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碩士論文

合成一個新型 NF-κB 相關啟動子藉此增加在腫瘤細胞中的基因表現

The creation of a novel NF-κB-base promoter to enhance transgene expression in tumor cell

研 究 生：林弘育

指導教授：廖光文　教授

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研 究 生：林弘育　　Student：Hong-Yu Lin

指導教授：廖光文　　Advisor：Kuang-Wen Liao, Ph.D.

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學生：林弘育　　指導教授：廖光文 博士

國立交通大學生物科技學系（研究所）碩士班

摘　要

利用腫瘤專一性起動子可以專一性的在腫瘤細胞中表現轉殖基因。但是因其活性太弱，所以治療效果有限。在此我們建構一個能快速得到具有高表現量或專一性的啟動子庫平台。近年來研究發現，NF-κB與腫瘤細胞的轉移、生長、抗細胞凋亡的機制有關。因此，本實驗以NF-κB為主軸並選用與NF-κB相關能直接或間接加強基因表現的轉錄因子，Sp1、CREB和MEF-2將之隨機黏合配對，做出一個NF-κB相關啟動子庫。再者，低效率的啟動子會妨礙高效率啟動子的篩選，所以我們嘗試利用已知的磁珠分離技術搭配競爭劑以降低表現量的啟動子。實驗證明在Balb/3T3利用這種改良的磁珠分離技術，的確可降低低效率啟動子1.59% 左右，而中效率啟動子的佔有率提高了7.99%。所以此種改良的磁珠分離技術的確可降低質體中，低效率啟動子的比率。

此技術可以在Balb/3T3，A549細胞株上挑選到高表現量，或是具腫瘤專一性的啟動子。雖然目前還有很多序列未定出（長度大於1 kb），但是可以看出在腫瘤細胞中具高表現量的兩個啟動子其中有一個有兩個NF-κB結合部位。另一個則無。這說明了腫瘤雖然有高活性的NF-κB，但是其它的轉錄因子或許也是很重要的。利用此種策略不但可以了解腫瘤細胞的最佳啟動子組成，也可以觀測出哪些轉錄因子在腫瘤細胞是重要的。藉此可以更深入的了解腫瘤細胞，進而找到治療癌症更好的方法。

The creation of a novel NF-κB base promoter to enhance transgene expression in tumor cells

Student：Hong Yu Lin Adviser: Dr. Kuang Wun Liao

College of Biological Science and Technology

National Chiao Tung University

Abstract

Tumor-specific promoters are useful tools to accomplish specific expression in targeted tumor cells. However, a low level of gene expression is the chief defect of tumor-specific promoters. We have set up a platform to quickly gain high active and specific promoter library. Recently, many researches have discovered that NF-κB is associated with cancer, including metastasis, tumor proliferation and anti-apoptosis. Therefore, we created a novel NF-κB based promoter library by randomly assembling Sp1, CRE, and MEF-2 binding sites.

Furthermore, low expressive promoters may interfere with the selection for the promoter with high expression activity. I used the techniques magnetic beads separation combing with the competitor (MBSCS) to solve this problem. In this experiment, promoter library transfected into Balb/3T3 from this modified separation system could decrease the expression of low expressive promoters by 1.59 % overall. Meanwhile, it increased the expression of medium expressive promoters by 7.99% overall. Therefore, the method can reduce the expression ratio of the low expressive promoters in plasmid.

Using this technology, high expressive or tumor-specific promoters were selected from A549 and Balb/3T3 cells. Although their sequences are not fully (over 1kb), there were sequenced still observed some results. There are two high and tumor-specific promoters from separation. One has two binding sites; the other has none. Let us which means other transcription factors may play an important role in cancers except for NF-κB. This strategy not only let us understand the context of high efficient promoter in tumor cells, but also let us knows which factors in the tumor cells are important. This may take advantage of it to more thorough understanding about tumor cells, and find a better treatment for cancer.

