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Identification of a putative caspase protein in S.Commune

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IDENTIFICATION

OF A

PUTATIVE CASPASE PROTEIN

IN

S.COMMUNE

by

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Abstract

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Caspases are the proteins responsible for apoptosis in cells.[1] Others have recently identified a caspase in *Saccharomyces cerevisiae*, a unicellular fungus.[2,3,4] Isolation of a caspase protein from *Schizophyllum commune* will allow for the examination of a caspase in a simple multicellular organism.[4] The *S.cerevisiae* caspase sequence was used to search the genomes of several fungi and identify similar sequences. Regions with high sequence identity in the aligned sequences were used to design degenerate PCR primers. The sequence of the resultant PCR product was then used to prepare primers for 3'RACE and determine the 3'end of the sequence. Through modified 5'-RACE PCR, 81 amino acids at the 5' end of the protein were identified. Approximately, 300 to 500 bp (approx. 20%) close to the 5' end remains to be identified by utilizing BD SMART ™ RACE synthesized cDNA and Failsafe PCR kit reagents. Once the entire gene has been cloned, the protein it encodes will be expressed and purified.

Acknowledgements

I would like to begin by extending my gratitude to the Union College Chemistry Department and the faculty for four years of steady and patient guidance through all of my chemistry and academic endeavors. A big thank you is also extended to the Union College Internal Education Fund as well as the Union College Faculty Research Fund that has provided the financial resources for the sustainability of this project.

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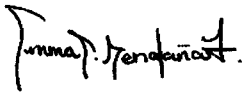
A last farewell and thanks to all of the undergraduate and graduate students that I met here at Union, chemistry and non-chemistry majors. We went through a lot, especially all of my peeps in the MM-Lab where we shared countless hours of exciting scientific learning late at night, but it was all worth it.

Lastly, an extended thanks to my family, especially my parents, for all of their guidance and support these past twenty-two years.



Emma Ester Bendaña Flores

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INTRODUCTION

Introduction

A. Overview

Cysteinyl Aspartate-Specific Proteases (caspases) are the proteins involved in the execution and regulation of apoptosis. Apoptosis is the most frequent mechanism used by cells to undergo programmed cell death, whereby the injured cell is destroyed through organelle dysfunction, nuclear disassembly, DNA fragmentation and protein degradation with little or no harm occurring to the surrounding healthy cells. One of the ways that the surrounding tissue is protected from inflammation is by the creation of apoptotic bodies that surround the destroyed organelles and cell components. In addition, harm to surrounding tissues is minimized by the regulated use of caspases and controlled gene expression. Historically, apoptosis has been examined in *Caenorhabditis elegans* (nematodes) and it has only recently been studied in *Saccharomyces cerevisiae* (yeast).[1] In this experiment, the goal is to identify a caspase protein in *Schizophyllum commune*, a simple multi-cellular organism, with the ultimate goal of purifying the protein and examining its enzymatic properties.

Caspases refers to a group of cysteine proteins that have been evolutionarily conserved and share a proximal histidine and distal cysteine in their active site.[19] In addition to caspases, two families of caspase-like proteins have been identified, paracaspases in metazoans and dictyostelium and metacaspases in plants, fungi and protozoa. Caspases, in themselves, are a unique type of enzyme that cleaves other proteins after recognizing aspartate residues, specifically the catalytic consensus sequence of (QAC(R/Q)G) for cleavage. [1]

Apoptosis has two general pathways. The first, extrinsic apoptosis, requires the interactions of membrane proteins, ligands and signaling molecules to activate caspases and other proteins. The second, intrinsic apoptosis, utilizes the participation of mitochondria to initiate the caspase cascade as well. The caspase cascade in both apoptotic pathways is contingent on the dual function of caspases as initiator and executioner caspases. Prior to apoptosis, initiator caspases are present in the cell as procaspases which are inactive. Once apoptosis is triggered, the initiator caspase binds to the caspase activation and recruitment domain (CARD) in surrounding activating molecules and starts cleaving downstream caspases, other non-caspase proteases and protein substrates, which fully initiates the apoptotic pathway. Executioner caspases are located downstream from initiator caspases because they require activation from the cleavage of other proteases. Cleavage of executioner caspases occurs in death-inducing signaling complexes (DISC) which are large protein combinations that include initiator caspases and apoptosomes. The proper functioning of several caspases in a predetermined order is critical for the execution of apoptosis in the cell. [1,18]

Schizophyllum commune, from the Basidiomycotina class, subclass Holobasidiomycetidae, order Aphyllophorales and family Schizophyllaceae, is a non-parasitic fungus found on fallen hardwoods (Figure 1).[2] *S.commune* is involved in the recycling of carbon in celluloses and xylans within fallen wood trees.[3] The majority of research pertaining to *S. commune* focuses on its sexual morphogenesis that is controlled through several genes and biochemical pathways. [4] *S.commune* is also a model organism for the identification of a caspase. Even though the genome of *S.commune* is not sequenced, a cDNA library and access to mRNA is available through Professor Stephen

Horton for various gene probing techniques. In addition, *S.commune* provides a platform to examine caspases in a simple, yet multi-cellular organism. Unlike yeast, which is unicellular, the action of apoptosis in *S.commune* may be more similar to humans and other more complex organisms. Apoptosis is meant to destroy injured cells without harming surrounding tissue with the overall goal of protecting the entire organism. This need is not present in yeast as a unicellular organism that upon proceeding forth with apoptosis, destroys itself. In addition, *S.commune*, as a fungus, is a lower eukaryote that is very closely related to humans, more so than bacteria and viruses. Fungi have larger and more complicated genomes than bacteria and have introns similar to introns in humans. However, fungi introns are about 1/300th the size of human introns, which facilitates genetic sequencing. Approximately 30% of human proteins are homologous to fungal proteins.[18]

Apoptosis is well –studied in humans, however it has been difficult to understand the intricacies involved due to the complexity of the human body, the overall complex process and the high number of caspases participating in the system. Therefore, *S.commune* is an ideal candidate for apoptosis research due to its simple, yet multi-cellular structure and the likelihood that only a few caspases are involved in the system. By reducing the complexity of the organism and system, the fundamental processes of apoptosis can be thoroughly understood.

Figure 1 ***S.commune***

S.commune **is a non-parasitic fungus that grows on fallen hardwoods. Genetic studies of** ***S.commune*** **have focused on its sexual morphogenesis that is controlled through several genes and biochemical pathways.** [4]

B. Screening by Colony Hybridization

Probing for a particular gene can be done with several techniques. One procedure, colony hybridization, involves using plates to grow *E.coli* bacteria that contain plasmids with sections of cDNA. The *E.coli* is plated and allowed to grow overnight. The following day, the colonies of *E.coli* are blotted onto filter paper that is then subjected to several washes in order to promote binding of these cDNA-containing plasmids. The appropriate stretch of cDNA is identified by the actual hybridization of a probe designed to bind to the gene of interest.

Each of the washes in the procedure is designed to improve the hybridization of the probe to the cDNA. The function of the pre-washing solution is to allow the probe to stick well to the plasmid DNA, thus preventing other non-probe elements from binding to the plasmid. Afterwards, the blocking buffer serves to reduce the probe's affinity for the filter paper. The filter paper by default is able to bind easily to DNA, the probe, and several other elements. Therefore, it is important to use the blocking buffer in order to increase the selectivity of the probe for the plasmid DNA, rather than the filter paper. Once the filter paper has been incubated with the probe, the results of the procedure can

be seen by exposing the filter to X-ray film overnight. The location of probe binding on the filters is represented by dots on the film. Theoretically, the dots on the film represent the colonies with the gene of interest, however the analysis of this procedure is not always so straight forward. In some cases noise and other factors can contribute to dots on the X-ray films that are in fact artefacts. Duplicate filters are normally made in order to reconfirm the presence of probe hybridization rather than noise.[5]

C. Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is one of the most frequent techniques used to probe for specific DNA sequences or specific genes. PCR in itself is used to replicate DNA through a cycle of three different steps: denaturation, annealing and extension (Figure 2). In the first step, denaturation, the double-stranded DNA template is separated into single-stranded DNA at a very high temperature, usually about 94° C. The second step, annealing, involves the attachment of primers or smaller pieces of DNA (20 to 30 bp) that are complementary to the original DNA template. This occurs at a lower temperature than denaturation. Each primer has an optimal annealing temperature that is determined by its nucleic acid composition. In the final step of PCR, extension, DNA polymerase completes the DNA sequence of the primer by adding complementary dNTPs to the template DNA strand with the attached primer.[6] Therefore, after one round of PCR, from one double stranded piece of DNA, two double stranded pieces of DNA are produced. In one run, PCR will cycle through denaturation, annealing and extension approximately 20 to 35 times depending on the specific protocol followed in order to generate many double stranded pieces of DNA.

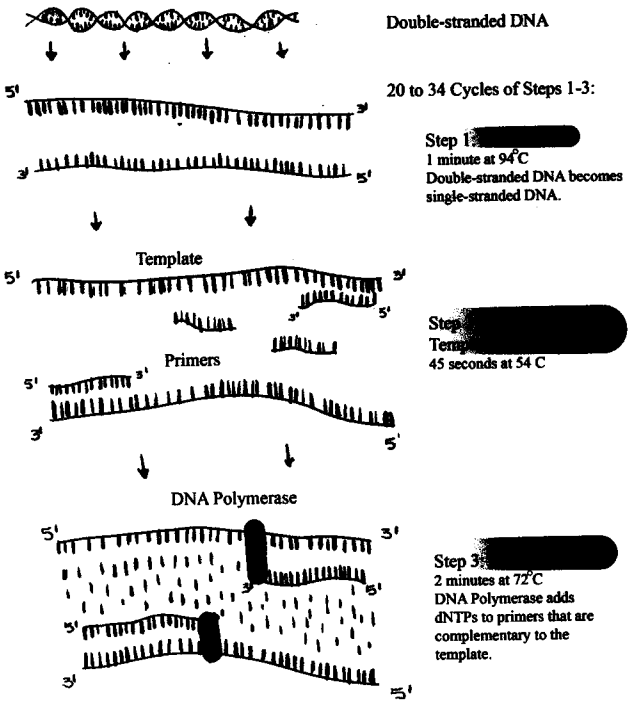


Figure 2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) is a technique used to replicate DNA through a cycle of three different steps: denaturation, annealing and extension. By designing primers that anneal to specific regions of the DNA, specific DNA genes or sequences can be replicated specifically.

PCR has many variations depending on the type of primers that are used. The primers can be designed to match the exact DNA sequence of the template. In many cases, the exact DNA sequence of the template might not be known and only the protein

sequence of the template is available. Since one amino acid is coded by three nucleic acids and each amino acid has more than one DNA code, a family of primers must be designed to encompass all of the possible combinations. This is known as degenerate PCR.[6]

PCR is a technique that can be done with either genomic DNA or cDNA. Rapid Amplification of cDNA ends (RACE-PCR) is a type of PCR designed to find the beginning and ending sequences of specific genes.[7] RACE-PCR becomes very useful when probing for specific proteins since cDNA is used and only contains exons. Messenger RNA (mRNA) is transcribed from genomic DNA. Before mRNA leaves the nucleus, it is spliced removing the introns; only the exons that contain the protein coding information remain.[8] The resulting mRNA is then used to synthesize cDNA that can be used in RACE-PCR. A variation of RACE-PCR, known as Modified RACE-PCR, is specifically designed to identify the 5' sequence of a gene. RACE-PCR is most successful with 3' RACE-PCR because the poly A tail of the mRNA is used as an annealing site for primers. The 5' end of a gene does not have the same anchor and is typically the most difficult end to sequence. In Modified-RACE PCR a cDNA library is constructed from the organism of interests by ligating pieces of cDNA into the multiple cloning site (MCS) of pMyr vectors.[5,13] In Modified-RACE PCR, two types of primers are needed, gene-specific and vector-specific (Figure 3).

Two sets of primers are designed, vector-specific and gene-specific, in Modified-RACE PCR. In addition, inner and outer PCR primers are designed in order to confirm the correct PCR products. Inner primers anneal to DNA sequence inside of the location to which the outer primers anneal (Figure 3). Initially, an outer PCR reaction containing

the outer gene-specific and outer vector-specific primers, is completed. In some instances, this reaction successfully produces several PCR products. In order to successfully identify the correct PCR product, an inner (nested) PCR reaction with the inner vector-specific and inner gene-specific vector is completed. The outer PCR products that contain the gene of interest will be further amplified through the inner PCR, but the size of the band will decrease because the inner primers anneal to the DNA closer to each other than the outer primers. The downward shift of the band will allow for its identification.

Inner PCR can also be used to further amplify outer PCR products. In some cases, the outer PCR products are not visible on a gel. Inner PCR can specifically amplify the gene of interest and give sufficient quantities of the products in order to see them on a gel. Various other combinations of inner and outer primers can be made in order to do hemi-nested PCR reactions. By manipulating the primer mix, several options for PCR reactions are possible.

