

Expression Arrest™ Mouse retroviral shRNA^{mir} individual constructs

Catalog # RMM1766

The laboratory of Dr. Greg Hannon at Cold Spring Harbor Laboratory (CSHL) has created an RNAi Library comprised of multiple short-hairpin RNAs (shRNAs) specifically targeting annotated mouse genes. The shRNA Library permits rapid, cost-efficient, loss-of-function genetic screens and rapid tests for genetic interactions to be performed in mammalian cells.

Each shRNA construct has been sequence verified and cloned into a retroviral vector to ensure a match to the target gene. The shRNA expression cassette is carried in a validated Murine Stem Cell Virus (MSCV) backbone. The pSHAG-MAGIC vector (See Figures 1 & 2) can be used both for transient and stable delivery by transfection and for stable delivery using the replication-deficient retrovirus as a delivery method.

shRNA construct storage

Each Expression Arrest™ Mouse shRNA construct is shipped as a bacterial culture of *E. coli* (DH10bpir116) in LBR broth with 8% glycerol and chloramphenicol (50µg/ml). Open Biosystems checks all cultures for growth prior to shipment.

Individual constructs are shipped on wet ice. All Expression Arrest™ Mouse shRNA constructs should be stored at -80°C.

Expression Arrest™ Mouse shRNA collection

Expression Arrest™ mouse shRNA library from Open Biosystems is a whole genome RNAi resource and the only choice for transient, stable and *in vivo* RNAi studies. This collection was developed in collaboration with Drs Greg Hannon (CSHL) and Steve Elledge (Harvard). The collection has several unique features that make it a very versatile and efficient tool for RNAi studies including large-scale screens (Paddison *et al* 2004).

These include:

(1) Unique MicroRNA-30 based hairpin design

Expression Arrest™ short hairpin RNA constructs are expressed as human microRNA-30 (miR30) primary transcripts. This design adds a Drosha processing site to the hairpin construct and has been shown to greatly increase knockdown efficiency (Boden *et al* 2004). The hairpin stem consists of 22-nt of dsRNA and a 19-nt loop from human miR30. Adding the miR30 loop and 125nt of miR30 flanking sequence on either side of the hairpin results in greater than 10-fold increase in Drosha and Dicer processing of the expressed hairpins when compared with conventional shRNA designs without microRNA. Increased Drosha and Dicer processing translates into greater siRNA/miRNA production and greater potency for expressed hairpins.

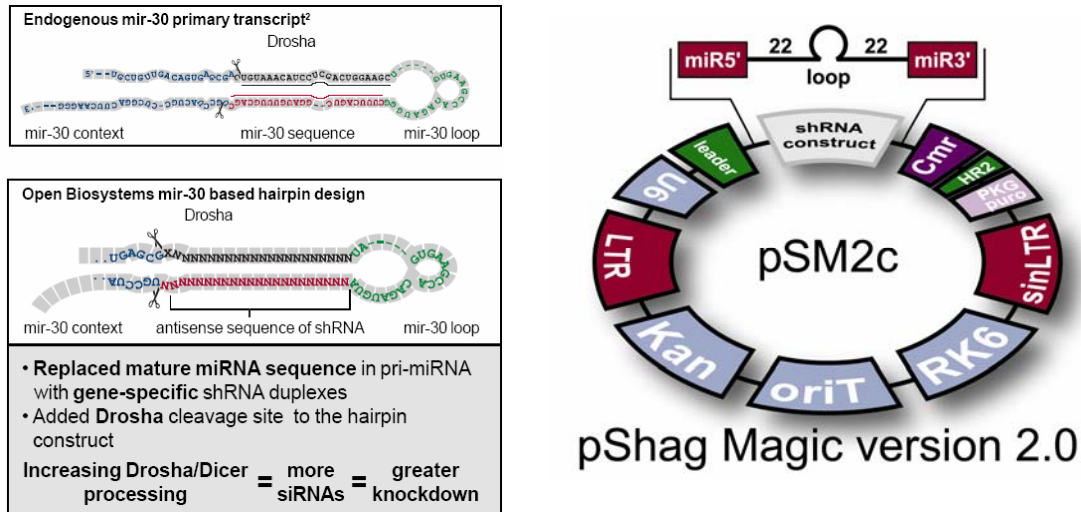


Figure 1: Expression Arrest™ shRNA are expressed as mir30 primary transcripts

Use of the miR30 design also allowed the use of **'rules-based' designs** for target sequence selection. One such rule is the destabilizing of the 5' end of the antisense strand which results in strand specific incorporation of miRNAs into RISC. The proprietary design algorithm targets coding regions and the UTR with the additional requirement that they contain greater than 3 mismatches to any other sequence in the human or mouse genomes.

Due to the placement of the RNA Polymerase III transcription terminator (four or more thymidines) downstream of the hairpin, each transcript is designed to precisely terminate. RNA Polymerase III terminates on the second thymidine, two uridines remain to create a 2 base overhang. Each shRNA construct has been sequence verified before being cloned into the retroviral vector to ensure a match to the target gene. To assure you the highest possibility of modulating the gene expression level, each gene is represented by multiple shRNA constructs, each covering a unique region of the target gene.

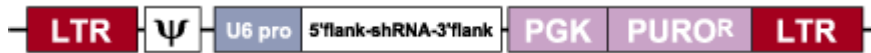
(2) Versatile vector design

Expression Arrest™ shRNA are already cloned into the pSHAG-MAGIC2 (pSM2) retroviral vector. This vector has a Murine Stem Cell Virus (MSCV) backbone. Features of the vector that make it a versatile tool for RNAi studies include:

- Ability to perform transfections (transient and stable) or transductions using the replication incompetent retrovirus
- Amenable to *in vitro* and *in vivo* applications
- Puromycin drug resistance marker for selecting stable cell lines
- Molecular barcodes enable complex screening in pools

The pSHAG-MAGIC2 cloning vector is roughly equivalent to pSHAG-MAGIC1 (see Paddison *et al.*, 2004) with a few notable exceptions. First, the cloning strategy has been changed. Previously “PCR-SHAG” was used to clone hairpins by adding the entire hairpin onto the end of a PCR primer. Now a single oligo is used, containing the hairpin

and common 5' and 3' ends, as a PCR template. That is, the oligo is PCR amplified using universal primers that contain *XhoI* (5' primer) and *EcoRI* (3' primer). These PCR fragments are then cloned into the hairpin cloning site of pSHAG-MAGIC2. The mir30-styled hairpins are still expressed from the human U6 promoter. The configuration of pSHAG-MAGIC2 is shown below. The 5' and 3' flanks are derived from 125 bases surrounding the Human miR30 microRNA.



Vector Element	Utility
U6 promoter	RNA generated with 4 uridine overhangs at each 3' end
Retroviral Signaling Sequence	Combined with packaging extract for mammalian cell infection
PGK-Puro	Selection for transfection stability in mammalian cells
Chloramphenicol/Kanamycin	Bacterial selection marker
Homologous recombination sites	Transfer shRNA cassette into new vectors through the MAGIC homologous recombination system
RK6	Conditional origin of replication. Requires the expression of pir1 gene within the bacterial host to propagate

Table 1: Features of the pSHAG-MAGIC2 Vector

Antibiotic Resistance

pSHAG-MAGIC2 contains 4 antibiotic resistance markers. (See Table 2)

Antibiotic	Concentration	Utility
Chloramphenicol	50ug/ml	Bacterial selection marker (shRNA insert)
Kanamycin	optional	Bacterial selection marker (vector)
Puromycin		Mammalian selectable marker

Table 2: Antibiotic Resistances Conveyed by pSHAG-MAGIC2

Culturing protocols and maintenance of pSM2

It is well known that viral vectors have a tendency to recombine producing background recombinants. Recombination occurs at the long terminal repeat regions (LTR's). The LTR recombination, which results in loss of most of the plasmid, can confer a growth advantage on the cells. It is therefore critical to maintain careful growth conditions when culturing viral vectors in *E.coli* in order to reduce the number and abundance of background recombinants.

The Expression Arrest™ shRNA Library version 2.3 is constructed in the multi-functional pSM2 vector. This vector allows for both transient and stable gene knockdown via the mechanism of RNA interference. The vector is capable of producing self-inactivating murine-stem-cell-virus (MSCV) particles when used in conjunction with retroviral packaging lines.

pSM2 is a viral vector that produces very little recombinant background product under careful growth and handling conditions. We have observed that greater than 24-hour incubation times increases recombination only slightly.

In order to obtain a good yield of cells in a short period of incubation, rich media (containing 8% glycerol) should be used to culture pSM2 constructs. An incubation period of 14-20 hours at 37°C with aeration is sufficient. It is recommended that the cultures remain frozen at -80°C when not in use. Freeze/thaw cycles do not seem to have any detrimental effect providing the cultures are not incubated at room temperature or higher, for long periods of time.

Gel images of plasmid isolated from cultures grown under the above conditions are shown below.

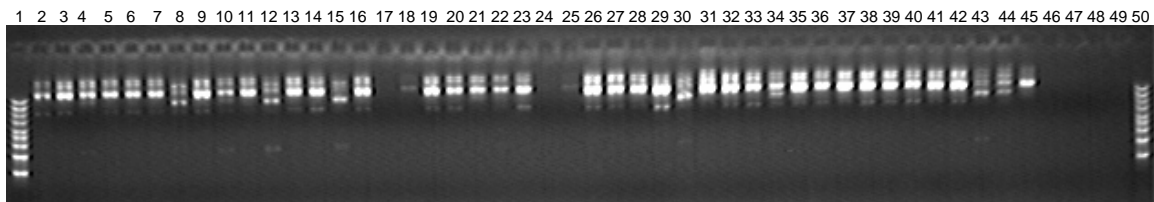


Figure 2. 1.5 ml cultures of 42 different shRNA constructs after 20 hours of incubation at 37°C with shaking (~170 rpm). 2X LB media (low-salt) with 8% glycerol was used for culturing. This vector is a stable retroviral vector and shows minimal recombinants. The pSM2 band usually runs around 7kb although it is not uncommon to see a band around 10kb or even around 5 kb. The presence of a faint recombinant band is seen around 1.8 kb in lanes 10 and 12. If the recombinant product is not a significant proportion (over 50%) of your plasmid prep the DNA is still acceptable for transfection since the LTR-LTR recombinant product does not contain the puromycin resistance gene or the shRNA construct.