誌謝（Acknowledgement)

在這兩年不到的歲月，所做的東西很多還是半成品，但是從一個只知道DNA,RNA和蛋白質是什麼到完成一個實驗學到很多課本沒教的東西。首先，我要感謝指導老師 廖光文教授的叮嚀，老師豐富的人生經歷在在於談話中表達出來。把原本認為實驗的結果認為是重要的觀念扭轉，有科學家的思考與創意，及懂得分析Data才是做實驗的王道。老師從不同的觀點看Data的能力，確實解決一些問題及更進一步地讓我更明白實驗可以朝何方向進行。

第二大恩人是博班的靜宜學姐，由於其前面的建構完善 (B2a 與phOx-BSA-FITC 的建構)，與宛如第二個老師般教導我一些實驗與知識。不虧是實驗室的第二把交椅（第一把交椅是老師)。接下我實驗的pei學妹，與接下耗材管理的厚厚，雖然我沒教什麼 不過憑過人的智慧輕鬆的接下來，著實讓我碩二下輕鬆不少。尤其pei學妹的吃苦耐勞著實是女中點範。上知同學的熱心公益(第三把交椅)，讓我生活無慮。平時的實驗討論也讓我有效的解決問題。田先生的熱情資助與討論，讓我對自己有新的體認。高小姐的在實驗 (pCMV-hrGFP 是的完成)與英文上幫助，不虧是實驗室水平最高的女王。正晟學弟好心提供地方讓我有容身之處,dg兄讓我在煩悶的實驗中有一些娛樂。還有RRT,James,Chenyu,寶尼,堅甫,Gill,Yu Shong等人不論實驗上或是生活上的協助。感覺大家像是我生命中的另一個家人。雖然平時吵吵鬧鬧，我相信大家都是為了別人好，為實驗室而奮鬥!!!

最後要謝謝口試委員 楊昀良與吳彰哲教授在百忙中抽空指導我的論文與修正。讓我明白自己實驗上或Data上不完整之處。最後要謝謝我的哥哥與父母在我讀書的時候提供我物資讓我順利完成學業。在此，希望大家都能順利完成自己的事情，上研究所的上研究所，讀書的順利畢業，工作的人順利升阡與賺大錢。雖然說離開交大心中有些許的悲傷，也有點高興。大家的協助不論是有形或是無形的，我想我只要翻開這本論文一定會浮現出來。結束前套用日劇女王的教室的一段話:**「不知道的東西，不必裝作一副瞭解的樣子接受。比起那個，更應該去想想現在。…可以想像嗎？我們的周圍，有許多美好的事物。全心的去感受，這就是所謂的生存。」**…在學習的路上,我們要學的還很多,不知道的還有很多！！大家一起加油吧！！！~~~~~~共勉之！ 2006/8/14 仲夏夜 筆

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Abbreviations

4-Ethoxymethyene-2-phenyl-2-oxazolin-5-one	phOx
α fetoprotein	AFP
Anemonia sulcata (red fluorescent protein)	AsRed2
activating transcription factor 1	ATF1
basic/leucine zipper	bZIP
Bombesin	BBS
base-pairs	bp
Albumin bovine Fraction V	BSA
cAMP response-element binding protein	CREB
carcinoembryonic antigen	CEA
clear-cell sarcomas of soft tissue	CCSST
coactivator protein CREB binding protein	CBP
cyclic AMP	cAMP
cytomegalovirus	CMV
Double distilled H_2O	ddH_2O
Decoy oligodeoxynucleotide	decoy ODNs
Dimethyl sulfoxide	DMSO
Dulbecco's Modified Eagle's Medium	DMEM
endothelial growth factor	VEGF
Interleukin-8	IL-8
interferon beta	IFN-β
IκB kinase	IKK
endothelial-specific kinase inserts domain receptor	KDR/flk-1
Escherichia coli	*E. coli*
Ewing sarcoma gene	EWSR1
Fas-associated death domain-like interleukin-1β converting enzyme	FLICE
Fas-associated death domain-like interleukin-1β converting enzyme -like inhibitory protein	cFLIP
Fluorescein isothiocyanate	FITC
Heat shock factor 1	HSF-1
humanized Renilla Green Fluorescent Protein	hrGFP
Human *Aspartyl-β-Hydroxylase*, *Junctin*, and *Junctate*	Human Aβ H-J-J locus
human germline Iγ1	human GL Iγ1