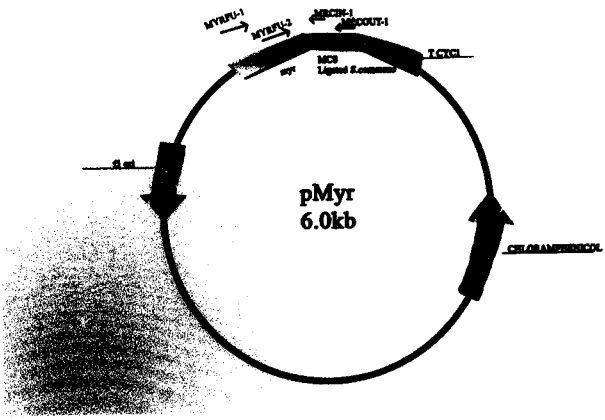


Figure 3 Modified RACE-PCR

In Modified RACE-PCR, pieces of cDNA from *S.commune* (or the organism of interest) are ligated into the multiple cloning site (MCS) of the pMyr vector. The gene of interest is probed by designing primers specific to the known sequence of the partial gene (rainbow). The 5' end of the gene is found by using the primers specific to the Myr sequence (blue). Inner and outer primers are designed specific to the gene and the vector sequence. The outer primers in this vector are the MRCOUT-1 and MYRFU-1. The inner primers are MRCIN-1 and MYRFU-2. [13]

In addition to the Modified RACE-PCR, a second type of RACE-PCR is available in order to determine the 5' end of a gene, the BD SMART ™ RACE cDNA Amplification Kit.[9] The novelty of the BD SMART ™ RACE[9] is the synthesis of the cDNA from mRNA or total RNA. Unlike the modified RACE-PCR done initially, the BD SMART ™ Kit[9] does not require that the synthesized cDNA be ligated into a vector. Instead, first strand cDNA can be used directly in the PCR reaction.[9]

The two distinguishing elements of the BD SMART ™ Kit[9] are the BD SMART II ™ A Oligonucleotide and BD PowerScript ™ Reverse Transcriptase (RT)[9] (Figure 4). Initially, the Modified 5' RACE CDS Primer anneals to the 3' region of the poly A tail. The RT begins to transcribe the mRNA into cDNA. At the end of the mRNA, the RT stutters and adds a series of three to five residues of dC to the 3' end of the first strand of the cDNA. The BD SMART II ™ A Oligonucleotide[9] anneals its GGG end to the awaiting C residues on the first strand of cDNA. The RT then starts using the BD SMART II ™ A Oligonucleotide[9] as the template, thus providing a specific region of annealing for the outer and inner primers included in the kit. This is the crucial step that avoids the need of ligating the cDNA into vectors. A complete cDNA from intact poly A is guaranteed because the RT is only able to add CCC to the 5' ends of full length transcripts of the mRNA strand. Upon the successful synthesis of the cDNA, the BD SMART ™ Kit PCR[9] functions like the other PCR reactions. Outer and inner PCR reactions can be done.

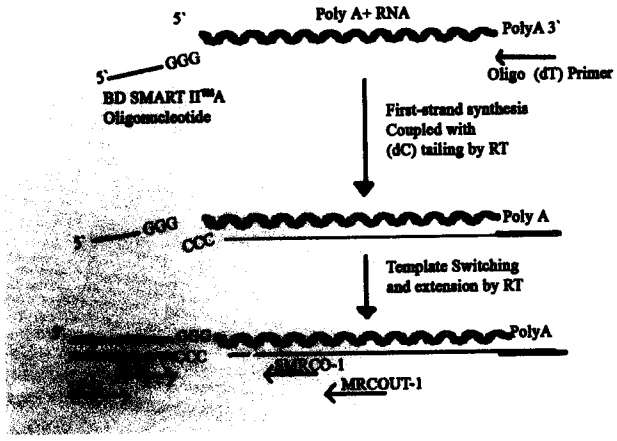


Figure 4 Synthesis of cDNA for BD SMART ™ RACE PCR [9]

The BD SMART ™ RACE PCR kit theoretically allows for the complete gene synthesis of cDNA from mRNA. This is achieved through the use of the BD SMART II ™ A Oligonucleotide and BD PowerScript ™ Reverse Transcriptase (RT). The ligation of cDNA into a vector, as done with Modified-RACE PCR is not necessary due to the addition of the BD SMART II ™ A Oligonucleotide which provides an anchor and annealing site for 5' primers. The Universal Primer Mix (UPM) and Nested Primer Mix (NPM) anneal to the BD SMART II ™ A Oligonucleotide. The gene-specific primers (SMRCO-1 and MRCOUT-1) anneal directly to the synthesized cDNA. [9]

D. Putative *S.commune* Caspase

The existence of a caspase protein in *S.commune* was determined by Professor Kristin Fox upon the successful identification of 279 amino acids through degenerate PCR and 3'-RACE PCR (Figure 5). A sequence alignment of three previously identified

caspase proteins in *N.crassa* and *S.cerevisiae* was used to design degenerate PCR primers (Figure 6). After the 279 amino acids of a putative caspase in *S.commune* were identified, a protein sequence alignment with the caspases in *N.crassa* and *S.cerevisiae* revealed that the identified protein in *S.commune* was very similar to the other caspases, especially in the inner regions of the protein (Figure 7). It was determined that additional 5' protein sequence was needed in order to complete the entire sequence of the protein. An ATG codon (transcription start codon) for Methionine was not present in the sequence, and the Kozak sequence C(A or G)CCATGGC surrounding the start codon was also missing. In addition, the identified sequence was too short to be a complete protein. The 5' end of the gene was not specifically targeted in the degenerate PCR, since the degenerate PCR primers were designed to anneal to the internal regions of the protein. The 3' region of the protein is complete since the last amino acid, cysteine, is followed by TGA, one of the DNA stop codons.[10] As a result, the purpose of this experiment will be to identify the complete 5' end of the putative caspase protein in *S.commune* through several PCR techniques including Modified-RACE PCR and the use of synthesized cDNA using the BD SMART ™ RACE technology. Once the 5' end and entire sequence of the protein has been confirmed, it will be made so that the protein can be purified in order to examine its enzymatic activities *in vitro*. The gene sequence can also be used to generate mutant forms of *S.commune* to determine the role of the caspase in the organism *in vivo*.

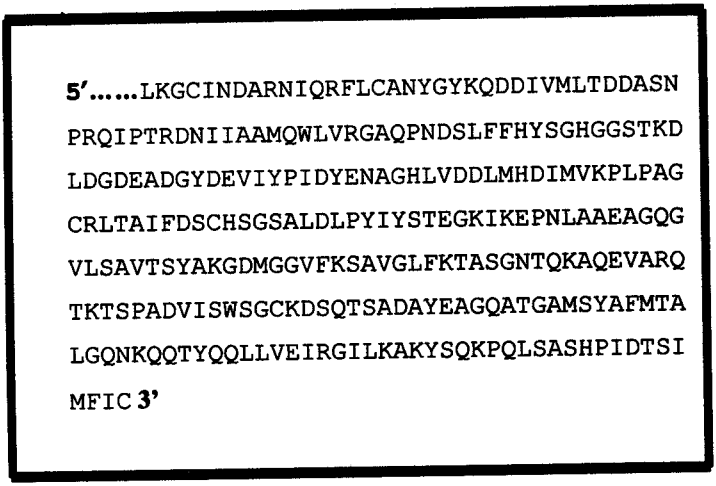


Figure 5 Partial Sequence of Putative Caspase Protein in *S.commune*

After completing a degenerate PCR and 3'RACE-PCR, 279 amino acids were identified for the putative caspase protein. The amino acids are color coded according to the method used to identify them.

Red Sequence determined through Degenerate Primer PCR
Blue Sequence determined with 3'RACE PCR
Pink Sequence overlap region between the two methods.

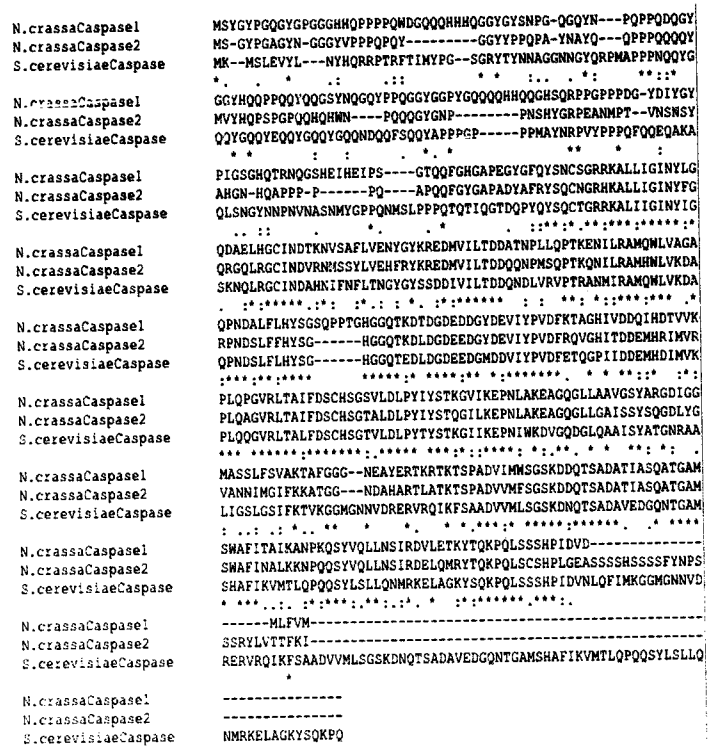


Figure 6 Protein Sequence Alignment of Caspase Proteins in *N.crassa* and *S.cerevisiae*

In order to design degenerate PCR primers, a protein sequence alignment was done with two known caspase proteins from *Neurospora Crassa* and one caspase protein from *Saccharomyces cerevisiae*. [10,15]

Figure 7 Protein Sequence Alignment of Caspases in *N.crassa*, *S.cerevisiae* and Partial Putative Caspase in *S.commune* found through Degenerate PCR and 3' RACE [10,15]

The protein sequence for *S.commune* determined through degenerate PCR and 3'RACE PCR aligns well with caspases from *N.crassa* and *S.cerevisiae*. Approximately 172 AA are missing in the *S.commune* caspase; if it is as long as the *S.cerevisiae* caspase.

EXPERIMENTAL PROCEDURES

Experimental Procedures

A. Screening by Colony Hybridization

The procedure and solutions used for screening by colony hybridization are outlined in Appendix 1: Procedure 1. The agar-plates used in this experiment were made with 25.0 grams of LB and 3.0 grams of Bacto-Agar in 1000 mL of Milli-Q water. The final concentration of chloramphenicol antibiotic was 0.1 mg/mL in the plates. A stock, with a concentration of 100 mg/mL, of the antibiotic chloramphenicol was used.

For the hybridization procedures in Day 3, a probe made by Professor Kristin Fox for other hybridization procedures involving the putative caspase in *S.commune* was used.

In the secondary screening by colony hybridization, the plated dilutions of the hybridized colonies from the primary screening did not exhibit an adequate number of colonies; as a result the stock solutions were used for the secondary hybridization procedure. A few modifications were made to the procedure followed in the primary screening. The volume of the probe was tripled. The amount of probe in the primary screening was set according to the approximate area of the two large circular filters used. Six colonies were analyzed, thus 12 smaller circular filter papers were used. Approximately, four smaller filter papers equal one larger filter paper. Thus, the amount of probe used in the secondary screening is three times the amount used in the primary screening. The hybridization temperature was decreased in the hybridization oven to 59°C in order to provide less stringent hybridization conditions.

Accidentally, some modifications were made to the secondary screening procedure during the experiment. At the last minute, there was no anti-digoxigenin as

needed in the Day 4 protocol, so the filter papers were left overnight in the washing solution. Afterwards, when the anti-digoxigenin was added, instead of adding 1 μL per 10 mL blocking buffer (total 15 μL), only 1 μL of anti-digoxigenin was added. As a result, the procedure was repeated with the same filters starting from the washing solutions in step 5 of the Day 4 protocol (Appendix 1: Procedure 1).

B. Modified RACE-PCR

Primers

The primers for the Modified RACE-PCR were designed using the algorithm on the Primer 3 website.[11] The specific properties and DNA sequences of the primer oligos used in this experiment can be found in Table 1. The location of the gene-specific primers in the partial DNA sequence of the putative caspase protein determined through degenerate PCR and 3' RACE-PCR [10] is close to the 5' end of the protein and they are approximately 100 bp apart (Figure 8).