Background recombination levels associated with pSM2

Although careful growth conditions were maintained when culturing this set, a small percentage of the whole set (~5%) still shows a low level of recombination. The following gel image is an example of what to expect after plasmid DNA preparation.

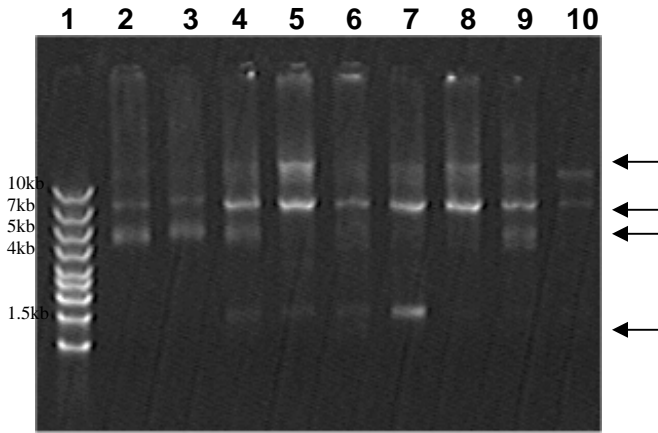


Figure 3: Lane 1– 10kb marker (10kb, 7kb, 5kb, 4kb, 3kb, 2.5kb, 2kb, 1.5kb, 1kb), Lanes 2 to 10– 10ul of plasmid prep product of nine different shRNA constructs.

A 1 μ l inoculum of 9 different shRNA constructs were cultured in 1ml of 2XLB medium (low-salt) in a bioblock with aeration by shaking at 200 RPM at 37° C for 16 hours. Plasmids were isolated and run uncut on a 0.9% agarose-TAE gel. The first three arrows from the top point to various forms of the correct plasmid pSM2, which when digested with restriction enzymes produces the correct band size. The last arrow from the top points to the recombinant product (~ 1.8kb). Samples on lanes 4, 5, 6 and 7 show varying levels of recombination. Samples 2, 3, 8, 9 and 10 show minimal to no recombination.

Protocols for culturing pSM2 shRNA constructs

2X-LB broth (low-salt) media preparation for plasmid DNA

Peptone	20 g/L
Yeast Extract	10 g/L
NaCl	5g/L
Chloramphenicol	50ug/ml
*Glycerol	8% for long term storage

Note: (1) LB media can be used instead of 2XLB

*(2) *Glycerol can be omitted from the media if you are culturing for plasmid preparation. If making copies of the constructs for long term storage at -80°C , 8% glycerol is required.*

Culture conditions for individual plasmid preparations

Most plasmid mini-prep kits recommend a culture volume of 1–10 ml for good yield. For shRNA constructs, 5ml of culture can be used for one mini-prep generally producing from 5–20 ug of plasmid DNA.

1. Upon receiving your glycerol stock(s) containing the shRNA of interest store at -80°C until ready to begin.
2. To prepare plasmid DNA first thaw your glycerol stock culture and pulse vortex to resuspend any *E. coli* that may have settled to the bottom of the tube.
3. Using a sterile loop or a pipette tip, streak the shRNA culture onto a LB agar plate containing 50 ug /ml Chloramphenicol. Incubate the plate overnight at 37°C . Return the glycerol stock(s) to -80°C .
4. The following day, pick 1 to 3 colonies from the agar plate and inoculate 6 ml of the 2XLB Chlor₅₀. Incubate at 37°C for 16-20 hrs with vigorous shaking (300 rpm).
5. The following day remove 1 ml of the culture and place in a sterile 2-ml sterile microcentrifuge tube. Place this tube at 4°C until the plasmid DNA from the remaining culture has been analyzed. Pellet the remaining 5-ml culture and begin preparation of plasmid DNA. We recommend preparing Ultra-pure DNA to ensure both high-purity and low endotoxin levels (Qiagen Catalog #12123) as required for transfection into eukaryotic cells.

If you wish to continue at a later time cell pellets can be kept frozen at -20°C overnight.

6. Run 3-5ul of the plasmid DNA on a 1% agarose gel. The uncut pSM2 shRNA constructs run at about 7-10kb while the most common product of a recombination event will run at ~ 1.5 -1.8kb. If recombination is present at a significant amount then return to the plate and pick another colony and repeat plasmid preparation. A small amount of recombination is acceptable during transfection since the LTR-LTR recombinant product does not contain the puromycin resistance gene or the shRNA.
7. Prepare an 8% glycerol stock culture using the 1ml of culture you removed prior to plasmid preparation. This culture can be used for future plasmid preparations but it is still recommended you streak isolate and work from a fresh colony. Store at -80°C .

Note: Due to the tendency of all viral vectors to recombine we recommend keeping the incubation times as short as possible and avoid subculturing. Return to your original glycerol stock or the colony glycerol stock for each plasmid preparation.

Restriction Digests of pSM2

You may wish to restriction digest a sample of your plasmid DNA following plasmid DNA preparation. The following is a protocol for dual restriction enzyme digestion using EcoRI and XhoI for quality control of pSM2 vectors (shRNA library and controls). The protocol for HindIII/XbaI digests is exactly the same except replace the EcoRI Buffer with the 10X Buffer 2 and exchange the enzymes used.

1. Using filtered pipette tips and sterile conditions add the following components, in the order stated, to a sterile PCR thin-wall tube.

Sterile, nuclease-free water	14.8µl
Restriction enzyme EcoR1 10X buffer	2µl
BSA (10X, 10mg/ml)	0.2µl
DNA sample 1µg, in water or TE buffer	1µl
Restriction enzyme EcoRI, 20U	1µl
Restriction enzyme XhoI, 20U	1µl
Final volume	20µl

2. Mix gently by pipetting.
3. Incubate in a thermalcycler at 37°C for 2.5 hours to digest then at 70°C for 20 minutes to kill the enzyme.
4. Add 4µl of 6X Loading Dye (or another appropriate DNA loading buffer), and proceed to gel analysis.
5. Load the gel with 20µl of each of the digested samples (a EcoRI/XhoI and HindIII/XbaI) on a 1% agarose gel. Also run 1µl (1µg) of the uncut sample combined with 16µl of water and 3µl of 6x dye alongside the digested samples.
6. The EcoRI/XhoI digest will release the 97-bp insert and leave an approximately 7-kb band. The XbaI/HindIII digests should have 3 bands: 3690bp, 2260bp and 1253bp.

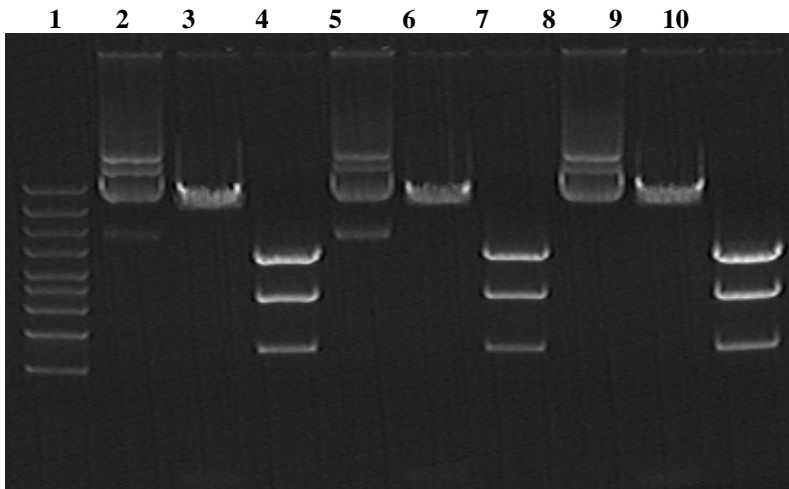
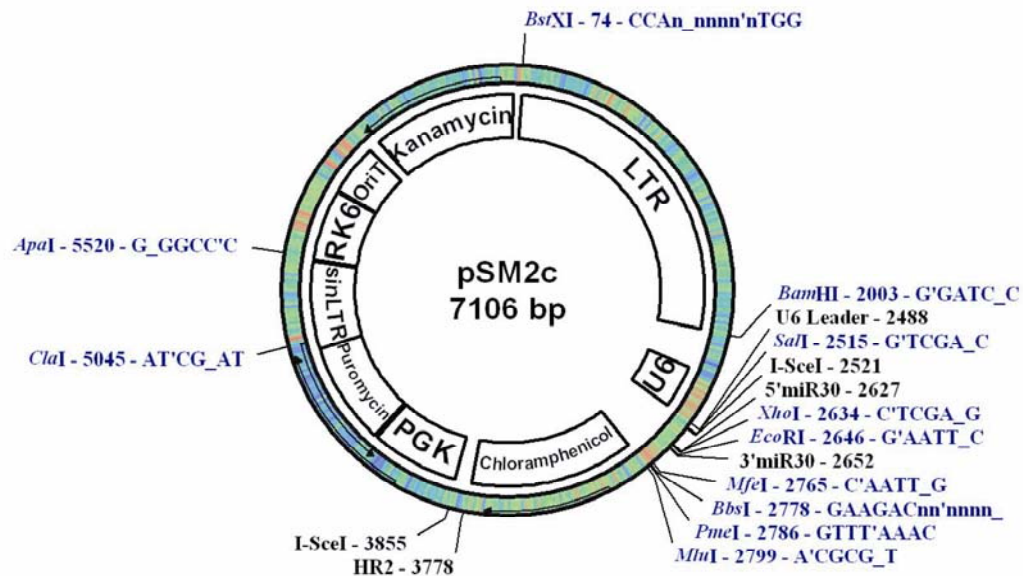


Figure 4. The 1% agarose gel above contains -10kb ladder followed by undigested

sample and restriction digests of the non-silencing shRNA control (lanes 2,3,4), the eGFP shRNA control (lanes 5,6,7) and the FFLuc shRNA control (lanes 8,9,10). For each sample the lanes are as follows: undigested sample, an EcoR1/Xho1 digest, then the Xba1/HindIII digest.

