

**SynapSys**  
*We Bridge the Gap®*

**pFlash™ Phagemids  
Technical Manual**

## I. Description

The pFlash™ phagemids are intended for the quantitative analysis of putative cis-elements and transacting factors that regulate eukaryotic gene regulation. The pFlash™ phagemids carry the coding region of the firefly (*Photinus pyralis*) luciferase gene which is used to monitor transcriptional activity in transfected eukaryotic cells. The assay of this reporter gene is rapid, sensitive and quantitative and represents the efficiency of transcription initiation utilizing the putative cis-elements cloned upstream/downstream of the reporter gene. In addition the pFlash™ phagemids contain numerous features that facilitate the characterization and mutagenesis of the putative cis-elements being studied.

## II. General Considerations

### A. Structure and Function

There are three types of pFlash™ vectors: pFlash I™, pFlash II™, and SV40-pFlash™. Each pFlash™ phagemid carries the luciferase gene (*luc*) followed by the SV40 t intron containing the polyadenylation [poly(A)] signals which is the generic donor for these signals and are used by every reporter gene vector that is currently available commercially. However the pFlash™ phagemids are distinguished by the fact that it is the only set in which the cryptic AP1 sequence (TGAGTCA) which is present in the wild type SV40 t intron has been specifically mutated by site-directed mutagenesis into an EcoRI (GAATTC) restriction site. The AP1 sequence is a strong eukaryotic enhancer element that is recognized by the ubiquitous Jun-Fos transcription factors as well as several others. The presence of this cryptic enhancer in the t intron results in spurious reporter gene expression and high background activity that has the potential to mask or otherwise interfere with the analysis of putative cis-elements that are being cloned upstream. The pFlash™ phagemids in which this cryptic site has been mutated are free of such activity and consistently exhibit extremely low signal-noise ratios. The vectors also contain a Col E1 origin of replication for maintenance in *E. coli*, an ampicillin resistance gene for antibiotic selection and a filamentous phage origin of replication (*f1 ori*) for single-stranded DNA production. The Multiple Cloning Sequences I (MCS I) present upstream of the reporter gene contains *Sac*I, *Bst*X I/*Sac* II, *Not* I/*Eag* I, *Spe* I, and *Bam*HI I unique restriction sites that allow easy cloning of putative regulatory sequences. The MCS II present downstream of the reporter gene cassette contains *Sma*I, *Pst* I, *Hind* III, *Sal* I/*Acc*I I/*Hinc* II, *Xho* I, *Apa*I I/*Dar* II and *Kpn* I unique restriction sites that allow cloning of regulatory elements downstream of the reporter gene cassette to test for positional effects. DNA fragments cloned in either MCS I or MCS II can be sequenced by dideoxy sequencing using primers that anneal to the T3 promoter sequence (upstream of MCS I) or to the T7 promoter sequence (downstream of MCS II). In addition, reporter gene specific primers that anneal to the first 100 bases of the *luc* gene or the last 100 bases of the polyadenylation signal cassette can also be used for sequencing. The sequences of these segments are included in the appendix for easy design of primers. Except for the inclusion of heterologous promoters, the three pFlash™ phagemids are structurally identical and are summarized below. Circle maps of SV40-pFlash™, pFlash I™ & pFlash II™ are shown.

#### pFlash I™

The pFlash I™ vector lacks eukaryotic promoter and enhancer sequences, allowing the researcher maximum flexibility in cloning putative regulatory elements. Expression of luciferase activity in cells transfected with this vector depends on insertion and proper orientation of a functional promoter upstream from *luc*. Potential enhancer elements can be cloned both upstream of the promoter as well as downstream of the *luc* reporter gene cassette. Alternatively the pFlash I™ vector may be used as such as a negative control, since in the absence of any promoter or enhancer, this vector gives no detectable *luc* gene expression.

#### pFlash II™

The pFlash II™ vector contains the TATA-box-containing heterologous promoter derived from the Herpes simplex virus-thymidine kinase gene (HSV-tk). This well characterized basal promoter contains a strong TATA-box and is located immediately upstream of the *luc* gene and is unlike the SV40 basal promoter

used in other commercially available reporter gene vectors which has an atypical and weak TATA-box. In addition the SV-40 promoter in these other vectors has been shown to exhibit silencing effects on some cytokine inducible enhancers such as the IFN- $\gamma$  inducible elements of the Fc $\gamma$ RI gene (Benech et. al. J. Exp. Med. 1992. Vol. 176: 1115-1123), while the HSV-tk promoter has been shown to be appropriately responsive to this and a wide variety of other enhancers. The pFlash II™ vector is hence an ideal candidate for the analysis of putative enhancer elements that can be cloned either upstream of the HSV-tk promoter or downstream of the *luc* reporter gene cassette.