human T-cell leukemia virus	HTLV-1
human telomerase reverse transcriptase	hTERT
human Telomerase RNA	hTR
Hypermethylated in cancer 1	*HIC-1*
hypoxia response elements	HREs
hypoxia-inducible factor-1	HIF-1
inhibitor of apoptosis proteins	IAPs
IκB kinase	IKK
lipopolysaccharide	LPS
magnetic beads separation combining with the competitor system	MBSCS
mucin-6	MUC6
Myocyte-specific enhancer binding factor-2	MEF-2
matrix metalloproteinase-1	MMP-1
NMDA receptor 1 subunit	NR1
Nuclear factor-kappaB	NF-κB
phosphate-buffered saline	PBS
Polyethylenimine	PEI
polymerase chain reaction	PCR
Potassium Acetate	KOAc
Propium iodide	PI
Penicillin-Streptomycin Amphotericin B Solution	PSA
prostate-specific antigen	PS antigen
prostate-specific enhancer	PSE
Regulatory Single-nucleotide polymorphism	rSNP
Rel-homology domain	RHD
room temperature	RT
Single-nucleotide polymorphism	SNP
Simian-virus-40-protein 1	Sp1
Sodium dodecylsulfat	SDS
transcription factor binding sites	TFBSs
Two-step transcriptional amplification system	TSTA

Chapter 1 Introduction

Cancer influenced people's health profoundly. In Taiwan, the top ten causes of death at 2004 were [1] cancers；[2] heart diseases；[3] cerebrovascular diseases；[4] diabetes；[5] accidental death；[6] pneumonia；[7] chronic hepatitis and cirrhosis；[8] nephritis, nephritic syndrome and nephropathy；[9] suicide and [10] hypertensive diseases (Department of Health 2005). Currently, cancer is considered as a lethal disease for human. However, efficiently therapeutic methods for malignant tumor now have not discovered until now.

Recently, gene therapy has facilitated tremendous progress in the technology of cancer treatment (McCormick 2001). But the greatest challenge in using it for the cancer treatment is to achieve the highest levels of specificity and efficacy for expression in tumor cells. Tumor-specific promoters are useful tools to accomplish specific expression in targeted tumor cells; however, a low level of gene expression is the chief defect of Tumor-specific promoters (Wu, Johnson et al. 2003). Consequently, many strategies improve transgene expression in tumor cells. In this chapter, I will focus on the transcription in tumor cells, and discuss strategies to amplify the magnitude of specific expression.

1.1. Tumor- specific promoter

Several promoters were reported to specifically regulate certain expression of transgenes in different tumor cells (Robson and Hirst 2003; Wu, Johnson et al. 2003; Papadakis, Nicklin et al. 2004).These promoters will be described as below (Robson and Hirst 2003):

1.1.1. Cancer-specific promoters

Cancer-specific promoters are specific for the malignant process without tissue specificity, such as telomerase related promoters. Usually, telomerases activation is considered as a critical step in cancer progression because of its role in cellular immortalization. Approximately 90% of human cancer cells re-gain activity of telomerases, but them are much lower or undetectable activity in normal somatic tissues (Kim, Piatyszek et al. 1994; Broccoli, Young et al. 1995; Yasui, Tahara et al. 1999). In human, telomerase consists of two fundamental components, an RNA component [human Telomerase RNA (hTR)] and a reverse transcriptase component [human telomerase reverse transcriptase (hTERT)] (Shaywitz and Greenberg 1999). The hTR is not translated and remains as RNA; hTERT add single-stranded telomere repeats into chromosome. At 2001, Keith's group measured hTR and hTERT expressions in a panel of 10 cell lines to demonstrate that the promoters of hTR and hTERT are tumor-specific active in tumor cells but not normal cells (Plumb, Bilsland et al. 2001). These promoters clearly have a real potential in targeting a wide range of different tumor types.