Note: pSM2 must be transformed into PIR1 competent bacteria. The pSM2 plasmid harbors a conditional bacterial origin of replication, which requires the expression of the "pir1" gene to be rendered functional.

Figure 1: Detailed Vector Map of pSHAG-MAGIC 2



The sequence of pSHAG-MAGIC2

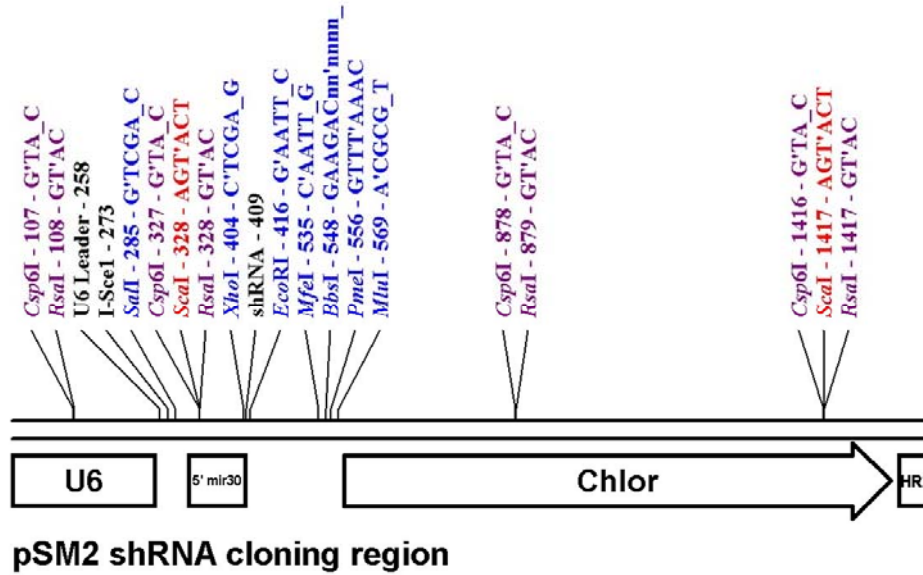
5' MSCV LTR- ~80-2000
 Packaging signal-
 U6 promoter + 27nt leader sequence...2003-2515
 5' mir30 context...2515-2634Cloning site [XhoI-EcoRI] 2634-2646
 3' mir30 context...2646-2760
 U6 terminator...2760-2764
 Barcode cloning site [MfeI-MluI]... 2765-2799
 Chloramphenicol resistance gene...2809-3763
 PGK-PURO-3862-4987 (3862-4374-pgk, 4391-4987-puro)
 3' MSCV-"SIN"-LTR-4988-5500

CTTCCCAACCTTACCAGAGGGCGCCCCAGCTGTCCGAAATATTATAAATTATCGCACACATAAAA
ACCATGCTGTTGGTGTGTCTATTAAATCGGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTT
CGCTATTACGCCAGCTGGCGAAAAGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAAACGCCAGGG
TTTTCCCAGTCACGACGTTGTA AAAACGACGGCGCAAGGAATGGTG CATGCAAGGAGATGGCGCCC
AACAGTCCCCCGGCCACGGGGCCTGCCACCATACCACGCCGAAA CAAGCGCTCATGAGCCCGAA
GTGGCGAGCCCGATCTTCCCATCGGTGATGTGCGCGATATAGGCGCCAGCAACCGCACCTGTGG
CGCCGGTGTATGCCGGCCACGATGCGTCCGGCGTAGAGGCGATTAGTCCAATTTGTTAAAGACAGG
ATATCAGTGGTCCAGGCTCTAGTTTTGACTCAACAATATCACCAGCTGAAGCCTATAGAGTACGA
GCCATAGATAAAAATAAAAGATTTTATTTAGTCTCCAGAAAAGGGGGGAATGAAAAGACCCACCT
GTAGGTTTGGCAAGCTAGCTTAAGTAACGCCATTTTGCAAGGCATGGAAAATACATAACTGAGAA
TAGAGAAGTTAGATCAAGGTTAGGAACAGAGAGACAGCAGAATATGGGCCAAAACAGGATATCTG
TGGTAAGCAGTTCTGCCCGGCTCAGGGCCAAGAACAGATGGTCCCAGATGCGGTCCCGCCCT
CAGCAGTTTCTAGAGAACCATCAGATGTTTTCCAGGGTGCSCCAAGGACCTGAAAATGACCCTGTGC
CTTATTTGAACTAACCAATCAGTTCGTTCTCGCTTCTGTTTCGCGCGCTTCTGCTCCCGAGCTC
AATAAAAGAGCCCAACCCCTCACTCGGCGCGCCAGTCCCTCCGATAGACTGCGTCCGCCGGGTA
CCCGTATTTCCCAATAAAGCCTCTTGCTGTTTG CATCCGAATCGTGGACTCGCTGATCCTTGGGAG
GGTCTCCTCAGATTGATTGACTGCCACCTCGGGGTCTTTTCATTTGGAGGTTCCACCGAGATTT
GGAGACCCCTGCCAGGGACACCGACCCCGCCCGCGGGAGGTAAGCTGGCCAGCGGTCTGTTTCG
TGTCTGCTCTGCTCTTTGTGCGTGTTTGTGCGGCATCTAATGTTTGCGCCTGCGTCTGTAAG
TTAGCTAACTAGCTCTGTATCTGGCGGACCCGTGGTGGAACTGACGAGTTCTGAAACCCCGCCG
CAACCCTGGGAGACGTCCCAGGGACTTTGGGGCCGTTTTTTGTGGCCCGACCTGAGGAAGGGAGT
CGATGTGGAATCCGACCCCGTCAGGATATGTGGTTCGGTAGGAGACGAGAACCTAAAACAGTTC
CCGCTCCGTCTGAATTTTTGCTTTCGGTTTTGGAACCGAAGCCGCGCTCTTGTCTGCTGCAGCG
CTGCAGCATCGTTCTGTGTTGTCTCTGTCTGACTGTGTTTTCTGTATTTGTCTGAAAATTAGGGCC
AGACTGTTACCACTCCCTTAAGTTTGACCTTAGGTCACCTGGAAAGATGTGAGCGGATCGCTCAC
AACCAGTCGGTAGATGTCAAGAAGAGACGTTGGGTTACCTTCTGCTCTGCAGAATGGCCAACCTT
TAACGTCGGATGGCCGCGAGACGGCACCTTTAACCAGACCTCATCACCCAGGTTAAGATCAAGG
TCTTTTACCTGGCCCGCATGGACACCCAGACCAGGTCCCCTACATCGTGACCTGGGAAGCCTTG
GCTTTTGACCCCCCTCCCTGGGTCAAGCCCTTTGTACACCCTAAGCCTCCGCCTCCTCTTCTCCTC
ATCCGCCCCCTCTCCCCCTTGAACCTCCTCGTTCGACCCCGCCTCGATCCTCCTTTATCCAG
CCCTCACTCCTTCTTAGGGCGCGAATTAGATCTCTCGATAATAGGGGACCCGATCCCCCGAG
TCCAACACCCGTGGAAATCCCATGGGCACCATGGCCCTCGCTCCAAAATGCTTTTCGCGTCTCG
CAGACACTGCTCGGTAGTTTTCGGGGATCAGCGTTTGTAGTAAGAGCCCGCTCTGAACCCTCCGCG
CCGCCCCGGCCAGTGGAAGACGCGCAGGCAAAACGCACCACGTGACGGAGCGTGACCGCGCGC
CGAGCGCGGCCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATAAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTAC
AAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTTAAAATTATGTTTTAA
AATGGACTATCATATGCTTACCGTAACCTGAAAGTATTTGATTTCTTGGCTTTATATATCTTGT
GGAAAGGACGAAAACCCGTGCTCGCTTCCGGCAGCACATATACTAGTCTGACTAGGGATAACAGGGT
AATTGTTTGAATGAGGCTTCAGTACTTTACAGAATCGTTGCCTGCACATCTTGGAAACACTTGCT
GGGATTACTTCTT CAGGTTAACCCAACAGAAGGCTCGAGCAACCAGAATTC AAGGGGCTACTTTA
GGAGCAATTTATCTTGTTTACTAAAACCTGAATACCTTGCTATCTCTTTGATACATTTTTTACAAAGC
TGAATTAATAATGGTATAAATAAATCACTTTTTTCAATTTGGAAGACTAATGCGTTTAAACACGCG
GCGACGCGTTTCGACCGAATAAAAACCTGTGACGGAAGATCACTTCGCAGAATAAATAAATCCTGGT
GTCCCTGTTGATAACGGGAAGCCCTGGGCCAACTTTTGGCGAAAATGAGACGTTGATCGGCACGT
AAGAGGTTCCAACCTTTCACCATAATGAAATAAGATCACTACCGGGCGTATTTTTTGTAGTTGTGCGA
GATTTTCAGGAGCTAAGGAAGCTAAAATGGAGAAAAAATCACTGGATATAACCACGTTGATATA
TCCAATGGCATCGTAAAGAACATTTTGAGGCATTTTCAGTCAGTTGCTCAATGTACCTATAACCA
GACCGTTCAGCTGGATATTACGGCCTTTTTTAAAGACCGTAAAGAAAAATAAGCACAAAGTTTTATC
CGGCTTTTATTCATTTCTTGCCCGCCTGATGAATGCTCATCCGGAATTACGTATGGCAATGAAA
GACGGTGAGCTGGTGATATGGGATAGTGTTCACCCCTTGTACACCGTTTTTCCATGAGCAAACCTGA
AACGTTTTTCATCGCTCTGGAGTGAATACCACGACGATTTCCGGCAGTTTTCTACACATATATTGCG
AAGATGTGGCGTGTACGGTGA AAAACCTGGCCTATTTCCCTAAAGGGTTTTATTGAGAATATGTTT
TTGCTCTCAGCCAATCCCTGGGTGAGTTTACCAGTTTTGATTTAAACGTGGCCAATATGGACAA
CTTCTTCGCCCCGTTTTTACCATGGGCAAAATATTATACGCAAGGCGACAAGGTGCTGATGCCGC
TGGCGATTCAGGTTTCATCATGCCGTTTTGTGATGGCTTCCATGTTCGCGAGAATGCTTAATGAATTA