Table 1 Properties of Primers used in Modified RACE-PCR and BD SMART ™ RACE PCR[9] Reactions

Oligo	DNA Sequence	Length (bp)	GC%	Tm (°C) by [%GC Method]
MRCIN-1	TGTGTGCGAACTACGGCTAC	20	55.00	70.30
MRCOUT-1	TGTTCTTCCATTACTCCGGC	20	50.00	68.25
SMRCO-1	ACCTGGATGGTGACGAGGCAGACGG	25	64.00	80.94
MYRFU-1	TAGCAGCTGTAATACGACTC	20	45.00	66.20
MYRFU-2	TCTAGAGCTTACTAGTATGG	20	36.84	61.08

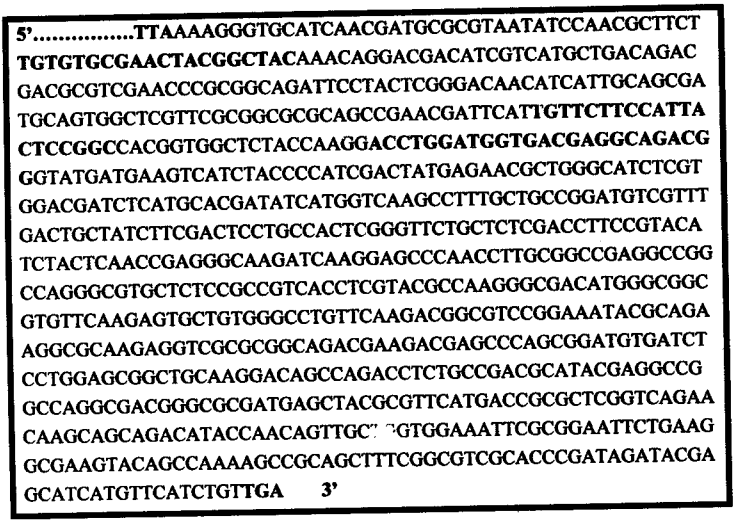


Figure 8 Location of PCR Primers in Partial DNA Sequence of Putative Caspase Protein in *S.commune* [10]

The DNA Sequence shown was determined through degenerate PCR and 3'-RACE PCR.[10] The DNA sequence determined through Modified-RACE PCR is not included. The three primers used in the various PCR reactions for this experiment are identified by color. SMRO-1, MRCOUT-1 and MRCIN-1. In addition, at the 5' region, **TTA** corresponds to the first amino acid known in the sequence which is Leucine (Figure 5). **TGA** at the 3' end of the gene is the stop codon for the protein.

Upon arrival the primers were hydrated with 100 mM Tris-Cl, pH 8.0 solution. Initially, the dry primer powder is centrifuged for a minute to ensure that the entire contents of the tube are at the bottom of the tube. Afterwards 100 µL of 100 mM of Tris-Cl, pH 8.0 is added. The solution was vortexed at low speed in order to ensure good

mixing. A dilution was made to a concentration of 10 pmol/μL in a total volume of 200 μL (Table 2). After dilution, the stock and diluted primers were stored at -20°C.

Table 2 Hydration of Primers used for Modified RACE-PCR and BD SMART ™ RACE[9] Reactions

Oligo	pmol (given from manufacturer)	[ ] in stock (pmol/μL) = pmol / 100μL	μL of stock needed to give 10 pmol/μl in 200 μL *	μL of 100 mM Tris-Cl, pH 8.0 Buffer needed
MRCIN-1	137449	1374.49	1.5	198.5
MRCOUT-1	122157	1221.57	1.6	198.4
SMRCO-1	141786	1417.86	1.41	198.59

* MV = MV
(10 pmol/μL) (200 μL) = (1374.49 pmol/μL) (x)
2000 pmol = (1374.49 pmol/μL) (x)
x = (1374.49 pmol/μL) * (1/2000 pmol)
x = 1.5 μL of MRCIN-1 stock needed

Outer Modified RACE-PCR

Once the primers were available, the Outer Modified RACE-PCR was set up. A library of ligated *S.commune* cDNA into pMyr vector constructed by Professor Stephen Horton was used. For purposes of this experiment, the FailSafe ™ PCR PreMix Selection kit[17] was used. The reagents in the FailSafe ™ PCR PreMix Selection Kit[17] provide several buffer mixes that allow a wide range of salt environments for the PCR reaction. The variety in salt environments provided by the FailSafe ™ PCR PreMix Selection Kit[17] is important for the success of PCR reactions with unknown preferences. The total volume of the PCR reaction was 20 μL (Table 1). A total of seven reactions were done. The first six reactions all contained the same ingredients as indicated in Table 3, but a different FailSafe[17] buffer was used (Buffer A-F). The seventh PCR reaction served as a control and did not contain any template, instead 0.2 μL of sterile Milli-Q

water was added. The program used for the Outer PCR is the Modified B Version of the Touchdown Program in the BIO-RAD iCycler ™ Thermal-Cycler as indicated in Table 4. After the Outer PCR reaction was completed, 10 μL of the reaction was loaded onto a 1.5% agarose gel and the remaining 10 μL of the PCR products through the QIAquick® PCR Purification Kit[12] to be used in the Inner PCR reaction of the Modified-RACE PCR. A diagnostic 1.5% agarose gel was run on the BIO-RAD Power Pac 300 for the seven reactions. The gel was run for 1.25 hours and was stained using 2 μL of a 10 mg/mL ethidium bromide stock mixed into the liquefied gel prior to solidification. For each reaction 10 μL of PCR product and 2 μL of loading dye were loaded. The loading dye used was composed of 0.25% bromophenol blue and 40% (w/v) of sucrose in water.[21] The gel was loaded with 2.5 μL of 1 KB DNA Ladder from Promega, 7.5 μL of water and 2 μL of dye.

Table 3 Reagents for Outer PCR Reaction for Modified RACE-PCR

Volume (μL)	Reactant	Indications
0.2	Template	*E.coli* containing ligated portions of cDNA of *S.commune* in plasmid vector prepared by Professor Stephen Horton on 9/25/03.
0.8	MYRFU-1 primer	The outer vector-specific primer in the plasmid the *S.commune* cDNA has been ligated.
0.8	MRCOUT-1	Outer Gene-Specific Primer
0.2	Failsafe Enzyme	Used from FailSafe RACE PCR Kit
10	Failsafe Buffer Mix	From FailSafe RACE PCR Kit : Comes in many varieties A through F.
8	Sterile MilliQ Water	
Total Volume of Reaction: 20 μL		

Table 4 Modified B Version of the Touchdown Program

Cycle 1 (1x) Step 1 94° C for 3:00
Cycle 2 (35x) Step 1 94° C for 0:40 Step 2 68 °C for 0:40 (Decrease Temperature by 0.5° C every 1 cycle) Step 3 72 °C for 1:00
Cycle 3 (1x) Step 1 72 °C for 10:00
Cycle 4 (1x) Step 1 4 °C for ∞

Inner PCR

The volumes and ingredients for the Inner Modified RACE-PCR (Table 5) were slightly modified from the volumes and ingredients used for the Outer Modified RACE-PCR (Table 3). The program used for the Inner PCR was the Modified B Version of the Touchdown Program as indicated in Table 4. A diagnostic 1.5% agarose gel was run as done with the Outer PCR Reaction.

Table 5 Inner PCR Reaction for Modified RACE-PCR

Volume (µL)	Reactant	Indications
3.5	Template	QIAquick® PCR Purified Outer PCR products
0.8	MYRFU-2 primer	Inner vector-specific primer to the vector containing the ligated portions of the *S.commune* cDNA
0.8	MRCIN-1	Inner gene-specific primer
0.2	Failsafe Enzyme	Used from FailSafe RACE PCR Kit
10	Failsafe Buffer Mix	From FailSafe RACE PCR Kit : Comes in many varieties A through F.
4.7	Sterile MilliQ Water	
Total Volume of Reaction: 20 µL		

As an additional source of PCR products, a combination of re-amplifications and hemi-nested PCR reactions were done (Table 6). The volumes of reagents used was the

same as the Inner PCR reactions done initially (Table 5). The Modified B version of Touchdown PCR Program was used (Table 4).

Table 6 Modified RACE PCR Reactions: Re-amplification and Hemi-Nested

Template	Buffer	Vector Specific	Gene Specific Primer	Indications
QIAquick® PCR Purified Outer PCR Product 2	B	MYRFU-1	MRCOUT-1	Re-amplification
QIAquick® PCR Purified Outer PCR Product 3	C	MYRFU-1	MRCOUT-1	Re-amplification
QIAquick® PCR Purified Outer PCR Product 4	D	MYRFU-1	MRCOUT-1	Re-amplification
No Template	B	MYRFU-1	MRCOUT-1	Control
S.commune cDNA*	B	MYRFU-2	MRCOUT-1	Hemi-Nested PCR Reaction
S.commune cDNA*	B	MYRFU-1	None	Control
S.commune cDNA*	B	None	None	Control
S.commune cDNA*	B	None	MRCOUT-1	Control

**E.coli* containing ligated portions of cDNA of *S.commune* in plasmid vector prepared by Professor Stephen Horton on 9/25/03.

PCR Amplification for Purposes of Sequencing

The PCR products produced through the initial Outer Modified-RACE PCR reaction (Table 3) were re-amplified in order to complete DNA sequencing (Table 7). Two reactions were done, one for the Outer PCR reaction of Product 3C and the other for Outer PCR product 4D. The numbering refers to the order that the reaction was in and the letter refers to the FailSafe17 Enzyme Buffer used.[8] The PCR Program was the Modified B version of the Touchdown Program. Afterwards, a 1.5% gel was run in a 6-lane gel. Each well was loaded with 50 μL of PCR reaction and 10 μL of dye.

Afterwards, each of the PCR products was cut out of the gel and underwent a Gene Clean III Procedure (Appendix I: Procedure 2). [20]

Table 7 Amplification PCR for Sequencing

Volume (μL)	Reactant	Indications
0.5	Outer PCR Products	Processed through QIAquick® PCR Purification Kit
2.0	MYRFU-1	Vector-Specific Outer Primer
2.0	MRCOUT-1	Gene-Specific Outer Primer
0.5	Failsafe Enzyme	FailSafe RACE-PCR Kit
25.0	Failsafe Buffer Mix	FailSafe RACE-PCR Kit
20.0	Milli-Q Water	
Total Volume of PCR Reaction: 50.0 μL		

The PCR products were processed through the Gene Clean III[20] procedure, ligated into the pGEM T-EASY vector from Promega [16] and the DNA was then precipitated to remove the salt. (Appendix I: Procedure 3). The precipitated DNA was used to transform *E.coli* DH5 alpha cells using electroporation (Appendix I: Procedure 4) and plated onto LB-Amp plates containing 40 μL of 20 mg/mL X-GAL and 35 μL of 100 mM IPTG.

The *E.coli* DH5 alpha cells that were successfully transformed with the pGEM T-Easy vector from Promega[16] were identified by their blue color. The blue colonies were picked with a toothpick and grown in 5.0 mL of LB broth and 5 μL of ampicillin (100 μg/μL) overnight. The cultures were then processed through the QIAprep ® Spin Miniprep Kit[14] in order to purify the plasmid DNA from the *E.coli* DH5 alpha cells. A restriction enzyme digest was set up with Eco-RI in order to confirm the presence of a ligated vector in the plasmid (Table 8). Several restriction enzyme digests were completed. The indications state that restriction enzyme digests can be done for 2 hours

or overnight. The most successful digest was for 12 hours. Confirmation of a digest was done by running a 1.5% agarose gel.

Table 8 Restriction Enzyme Digest of Ligation

Volume (μL)	Reactant
1.0	DNA processed after Mini-prep
1.0	10x Buffer H
0.3	Restriction Enzyme – Eco-RI
7.7	Deionized water
Total Volume : 10.0 μL	

The final portion of the experiment requires that the concentration of plasmid DNA be determined through absorbance. The absorbance was taken at 260 and 280 nm. For sequencing two numbers must be determined, A_{260}/A_{280} and the value of [DNA] = $A_{260}/(0.02\ mL/\mu L*cm)$.

C. BD SMART™ RACE cDNA Amplification Kit

Preparation of cDNA from poly A+

The synthesis of cDNA was done on poly A+ RNA made by Professor Stephen Horton on August 11, 1999 from *S.commune* and had a concentration of 0.69 μg/μL. The procedure for this was followed as outlined in the BD SMART ™ RACE cDNA Amplification Kit User Manual from CLONTECHniques.[9] The procedure required 1 μg of mRNA to be used for the cDNA synthesis. Therefore, 1.44 μL of the 0.69 μg/μL mRNA from *S.commune* was used. In addition, cDNA was synthesized from Control Human Placental Total RNA from CLONTECHniques.[9] The synthesized cDNA made was specifically for 5' RACE PCR.

Positive Control PCR Experiment

The control human placental synthesized cDNA was used to confirm that the PCR program detailed in the BD SMART ™ RACE cDNA Amplification User Manual from CLONTECHniques[9] was appropriate for the thermal cycler in the lab. For this PCR reaction and others, the BD Advantage 2 Polymerase Mix in the kit allows for long distance (LD) PCR. The replication of longer sequences of DNA is guaranteed due to the inclusion of BD Titanium ™ Taq DNA Polymerase and BD TaqStart ™ Antibody. LD PCR allows for longer sequences of cDNA to be amplified, thus intact genes from the synthesized cDNA.[9]

BD SMART™ RACE Primers

The primer for the PCR reactions was designed as outlined in the BD SMART ™ RACE cDNA Amplification User Manual CLONTECHniques[9] (Table 1 & Figure 8).