1.1.2. Tumor-type-specific promoters

Tumor-type-specific promoters are oncofetally related with tissue specificity. Certain types of tumor often overexpress genes of oncofetal origin that are silent in normal tissue. The most well-characterized promoters of these tumor-specific genes are the *carcinoembryonic antigen (CEA)* (Cao, Kuriyama et al. 1999; Qiao, Doubrovin et al. 2002) and *α fetoprotein (AFP)*(Sato, Tanaka et al. 1998; Ishikawa, Nakata et al. 1999), which are expressed in adenocarcinomas and hepatocellular carcinomas, respectively. There are also many other genes that are over expressed in certain tumor types and, as such, their promoters are being utilized for transcriptional regulation of therapeutic genes.

1.1.3. Tumor microenvironment-related promoters

Tumor microenvironment-related promoters are responding to the tumor microenvironment and physiology, for example, hypoxia and glucose regulation. Many genes transcriptionally upregulated in response to hypoxia are mediated by the inducible transcription complex, hypoxia-inducible factor-1 (HIF-1). HIF-1 binds to DNA motifs known as hypoxia response elements (HREs) within these genes. Therefore, HREs can be used to drive transgene expression specifically within areas of tumor hypoxia. It is extremely important to target this population of cells since they are highly resistant to other forms of treatment, such as radiotherapy and chemotherapy (Hockel and Vaupel 2001).

In addition to oxygen starvation, tumors can also be deprived of

glucose that leads to the increased expression of genes involved in glucose metabolism. The promoters of these genes are also used to drive transgene expression specifically within a tumor (Little, Ramakrishnan et al. 1994; Gazit, Hung et al. 1999).

1.1.4. Tumor vasculature-related promoters

Tumor vasculature-related promoters are more active in the tumor vasculature than normal one. Because their genes have been reported to be upregulated in proliferating endothelium cells of tumor blood vessels (Walton, Wang et al. 1998). The endothelial-specific kinase inserts domain receptor (KDR/flk-1) and E-selectin promoter have been enhanced transgene expression in tumor endothelium (Jaggar, Chan et al. 1997). More recently, it was demonstrated that the KDR/flk-1 promoter, is not only endothelial cell-specific, but also actives in human ovarian cancer cell lines (Szary, Kalita et al. 2001).

Although the use of tumor specific promoters will likely improve the safety of gene-based therapy, the activities of specific promoters are weaker than the current benchmark CMV promoter (Wu, Johnson et al. 2003). Given that gene delivery to the tumor cells in vivo might be limited, a concern for employing weak specific promoters is that therapeutic efficacy might decline. Therefore, controlling promoter activity is very important.

1.2. Regulation of promoter activity

Modular elements, DNA curvature and flexibility are crucial effects on promoter activity. Modular elements can be bound by transcription factors to start transcribing. DNA curvature and flexibility are important in formation of protein-DNA complexes (Nikolov, Chen et al. 1996). These may lead to greater/ weaker binding activity of transcription factors or RNA polymerase II. Usually, modification of modular elements and change of DNA structure can increase/decrease gene expression. These are discussed as below:

1.2.1. Regulatory Single-nucleotide polymorphism

Single-nucleotide polymorphism, SNP, is a small genetic change or variation. This variation occurs when a single nucleotide, such as an A, replaces one of the other three-nucleotide letters—C, G, or T. If the variation occurs within promoters, it may affect the regulation of gene expression; this phenomenon is called regulatory SNP (rSNP). rSNP lies within known TFBSs and other location. Thus, rSNP can change the conformation of DNA and the sequences of TFBS to affect transcriptional ability (Buckland 2006).

rSNP also causes human cancers. In non-small cell lung cancer, rSNP lies on -245 upstream of the hTERT promoter, so that interferes with Ets2 (one of the transcription factors) binding and telomerase activity (Hsu, Hsu et al. 2006). In addition, it can be found in other cancers, like breast cancer (Tower, Coon et al. 2003). In breast cancer, highly constitutive levels of matrix metalloproteinase-1 (MMP-1) correlate with metastasis,

because MMP-1 can degrade stromal collagens in the extracellular matrix (Brinckerhoff, Rutter et al. 2000; Tower, Coon et al. 2003). Cancer's metastasis associates with MMP-1, but how can cancers cause high levels of MMP-1? The major mechanism is by adding an additional ETS (one of the transcription factors) site in the MMP-1 promoter which is conferred by the rSNP at -1607 bp upstream to induce higher levels of transcription (Tower, Coon et al. 2003) .

1.2.2 Methylation

DNA methylation refers to the covalent addition of a methyl group on nucleotides, and that is an epigenetic event that affects cell function by altering gene expression. When DNA is methylated in the promoter regions of genes where transcription is initiated, genes are inactivated and silenced (Siegfried and Cedar 1997; Baylin 2005; Baylin and Ohm 2006) .

In cancer cells, methylation within promoters serves to turn off critical genes that could otherwise suppress tumorigenesis (Weichselbaum, Hallahan et al. 1991; Jones and Baylin 2002; Baylin 2005; Baylin and Ohm 2006), such as *Hypermethylated in cancer 1(HIC-1)*. *HIC-1* is ubiquitously expressed in normal tissues, and it can suppress cell growth. In different tumor cells, it is usually underexpressed due to hypermethylation. Constitutive expression of *HIC-1* by stable transfection in various cancer cell lines results in a significant decrease in their survival (Wales, Biel et al. 1995; Guerardel, Deltour et al. 2001).

1.2.3 Transcription factor binding sites and transcription factors

Eukaryotic transcriptional regulatory factors are conducted synergistically by multiple transcriptional regulatory factors (Stormo 2000). These factors can bind to the promoter regions called transcription factor binding sites (TFBSs). TFBSs are usually short [around 5-15 base-pairs (bp)] and they are frequently degenerate sequence motifs (Bulyk 2003). The sequence degeneracy of TFBSs has been selected through evolution and is beneficial, because it confers different levels of activity upon different promoters, causing certain genes in specific cells to be transcribed at higher levels than other cells (Bulyk 2003). Although the sequences of TFBSs are degenerated, they still have consensus sequences (usually < 10 bp), such as NF-κB element [consensus sequence 5'-GGGPuNNPyPyCC-'3 (Shankar, Cheng et al. 2005)], which can be recognized by specific transcription factors.

The orientations and functions of TFBSs are not absolutely correlated. In yeast, their positions within a promoter can be varied, and in higher eukaryotes they can be placed upstream, downstream, or in the introns of the genes that they regulate; in addition, they can be placed close to or far away from regulated gene(s) (Bulyk 2003).

One transcription factor can interact with other transcription factors. If this interaction results in high levels of a transcriptional activation, it is known as **"synergism or synergistic effect"**. The phenomenon usually forms a ternary protein-protein-DNA complex (Moreno, Emery et al. 1995; Brass, Kehrli et al. 1996; Butscher, Powers et al. 1998), which leads to altered DNA conformation and allowed other factors to bind on DNA. Interactions between two factors may be direct (Brass, Kehrli et al. 1996;

Shankar, Cheng et al. 2005) or mediated by coactivators. For instance, the coordination of c-Rel and ATF-1/CREB2 is mediated by p300/CREB-BP (Butscher, Powers et al. 1998).