CAACAGTACTGCGATGAGTGGCAGGGCGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTA
AACGCCTGGTTGCTACGCCTGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGGATCTCG
ACCGCGTTTTGGGCGGTGGCTCCCTGCCACGCGGCTCCGAACAGAAGCTGATCTCCGAAGAGGATC
TGATTACCCTGTTATCCCTACCTAAAATTCTACCGGGTAGGGGAGGCGCTTTTTCCCAAGGCAGT
CTGGAGCATGCGCTTTAGCAGCCCCGCTGGGCACTTGGCGCTACACAAGTGGCCTCTGGCCTCGC
ACACATTTCCACATCCACCGGTAGGCGCAACCGGCTCCGTTCTTTGGTGGCCCCCTTCGCGCCACC
TTCTACTCCTCCCCTAGTCAGGAAGTTCCCCCGCCCCGAGCTCGCGTCTGTCAGGACGTGAC
AAATGGAAGTAGCACGTCTCACTAGTCTCGTGCAGATGGACAGCACCGCTGAGCAATGGAAGCGG
GTAGGCCTTTGGGGCAGCGCCAATAGCAGCTTTGCTCCTTCGCTTTCTGGGCTCAGAGGCTGGG
AAGGGGTGGGTCCGGGGGCGGGCTCAGGGGCGGGCTCAGGGGCGGGGCGGGCGCCGAAGGTCTT
CCGGAGGCCCGGCATTTCTGCACGCTTCAAAGCGCACGTCTGCCGCGCTGTTCTCCTCTTCTCA
TCTCCGGGCTTTTCGACCTGCAGCCCAAGCTTACCATGACCGAGTACAAGCCACGGTGCGCCTC
GCCACCCGCGACGACGTCCCAGGGCCGTACGCACCCTCGCCGCGCGTTCGCCGACTACCCCGC
CACGCGCCACACCGTCGATCCGGACCGCCACATCGAGCGGGTACCGAGCTGCAAGAACTCTTCC
TCACGCGCGTCCGGCTCGACATCGGCAAGGTGTGGGTTCGCGGACGACGGCGCCGCGGTGGCGGT
TGGACCACGCCGGAGAGCGTCAAGCGGGGGCGGTGTTCCGCCGAGATCGGCCCGCGCATGGCCGA
GTTGAGCGGTTCCCGGCTGGCCGCGCAGCAACAGATGGAAGGCCTCTGGCGCCGCACCGGCCCA
AGGAGCCCGCGTGGTTCCTGGCCACCGTCGGCGTCTCGCCCGACCACCAGGGCAAGGGTCTGGG
AGCGCCGTCTGTCTCCCAGGAGTGGAGCGGCGAGCGCGCCGGGGTGCCTCGCTTCTTGGAGAC
CTCCGCGCCCGCAACCTCCCCTTCTACGAGCGGCTCGGCTTACCCTACCGCCGACGTGAGG
TGCCGAAGGACCGCGACCTGGTGCATGACCCGCAAGCCCGGTGCTGACGCCCGCCACGAC
CCGACGCGCCGACCAGAAAGGAGCGCACGACCCCCATGCATCGATAAAAATAAAAGATTTTTATTAG
TCTCCAGAAAAAGGGGGGAATGAAAAGCCCCACCTGTAGGTTTTGGCAAGCTAGAGAACCATCAGA
TGTTTTCCAGGGTGCCTCAAGGACCTGAAATGACCCTGTGCCTTATTTGAACTAACCAATCAGTTC
GCTTCTCGCTTCTGTTTCGCGCGCTTCTGCTCCCCGAGCTCAATAAAAGAGCCCAACCCCTCAC
TCGGCGCGCCAGTCTCCGATAGACTGCGTTCGCCGGGTACCCGTGTATCCAATAAACCCCTCTTG
CAGTTGCATCCGACTTGTGGTCTCGCTGTTCTTGGGAGGGTCTCCTCTGAGTGATTGACTACCC
GTCAGCGGGGGTCTTTCATGGGTAACAGTTTTCTTGAAGTTGGAGAACAACATTCTGAGGGTAGGA
GTCAATCGACGTGGGCCCAAT
CTGTGACCCGTTAAGTGTTCCTGTGTCACTGAAAATTGCTTTGAGAGGCTCTAAGGGCTTCTCAG
TGCGTTACATCCCTGGCTTGTGTCCACAACCGTTAAACCTTAAAAGCTTAAAAGCCTTATATA
TTCTTTTTTTTTTATAAAAACCTTAAAACCTTAGAGGCTATTTAAGTTGCTGATTTATATTAATTT
TATTGTTCAAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTTAGCCATGAGAGCT
TAGTACGTTAGCCATGAGGGTTTTAGTTCGTTAAACATGAGAGCTTAGTACGTTAAACATGAGAGC
TTAGTACGTGAAACATGAGAGCTTAGTACGTACTATCAACAGGTTGAACTGCTGATCAACAGATC
CTCTACTAGAAAGGACGCACCGCTAGCAGCGCCCTAGCGGTATCCTATAAAAAAACACACCG
CGCCGCTAGCAGCACCCCTAATATAAAATAATGTTTTTTATAAAAAATAGTCAGTACCACCCCTAC
AAAACGGTGTTCGGCGCTTGTGTAGCCGCGCCGACACCGCTTTTTTTAAATATCATAAAGAGAGT
AAGAGAACTAATTTTTTCATAACACTCTATTTATAAAGAAAAATCAGCAAAAACCTGTTTTTTGCG
TGGGGTGTGGTGCTTTTGGTGGTGAGAACCACCAACCTGTTGAGCCTTTTTTGTGGAGTGGGTAA
ATTATACTAGCGCTTTCGAACCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAA
GGCGATGCGCTGCGAATCGGGAGCGCGATAACCGTAAAGCACGAGGAAGCGGTGAGCCCATTCG
CGCCAAGCTCTTCAAGCAATATCACGGGTAGCCAACGCTATGTCTGATAGCGGTCCGCCACACCC
AGCCGCGCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTGCGCAAGCAGGC
ATCGCCATGTGTACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGCGCAACAGTT
CGGCTGGCGCGAGCCCTGATGCTTTCGTTCCAGATCATCCTGATCGACAAGACCGGCTTCCATC
CGAGTACGTGCTCGCTCGATGCGATGTTTTCGCTTGGTGGTGAATGGGCAGGTAGCCGGATCAAG
CGTATGCAGCCGCGCATTCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATG
ACAGGAGATCCTGCCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCCTTCACTGACAACG
TCGAGCACAGCTGCGCAAGGAACGCCCGTCTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTCTG
CAGTTCATTAGGGCACCAGGACAGGTCGGTCTTGACAAAAGAACCAGGCGCCCTGCGCTGACA
GCCGGAACACGGCGGCATCAGAGCAGCCGATTGTCTGTTGTGCCAGTCATAGCCGAATAGCCTC
TCCACCCAAGCGGCCGAGAACCTGCGTGAATCCATCTTGTTCAATCATGCGAAACGATCCTCA
TCCTGTCTCTTGATCAGATCT

See Appendix 1 for restriction analysis

Figure 5: pSM2 cloning region



See Appendix 2 for restriction analysis

Appendix1

Restriction analysis of pSM2 vector

SspI

1 CTTCCCAACC TTACCAGAGG GCGCCCCAGC TGTCCGAAAT ATTATAAATT
GAAGGGTTGG AATGGTCTCC CGCGGGGTCG ACAGGCTTA TAATATTTAA

BstXI

51 ATCGCACACA TAAAACCAT GCTGTTGGTG TGTCTATTAA ATCGGCAACT
TAGCGTGTGT ATTTTGGTA CGACAACCAC ACAGATAATT TAGCCGTTGA

PvuI

101 GTTGGGAAGG GCGATCGGTG CGGGCCTCTT CGCTATTACG CCAGCTGGCG
CAACCTTCC CGCTAGCCAC GCCCGGAGAA GCGATAATGC GGTCGACCGC

151 AAAGGGGGAT GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTTC
TTCCCCCTA CACGACGTTT CGCTAATTCA ACCCATTGCG GTCCCAAAG