### **SV40-pFlash™**

The SV40-pFlash™ vector contains the SV40 promoter and enhancer sequences, resulting in strong, constitutive *luc* gene expression in most eukaryotic cells, and is hence useful as a positive control for monitoring transfection efficiency in general.

### **Transformation**

These vectors contain intron sequences and recombinant reporter genes. It is strongly recommended that the investigator amplify these phagemids by transformation into a recA<sup>-</sup> strain of competent E.coli eg., HB101 or JM109. This will minimise the risk of recombinational deletion or mutation during amplification. In the event that a recombinational event is suspected to have occurred, the most appropriate course of action would be to order a fresh batch of vector DNA from SynapSys Corp.

### **B. Luciferase Reporter gene Expression Assay**

Expression of luciferase activity is evaluated following transient transfection into eukaryotic cells. Constructs of pFlash™ vectors containing putative promoters and enhancer elements may be transfected by Calcium phosphate, DEAE-Dextran, lipofection or Electroporation protocols as described in the standard molecular biology laboratory manuals (e.g., Current Protocols in Molecular Biology ed. Ausubel et. al., Wiley Interscience, 1991). The choice of protocol and the precise transfection conditions will vary according to the cell into which the constructs are being introduced as well as individual preferences. The researcher is advised to determine the best method and conditions by a series of pilot experiments using the CMVpFlash™ positive control vector provided. Assay of luciferase activity is usually performed 24 to 48 hr. after transfection, except in the case of myeloid cells of human or murine origin, such as, U937, THP1, HL-60, RAW 264.7 and J774 cell-lines. In these cases, it is best to perform the luciferase assay 7-8 hr. after transfection since the luciferase enzyme is rapidly degraded by these cells. The luciferase assay measures the emission of light catalyzed by the action of the luciferase enzyme on the substrate luciferin in the presence of ATP. The light emission is quantified using a luminometer and as such this assay is more sensitive and rapid than the older, conventional chloramphenicol acetyltransferase (CAT) assays. In addition the measurement of luciferase activity is non-radioactive.

### **C. Mapping Cis-Elements within DNA fragments**

The location of functional cis-elements within a DNA fragment can be determined by generating a set of serial unidirectional deletions (nested deletions) by the method of Henikoff and assaying for biological activity. The method takes advantage of the unique property of Exonuclease III (Exo III) which will digest 5'-overhangs, but not 3'-overhangs or  $\alpha$ -phosphorothiorate nucleotide filled in overhangs. This digestion proceeds in a processive manner at a fixed rate of bases per minute at a given temperature. By terminating the digestion at different time points a library of nested deletions of the insert can be directly generated in pFlash™ phagemids using this method, eliminating the need to create the deletions in a conventional cloning vector followed by the tedious task of individually subcloning them into the reporter gene plasmid. The upstream SacI, BstXI and Sac II in the MCS I region and the downstream Kpn I, ApaI and Pst I in the MCS II region generate 3' overhangs that resist Exo III digestion. The unique Not I, Spe I, BamHI, and Eag I sites in MCS I and the HindIII, Cla I, Sal I and Xho I sites in MCS II generate 5' overhangs that are susceptible to Exo III attack. After Exo III digestion, the resultant undigested single strand is removed

by the addition of S1 nuclease that digests the single strand leaving behind blunt ends at both ends. DNA ligase is now added to religate the ends resulting in a vector containing an insert that is shorter at the 5' end by a finite number of bases. Deletion clones can be screened by gel electrophoresis of miniprep DNA and the precise deletion endpoints can be determined by dideoxy sequencing using the T3 / T7 primers or the pFlash-1™, pFlash-2™ or pFlash-3™ primers. The deletion clones can then be directly transfected into the appropriate cell and assayed for luciferase activity. The precise deletion that results in either loss of reporter gene expression (the usual case) or sudden increase in reporter gene expression (in the event of removal of an active repressor element by the deletion) enables the researcher to map the location of the cis-element to within 50-100 bases (depending on the size of each deletion). Detailed protocols for generation of nested deletions by Exo III/S1 digestion may be found in standard laboratory manuals of Molecular Biology.