5' Amplification of S.commune synthesized BD SMART™ Kit cDNA

The 5' amplification of the *S.commune* cDNA is outlined in the BD SMART ™ RACE cDNA Amplification Kit User Manual from CLONTECHniques.[9] The modifications and exact number of reactions are indicated in Table 9. The PCR Programs used were found in the BD SMART ™ RACE cDNA Amplification User Manual[9] and the modifications made are outlined in Table 10. The volumes of PCR reagents for re-amplification are outlined in Table 11.

Table 9 Summary of 5' Amplification of *S.commune* synthesized cDNA
*1.25 hours Run-time, all gels were stained for 20 minutes in a 0.5 µg/mL solution of ethidium bromide.

Run	PCR Program[9]	PCR Reactions and Primers	Analysis*
1	Program 1	A. *S.commune* cDNA w/ UPM and SMRO-1 B. *S.commune* cDNA w/UPM (control) C. *S.commune* cDNA w/SMRO-1 (control)	2% gel 5 µL of sample + 5 µL of deionized water + 2 µL of dye
1	Reanalysis of Initial Products		2% gel Samples loaded in duplicates 5 µL or 2 µL of sample + 5 µL or 8 µL of deionized water + 2 µL of dye for a total volume of 12 µL.
2	Re-amplification of Run #1 with Program 2 Outer PCR Products processed through QIAquick® PCR Purification Kit.	A. Control – no cDNA B. Hemi-Nested: PCR Product A1 w/NUPM + SMRO-1 C. Full Nested: PCR Product A1 w/NUPM + MRCOUT-1 D. Hemi-Nested: PCR Product A1 w/UPM + SMRO-1 E. Hemi-Nested: PCR Product A1 w/ UPM + MRCOUT-1 F. PCR Product B1 w/ NUPM + SMRO-1 G. PCR Product C1 w/ NUPM + SMRO-1	2% agarose gel Wells loaded with 5 µL of sample + 2 µL of dye + 5 µL of deionized water.
2	Reanalysis of Re-amplification PCR		2% Agarose Gel Samples 2B, 2C run in duplicate, 5 µL and 2 µL of sample.
3	Gradient 5' BDSMART RACE Modified Program 1 (Outer PCR)	A. *S.commune* cDNA w/UPM + SRI2 B. *S.commune* cDNA w/UPM + MRCOUT-1 C. *S.commune* cDNA w/ UPM + SMRO-1 Each reaction ran in triplicate, so that each set was at a different temperature.	2% Gel 5 µL of PCR Product Loaded
4 Outer PCR Products diluted in 245 µL of 10 mM Tricine-KOH, pH 8.5 & 1.0 mM EDTA buffer.	Gradient 5' BDSMART RACE Modified Program 2 (Inner PCR and Re-amp)	A. Reamp 3A B. Reamp 3B C. Reamp 3C D. Product 3A w/ MRCOUT-1 + NPM E. Product 3B w/SMRO-1 + NPM F. Control no cDNA	2% Gel 5 µL of PCR Product Loaded

Table 10 PCR Programs for Thermal Cycler using BD SMART ™ synthesized cDNA

Program 1 (citation)	Program 1 (Modified)	Program 2 (citation)	Program 2 (Modified)
A. 5 Cycles 94 °C 30 sec 72 °C 3 min	A. 5 Cycles 94 °C 30 sec 72 °C 3 min	20 cycles 94 °C 30 sec 68 °C 30 sec 72 °C 3 min	20 Cycles 94 °C 30 sec 60-68 °C 30 sec 72 °C 3 min
B. 5 Cycles 94 °C 30 sec 70 °C 30 sec 72 °C 3 min	B. 5 Cycles 94 °C 30 sec 60-70 °C 30 sec 72 °C 3 min		
C. 20 Cycles 94 °C 30 sec 68 °C 30 sec 72 °C 3 min	C. 20 Cycles 94 °C 30 sec 60-68 °C 30 sec 72 °C 3 min		

Table 11 Re-amplification of Outer PCR Products (BD SMART ™ cDNA)

Volume (µL)	Reactant
27.6	PCR Grade Water
4.0	10x BD Advantage 2 PCR Buffer
0.8	DNTP Mix (10mM)
0.8	50x BD Advantage 2 Polymerase Mix
0.8	NPM
0.8	SMRO-1
4.0	Template: Inner PCR Product 4E
Total Volume : 50.0 µL	

D. BD SMART ™ RACE cDNA and FailSafe RACE PCR Kit Reagents

The synthesized cDNA from the BD SMART ™ RACE Kit[9] was used in many reactions using the FailSafe RACE PCR Kit Reagents[17]. Several combinations were done between the gene-specific primer designed for the BD SMART ™ RACE[9] Reactions and the inner and outer gene-specific primers used in the Modified RACE-

PCR. The PCR Programs used for the reactions are outlined in Table 10. The volumes used for reagents are listed in Table 12.

Table 12 PCR Ingredients for BD SMART ™ RACE cDNA[9] and FailSafe RACE PCR Kit Reagents[8]

Initial PCR (Outer PCR)		Inner PCR or Re-amplification	
Volume (µL)	Reagents	Volume (µL)	Reagents
1	Template cDNA	3.5	Template (Outer PCR Product)
2.5	UPM	0.8	NPM
2.5	Outer Primer	0.8	Inner Gene Specific Primer
0.25	Failsafe Enzyme	0.2	Failsafe Enzyme
12.5	Failsafe Buffer Mix	10	Failsafe Buffer Mix
6.25	Sterile Milli-Q Water	7.7	Sterile Milli-Q Water

RESULTS

Results

A. Screening by Colony Hybridization

Primary Screening by Colony Hybridizations

Two plates with the *E.coli* cultures were used for the colony hybridizations (A and B). The X-rays films only revealed reproducible hybridization points to the probe on one plate (B) (Figure 10). The A plate demonstrated some hybridization, however the hybridization points were not replicated in the double X-rays taken, thus indicating that the observed hybridizations were most likely noise (Figure 9).

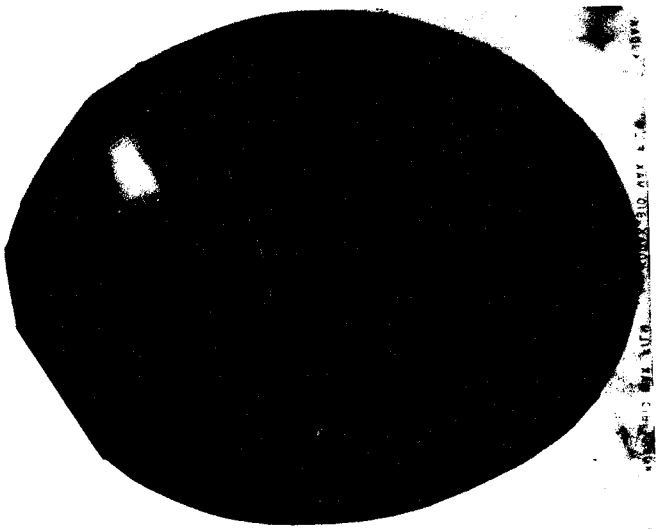


Figure 9 Unsuccessful Primary Screening by Colony Hybridization: Plate A

The X-ray films taken of Plate A did not reveal reproducible hybridization points to the probe. As a result, the identified hybridization sports observed were attributed to noise rather than signal.

In Plate B, the X-ray films demonstrated reproducible hybridization points (Figure 10). These colonies were then used for the secondary screening by colony hybridization (Figure 10).

Secondary Screening by Colony Hybridization

Of the six different hybridization spots identified in the primary screening (Figure 10), the developed X-ray films in the secondary screening did not identify any hybridizations spots. The developed films for all six colonies were dark and overwhelmed with noise (Figure 11).

B. Modified-RACE PCR

Two different sized outer PCR products were amplified using the outer gene-specific primer MRCOUT-1 and the outer pMyr vector-specific primer MYRFU-1 (Figure 12). The size of the outer PCR products is not given due to the distorted KB ladder. Further re-amplification through the inner Modified-RACE PCR was not successful (Figure 13).

The isolation of the original two outer PCR products (Lane 1 & 3 from Figure 12) through Gene Clean III [20] and then running the products on a gel, determined their size to be 400 bp and 700 bp (Figure 14). For purposes of sequencing, only one outer PCR product (400 bp) was successfully ligated for further replication in *E.coli* as evidenced in the successful Eco-RI digest (Figure 15).

The capillary electrophoresis elution chromatogram for the DNA sequence identified through Modified-RACE PCR is in Figure 16. A total of 400 bp of pMyr vector and gene sequence were identified. The additional gene sequence identified was 243 bp (81 amino acids) in the partial putative caspase in *S.commune* (Figure 17). The 5' end of the cDNA segment was confirmed by identifying the Myr sequence. A protein sequence alignment of the additional sequence determined through the Modified-RACE PCR reveals that it is still 91 Amino Acids shorter than the *S.cerevisiae* protein (Figure 18).

C. BD SMART ™ RACE PCR Reactions

The successful synthesis of cDNA was confirmed through a PCR reaction using the control human placental total RNA. Two bands were identified in the gel of 2600 bp and 300 bp (Figure 19).

The outer PCR reaction between the BD SMART UPM and the gene specific outer MRCOUT-1 primer did not reveal any bands in the region between 500 bp and 750 bp (Figure 20A). This would correspond to 172 amino acids. Only 91 amino acids of the 172 amino acids would be "new" amino acids. The other 81 amino acids would correspond to the amino acids identified through Modified-RACE PCR (Figure 17). The inner PCR reaction gave a band at 6000 bp using the outer PCR as a template and the nested primers BD SMART NPM and the gene specific SMRO-1 primer (Figure 20A). A 40 µL PCR re-amplification of the 6000 bp inner PCR product did not result in a band (Figure 20B).

Several bands were successfully seen when the cDNA from the BD SMART ™ RACE kit was used in conjunction with the Failsafe PCR Reagents (Figure 21). The bands ranged in size from 1 Kb to less than 250 bp. The PCR reactions in Lanes 4-7 are hemi-nested reactions to the PCR reactions in Lanes 1-3. A slight downward shift can be seen in the bands indicated by the arrows, which gives an indication that it might be the band containing the gene of interest. The shift in bands is also seen in the hemi-nested PCR in Figure 22 as indicated by the arrows. Unfortunately, the full-nested PCR was not successful.

DISCUSSION

Discussion

A. Screening by Colony Hybridization

Even though the primary screening by colony hybridization successfully identified six potential *E.coli* colonies on Plate B containing the sequence of the putative *S.commune* caspase protein (Figure 10), not one colony successfully produced hybridization points in the secondary screening (Figure 11). As a result, no additional sequence of the putative caspase in *S.commune* was identified through screening by colony hybridization.

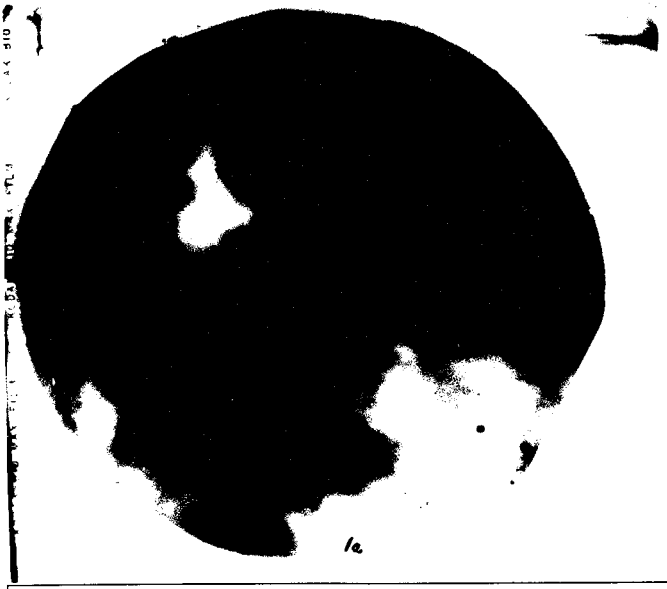


Figure 10 Reproducible Hybridization Points in Primary Screening by Colony Hybridization: Plate B
The X-ray films taken of Plate B revealed six reproducible hybridization points to the probe. These hybridization points are circled in red and numbered. These colonies were then used to plate the six cultures used for the secondary screening by colony hybridization.

Several reasons can be given to explain the unsuccessful secondary screening by colony hybridization. One of the most obvious places for the possibility of error, is that the hybridization spots from Plate B were not picked in such a way to encompass the amount of uncertainty in their identification. As a result, the secondary plates would not contain the appropriate bacterial colonies and hybridization to the probe would not be possible. In order to move forward into the secondary screening, the hybridization spots had to be accurately picked and plated.