201 CCAGTCACGA CGTTGTAAAA CGACGGCGCA AGGAATGGTG CATGCAAGGA
GGTCAGTGCT GCAACATTTT GCTGCCGCGT TCCTTACCAC GTACGTTCT

251 GATGGCGCCC AACAGTCCCC CGGCCACGGG GCCTGCCACC ATACCCACGC
CTACCGCGGG TTGTCAGGGG GCCGGTGCCG CGGACGGTGG TATGGGTGCG

AfeI BspHI

301 CGAAACAAGC GCTCATGAGC CCGAAGTGGC GAGCCCGATC TTCCCCATCG
GCTTTGTTTCG CGAGTACTCG GGCTTCACCG CTCGGGCTAG AAGGGGTAGC

SgrAI

351 GTGATGTCGG CGATATAGGC GCCAGCAACC GCACCTGTGG CGCCGGTGAT
CACTACAGCC GCTATATCCG CGGTCGTTGG CGTGGACACC GCGGCCACTA

401 GCCGGCCACG ATGCGTCCGG CGTAGAGGCG ATTAGTCAA TTTGTAAAG
CGGCCGGTGC TACGCAGGCC GCATCTCCGC TAATCAGGTT AAACAATTC

EcoRV

451 ACAGGATATC AGTGGTCCAG GCTCTAGTTT TGA CTCAACA ATATCACCAG
TGTCTATAG TCACCAGGTC CGAGATCAA ACTGAGTTGT TATAGTGTC

501 CTGAAGCCTA TAGAGTACGA GCCATAGATA AAATAAAAGA TTTTATTTAG
GACTTCGGAT ATCTCATGCT CGGTATCTAT TTTATTTTCT AAAATAAATC

551 TCTCCAGAAA AAGGGGGGAA TGAAAGACCC CACCTGTAGG TTTGGCAAGC
AGAGGTCTTT TTCCCCCTT ACTTTCTGGG GTGGACATCC AAACCGTTCC

AfIII

601 TAGCTTAAGT AACGCCATTT TGCAAGGCAT GGAAAATACA TAACTGAGAA
ATCGAATTCA TTGCGTAAA ACGTTCCGTA CCTTTTATGT ATTGACTCTT

651 TAGAGAAGTT CAGATCAAGG TTAGGAACAG AGAGACAGCA GAATATGGGC
ATCTCTCAA GTCTAGTCC AATCCTGTC TCTCTGTCGT CTTATACCCG

GACCATCCTC TGCTCTTGA TTTTGTCAAG GCGGAGGCA GACTTAAAA

AfeI

1451 GCTTTCGGTT TGAACCGAA GCCGCGCCTC TTGTCTGCTG CAGCGCTGCA
CGAAAGCCAA ACCTTGCTT CCGCGCGCAG AACAGACGAC GTCGCGACGT

1501 GCATCGTTCT GTGTTGTCTC TGTCTGACTG TGTTTCTGTA TTTGTCTGAA
CGTAGCAAGA CACAACAGAG ACAGACTGAC ACAAAGACAT AACAGACTT

AflII Bsu36I

1551 AATTAGGGCC AGACTGTTAC CACTCCCTTA AGTTTGACCT TAGGTCAGTG
TTAATCCCGG TCTGACAATG GTGAGGGAAT TCAAAGTGA ATCCAGTGAC

1601 GAAAGATGTC GAGCGGATCG CTCACAACCA GTCGGTAGAT GTCAAGAAGA
CTTTCTACAG CTCGCCTAGC GAGTGTGGT CAGCCATCTA CAGTTCTTCT

BstEII

1651 GACGTTGGGT TACCTTCTGC TCTGCAGAAT GGCCAACCTT TAACGTCGGA
CTGCAACCCA ATGGAAGACG AGACGTCTTA CCGGTTGGAA ATTGCAGCCT

1701 TGGCCGCGAG ACGGCACCTT TAACCGAGAC CTCATCACCC AGGTTAAGAT
ACGGCGCTC TGCCGTGGAA ATTGGCTCTG GAGTAGTGGG TCCAATTCTA

SexAI

1751 CAAGGTCTTT TCACCTGGCC CGCATGGACA CCCAGACCAG GTCCCCTACA
GTTCCAGAAA AGTGGACCGG GCGTACCTGT GGGTCTGGTC CAGGGGATGT

1801 TCGTGACCTG GGAAGCCTTG GCTTTTGACC CCCCTCCCTG GGTCAAGCCC
AGCACTGGAC CTTTCGGAAC CGAAAAGTGG GGGGAGGGAC CCAGTTCGGG

BsrGI

1851 TTTGTACACC CTAAGCCTCC GCCTCCTCTT CCTCCATCCG CCCCGTCTCT
AAACATGTGG GATTCCGAGG CGGAGGAGAA GGAGGTAGGC GGGGCAGAGA

1901 CCCCTTGAA CTCCTCGTT CGACCCCGCC TCGATCCTCC CTTTATCCAG
GGGGAACTT GGAGGAGCAA GCTGGGGCGG AGCTAGGAGG GAAATAGGTC

EcoNI

BglII

1951 CCCTCACTCC TTCTCTAGGC GCCGGAATTA GATCTCTCGA TAATAGGGGA
GGGAGTGAGG AAGAGATCCG CGGCCTTAAT CTAGAGAGCT ATTATCCCCT

CspCI

BamHI

CspCI

XcmI

2001 CCGGATCCC CCGAGTCAA CACCCGTGGG AATCCCATGG GCACCATGGC
GGCCTAGGGG GGCTCAGGTT GTGGGCACCC TTAGGGTACC CGTGGTACCG

BtsI

2051 CCCTCGCTCC AAAAATGCTT TCGCGTCTCG CAGACACTGC TCGGTAGTTT
GGGAGCGAGG TTTTACGAA AGCGCAGAGC GTCTGTGACG AGCCATCAA

2101 CGGGGATCAG CGTTTGAGTA AGAGCCCGCG TCTGAACCCT CCGCGCCGCC
GCCCTAGTC GCAAACATCAT TCTCGGGCGC AGACTTGGGA GCGCGGGCGG

PmlI

DrallI

2151 CCGGCCAGT GGAAAGACGC GCAGGCAAAA CGCACCACGT GACGGAGCGT
GGCCGGGTCA CCTTTCTGCG CGTCCGTTTT GCGTGGTGCA CTGCCTCGCA

2201 GACCGCGCGC CGAGCGCGCG CCAAGGTCGG GCAGGAAGAG GGCCTATTTT
CTGGCGCGCG GCTCGCGCGC GGTTCAGCC CGTCCTTCTC CCGGATAAAG

2251 CCATGATTCC TTCATATTTG CATATACGAT ACAAGGCTGT TAGAGAGATA
GGTACTAAGG AAGTATAAAC GTATATGCTA TGTTCCGACA ATCTCTCTAT

AseI

2301 ATTAGAATTA ATTTGACTGT AAACACAAAG ATATTAGTAC AAAATACGTG
TAATCTTAAT TAAACTGACA TTTGTGTTTC TATAATCATG TTTTATGCAC

2351 ACGTAGAAAG TAATAATTTT TTGGGTAGTT TGCAGTTTTT AAAATTATGT
TGCATCTTTC ATTATTAAG AACCCATCAA ACGTCAAAAA TTTTAATACA

NdeI

2401 TTTAAATGG ACTATCATAT GCTTACCGTA ACTTGAAAGT ATTTGATTT
AAATTTTACC TGATAGTATA CGAATGGCAT TGAACCTTCA TAAAGCTAAA

2451 CTTGGCTTTA TATATCTTGT GGAAAGGACG AAACACCGTG CTCGCTTCGG
GAACCGAAAT ATATAGAACA CCTTCTCTGC TTTGTGGCAC GAGCGAAGCC

Sall

HincII

AccI

2501 CAGCACATAT ACTAGTCGAC TAGGGATAAC AGGGTAATTG TTTGAATGAG
GTCGTGTATA TGATCAGCTG ATCCCTATTG TCCCATTAAC AAACCTACTC

Scal

2551 GCTTCAGTAC TTTACAGAAT CGTTGCCTGC ACATCTTGGA AACACTTGCT
CGAAGTCATG AAATGTCTTA GCAACGGACG TGTAGAACCT TTGTGAACGA

HpaI

HincII

XhoI

PspXI

EcoRI

2601 GGGATTACTT CTTCAAGTTA ACCCAACAGA AGGCTCGAGC AACCGAATT
CCCTAATGAA GAAGTCCAAT TGGGTTGTCT TCCGAGCTCG TTGGTCTTAA

2651 CAAGGGGCTA CTTTAGGAGC AATTATCTTG TTTACTAAAA CTGAATACCT
GTTCCCGAT GAAATCCTCG TTAATAGAAC AAATGATTTT GACTTATGGA

2701 TGCTATCTCT TTGATACATT TTTACAAAGC TGAATTAATAA TGGTATAAAT
ACGATAGAGA AACTATGTAA AAATGTTTCG ACTTAATTTT ACCATATTTA

MluI

MfeI

BbsI

PmeI

AflIII

2751 TAAATCACTT TTTTCAATTG GAAGACTAAT GCGTTTAAAC ACGCGGCGAC
ATTTAGTGAA AAAAGTTAAC CTTCTGATTA CGCAAATTTG TGCGCCGCTG

2801 GCGTTCGACC GAATAAAACC TGTGACGGAA GATCACTTCG CAGAATAAAT
CGCAAGCTGG CTTATTTTGG AACTGCCTT CTAGTGAAGC GTCTTATTTA

PfIMI

2851 AAATCCTGGT GTCCCTGTTG ATACCGGGAA GCCCTGGGCC AACTTTTGGC
TTTAGGACCA CAGGGACAAC TATGGCCCTT CGGGACCCGG TTGAAAACCG

MslI

2901 GAAAATGAGA CGTTGATCGG CACGTAAGAG GTTCCAACCT TCACCATAAT
CTTTACTCT GCAACTAGCC GTGCATTCTC CAAGGTTGAA AGTGGTATTA

2951 GAAATAAGAT CACTACCGGG CGTATTTTTT GAGTTGTCGA GATTTTCAGG
