For procaspase-3, both zymogen as well as mature enzyme were synthesized from the same construct under different synthesis conditions. The estimated yields were ~70µg/ml for procaspase-3 zymogen in batch mode and ~400–500µg/ml mature caspase-3 in dialysis mode. Finally, we showed that the FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System does not significantly interfere with the activity of procaspase-3, allowing zymogen activation to be studied without radioactive labeling.

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PROTOCOL

- *TNT® SP6 High-Yield Protein Production System Technical Manual* #TM282, Promega Corporation. (www.promega.com/tbs/tm282/tm282.html)

ORDERING INFORMATION

Product	Size	Cat.#
TNT® SP6 High-Yield Protein Expression System	40 × 50µl reactions	L3260
	10 × 50µl reactions	L3261
FluoroTect™ $Green_{Lys}$ in vitro Translation Labeling System*	40 reactions	L5001

* For Laboratory Use.

[a]For Research Use. Any use of the product for diagnostics requiring clearance or approval by the FDA may require a license under Mayo Clinic U.S. Pat. Nos. 6,027,913 and 6,361,949.

[b]U.S. Pat. Nos. 5,324,637 and 5,492,817, Australian Pat. No. 660329 and other patents.

IN THE LITERATURE

Suzuki, T. *et al.* (2003) LXXLL-related motifs in Dax-1 have target specificity for the orphan nuclear receptors Ad4BP/SF-1 and LRH-1. *Mol. Cell. Biol.* **23**, 238–49.

In this study, the authors investigated the interaction between the orphan receptors Dax-1 and Ad4bp/SF-1. Specific interaction was detected when the two proteins were used in both yeast and mammalian two-hybrid systems. To further characterize this interaction, an in vitro pull-down assay was performed. Dax-1 protein or a dax-1 mutant lacking an LXXLL motif were transcribed and translated in vitro using the TNT® T7 Quick System and FluoroTect™ $Green_{Lys}$ tRNA. The fluorescently labeled protein interacted with a Ad4BP/SF-1-maltose binding protein (MBP) fusion protein immobilized to an amylose resin. After elution and SDS-PAGE, the fluorescently labeled protein was detected using a Hitachi FMBIO® II Fluorescence Imaging System. The interaction between Dax-1 and Ad4BP/SF-1 was dependent on the LXXLL motif.

See more citations at: www.promega.com/citations/

For procaspase-3, both zymogen as well as mature enzyme were synthesized from the same construct under different synthesis conditions.