Theoretically, if the picking of the hybridized colonies in the primary screening successfully encompassed the uncertainty, other sources of error could have arisen in the secondary screening. Potentially, the probe, since it was made previously for other hybridization procedures may have become ineffective, thus not allowing for adequate hybridization to the DNA. However, this possibility does not appear to be of great concern, since the same probe was used in the primary screening and worked well. Lack of selectivity appears to be the main problem for the secondary screening as seen through the dark spots. The filter paper is designed to allow everything to bind. It is through the various washes that one establishes the selectivity of hybridization. In the secondary screening, three times the amount of probe was used since the area of the combined filters was three times as that in the primary screening. In addition, the hybridization oven temperature was reduced from 68°C to 59°C which allowed for less stringent hybridization conditions. Overall, the increased amount of probe and the less stringent hybridization temperature allowed for greater binding to the filter paper. The blocking buffer that is used to decrease the probe's affinity to the filter paper was kept at the same concentration (1.5%) as used for the primary screening. This might not have

been sufficient to selectively allow the additional probe to hybridize specifically to the DNA.

In addition, due to missing anti-digoxigenin, the filters were left overnight in the pre-washing solution that is meant to improve probe binding. The following day, only 1 μL of anti-digoxigenin was added instead of the required 15 μL. As a last minute attempt to rectify this, the procedure was started again with the same filters at the pre-washing solution phase and then the 15 μL of anti-digoxigenin was added. The anti-digoxigenin is responsible for the chemiluminescence of the probe which allows its viewing on the X-ray films. Overall, the procedural changes and mishaps may have resulted in the overall non-selective hybridization of the probe throughout the filter paper. Even if the probe did hybridize selectively, the chemiluminescence may not have worked due to the procedural errors in adding the anti-digoxigenin. As a result, the secondary screening by colony hybridization did not produce identifiable hybridization points (Figure 11). The procedure was not repeated because the time necessary to complete the procedure was not available.

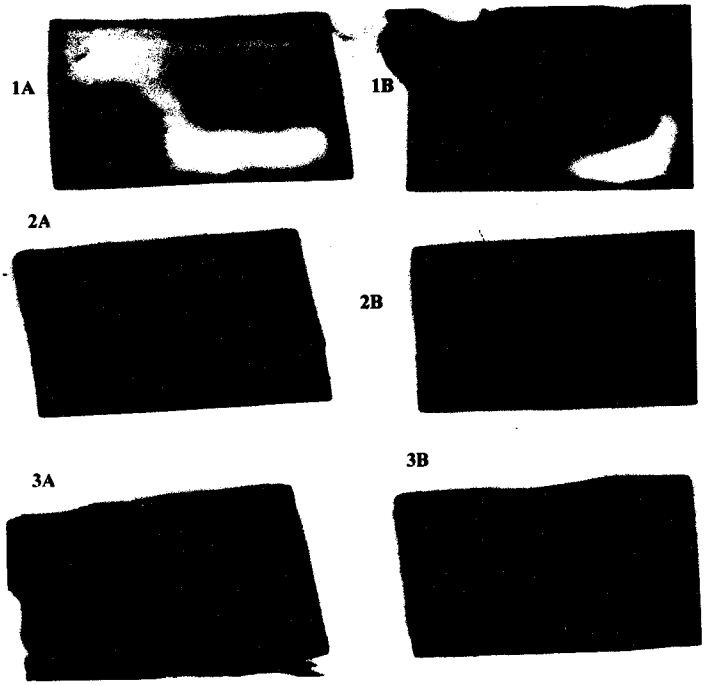


Figure 11 Secondary Screening by Colony Hybridization

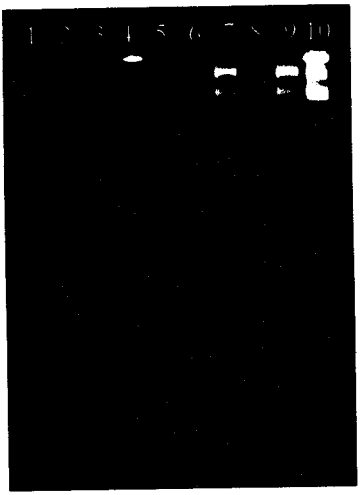
The X-ray films taken of the cultures for all six hybridized colonies identified in Plate B (Figure 10) did not give any reproducible hybridization points. The developed X-ray films were dark and areas of hybridization could not be identified. Figure 11 contains the replicate X-ray films of Colonies 1, 2 and 3. A and B identifies the two filter papers taken per culture.

B. Modified-RACE PCR

The Modified-RACE Outer PCR reaction using the pMyr vector with ligated *S.commune* cDNA as the template, successfully amplified two differently sized products (Figure 12). The Failsafe Enzyme Buffers that successfully worked included Buffers B, C, and D with the primers MRCOUT-1 and MYRFU-1. The KB Ladder came out distorted in the gel, perhaps because it was too dilute, and thus could not be used to determine the size of the bands. It was thought that the inner vector-specific primer might work well with the outer gene-specific primer and produce PCR products that were slightly shifted. A hemi-nested PCR between the inner vector primer, MYRFU-2, and MRCOUT-1 (gene-specific primer) was not successful as seen in Lane 7 (Figure 12). Lane 7 in fact looks just like Lane 9 where no primers were used, thus indicating that little annealing if any occurred with the primers and only the cDNA is being seen.

The Outer PCR reaction was completed successfully as seen through the controls (Figure 12). One of the controls in Lane 6 only contains primers, but no cDNA. No bands were seen in Lane 6, thus suggesting that the products seen in Lanes 1 through 3 are not arbitrary. In lanes 8 and 10, only one primer was mixed with the cDNA. It was expected that only the cDNA would be seen, which is the case in lane 10. The only control that did not work well was in lane 8 where MYRFU-1 and cDNA were mixed. No bands are seen, thus it is thought that the cDNA may not have been added to the PCR tube. The cDNA in the gel is seen as three bands because it is not linear, it is pieces of cDNA ligated into a vector (Lane 9). The smallest band that travels the furthest represents any vector pieces that were nicked during the procedure and were able to

travel as linear cDNA. In the gel this band is extremely faint. The next band represents the super coiled vector and the top band that travels as a vector (Figure 12).



Legend:

Lanes 1: Buffer B (MRCOUT-1 & MYRFU-1)
Lane 2: Buffer C (MRCOUT-1 & MYRFU-1)
Lane 3: Buffer D (MRCOUT-1 & MYRFU-1)
Lane 4: KB Ladder (Distorted)
Lane 5: Blank
Lane 6: No cDNA w/ Primers (MRCOUT-1 & MYRFU-1)
Lane 7: Buffer D (MYRFU-2 & MRCOUT-1)
Lane 8: MYRFU-1 alone
Lane 9: cDNA (no primers)
Lane 10: MYRFU-1 Alone with cDNA

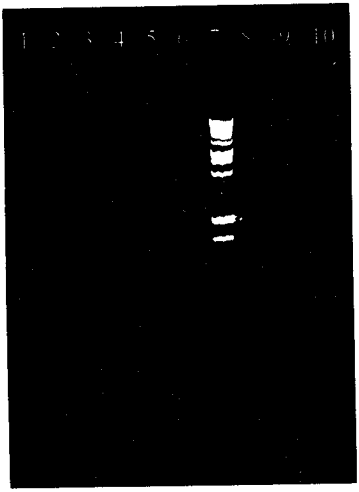
1.5% Agarose & 1.25 hours Runtime
Gel stained with 2 µL of 10 mg/mL ethidium bromide in liquid gel.

Figure 12 Outer PCR Reaction for Modified-RACE PCR

Two different sized outer PCR products were generated using MRCOUT-1 and MYRFU-1 (Lane 1,2 & 3). The size of the two products cannot be determined from the gel due to the distorted KB ladder in Lane 4.

Overall, the Outer PCR with the Modified-RACE PCR was able to confirm that two products were possible containing the DNA in question. In an attempt to further confirm these results and verify the products as specific to the gene, nested PCRs were tried with the products from Lane 1 through 3 from Figure 12. Not one of the inner PCR reactions successfully produced any bands (Figure 13). This may be attributed to several

reasons. First, the combination of primers used may not be ideal with the Failsafe Enzyme Buffers that were used. Secondly, we know the gel ran well, since the KB ladder is visible in Lane 7. In addition, no bands were seen in lane 9 where primers were added with no cDNA (Figure 13). Even if the PCR reactions did not work, a DNA smear should appear in the lanes. Therefore, we believe that the purified PCR products used for the template in the inner PCR reaction may have been too dilute and thus not have produced sufficient product to be seen on the gel (Figure 13).



Legend:

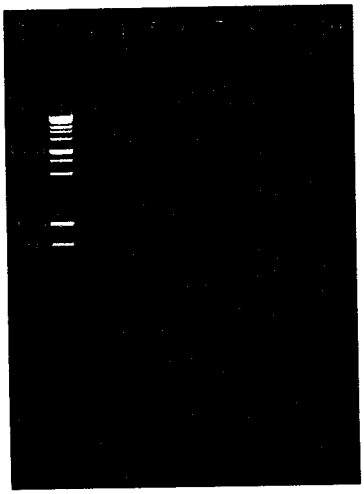
Lane 1: Template: Outer Lane 1 Buffer A (MYRFU-2 & MRCIN-1)
Lane 2: Template: Outer Lane 2 Buffer B (MYRFU-2 & MRCIN-1)
Lane 3: Template: Outer Lane 1 Buffer C (MYRFU-2 & MRCIN-1)
Lane 4: Template: Outer Lane 3 Buffer D (MYRFU-2 & MRCIN-1)
Lane 5: Template: Outer Lane 1 Buffer E (MYRFU-2 & MRCIN-1)
Lane 6: Blank
Lane 7: KB Ladder
Lane 8: Template: Outer Lane 1 Buffer F (MYRFU-2 & MRCIN-1)
Lane 9: No cDNA w/(MYRFU-2 & MRCIN-1)
Lane 10: Blank

1.5% Agarose Gel & Run-time 1.25 hrs.
Gel stained with 2 µL of 10 mg/mL ethidium bromide in liquid gel.

Figure 13 Inner PCR for Modified RACE-PCR

The inner PCR reaction using the outer PCR products in Lane 1-3 from Figure 8 as the templates, did not successfully produce any products.

Since the inner PCR reaction did not successfully produce any re-amplified products, the outer PCR products were used for sequencing. If several bands had been present, an inner PCR would have reconfirmed the gene-specific products, but since only two different products were present it was best to move forward with the sequencing. After a 40 μL re-amplification PCR reaction, the two outer PCR products were successfully isolated and processed through Gene Clean III. The gel confirming the isolated Outer PCR products identified them as being 400 bp as produced with Buffer C and 700 bp with Buffer D (Figure 14). Since approximately 516 bp were missing if the *S.commune* putative caspase was as long as the *S.cerevisiae*, bands of this size could be consistent with obtaining the 5' end of the gene. Unfortunately, after the ligation procedure, only the 400 bp PCR fragment was successfully ligated into the pGEM-T easy plasmid, as demonstrated through the l RI digest (Figure 15). If the *S.commune* putative caspase is as long as the *S.ce isiae*, then this outer PCR product will only give a portion of the 5' end.



Legend:

Lane 2: KB Ladder

Lane 5: Outer PCR Product A (Lane 1 Figure 8) Buffer C

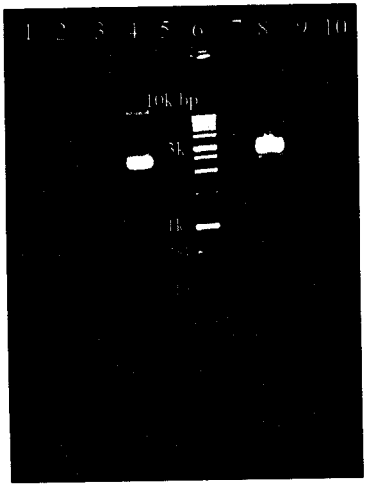
Lane 6: Outer PCR Product B (Lane 3 in Figure 8) Buffer D

All other lanes are blank.

1.5% Agarose Gel, 1.25 Hrs. Runtime
Gel stained with 2 μL of 10 mg/mL ethidium bromide in liquid gel.

Figure 14 Gene Cleaned III Outer PCR Products from Modified RACE-PCR

This gel was used to confirm the isolation of the outer PCR products identified in Figure 8. In addition, the size of the products was determined to be 400 bp and 700 bp.



Legend:

Lane 3: no Plasmid

Lane 4: Uncut Plasmid

Lane 6: KB Ladder

Lane 8: Eco-RI digest of Plasmid

All other lanes are blank.

1.5% Agarose Gel & 1.25 Hrs. Runtime
Gel stained with 2 µL of 10 mg/mL ethidium bromide in liquid gel.

Figure 15 Successful Ligated Outer PCR Product from Modified RACE-PCR Confirmed through Eco-RI Digest

The Outer PCR product (Lane 5 in Figure 14) successfully ligated into the pGEM-T easy plasmid and is 400 bp as seen in Lane 8. Only one of the two original Outer PCR products successful ligated into the plasmid vector. The 700 bp Outer PCR product (Lane 8 in Figure 14) was not successfully ligated into the vector. The 3000 bp band in Lane 8 represents the pGEM-T easy plasmid DNA.