CTTTATTCTA GTGATGGCCC GCATAAAAAA CTCAACAGCT CTAAGGTC

3001 AGCTAAGGAA GCTAAAATGG AGAAAAAAT CACTGGATAT ACCACCGTTG
TCGATTCTT CGATTTTACC TCTTTTTTA GTGACCTATA TGGTGGCAAC

3051 ATATATCCCA ATGGCATCGT AAAGAACATT TTGAGGCATT TCAGTCAGTT
TATATAGGGT TACCGTAGCA TTTCTTGTA AACTCCGTAA AGTCAGTCAA

3101 GCTCAATGTA CCTATAACCA GACCGTTCAG CTGGATATTA CGGCCTTTTT
CGAGTTACAT GGATATTGGT CTGGCAAGTC GACCTATAAT GCCGGAAAAA

3151 AAAGACCGTA AAGAAAAATA AGCACAAGTT TTATCCGGCC TTTATTCA
TTCTGGCAT TTCTTTTTAT TCGTGTTCAA AATAGGCCGG AAATAAGTGT

SnaBI

3201 TTCTTGCCCG CCTGATGAAT GCTCATCCGG AATTACGTAT GGCAATGAAA
AAGAACGGGC GGACTACTTA CGAGTAGGCC TTAATGCATA CCGTTACTTT

3251 GACGGTGAGC TGGTGATATG GGATAGTGTT CACCCTTGTT ACACCGTTTT
CTGCCACTCG ACCACTATAC CCTATCACAA GTGGGAACAA TGTGGCAAAA

AclI

3301 CCATGAGCAA ACTGAAACGT TTTCATCGCT CTGGAGTGAA TACCACGACG
GGTACTCGTT TGACTTTGCA AAAGTAGCGA GACCTCACTT ATGGTGCTGC

3351 ATTTCCGGCA GTTTCTACAC ATATATTCGC AAGATGTGGC GTGTTACGGT
TAAAGCCGT CAAAGATGTG TATATAAGCG TTCTACACCG CACAATGCCA

3401 GAAAACCTGG CCTATTTCCC TAAAGGGTTT ATTGAGAATA TGTTTTTCGT
CTTTTGACC GGATAAAGGG ATTTCCCAA TAACTCTTAT AAAAAAGCA

PfIMI

3451 CTCAGCCAAT CCCTGGGTGA GTTTCACCAG TTTTGATTTA AACGTGGCCA
GAGTCGGTTA GGGACCCACT CAAAGTGGTC AAACTAAAT TTGCACCGGT

SspI

3501 ATATGGACAA CTTCTTCGCC CCCGTTTTCA CCATGGGCAA ATATTATACG
TATACCTGTT GAAGAAGCGG GGGCAAAAGT GGTACCCGTT TATAATATGC

3551 CAAGGCGACA AGGTGCTGAT GCCGCTGGCG ATTCAGGTTT ATCATGCCGT
GTTCCGCTGT TCCACGACTA CGGCGACCGC TAAGTCCAAG TAGTACGGCA

Scal

3601 TTGTGATGGC TTCCATGTCG GCAGAATGCT TAATGAATTA CAACAGTACT
AACACTACCG AAGGTACAGC CGTCTTACGA ATTAATTAAT GTTGTCTATGA

3651 GCGATGAGTG GCAGGGCGGG GCGTAATTTT TTTAAGGCAG TTATTGGTGC
CGCTACTCAC CGTCCCGCCC CGCATTAATA AAATTCCGTC AATAACCACG

3701 CCTTAAACGC CTGGTTGCTA CGCCTGAATA AGTGATAATA AGCGGATGAA
GGAATTTGCG GACCAACGAT GCGGACTTAT TCACTATTAT TCGCCTACTT

3751 TGGCAGAAAT TCGGATCTCG ACCGCGTTTG GGCGGTGGCT CCCTGCCACG
ACCGTCTTTA AGCCTAGAGC TGGCGCAAAC CCGCCACCGA GGGACGGTGC

3801 CGGCTCCGAA CAGAAGCTGA TCTCCGAAGA GGATCTGATT ACCCTGTTAT
GCCGAGGCTT GTCTTCGACT AGAGGCTTCT CTTAGACTAA TGGGACAATA

3851 CCCTACCCTA AAATTCTACC GGGTAGGGGA GGCGCTTTTC CCAAGGCAGT
GGATGGGAT TTTAAGATGG CCCATCCCCT CCGCGAAAAG GGTTCCGTC

3901 CTGGAGCATG CGCTTTAGCA GCCCCGCTGG GCACTTGGCG CTACACAAGT
GACCTCGTAC GCGAAATCGT CGGGGCGACC CGTGAACCGC GATGTGTTCA

AgeI

3951 GGCCTCTGGC CTCGCACACA TTCCACATCC ACCGGTAGGC GCCAACCGGC
CCGGAGACCG GAGCGTGTGT AAGGTGTAGG TGGCCATCCG CGGTTGGCCG

4001 TCCGTTCTTT GGTGGCCCCT TCGCGCCACC TTCTACTCCT CCCCTAGTCA
AGGCAAGAAA CCACCGGGGA AGCGCGGTGG AAGATGAGGA GGGGATCAGT

4051 GGAAGTTCCC CCCC GCCCCG CAGCTCGCGT CGTGCAGGAC GTGACAAATG
CCTTCAAGGG GGGGCGGGG GTCGAGCGCA GCACGTCCTG CACTGTTTAC

BssSI

B1pI

4101 GAAGTAGCAC GTCTACTAG TCTCGTGCAG ATGGACAGCA CCGCTGAGCA
CTTCATCGTG CAGAGTGATC AGAGCACGTC TACCTGTCGT GGCGACTCGT

StuI

4151 ATGGAAGCGG GTAGGCCTTT GGGGCAGCGG CCAATAGCAG CTTTGCTCCT
TACCTTCGCC CATCCGGAAA CCCCCTCGCC GGTTATCGTC GAAACGAGGA

4201 TCGCTTTCTG GGCTCAGAGG CTGGGAAGGG GTGGGTCCGG GGGCGGGCTC
AGCGAAAGAC CCGAGTCTCC GACCCTTCCC CACCCAGGCC CCCGCCGAG

4251 AGGGGCGGGC TCAGGGGCGG GCGGGCGCC CGAAGGTCCT CCGGAGGCC
TCCCCGCCCG AGTCCCCGCC CCGCCCGCGG GCTTCCAGGA GGCCTCCGGG

4301 GGCATTCTGC ACGTTCAAAG AGCGCACGTC TGCCGCGCTG TTCTCCTCTT
CCGTAAGACG TCGAAGTTT TCGCGTGCAG ACGGCGCGAC AAGAGGAGAA

HindIII

4351 CCTCATCTCC GGGCCTTTTCG ACCTGCAGCC CAAGCTTACC ATGACCGAGT
GGAGTAGAGG CCCGGAAGC TGGACGTCGG GTTCGAATGG TACTGGCTCA

BsiWI

4401 ACAAGCCAC GGTGCGCCTC GCCACCCGCG ACGACGTCCC CAGGGCCGTA
TGTTCCGGTG CCACGCGGAG CGGTGGGCGC TGCTGCAGGG GTCCCCGCAT

4451 CGCACCTCG CCGCCGCGTT CGCCGACTAC CCCGCCACGC GCCACACCGT
GCGTGGGAGC GCGGCGCAA GCGGCTGATG GGGCGGTGCG CGGTGTGGCA

RsrlI BstEII Ppil
4501 CGATCCGGAC CGCCACATCG AGCGGGTCAC CGAGCTGCAA GAACTCTTCC
GCTAGGCCTG GCGGTGTAGC TCGCCAGTG GCTCGACGTT CTTGAGAAGG

4551 TCACGCGCGT CGGGCTCGAC ATCGGCAAGG TGTGGGTCGC GGACGACGGC
AGTGCGCGCA GCCCGAGCTG TAGCCGTTCC ACACCCAGCG CCTGCTGCC

SacII
4601 GCCGCGGTGG CGGTCTGGAC CACGCCGGAG AGCGTCGAAG CGGGGGCGGT
CGGCGCCACC GCCAGACCTG GTGCGGCCTC TCGCAGCTTC GCCCCCGCCA

4651 GTTCGCCGAG ATCGGCCCGC GCATGGCCGA GTTGAGCGGT TCCCGGCTG
CAAGCGGCTC TAGCCGGGCG CGTACCGGCT CAACTCGCCA AGGGCCGACC

Stul BsaXI
4701 CCGCGCAGCA ACAGATGGAA GGCCTCCTGG CGCCGCACCG GCCCAAGGAG
GGCGCGTCGT TGTCTACCTT CCGGAGGACC GCGGCGTGGC CGGGTTCTC

4751 CCCGCGTGGT TCCTGGCCAC CGTCGGCGTC TCGCCCGACC ACCAGGGCAA
GGGCGACCA AGGACCGGTG GCAGCCGCAG AGCGGGCTGG TGTCCCGTT

Bcgl
4801 GGGTCTGGGC AGCGCCGTCG TGCTCCCCGG AGTGGAGGCG GCCGAGCGCG
CCCAGACCCG TCGCGGCAGC ACGAGGGGCC TCACCTCCGC CGGCTCGCGC

PfoI
4851 CCGGGGTGCC CGCCTTCTG GAGACCTCCG CGCCCCGCAA CCTCCCCTC
GGCCCCACGG GCGGAAGGAC CTCTGGAGGC GCGGGGCGTT GGAGGGGAAG

4901 TACGAGCGGC TCGGCTTAC CGTCACCGCC GACGTGAGG TGCCCGAAGG
ATGCTCGCCG AGCCGAAGTG GCAGTGGCGG CTGCAGCTCC ACGGGCTTC

SexAI
DrallI
4951 ACCGCGCACC TGGTGCATGA CCCGCAAGCC CGGTGCCTGA CGCCCGCCCC
TGGCGCGTGG ACCACGTACT GGGCGTTCGG GCCACGGACT GCGGGCGGGG

Nsil ClaI
BfrBI
5001 ACGACCCGCA GCGCCCGACC GAAAGGAGCG CACGACCCCA TGCATCGATA
TGCTGGGCGT CGCGGGCTGG CTTTCTCGC GTGCTGGGGT ACGTAGCTAT