#### **D. Site-directed Mutagenesis of putative cis-elements**

Once a cis-element has been mapped to precision (ie., within 10 bases), site-directed mutagenesis may be used to specifically mutate the element while retaining the rest of the DNA fragment intact. Such mutagenetic manipulation allows the researcher to dissect the individual contributions and interactions of each cis-element to the overall transcriptional activity of the promoter. There are several ways in which such a mutagenesis can be performed and the researcher is advised to review the discussion of these protocols in *Molecular Cloning: A laboratory manual 2nd. ed.*, Sambrook et. al, Cold Spring Harbor Laboratory Press 1989; *Current Protocols in Molecular Biology ed.* Ausubel et. al, Wiley Interscience 1991. However the pFlash™ vectors contain the filamentous phage origin of replication (f1 ori). This allows recovery of the single stranded phagemid DNA upon super-infection of pFlash™ containing E.coli with the appropriate helper phage. A detailed discussion of site-directed mutagenesis with Kunkel's dut<sup>-</sup> / ung<sup>-</sup> method of selection of mutant progeny may be found in conventional manuals of molecular biology laboratory techniques.

# Appendix

## 1. PRIMER SEQUENCES :-

### pFLASH I<sup>TM</sup> MCS I region:

T3 PRIMER

5' -AATTAACCTCACTAAAGGG-3' -> SacI SacII/BstXI/EaqINot

I

GCTCGAAATTAACCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGC  
 CGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTTCGACCTCGAGGTGGCGCCACCGCCGGCG

Xba I Spe I BamHI Luc translation start

TCTAGAACTAGTGGATCCAGATCCAAATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGC  
 AGATCTTGATCACCTAGGTCTAGGTTTACCTTCTGCGGTTTTTGTATTTCTTTCCGGGCCG

GCCATTCTATCCTCTAGAGGATGGAACCGCT  
 CGGTAAGATAGGAGATCTCCTACCTTGCGCA  
 3' -AGATAGGAGATCTCCTACCT-5' -----pFlash 1<sup>TM</sup> primer

### pFLASH I<sup>TM</sup> MCS II region:

pFlash 2<sup>TM</sup> primer

5' -

GCATTTTTTTCCTGCAATTCTA-3'  
 GGTACAAATAAAGCAATAGCATCACAAATTTACAAATAAAGCATTTTTTTTCCTGCAATTCT  
 CCAATGTTTTATTTGTTATCGTAGTGTAAAGTGTATTTTCGTAAAAAAGTGACGTAAGA

SmaI PstI

AGTTGTGGTTTGTCCAACTCATCAATGTATCTTATCATGTCTGGATCGATCCCCGGGCTGCA  
 TCAACACCAAACAGGTTTGAGTAGTTACATAGAATAGTACAGACCTAGCTAGGGGCCCGACGT

HincII  
 AccI ApaI

EcoRI EcoRV HindIII/ClaI SalI XhoI DraII KpnI

GGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATT  
 CCTTAAGCTATAGTTTGAATAGCTATGGCAGCTGGAGCTCCCCCGGGCCATGGGTTAA

CGCCCTATAGTGAGTCGTATTACAATTCCTGCGCGTCGTTTTA  
 GCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAAT  
 <-3'GATATCACTCAGCATAA-5'--- T7 PRIMER

pFlash II™ :

pFLASH II™ MCS I region:

5'-AATTAACCCTCACTAAAGGG-3'->T3 PRIMER SacII/BstXI/EagINot I  
 GCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGC  
 CGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTTCGACCTCGAGGTGGCGCCACCGCCGGCG

<-----HSV-tk Basal Promoter----->

Xba I Spe I BamHI Y-box(inverted CCAAT box)  
 TCTAGAACTAGTGGATCCGGCCCCGCCAGCGTCTTGTTCATTGGCGAATTTCGAACACGCAG  
 AGATCTTGATCACCTAGGCCGGGGCGGGTCCGAGAACAGTAACCGCTTAAGCTTGTGCGTC

----->  
 HSV-TK TATA box  
 ATGCAGTCGGGGCGGCGCGGTCCGAGGTCCACTTCGCATATTAAGGTGACGCGTGTGGCCTC  
 TACGTCAGCCCCGCCGCGCCAGGCTCCAGGTGAAGCGTATAATTCCAGTGCGCACACCGGAG

GAACACCGAGCGACCCTGCAGCGACCCGCTTAACAGCGTCAACAGCGT  
 CTTGTGGCTCGCTGGGACGTCGCTGGGCGAATTGTCGCAGTTGTCGCA  
 3'-GTGGCTCGCTGGGACGTCGCT-5'---pFlash 3™ primer

Note : pFlash II™ MCS II region is identical to that of pFlash I™.

7, Bradford Road, Burlington, MA 01803

Tel: (508)448-9561

Fax: (508)448-9853

E-mail: [synapsys@world.std.com](mailto:synapsys@world.std.com)

(+) Indicates that coding strand of CAT gene will be rescued on infection with helper phage