In some cases, two factors binding to DNA independently, can still activate transcription synergistically (Zaiman and Lenz 1996; Ohmori, Schreiber et al. 1997; Cantwell, Sterneck et al. 1998). A number of factors are known to bend DNA and thus permit binding of other factors (Kerppola and Curran 1991; Stros, Stokrova et al. 1994). For example, Fos and Jun can induce a corresponding alteration in the conformation of the DNA helix (Kerppola and Curran 1991).

A variety of elements can contribute to promoter activity, but none is essential for all promoters (Lewin 2004). All promoters probably require one or more elements in order to function efficiently. Some transcription factors have tissue-specificity, and they can affect cells development (Fukamachi 2006). In the tumor development, the transcription factors play a major role. For example, NFκB promotes cell cycle progression, regulates apoptosis, and facilitates cell adhesion (Ravi and Bedi 2004). The tumor development co-relates to above actions, so that tumor can escape from immunity, and then survive and metastasis.

Recently, many strategies have been used to enhance the potency of promoters needed to retain the tumor specificity in order to maintain potential therapeutic benefits. A principle is that the transcription factor can recognize DNA sequence specifically. In the next section I will discuss how to increase the potency of tumor-specific promoters.

1.3. Augmented transgenes expression of tumor-specific promoters

Many strategies were designed to augment the expression of tumor-specific promoters because tumor-specific promoters are usually underexpressed. These strategies will be described below.

1.3.1 To insert or remove transcription factor binding sites of tumor-specific promoters

To insert or remove TFBSs of tumor-specific promoters can promote synergistic interactions of transcription factors to enhance transcription. Many investigations have showed the increase of gene performance. In clinical, prostate-specific (PS) antigen is used as a biomarker for benign prostatic hyperplasia and prostatic carcinoma (Catalona, Smith et al. 1991). PS antigen promoter and enhancer (PSE) can be augmented by intrinsic androgen receptor elements (Schuur, Henderson et al. 1996; Pang, Dannull et al. 1997). Chimeric PSE activity improved nearly 20-fold over the parental PSE in prostate cancer by insertion of four tandem copies of the synthetic androgen-responsive element, or by duplication of a 400-base pairs enhancer core element (Wu, Matherly et al. 2001). Similar approaches have been successful in improving the activity of the CEA promoter (Richards, Austin et al. 1995) and the tyrosinase promoter (Siders, Halloran et al. 1996).

1.3.2 Two-step transcriptional amplification system (TSTA)

The other strategy that has frequently been employed to amplify weak promoter activity in a two-tiered manner is called two-step transcriptional amplification (TSTA) system. TSTA system needs two differential promoters to drive differential genes. One specific promoter drives expression of a synthetic transcription factor, like GAL4-VP16, which in turn binds to the second promoter, such as GAL4 responsive promoter, and activates the reporter or therapeutic gene. This system has been employed to improve PSA promoter efficiency of transgene expression for cancer imaging (Wu, Matherly et al. 2001; Zhang, Adams et al. 2002). In addition to PSA promoter, the system can boost expression from a CEA promoter (Koch, Guo et al. 2001; Qiao, Doubrovin et al. 2002).

1.3.3 Cre/loxP site-specific recombinase system

In another approach, a tumor-specific promoter controls the expression of Cre site-specific recombinase (Richards, Austin et al. 1995), then activates the reporter gene expression. A transgene is linked to a strong constitutive promoter that is interrupted by expression termination sequences flanked by two loxP sites, the cognate site of Cre. Cre-dependent activation of transgene expression can enhance many tumor-specific promoters efficacy, such as improving CEA-promoter efficacy in gastric carcinoma (Bertran, Miller et al. 1996), and enhancing AFP-promoter-based therapy for liver tumor (Sakai, Kaneko et al. 2001).