5051 AAATAAAAGA TTTTATTTAG TCTCCAGAAA AAGGGGGGAA TGAAAGACCC
TTTATTTTCT AAAATAAATC AGAGGTCTTT TTCCCCCTT ACTTTCTGGG

5101 CACCTGTAGG TTTGGCAAGC TAGAGAACCA TCAGATGTTT CCAGGGTGCC

GTGGACATCC AAACCGTTCG ATCTCTTGGT AGTCTACAAA GGTCCCACGG

5151 CCAAGGACCT GAAATGACCC TGTGCCTTAT TTGAACTAAC CAATCAGTTC
GGTTCCTGGA CTTTACTGGG ACACGGAATA AACTTGATTG GTTAGTCAAG

Sacl
EcoICRI

5201 GCTTCTCGCT TCTGTTGCGC CGCTTCTGCT CCCCAGGCTC AATAAAAGAG
CGAAGAGCGA AGACAAGCGC GCGAAGACGA GGGGCTCGAG TTATTTTCTC

Xmal
SmaI

AscI

5251 CCCACAACCC CTCACTCGGC GCGCCAGTCC TCCGATAGAC TCGTTCGCCC
GGGTGTTGGG GAGTGAGCCG CGCGGTCAGG AGGCTATCTG ACGCAGCGGG

BaeI
BaeI
KpnI
BaeI
BaeI
Acc65I

5301 GGGTACCCGT GTATCCAATA AACCTCTTG CAGTTGCATC CGACTTGTGG
CCCATGGGCA CATAGGTTAT TTGGGAGAAC GTCAACGTAG GCTGAACACC

5351 TCTCGCTGTT CTTGGGAGG GTCTCCTCTG AGTGATTGAC TACCCGTCAG
AGAGCGACAA GGAACCCTCC CAGAGGAGAC TACTAACTG ATGGGCAGTC

5401 CGGGGGTCTT TCATGGGTAA CAGTTTCTTG AAGTTGGAGA ACAACATTCT
GCCCCAGAA AGTACCCATT GTCAAAGAAC TTCAACCTCT GTTTGTAAGA

5451 GAGGGTAGGA GTCGAATCGA GAGAGAGAGA GAGAGAGAGA GAGAGAGAGA
CTCCATCCT CAGCTTAGCT CTCTCTCTCT CTCTCTCTCT CTCTCTCTCT

PspOMI
ApaI

5501 GAGAGAGAGA GACGTGGGCC CAATTCTGTC AGCCGTTAAG TGTTCTGTG
CTCTCTCTCT CTGCACCCGG GTTAAGACAG TCGGCAATTC ACAAGGACAC

5551 TCACTGAAAA TTGCTTTGAG AGGCTCTAAG GGCTTCTCAG TCGTTACAT
AGTGACTTTT AACGAACTC TCCGAGATTC CCGAAGAGTC ACGCAATGTA

HindIII

5601 CCCTGGCTTG TTGTCCACAA CCGTTAAACC TTAAAAGCTT TAAAAGCCTT
GGGACCGAAC AACAGGTGTT GGCAATTTGG AATTTTCGAA ATTTTCGGAA

5651 ATATATTCTT TTTTTCTTA TAAAACCTAA AACCTTAGAG GCTATTTAAG
TATATAAGAA AAAAAAGAAT ATTTTGAATT TTGGAATCTC CGATAAATTC

AseI

5701 TTGCTGATTT ATATTAATTT TATTGTTCAA ACATGAGAGC TTAGTACGTG
AACGACTAAA TATAATTAATA ATAACAAGTT TGTACTCTCG AATCATGCAC

5751 AAACATGAGA GCTTAGTACG TTAGCCATGA GAGCTTAGTA CGTTAGCCAT

TTGTACTCT CGAATCATGC AATCGGTACT CTCGAATCAT GCAATCGGTA

5801 GAGGGTTT TAG TTCGTAAAC ATGAGAGCTT AGTACGTAA ACATGAGAGC
CTCCCAAATC AAGCAATTTG TACTCTCGAA TCATGCAATT TGTACTCTCG

SnaBI

5851 TTAGTACGTG AACATGAGA GCTTAGTACG TACTATCAAC AGGTTGAACT
AATCATGCAC TTTGTACTCT CGAATCATGC ATGATAGTTG TCCAACCTGA

BclI

5901 GCTGATCAAC AGATCCTCTA CACTAGAAGG GACGCACCGC TAGCAGCGCC
CGACTAGTTG TCTAGGAGAT GTGATCTTCC CTGCGTGGCG ATCGTCGCGG

5951 CCTAGCGGTA TCCTATAAAA AAACACACCG CGCCGCTAGC AGCACCCCTA
GGATCGCCAT AGGATATTTT TTTGTGTGGC GCGGCGATCG TCGTGGGGAT

6001 ATATAAATA ATGTTTTTTA TAAAAATAGT CAGTACCACC CCTACAAAAC
TATATTTTAT TACAAAAAAT ATTTTTATCA GTCATGGTGG GGATGTTTTG

6051 GGTGTCGGCG CGTTGTTGTA GCCGCGCCGA CACCGCTTTT TAAATATCA
CCACAGCCGC GCAACAACAT CGGCGCGGCT GTGGCGAAAA AATTTATAGT

6101 TAAAGAGAGT AAGAGAACT AATTTTTCAT AACACTCTAT TTATAAAGAA
ATTTCTCTCA TTCTCTTGA TAAAAAAGTA TTGTGAGATA AATATTTCTT

6151 AAATCAGCAA AAACCTGTTT TTGCGTGGGG TGTGGTGCTT TTGGTGGTGA
TTTAGTCGTT TTTGAACAAA AACGCACCCC ACACCACGAA AACCACCACT

6201 GAACCACCAA CCTGTTGAGC CTTTTTGTGG AGTGGGTAA ATTATACTAG
CTTGGTGGTT GGACAACCTG GAAAAACACC TCACCCAATT TAATATGATC

BstBI BpI

6251 CGCGTTTCGA ACCCCAGAGT CCCGCTCAGA AGAACTCGTC AAGAAGGCGA
GCGCAAAGCT TGGGGTCTCA GGGCGAGTCT TCTTGAGCAG TTCTTCCGCT

BssSI

6301 TAGAAGGCGA TCGCTGCGA ATCGGGAGCG GCGATACCGT AAAGCACGAG
ATCTTCCGCT ACGCGACGCT TAGCCCTCGC CGCTATGGCA TTTCTGTGCTC

SapI

6351 GAAGCGGTCA GCCCATTGCG CGCCAAGCTC TTCAGCAATA TCACGGGTAG
CTTCGCCAGT CGGGTAAGCG GCGGTTGAG AAGTCGTTAT AGTGCCCATC

RsrII

6401 CCAACGCTAT GTCCTGATAG CGGTCCGCCA CACCCAGCCG GCCACAGTCG
GGTTGCGATA CAGGACTATC GCCAGGCGGT GTGGGTCGGC CGGTGTCAGC

MsiI

6451 ATGAATCCAG AAAAGCGGCC ATTTTCCACC ATGATATTCG GCAAGCAGGC
TACTTAGGTC TTTTCGCCGG TAAAAGGTGG TACTATAAGC CGTTCGTCCG

BpuEI

6501 ATCGCCATGT GTCACGACGA GATCCTCGCC GTCGGGCATG CGCGCCTTGA
TAGCGGTACA CAGTGCTGCT CTAGGAGCGG CAGCCCGTAC GCGCGGAACT

SapI

6551 GCCTGGCGAA CAGTTCGGCT GGC GCGAGCC CCTGATGCTC TTCGTCCAGA
CGGACCGCTT GTCAAGCCGA CCGCGCTCGG GGACTACGAG AAGCAGGTCT

FalI

6601 TCATCCTGAT CGACAAGACC GGCTTCCATC CGAGTACGTG CTCGCTCGAT
AGTAGGACTA GCTGTTCTGG CCGAAGGTAG GCTCATGCAC GAGCGAGCTA

6651 GCGATGTTTC GCTTGGTGGT CGAATGGGCA GGTAGCCGGA TCAAGCGTAT
CGCTACAAAG CGAACCACCA GCTTACCCGT CCATCGGCCT AGTTCGCATA

6701 GCAGCCGCCG CATTGCATCA GCCATGATGG ATACTTTCTC GGCAGGAGCA
CGTCGGCGGC GTAACGTAGT CGGTACTACC TATGAAAGAG CCGTCCTCGT

6751 AGGTGAGATG ACAGGAGATC CTGCCCCGGC ACTTCGCCCA ATAGCAGCCA
TCCACTCTAC TGTCTCTAG GACGGGGCCG TGAAGCGGGT TATCGTCGGT

FspI

6801 GTCCCTTCCC GCTTCAGTGA CAACGTCGAG CACAGCTGCG CAAGGAACGC
CAGGGAAGGG CGAAGTCACT GTTGACGCTC GTGTCGACGC GTTCCTTGCG

6851 CCGTCGTGGC CAGCCACGAT AGCCGCGCTG CCTCGTCCTG CAGTTCATTC
GGCAGCACCG GTCGGTGCTA TCGGCGCGAC GGAGCAGGAC GTCAAGTAAG

DrdI

6901 AGGGCACCGG ACAGGTCGGT CTTGACAAAA AGAACCGGGC GCCCTGCGC
TCCCGTGGCC TGTCAGCCA GAACTGTTTT TCTTGCCCG CGGGGACGCG

6951 TGACAGCCGG AACACGGCGG CATCAGAGCA GCCGATTGTC TGTTGTGCC
ACTGTCGGCC TTGTGCCGCC GTAGTCTCGT CGGCTAACAG ACAACACGGG

7001 AGTCATAGCC GAATAGCCTC TCCACCCAAG CGGCCGAGGA ACCTGCGTGC
TCAGTATCGG CTTATCGGAG AGGTGGGTTT GCCGGCCTCT TGGACGCACG

BsaBI

BclI

7051 AATCCATCTT GTTCAATCAT GCGAAACGAT CCTCATCCTG TCTCTTGATC
TTAGGTAGAA CAAGTTAGTA CGCTTTGCTA GGAGTAGGAC AGAGAAGTAC

BglII

7101 AGATCT

Appendix 2: Restriction Analysis of pSM2 cloning region

EcoO109I

EarI Sau96I

1 GCAGGAAGAG GGCCTATTC CCATGATTCC TTCATATTTG CATATACGAT
CGTCCTTCTC CCGGATAAAG GGTACTAAGG AAGTATAAAC GTATATGCTA

AseI

51 ACAAGGCTGT TAGAGAGATA ATTAGAATTA ATTTGACTGT AAACACAAAG
TGTTCCGACA ATCTCTCTAT TAATCTTAAT TAAACTGACA TTTGTGTTTC

Tsp45I

101 ATATTAGTAC AAAATACGTG ACGTAGAAAG TAATAATTC TGGGGTAGTT
TATAATCATG TTTTATGCAC TGCATCTTTC ATTATTAAAG AACCCATCAA

NdeI

151 TGCAGTTTTT AAAATTATGT TTTAAAATGG ACTATCATAT GCTTACCGTA
ACGTCAAAAA TTTTAATACA AAATTTTACC TGATAGTATA CGAATGGCAT

201 ACTTAAAAGT ATTTGATTT CTTGGCTTTA TATATCTTGT GGAAAGGACG
TGAACCTTCA TAAAGCTAAA GAACCGAAAT ATATAGAACA CCTTTCCTGC

Sall

HincII

AccI

Bsp1286I

TseI

Bfal

BsiHKAI

BbvI

SpeI

Bfal

251 AACACCGTG CTCGCTTCGG CAGCACATAT ACTAGTCGAC TAGGGATAAC
TTTGTGGCAC GAGCGAAGCC GTCGTGTATA TGATCAGCTG ATCCCTATTG

Scal

AcuI

BsgI

301 AGGGTAATTG TTTGAATGAG GCTTCAGTAC TTTACAGAAT CGTTGCCTGC
TCCCATTAAC AAACCTACTC CGAAGTCATG AAATGTCTTA GCAACGGACG

HpaI

BseYI

AcuI HincII

351 ACATCTTGA AACACTTGCT GGGATTACTT CTTCAAGTTA ACCCAACAGA
TGTAGAACCT TTGTGAACGA CCCTAATGAA GAAGTCCAAT TGGGTTGTCT

XhoI

SmlI

AvaI

EcoRI

PspXI

ApoI

401 AGGCTCGAGC AACCAGAATT CAAGGGGCTA CTTTAGGAGC AATTATCTTG
TCCGAGCTCG TTGGTCTTAA GTTCCCCGAT GAAATCCTCG TTAATAGAAC

451 TTTACTAAAA CTGAATACCT TGCTATCTCT TTGATACATT TTTACAAAGC
AAATGATTTT GACTTATGGA ACGATAGAGA AACTATGTAA AAATGTTTCG

MfeI BbsI

501 TGAATTAATA TGGTATAAAT TAAATCACTT TTTTCAATTG GAAGACTAAT
ACTTAATTTT ACCATATTTA ATTTAGTGAA AAAAGTTAAC CTTCTGATTA

MluI
AflIII
HgaI TaqII TspGWI
PmeI Hpy99I BsiEI Tsp45I
551 GCGTTTAAAC ACGCGGCGAC GCGTTCGACC GAATAAAACC TGTGACGGAA
CGCAAATTTG TGCGCCGCTG CGCAAGCTGG CTTATTTTGG ACACTGCCTT