Figure 16 Capillary Electrophoresis Elution chromatogram for the DNA sequence of the Putative Caspase in *S.commune* identified through Modified-RACE PCR (Next Page)

The DNA sequencing was completed at the Center for Comparative Functional Genomics at the University of Albany. This is a capillary electrophoresis elution chromatogram of DNA and it outlines the raw data DNA sequence of the outer PCR product generated through the Modified-RACE PCR procedure (Figure 15). In DNA sequencing, each of the different DNA bases are labeled with a different Fluorescence color: blue is C, red, T, black G and green A. The presence of a specific base is then identified on the chromatogram by the colored peak.

ABI PRISM

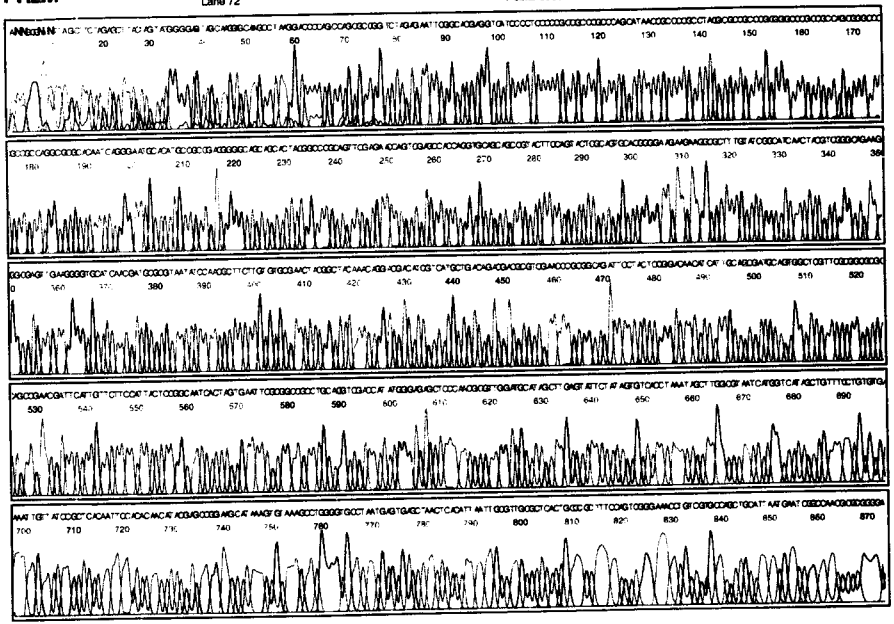
Model 3700
BC 1.3.0.0

DSR650_C10_B10392_072.ab1
MRC6 MYRFU1
B10392
Lane 72

Signal G:499 A:468 T:294 C:663
DT3700POP6{BDv3}v1.mob

Points 3930 to 21386 Base 1: 3930

Mon, Dec 8, 2003 9:50 AM
Fri, Dec 5, 2003 3:21 PM
Spacing: 11.50



After sequencing an additional 81 amino acids were identified in the *S.commune* putative caspase protein (Figure 17). For purposes of sequencing, the SP6 primer (3' primer) worked well and gave an additional 400 bps of *S.commune* gene sequence and pMyr vector sequence. The T7 primer (5' primer) did not work during the sequencing procedure by showing only the portion of the *S.commune* gene we already knew. The second round of sequencing worked (Figure 16).

The intact 5' end of the gene was not determined through the Modified-RACE PCR for several reasons. First, the outer PCR product (400 bp) that was sequenced was smaller than the theorized missing segment of the gene, estimated to be 516 bp. A portion of the outer PCR product sequenced had to contain some of the vector sequence in addition to the ligated cDNA segment. Upon examination of the sequence, several key features were missing. An ATG codon (transcription start codon) was not present in the new sequence, and the Kozak sequence C(A or G)CCATGGC surrounding the start codon was also missing. Since the protein in question is a *S.commune* protein, one of the species specific properties of the protein is the Kozak sequence (Figure 17).

Several reasons can explain the fact that the full 5' region of the protein is missing in addition to the small size of the PCR product. First of all the cDNA library may not contain the full-length gene ligated into the vector. The pMyr library of *S.commune* cDNA is made up of synthesized cDNA. This synthesized cDNA must be transcribed from mRNA. The first portion that is made is the poly A+ tail which is the 3' region of the protein. Many times the synthesis is not complete because the DNA polymerase is not able to transcribe the entire gene, thus leaving many cDNA segments with incomplete terminal 5' ends. Therefore, even if another outer PCR was completed, if the cDNA

fragment does not have the complete gene, new sequence will not be determined. The other possibility is that the 700 bp fragment contained the remaining portion of the gene, or at least additional sequence. This may be the case, but the possibility was not pursued and instead it was felt that new cDNA should by synthesized to ensure a complete transcribed gene.

5'.....SSPPPPPAQHNRPAYAPPAGPPPGAHNQGMHMPPTGQQHYGPQFE
NQSSHQVQQPYFQYSQCTGKKKALCIGINYVGQKGELKGCINDARNIQRFLCAN
YGYKQDDIVMLTDDASNPRQIPTRDNIIAAMQWLVRGAQPNDSLFFHYSGHGGS
TKDLDGDEADGYDEVIYPIDYENAGHLVDDLMHDIMVKPLPAGCRLTAIFDSCH
SGSALDLPYIYSTEGKIKEPNLAAEAGQGVLSAVTSYAKGDMGGVFKSAVGLFK
TASGNTQKAQEVARQTKTSPADVISWSGCKDSQTSADAYEAGQATGAMSYAFM
TALGQNKQQTYQQLLVEIRGILKAKYSQKPQLSASHPIDTSIMFIC 3'

Figure 17 Additional Protein Sequence of Putative Caspase Protein in *S.commune* identified through Modified-RACE PCR (360 amino acids)

The protein sequence determined through Modified-RACE PCR identified an additional 81 Amino Acids at the 5' region of the Putative Caspase. The Modified-RACE PCR sequence is identified in green. The remaining sequence shown was identified by degenerate PCR and 3'RACE PCR.[10]
Red Sequence determined through Degenerate Primer PCR,
Blue Sequence determined with 3'RACE PCR
Pink Sequence overlap region between the two methods.

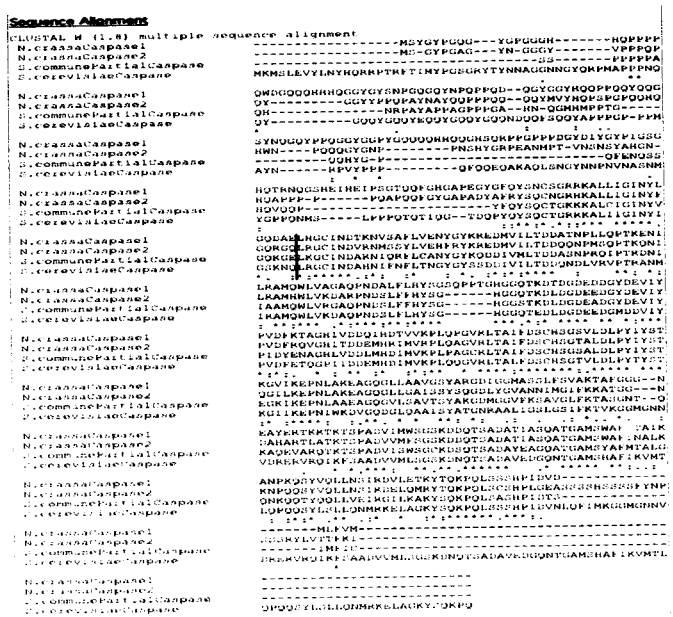
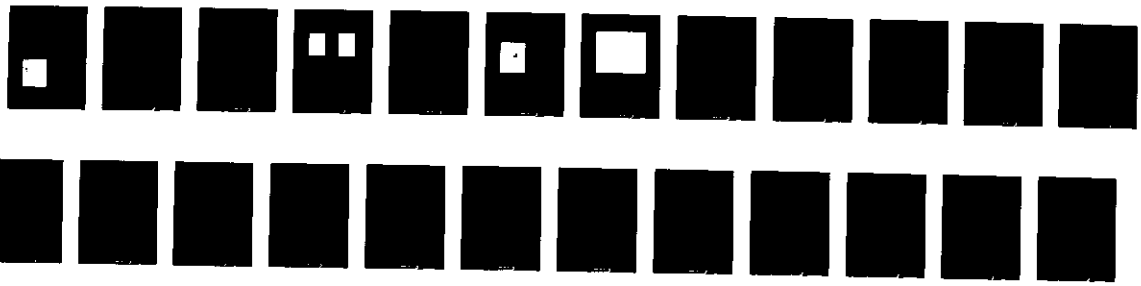


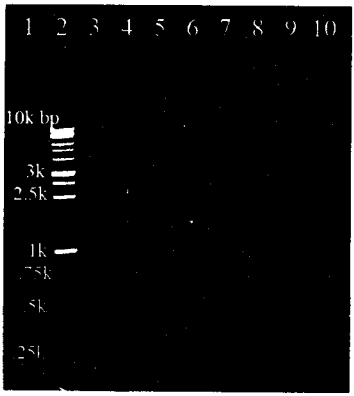
Figure 18 Sequence Alignment of Partial Putative Caspase in *S.commune* identified through Modified-RACE PCR with *N.crassa* and *S.cerevisiae* Caspases

The two *N.crassa* caspases have 460 and 441 amino acids, respectively. The *S.cerevisiae* caspase has 537 amino acids. An additional 81 amino acids were identified in the *S.commune* putative caspase protein and can be found to the left of the Red Line. Leucine is the first amino acid identified through degenerate PCR.[10] A total of 360 amino acids have been identified in the putative caspase in *S.commune*. From the sequence alignment, one can see that the 5' ends of the proteins do not have a great deal of sequence similarity. Overall, the 5' region of the caspases appears to be highly variable. Of all the caspases in the alignment, the longest is the *S.cerevisiae* caspase. An additional, 91 amino acids must be identified if the putative caspase in *S.commune* is as long as the *S.cerevisiae* caspase.



C. BD SMART ™ RACE cDNA Amplification Kit[9] Reactions

In order to complete the sequence of the missing 5' region of the putative caspase in *S.commune*, the BD SMART ™ Kit[9] was purchased because it claimed to ensure the synthesis of intact cDNA. The synthesis of the cDNA was confirmed through a control PCR reaction with the synthesized cDNA from the Human Placental Total RNA which was performed in parallel with the synthesis of the *S.commune* cDNA. The two bands appeared in the gel at 2600 bp and 300 bp, the sizes indicated in the BD SMART ™ Kit[9] (Figure 19). The 2600 bp band is a bit strange because it has a smiley shape, however it is located at the appropriate location. Also, the cDNA synthesized for *S.commune* was used by Professor Stephen Horton in a PCR reaction that successfully produced expected PCR products, thus reconfirming proper synthesis of the cDNA.



Legend:

Lane 2: KB Ladder

Lane 4: 5' RACE Control (UPM & Placental Gene Specific Primer)

Lane 7: Internal Control (Two Placental Gene Specific Primers)

1.2% Agarose Gel: 1.25 Hrs. Runtime
The gel was stained 20 minutes in a 0.5 µL/mL solution.

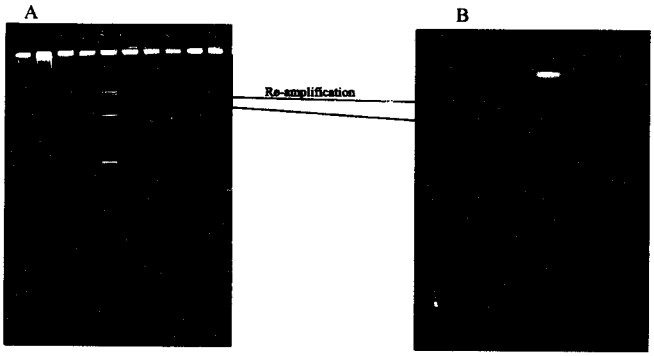
Figure 19 Control BD SMART PCR Reaction with Human Placental Total RNA

The control PCR reaction with the synthesized Human Placental cDNA from total RNA successfully produced the two indicated bands 2.6 KB (Lane 4) and 0.3 KB (Lane 7). This indicates that the cDNA was successfully synthesized from the RNA.

As stated earlier, it is expected that 91 amino acids (273 bp) are missing from the putative *S.commune* caspase protein. However, the primers used in this PCR reaction are the same ones used from the Modified RACE-PCR (Table 1 & Figure 8). The template used for these reactions is the cDNA synthesized from the BD SMART ™ Kit.[9] Therefore, the 81 amino acids (243 bp) determined through the Modified RACE-PCR plus the hypothesized missing 91 amino acids (273 bp) are expected. This would result in a total of 172 amino acids (516 bp). Therefore, a band is expected between 500 and 750 bp to account for additional untranslated sequence (Figure 20A). In this PCR reaction, the outer PCR did not give any bands. The only band that was produced was through an inner PCR reaction of the Outer PCR products (Figure 20A). This band was a bit abnormal in Lane 8 due to its sharp v-character. In order to reconfirm the abnormal v-shaped band, a re-amplification was done for a total PCR reaction of 40 µL, however no bands were visible (Figure 20B).