1.3.4 The transcriptional feedback loop system

The construction of transcriptional feedback loop system enhances trangene expression in tumor cells is also the effective strategy. The strategy needed to set up the vector that has two parts. One is an upstream tumor-specific promoter incorporated with other regulatory elements; another is a downstream gene including the report gene and regulatory factors. When tumor-specific promoter expresses a report gene and regulatory factors concurrently, regulatory factors can bind to regulatory elements and improve the tumor-specific promoter efficacy.

Someone used transcriptional feedback loop system to improve tyrosinase promoter efficacy in melanomas. Heat shock factor 1 (HSF-1) binds to a heat shock-binding element nearby upstream of the tyrosinase promoter. This enhances promoter activation and completes the transcriptional feedback loop. This strategy has achieved long-term eradication of melanomas in mice (Emiliusen, Gough et al. 2001).

In addition to insert or remove TFBSs, these systems usually need two independent vectors. However, the activation of this system requires co-delivery of two vectors into the same cell, which could be inefficient in vivo (Sakai, Kaneko et al. 2001). To insert or remove TFBSs may enhance tumor-specific promoters, but the efficacy is limited, because this merely modifies origin promoter sequence. Therefore, I create an artificial promoter instead of a nature one to avoid limitary efficacy of origin promoter sequence.

1.4 Experimental strategy and purpose

I tried to create a new tumor specific promoter with the concept of DNA shuffling technology. DNA shuffling was first established by *Stemmer et al.* at 1994 (Wright, Semyonov et al. 2005). DNA shuffling is a method to obtain mutant gene pools via in vitro homologous recombination by random fragmentation and polymerase chain reaction (PCR) reassembly. If the new promoter sequences were created by the above method it is called **"promoter shuffling"**. The process can produced a promoter library for picking the most efficient promoter.

Hence, the preparations of four different TFBSs (NF-κB, CRE, Sp-1, and MEF-2) rearrange DNA fragments and create new promoters. Then the modified magnetic beads separation system isolate lower gene expression and medium / high expression of promoters. The highly expressed tumor promoter can be used to enhance the potency of tumor therapy. The tumor specific promoters manufacture procedures made and obtained procedures are shown in **Figure 1-2**.The experiment includes three systems: promoter shuffling system, report gene system and magnetic beads separation combining with thecompetitor system (MBSCS). These systems will be illustrated the next section **(Figure 3, 4 and 5)**.

1.4.1 **Using promoter shuffling system to construct NF-κB based promoter library**

Recently, many cases enhance promoter efficacy by promoter

shuffling. For example, cytomegalovirus (CMV) promoter could use this strategy to enhance trangene expression. CMV promoter is one of the most potent promoters and used in many experiments. By the promoter shuffling, CMV promoter can increase approximately 2-fold level luciferase reporter gene expression and anti-β-galactoside antibody response *in vivo* when compared with wild-type promoters (Wright, Semyonov et al. 2005).

In this experiment, a novel promoter shuffling system was used to crate a new promoter that is ever not exit in nature. This novel system applies random assembly of known and natural TFBSs to form artificial promoter library. *Li et al.* apply this novel promoter shuffling system to create muscle specific promoters that are twofold to six-fold higher expression than CMV promoter in vitro. Analysis of direct intramuscular injection of DNA plasmids in normal muscle after 2~4 weeks revealed a 6~8-fold increase over the CMV promoter (Li, Eastman et al. 1999).

In due to design specific tumor promoters, I researched into the relativity of transcription factors and tumors. In my research, NF-κB is an important role in the tumor development (discuss in section 1.5), but NF-κB alone maybe isn't powerful efficiency on eukaryotic gene expression. So that, the creation of NF-κB based promoter combine with other interactive factors to enhance transgene expression in tumor cells. Perhaps, there is the strongest tumor-specific promoter in promoter library, and it can used on detect efficiently tumors in vivo and cure cancer completely!