BsmFI
BslFI
BslFI NciI
601 GATCACTTCG CAGAATAAAT AAATCCTGGT GTCCCTGTTG ATACCGGGAA
CTAGTGAAGC GTCTTATTTA TTTAGGACCA CAGGGACAAC TATGGCCCTT

Sau96I BsmBI
PaeI PfiMI BsmAI
651 GCCCTGGGCC AACTTTTGGC GAAAATGAGA CGTTGATCGG CACGTAAGAG
CGGGACCCGG TTGAAAACCG CTTTACTCT GCAACTAGCC GTGCATTCTC

MmeI MspI NciI
701 GTTCCAATT TCACCATAAT GAAATAAGAT CACTACCGGG CGTATTTTTT
CAAGGTTGAA AGTGGTATTA CTTTATTCTA GTGATGGCCC GCATAAAAAA

DdeI
Bpu10I TspRI
751 GAGTTGTCGA GATTTTCAGG AGCTAAGGAA GCTAAAATGG AGAAAAAAT
CTCAACAGCT CTAAAAGTCC TCGATTCTT CGATTTTACC TCTTTTTTTA
M E K K I Frame 1

BsrI
TspRI SfaNI
801 CACTGGATAT ACCACCGTTG ATATATCCCA ATGGCATCGT AAAGAACATT
GTGACCTATA TGGTGGCAAC TATATAGGGT TACCGTAGCA TTTCTTGTA
T G Y T T V D I S Q W H R K E H F Frame 1

PvuII
MspA1I
851 TTGAGGCATT TCAGTCAGTT GCTCAATGTA CCTATAACCA GACCGTTCAG
AACTCCGTAA AGTCAGTCAA CGAGTTACAT GGATATTGGT CTGGCAAGTC
E A F Q S V A Q C T Y N Q T V Q Frame 1

BceAI
901 CTGGATATTA CGGCCTTTTT AAAGACCGTA AAGAAAAATA AGCACAAGTT
GACCTATAAT GCCGGAAAAA TTTCTGGCAT TTCTTTTTAT TCGTGTTCAA
L D I T A F L K T V K K N K H K F Frame 1

BspEI
BsaWI
FokI
FauI BsmI BstF5I
951 TTATCCGGCC TTTATTCACA TTCTTGCCCG CCTGATGAAT GCTCATCCGG
AATAGGCCGG AAATAAGTGT AAGAACGGGC GGACTACTTA CGAGTAGGCC
Y P A F I H I L A R L M N A H P E Frame 1

SnaBI BsrDI
1001 AATTACGTAT GGCAATGAAA GACGGTGAGC TGGTGATATG GGATAGTGTT
TTAATGCATA CCGTTACTTT CTGCCACTCG ACCACTATAC CCTATCACAA
L R M A M K D G E L V I W D S V Frame 1

AclI BtgZI
1051 CACCCTTGTT ACACCGTTTT CCATGAGCAA ACTGAAACGT TTTCATCGCT
GTGGGAACAA TGTGGCAAAA GGTACTCGTT TGACTTTGCA AAAGTAGCGA
H P C Y T V F H E Q T E T F S S L Frame 1

BpmI Hpy99I
1101 CTGGAGTGAA TACCACGACG ATTTCCGGCA GTTCTACAC ATATATTCGC
GACCTCACTT ATGGTGCTGC TAAAGGCCGT CAAAGATGTG TATATAAGCG
W S E Y H D D F R Q F L H I Y S Q Frame 1

1151 AAGATGTGGC GTGTTACGGT GAAAACCTGG CCTATTTCCC TAAAGGGTTT
TTCTACACCG CACAATGCCA CTTTTGGACC GGATAAAGGG ATTTCCCAA
D V A C Y G E N L A Y F P K G F Frame 1

DdeI
BspCNI
BseMII
BsmAI TaqII
BsmBI PfiMI PstI BsrI
1201 ATTGAGAATA TGTTTTTCGT CTCAGCCAAT CCCTGGGTGA GTTTCACCAG
TAACTCTTAT ACAAAAAGCA GAGTCGGTTA GGGACCCACT CAAAGTGGTC
I E N M F F V S A N P W V S F T S Frame 1

MscI
EaeI
1251 TTTTGATTTA AACGTGGCCA ATATGGACAA CTTCTTCGCC CCCGTTTTCA
AAAATAAAT TTGCACCGGT TATACCTGTT GAAGAAGCGG GGGCAAAAGT
F D L N V A N M D N F F A P V F T Frame 1

StyI
NcoI MspA1I
BtgI SspI SfaNI
1301 CCATGGGCAA ATATTATACG CAAGGCGACA AGGTGCTGAT GCCGCTGGCG
GGTACCCGTT TATAATATGC GTTCCGCTGT TCCACGACTA CGGCGACCGC
M G K Y Y T Q G D K V L M P L A Frame 1

BceAI BclI BsmI
1351 ATTCAGGTTT ATCATGCCGT TTGTGATGGC TTCCATGTCG GCAGAATGCT
TAAGTCCAAG TAGTACGGCA AACACTACCG AAGGTACAGC CGTCTTACGA
I Q V H H A V C D G F H V G R M L Frame 1

Scal BtgZI Faul
1401 TAATGAATTA CAACAGTACT GCGATGAGTG GCAGGGCGGG GCGTAATTTT
ATTACTTAAT GTTGTGATGA CGTACTCAC CGTCCC GCCC CGCATTAAAA
N E L Q Q Y C D E W Q G G A * Frame 1

Bsp1286I
Bme1580I
BamI

1451 TTTAAGGCAG TTATTGGTGC CCTTAAACGC CTGGTTGCTA CGCCTGAATA
AAATTCCGTC AATAACCACG GGAATTTGCG GACCAACGAT GCGGACTTAT

FokI BstYI
BstF5I ApeI AlwI BsiEI

1501 AGTGATAATA AGCGGATGAA TGGCAGAAAT TCGGATCTCG ACCGCGTTTG
TCACTATTAT TCGCCTACTT ACCGTCTTTA AGCCTAGAGC TGGCGCAAAC

AlwNI EarI

1551 GGCGGTGGCT CCCTGCCACG CGGCTCCGAA CAGAAGCTGA TCTCCGAAGA
CCGCCACCGA GGGACGGTGC GCCGAGGCTT GTCTTCGACT AGAGGCTTCT

BstYI
AlwI

1601 GGATCTGATT
CCTAGACTAA

Useful References:

Carmell, M.A., Zhang, L., Conklin, D.S., Hannon, G.J., Rosenquist, T.A., **Germline transmission of RNAi in mice**. Nat Struct Bio 10, 91-92. (2003).

Conklin, D., Hannon, G., Bernstein, E., Caudy, A., Paddison, P., **Short Hairpin RNAs (shRNAs) induce sequence-specific silencing in Mammalian Cells**. Genes & Development. 16:948-958. (2002).

Hannon, G., Caudy, A., Paddison, P., **Stable Suppression of Gene Expression by RNAi in Mammalian Cells**. PNAS 99(3): 1443-1448 (2003).

Hemann, M.T., Friedman, J.S., Zilfou, J.T., Hernando, E., Paddison, P., Cordon-Cardo, C., Hannon, G.J., Lowe, S.W. **An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo**. Nat Genet 33, 396-400. (2003).

McCaffrey A.P., Meuse L., Pham T.T., Conklin D.S., Hannon G.J., Kay M.A. **RNA interference in adult mice**. Nature (418) 38-39. (2002).

Paddison, P.J., Silva, J.M., Conklin, D.S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K. Chang, K., Westbrook, T., Sachidanandam, R., McCombie W.R., Elledge, S.J., & Hannon, G.J. **A resource for large-scale RNAi based screens in mammals**. Nature, March 25; 427-431. (2004)

Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. & Conklin, D.S. **Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells**. Genes Dev 16,948-58. (2002).

Taira, K., Miyagishi, M. **U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells**. Nature Biotechnology 19, 497-500. (2002).

Zeng, Yan., Cullen, B. **Sequence requirements for micro RNA processing and function in human cells**. RNA 9, 112-123. (2003).

Limited Use License:

This product is covered by several patent applications owned by Cold Spring Harbor Laboratory. The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to resell, repackage, or further sublicense) under these patent rights to perform the RNAi knockdown methods using the RNAi-inducing vectors claimed in those patent applications for research purposes solely in conjunction with this product. No other license is granted to the buyer whether expressly, by implication, by estoppel or otherwise. In particular, the purchase of this product does not include nor carry any right or license to use, develop, or otherwise exploit this product commercially, and no rights are conveyed to the buyer to use the product or components of the product for any other purposes, including without limitation, provision of services to a third party, generation of commercial databases, or clinical diagnostics or therapeutics.

This product is sold pursuant to a license from CSHL, and CSHL reserves all other rights under these patent rights. For information on purchasing a license to the patent rights for uses other than in conjunction with this product or to use this product for purposes other than research, please contact the CSHL Office of Technology Transfer at, 516-367-8312.

Phone: 1-888-412-2225

FAX: 1-256-704-4849

info@openbiosystems.com

V010304

For Research Use Only