Several PCR reactions were done with the BDSMART reagents and synthesized cDNA, however, no bands were ever seen. If a band was observed, it was always irregular (v-shaped or smiley faced) and upon re-amplification for sequencing, produced no bands, only smears. This supports the fact that a product is not really present at any concentration, since re-amplification should give enough product to make it visible on a gel. If the mRNA used to make the cDNA was defective, then we would expect a large irregular band at the bottom of each lane corresponding to the degraded mRNA. However, this is not seen (Figure 20A). Since the cDNA was confirmed to be synthesized properly by our lab and Professor Stephen Horton's lab, it was thought that the reagents in the BD SMART kit as well as their suggested PCR program may not be

working as well with our cDNA or the gene-specific primer (SMRCO-1). The fact that MRCOUT-1, the successful gene-specific primer used in the Modified-RACE PCR was also not successful (Figure 20A), is consistent with this idea. Even though the BD SMART kit reagents worked well with the control human placental cDNA, it does not necessarily mean that the *S.commune* cDNA will optimally replicate under the same conditions. As a result, the best option was to move forward with the synthesized cDNA and determine conditions related to those that worked well before, namely, Failsafe PCR kit reagents.



Legend Gel A:

Lane 1-3: ReAmp of cDNA with UPM & MRCOUT-1 (Temp: 68°C)
Lane 5: KB Ladder
Lane 8: Inner PCR
Template: Amp of UPM & MRCOUT-1
Primers: NPM & SMRO-1 (Temp: 65°C)
1.5% Agarose Gel & Runtime 1.25 hrs.

The gel was stained 20 minutes in a 0.5 µL/mL solution

Legend Gel B:

Lane 2: Reamp of Lane 8 (Gel A)
Lane 4: Reamp of Lane 8 (Gel A)
Lane 6: KB Ladder Distorted
1.5% Agarose Gel & Runtime 1.25 hrs.

The gel was stained 20 minutes in a 0.5 µL/mL solution

Figure 20 BD SMART ™ RACE Outer and Inner PCR

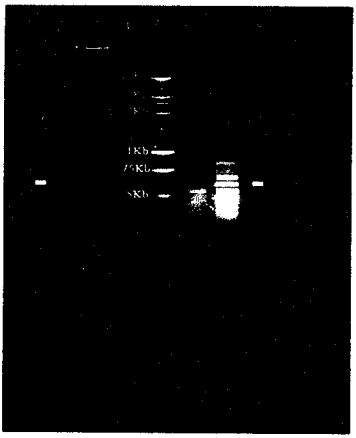
A band was expected between 500 and 750 bp in the outer PCR reaction (Lane 1-3) in Gel A. Specifically, 516 bp are expected if the putative caspase in *S.commune* is as long as the *S.cerevisiae*. Since the primers are in the same location as used in the Modified-RACE PCR, the same band size is expected. A band of 516 bp, would correspond to 172 amino acids, 81 amino acids that have already identified through Modified-RACE PCR and the additional 91 amino acids that are predicted to be missing. No band was observed in this region. The Inner PCR reaction of the outer PCR reaction produced a V-shaped band (Lane 8 Gel A). Upon Re-amplification of the Inner PCR Product, no bands were visible (Gel B).

D. BD SMART ™ Kit[9] Synthesized cDNA with FailSafe ™ PCR PreMix Selection Kit[17]

Several bands were successfully produced when the FailSafe ™ PCR [17] reagents were used with the BD SMART ™ Kit[9] synthesized cDNA (Figure 21). The basis of this PCR is similar to the logic used in the Modified RACE PCR. Just as before, one primer is specific to the "vector", in this case the UPM that anneals specifically to a sequence in the cDNA located upstream to the 5' end of the gene and the gene-specific primer within the gene of interest. The gene-specific primers were MRCOUT-1, SMRCO-1 and SCPR12 (Table 1). For this PCR, several conditions were modified, including annealing temperature and buffers used. The successful primers included the SMRCO-1 and MRCOUT-1 in conjunction with the UPM.

Since several bands were produced, it is important to determine which bands are specifically produced from the two primers. Since MRCOUT-1 is located outside of SMRCO-1, it is possible to compare the gel and establish shifted bands. The shift would be extremely small since the two primers are only 19 bps apart (Figure 8). There appears to be one set of bands that are slightly shifted at 700 bp (Figure 21) in Lanes 3 and 6. The band at 700 bp is very promising, since a band of approximately 516 bps is expected corresponding to 172 amino acids (Figure 20A) corresponding to the 81 amino acids found through the Modified RACE-PCR and the hypothesized 91 amino acids that are needed for the putative *S.commune* caspase to be the same length as the *S.cerevisiae* caspase. The additional sequence is likely close to the 5' untranslated region. The Inner PCR done on the Outer PCR products from Figure 21 is also promising due to the clear shift in the 700 bp bands seen in Lanes 2 and 3 (Figure 22). The shifted band is hypothesized to contain the gene of interest since a hemi-nested PCR resulted in a

downward shift in the band (Lane 2), and the size is of the band is consistent with the hypothesis.



Legend:

- Primers: UPM + MRCOUT-1
  - Lane 1: Buffer A 70° C
  - Lane 2: Buffer B 68.1 °C
  - Lane 3: Buffer C 65° C
- Primers: UPM + SMRCO-1
  - Lane 4: Buffer D 70° C
  - Lane 6: Buffer E 68.1 °C
  - Lane 7: Buffer F 65 °C
- Primers: SCPR12 + UPM
  - Lane 8: Buffer G 70 °C
  - Lane 9: Buffer H 68.1 °C
  - Lane 10: Buffer A 65 °C

Temperature Indications Refer to the Annealing Temperature used during the PCR Rxn.
1.5% Agarose Gel & 1.25 Hrs. Run-time. The gel was stained 20 minutes in a 0.5 µL/mL solution

Figure 21 Outer PCR of Synthesized cDNA from BD SMART ™ Kit and Failsafe PCR Reagents

The synthesized cDNA from the BD SMART Kit successfully produced bands when used with the Failsafe PCR reagents. Several bands were produced of varying sizes from 1kb to less than 250 bp using two different sets of primers. The bands indicated by the arrows are promising because of the expected missing sequence between 500 and 750 bp.

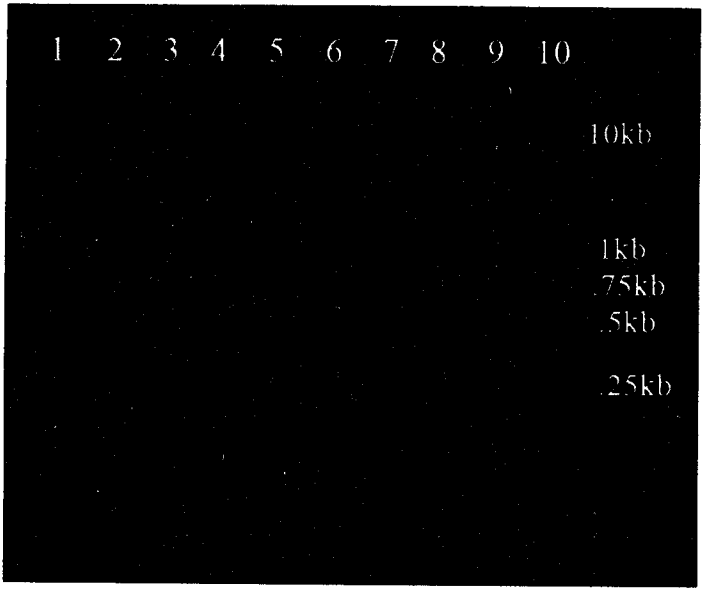


Figure 22 Inner PCR of Synthesized cDNA from BD SMART ™ Kit and Failsafe PCR Reagents

The Inner PCR reaction of the Outer PCR products from Lane 3 in Figure 21 successfully produced several bands. Lane 4 is a re-amplification of UPM and MRCOUT-1 PCR product. A hemi-nested PCR reaction of this product in Lane 3 produced a band slightly smaller that its corresponding band (indicated with arrows). This band shift suggests that the gene of interest has been probed.

1.5% Agarose Gel & 1.25 Hrs. Run-time. The gel was stained 20 minutes in a 0.5 µL/mL solution

- **Legend**
- Lane 1: NPM & MRCOUT-1
- Lane 2: NPM & SMRCO-1
- Lane 3: NPM & MRCOUT-1
- Lane 4: UPM & MRCOUT-1 (Re-Amp)
- Lane 5: UPM & MRCOUT-1 (Re-Amp)
- Lane 6: NPM & SMRO-1
- Lane 7: NPM & SMRO-1
- Lane 8: UPM & SMRO-1 (Re-AMP)
- Lane 9: UPM & SMRO-1 (Re-AMP)
- Lane 10: KB Ladder

CONCLUSION

Conclusion

This experiment successfully identified an additional 81 amino acids at the 5' end of the putative caspase protein in *S.commune* through Modified-RACE PCR. The use of the synthesized BD SMART[9] cDNA with the FailSafe Kit[17] reagents, shows great promise in the identification of the 91 amino acids at the 5' end of the gene that are hypothesized to be missing. The outer PCR products generated with the FailSafe Kit[17] reagents and BD SMART[9] cDNA suggests that the theorized missing segment of the gene is in the 700 bp band observed (Figure 21). This suggestion is only reconfirmed by the shift observed in the 700 bp band observed in the hemi-nested PCR reactions with the NPM and SMRCOUT-1 primers (Figure 21 & 22).

As a continuation to this project, a 40 µL nested-PCR reaction should be done in order to cut-out the 700 bp band and isolate it for sequencing. Before sequencing, the 700 bp band can be reconfirmed by designing a more suitable gene-specific primer. Ideally, in order to more accurately determine a shift in bands from an outer PCR product to an inner, it would be best to have a significant distance between the primers, like 100 bp. A clearer fully nested PCR would sufficiently reconfirm the 700 bp band and save a great deal of time in the ligation procedure necessary for processing the PCR product prior to sequencing.

If the BD SMART[9] cDNA with the FailSafe Kit[17] reagents do not successfully produce the complete missing sequence, it might be worth exploring a few PCR reactions with the Modified-RACE PCR procedure. In this procedure, a 700 bp PCR product was generated in addition to the 400 bp product used for sequencing (Figure 12). The 700 bp product might contain the full missing gene sequence and might be worth isolating.

Overall, from this experiment, it was found that the most effective method of probing for the putative caspase gene in *S.commune* has been through PCR rather than screening by colony hybridization. The most successful PCR reactions have been those that utilize the *S.commune* cDNA as a template (ligated into pMyr or synthesized through BD SMART Kit[9]) and in combination with FailSafe Kit[17] Reagents. It appears that the *S.commune* cDNA is prone to better hybridization and overall PCR success with the conditions provided by the FailSafe Kit[17] Reagents. This should be kept in mind with future PCR reactions.

APPENDIX I

Appendix
Procedure 1: Colony Screening by Hybridization

Primary Screen

Day 1

Take 1° cDNA library "A" T26 12/99 out of -80°C. Must be taken out of –80°C right before it is to be used. Thaw stock on ice. Dilute using the following schedule.

mix 1 = 50 µL T26 + 450 µL LB
mix 2 = 50 µL mix 1 + 950 µL LB
mix 3 = 100 µL mix 3 + 900 µL LB
mix 4 = 250 µL mix 4 + 750 µL LB

Plate 100 µL of Mix 4 on each of 2 large LB/CHL plates.
Grow overnight at 37°C. (Big room next to Biochemistry lab) Don't forget to label the plates. Store upside down.

Sterilize 2 filters /plate (label before autoclaving 1a, 1b, 2a, 2b with a pencil). Filters should be cut to the size of the plate. Only touch filter paper with gloves.
Autoclave filters in a glass dish. Keep blue filter paper coverings and stack in glass dish in between paper towels. Cover glass dish with foil and then autoclave.

Autoclave 2 forceps/plate. Forceps should be covered with foil and then autoclaved.

Day 2

Transfer plates to 4°C for at least 1 hour prior to continuing experiment.

Lay out 5 half sheets of 3MM paper with gloves on saran wrap.
Saturate with the solutions.

1= 10% SDS
2= denaturing buffer
3= neutralizing buffer
4= 2X SSC

Be sure that there is plenty of buffer, but that they are not dripping.

Lift colonies

Using sterile, blunt forceps, bend the filter (A) and then lower onto the plate so that the center of the filter touches the center of the plate first.

Let it sit 30 seconds. Don't push down on the paper with the forceps.

While it is sitting, mark with needle asymmetrically in a coding like fashion to distinguish between the two plates. Filters 1a and 1b will have the same coding scheme.
Remove filter and set right side up on 3MM paper to dry for 5-10 min. Make sure the plate is covered, it is important to maintain the sterility of the bacteria filled plate for use later.
Go back and mark the location of the needle pricks with pen on the bottom of the plate.
Repeat with a second filter (B).

Put the filters face up on the paper with the 10% SDS solution for 3 min.
Transfer to neutralizing buffer for 5 min.
Transfer to denaturing buffer for 5 min.
Transfer to 2X SSC for 5 min.

Put right side up on 3MM paper to dry for 1 hr.

UV Crosslink by exposing to UV light for 2 min. on UV box in darkroom.
Store between 3MM paper overnight.

Prepare Probe for tomorrow.

Day 3

Wet filters one at a time in 2X SSC, then allow to soak for 5 minutes. Transfer to Prewashing Solution in a rubbermaid container (all filters can go in the same container as long as they are wetted individually).
Place in 42°C incubator with shaking for 1-2 hours. Can stay longer if necessary.

Prewarm prehybridization solution to 60°C.
Transfer filters to seal a meal bags and add prehybridization solution at 68°C, incubate for 1-2 hours with agitation in hybridization oven.

Purify Probe using a Modified Spin Column

1. Cut off the tops of a 1.5 mL and 0.5 mL microfuge tubes
2. Poke a hole at the bottom of the 0.5 mL tube with a needle so that water can pass through
3. To the 0.5 mL tube add approximately 100-200 µL of glass beads. The glass beads are located in the top left of the reagent shelf in the Horton laboratory.
4. Using a sterile-transfer pipet, pipet hydrated sterile sephadex G-50 to the 0.5 mL tube until full. Make sure to use the solution at the bottom of the bottle. The solution can be found in the door of the fridge in the Horton lab.
5. Microfuge at 2000 rpm for 1 minute with larger tube as a liquid catcher.
6. Drop out the flow-through.

7. Repeat steps 4, 5 & 6 until the small tube is full. Usually takes about 2 or 3 rounds.
8. Microfuge for 4 minutes at 2000 rpm. Dispose of flow through.
9. Add 20 µL (all) of the probe solution to the column. Spin for 4 minutes at 2000 rpm and now SAVE flow through.

10. Using pipet estimate/measure the volume of the flow through.
11. Of the measured volume of the flow through (probe), take 1/10 of the volume and pipet into a microfuge tube. Add 500 µL of Salmon Sperm DNA (in probe box in fridge) of concentration 2 mg/mL. Boil at 95° C for 7 minutes. This solution will then be added to the filters and pre-hybridization solution in order to perform the hybridization.

Add Purified Probe to filters, reseal and incubate overnight at 68°C. with agitation in hybridization oven.

Day 4

1. Cut open bags and remove filters (do not allow to dry). Place in rubbermaid container with 300 mL 2X SSC. Incubate 15 min. with agitation in hybridization oven.
2. Repeat step 1.
3. Transfer to 300 mL 2X SSC + 0.1% SDS. Incubate 30 min. with agitation in hybridization oven.
4. Transfer to 300 mL 0.4X SSC + 0.1% SDS. Incubate 10 min. with agitation in hybridization oven.
5. Transfer to 150 mL washing solution. Incubate for 5 min. at room temperature.
6. Transfer to 150 mL 1.5% blocking solution. Incubate for 1 hr. (or more) at room temperature.
7. Centrifuge tube of anti-digoxigenin 2 min. at 4°C, then add 1 µL anti-digoxigenin per 10 mL 1.5% blocking buffer (150 mL). Add to filters, and incubate 30 min.
8. Empty dish and add some wash solution to rinse. Put the filters in a new dish with 150 mL wash solution. Incubate with agitation 15 min. (Can be left longer if necessary)
9. Repeat step 8 in same dish.
10. Replace buffer with 1X detection buffer for 2 min. with agitation.
11. Combine 800 µL detection buffer and 8 µL CSPD. Remove filters and place in pairs between transparency film sheets. Squeeze out most of the detection buffer and bubbles. Add all of detection solution, and spread evenly over the two filters. Tape edges of transparency sheets to prevent leaking of solution. Tape to an old sheet of x-ray film.
12. Incubate at 37°C for 1 hour.
13. Place in cassette with full sheet of x-ray film. Incubate overnight. Limit exposure to no more than 12 hours. Films were exposed overnight for 10 hours.

Day 5

1. Remove film in darkroom and develop.
   3 min. in developer
   3 min. in stop
   3 min. in fix
   all with mixing

2. Rinse thoroughly in running tap water (30 min.).
3. Rinse in DI water.
4. Dry 1-2 hours.
5. Cut out and overlay film portion corresponding to each filter.
6. Figure out how they overlap (pattern should be similar on both).
7. Transfer registration marks from filter to film.
8. Put small arrows next to colonies that appear to have reacted on both films.
9. Compare the film with the plate. Put dots on colonies that should be picked, then put a circle around the dot.

Secondary Screen

Day 7-8

1. Using a sterile toothpick, pick colony off of plate and swirl in 100 μL of LB broth in a microfuge tube. Note: Many plates will be used to determine the adequate dilution of colony. For this procedure dilutions of 1/100, 1/500, 1/1000, and 1/10,000 were made and plated. Make approximately 6 plates per colony.
2. Use a serious of dilutions to determine the adequate dilution for each colony.
3. Incubate overnight at 37° C.
4. Determine "correct solution" for each colony. Colonies should be about "o" and should be well separated (approx. 250-300/plate).
   If there are too many or too few, repeat above procedure with a different dilution of bacteria stock.
5. Note: Make sure all necessary solutions from Primary Screening are still available, if not make more.

Day 8-12

Once an adequate dilution of the colonies has been determined, follow the procedure for hybridization as described for Days 2-5. The only modification is that filters should be cut in a square/rectangle form, rather than circle form. This makes identifying the colonies easier. Instead of using sealed bags, one can use small Rubbermaid containers and seal them with parafilm to prevent evaporation in the hybridization oven. The amount of probe was also tripled. The temperature of the hybridization oven was decreased to 59°C. Films were left to expose overnight for 8 hours.

Denaturation Solution

21.92 g NaCl
5 g NaOH
bring up to 250 mL with Milli-Q water

Neutralizing Solution

125 mL 1 M Tris, pH 7.2
21.92 g NaCl
0.5 mL 0.5 M EDTA
bring up to 250 mL with Milli-Q water

20X SSC
3 M NaCl
300 mM sodium citrate, pH 7.0

Prewashing Solution (250 mL)
125 mL 10X SSC
125 mL water
1.25 mL 10% SDS
0.502 mL 0.5 M EDTA, pH 8.0

Washing Solution
50 mL 10 X maleic acid buffer (autoclaved)
1.5 mL Tween 20 (0.3% Tween solution) Pipet slowly because it is very viscous
up to 500 mL with autoclaved water in sterile container.

Blocking Solution
15 mL 10% blocking buffer
bring up to 100 mL with 1X maleic acid buffer

10x Maleic Acid buffer, pH 7.5
Total volume: 1000 mL
1 mole x 166.07 g/mole = 116.07 g of Maleic Acid
1.50 moles x 58.44 g/mole = 87.66 g of NaCl
Approximately: 40 grams of NaOH to adjust pH
Final titration with 4 M NaOH solution
Initial titration with approximately 500 mL and after reaching pH of 7.5 raise to 1000 mL.
Autoclave solution

Detection Buffer (1 M Tris-Buffer, pH 9.5
Total volume: 100 mL
0.1 mole x 121.4 g/mole = 12.11 g of Tris needed
pH adjusted with concentrated HCl
autoclaved

10% blocking buffer
10 g blocking reagent
100 mL water
stir over heat, but not boiling for 1 hour
autoclave

Prehybridization Solution

1.76 g SDS (add last after heating rest to 60°C)
6.27 mL 20X SSC
5.02 mL 10% blocking solution
1.25 mL 1 M sodium phosphate, pH=7.0
250.8 µL 10% sarcosyl
11.79 mL water

Preparation of Probe (Day 3)

need 75 micrograms of Gene Cleaned DNA

mix DNA plus sterile water to give 15 µL
Heat at 95°C for 7 min.
cool immediately on ice
add 2 µL 10X hexanucleotide mix
2 µL 10X DIG DNA mix
1 µL Klenow enzyme
incubate overnight at 37°C

Appendix
Procedure 2: Gene Clean III Procedure
(from JSH 10/28/03) Modified 04/16/04

1. After the gel has run for an appropriate amount of time, use a handheld UV light to identify the appropriate bands to cut out. Do not expose the gel to large amounts of UV light because it will disintegrate the DNA.
2. The appropriate band should be cut out with a razor blade that has been cleaned with DNase and afterwards wiped with water. Carefully cut out the appropriate band, chop into small pieces, and put into a clean, sterile microfuge tube that has been pre-weighted.
3. Weigh gel slice and determine volume (volume in mL = mass in g)
4. Add 3 gel volumes of NaI solution and melt at 55 °C for 5 minutes or until melted, mixing every few minutes.
5. Add 0.1 volumes TBE modifier. Mix.
6. Add 5 μL of well-mixed glassmilk, wrap tube with parafilm, mix every minute for approximately 10 minutes. Mix sure you wrap glassmilk bottle with parafilm when finished.
7. Spin 5 seconds at maximum in microfuge. Remove supernatant and discard. Spin again. Remove and discard remaining supernatant with smallest pipet tip.
8. Wash with New Wash solution by adding 600 μL, resuspend by light vortexing, spin 5 seconds, remove and discard supernatant. Repeat 2 more times (3 total washes).
9. Spin after last wash, remove last bit of supernatant. Let dry in hood 5 minutes.
10. Elute with Elution Buffer by adding 10 μL, mush around, spin 1-2 minutes, take off supernatant and transfer to a new tube. You want to save supernatant.
11. Before use, spin to pellet any remaining glassmilk.
12. After the band has been cut out, the gel can be reconstructed and a picture can be taken for the purpose of keeping a record.
13. An additional gel should be run with the 2 μL of the Gene Cleaned Products in order to confirm that the appropriate band has been isolated.

Appendix
Procedure 3: Ligation and DNA Precipitation

A. Ligation Procedure

Should have approximately 5:1 insert to vector ratio.

Ligation buffer should not be repeatedly freeze-thawed, so it is divided into 5 µL aliquots in the Enzyme Buffers freezer box. It is OK if you use 1 tube/ligation, there is plenty of buffer. Make sure ligation buffer does not have solid material in it. Warm to RT and vortex briefly to dissolve before using.

Mix:
0.5 µL of 10x ligation buffer – Enzyme Buffers – warm up well, mix well
1 µL of pGEM-T easy (plasmid DNA) yellow box/yellow tube in freezer
2 µL of PCR gene cleaned Product
0.5 µL of T4 DNA ligase – Blue Frozen Box – green tube
1 µL of DI water

Mix and incubate at 14 °C overnight. (For not so good results, do for 30 minutes at room temperature) The most successful ligation was done for 13.5 hours.

Before doing transformation precipitate DNA to purify.

B. Procedure for Precipitation of DNA to remove salt

1. Combine sample with
   1-1.5 µL glycoblue (misc DNA reagents box –20 °C)
   0.5 volumes 7.5 M NH_4OAC (2.5 µL)
   2+ volumes cold 95 % ethanol (12 µL)
2. Mix well incubate at –20 °C for 30 minutes to one hour.
3. Spin at maximum in microfuge at 4 °C for 30 minutes.
4. Remove supernatant and add 20 µL cold 70% ethanol. Spin 10 minutes at maximum at 4 °C. Repeat.
5. Remove supernatant. Spin briefly at RT. Remove any remaining supernatant.
6. Let dry in hood in open tube for approximately 5 minutes.
7. Add 5 µL sterile milliQ water and resuspend by pipetting up and down.

Appendix
Procedure 4: Transformation of *E. coli*

1. Transfer cells (DH5 alpha) from –80° C freezer to an ice bucket. Thaw on ice 5-10 minutes.
2. Put a cuvet (0.1 cm gap) on ice for each sample. Have available SOC medium at RT and a 1 mL pipettor.
3. Mix 1 µL DNA and 40 µL cells in a 1.5 mL tube on ice.
4. Set up "big bopper"
   Turn on-off-on
   200 Ohms a knob
   25 uFD a knob
   1.8 kV press "set volts", "then raise^"
5. Get sample holder out of refrigerator and set up.
6. Add DNA and cells to cuvet. Tap on table to get cells between plates. Put 1.5 mL tube in rack at room temperature.
7. Put cuvet in sample holder and slide in. Press "Time Const" key.
8. Press both pulse buttons in at the same time.
   Chg flashes
   Beeps
   Let go of buttons
   Record the time constant (should be 3.5 – 4.0)
9. Remove cuvet and add 1 mL SOC. Mix gently.
10. Transfer to 1.5 mL tube at RT.
11. Incubate 1 hr. at 37° C taped to the rotating tube holder. **Make special LB Amp plates.
12. Plate 100 µL on an LB (Special plates)* , then spin the remainder 10 min at 5000 x g to pellet cells.
13. Remove all but 100 µL SOC. Resuspend cells gently.
14. Plate 100 µL on a second LB – (Special plates)*
15. Grow Overnight at 37 °C. (12-18 hours)

** To Make these plates from LB-AMP plates, spread 40 µL 20 mg/mL X-GAL and 35 µL 100 mM IPTG on plates and let soak for approximately 30 minutes. (Located in Miscellaneous Reagents Box)

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