1.4.2 **Construction of anti-phox-B7e transmembrane domain takes as**

our reporter gene system

The anti-phOx single chain antibody fused with B7e transmembrane domain as my reporter gene (anti-phOx-eB7), which is constructed and published in *cancer gene therapy* by Dr. Liao (Schaefer, Brachwitz et al. 2004). The 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (phOx) is a hapten, it can recognize for the specific antibody (anti-phOx) (Nakela, Kaartinen et al. 1978; Clackson, Hoogenboom et al. 1991; Rode, Little et al. 1996; Gruel, Fridman et al. 2001; Shankar, Cheng et al. 2005). Therefore, it can apply to stain cells that are expressing anti-phOx-eB7. The phOx is modified to other materials due to it has attacked by amine group of substrates (involving lone pair electrons). The property can create many derivatives, for instance, BSA-phOx-FITC, Bead-phOx, and glycine-phOx (Nakela, Kaartinen et al. 1978) **(Figure 6)**. These derivatives are applied in my experiment and used to screen high or low expression of promoters.

Other reporter genes, such as green fluorescent protein, ß-galactosidase or luciferase, can detect gene expression levels. But the collection of high expression promoters must need cell sorter to isolate and collect, because these report genes aren't transmembrane proteins. The anti-phOx-eB7 can isolate different expression levels of cells by magnetic beads separation. The reason in the next section will explain why does use magnetic beads separation to isolate different expression cells.

1.4.3 Selecting library by magnetic beads separation combining with the competitor system (MBSCS)

The traditional magnetic bead separation system can isolate expression and non-expression surface antigens of cells by the strong magnetic filed. But it does not isolate high-expression and low-expression surface antigens of cell efficiently. Therefore, add the competitors to remove low-expression anti-phOx-eB7 proteins of cells, leading to select high-efficient promoters easily. In this system, the competitor is glycine-phOx, because glycine-phOx is more stable than phOx and glycine does not affect binding activity of phOx (glycine smaller than phOx).

Why not I use other separated technology, such as cell sorter? Because cell sorter distinct sensitivity is only 1%, if target cells in overall cells are lower than 1%, cell sorter doesn't isolate and collect them. These specific or high expression tumor promoters are created by above technologies and they can help us to investigate mechanism of carcinogenesis and cure for cancers.

The next section will discuss that NF-κB relate with cancers and NF-κB interact to other factors.

1.5 Nuclear factor-kappaB (NF-κB)

Nuclear factor-kappaB (NF-κB) is a common transcriptional factor. So that, it can regulates many gene expressions. Many diseases relates to NF-κB, such as cardiovascular diseases (Schaefer, Brachwitz et al. 2004), muscular dystrophy (Baghdiguian, Martin et al. 1999), inflammatory diseases (Dijkstra, Moshage et al. 2002), and cancers (Gilmore, Gapuzan et al. 2002) etc. In this section will discuss the relationship of NF-κB and

cancers.

1.5.1 NF-κB

NF-κB was first found in B-lymphocytes (Sen and Baltimore 1986). But NF-κB didn't restrict to B-lymphocytes, lipopolysaccharide (LPS) or phorbol ester could stimulate NF-κB was observed in a T cell line (Jurkat) and a nonlymphoid cell line (HeLa) (Sen and Baltimore 1986). NF-κB belongs to the Rel family transcriptional factors, including Rel-A (also known as p65), Rel-B, c-rel, p50/p105 and p52/p100 (Zhang, Adams et al. 2002). The mature DNA-binding forms of p105 and p100 are shortened forms called p50 and p52, respectively. Unlike most transcriptional factors, proteins of this family reside in the cytoplasm and must translocate into the nucleus to work (Legrand-Poels, Schoonbroodt et al. 1998).

All NF-κB proteins contain a highly conserved Rel-homology domain (RHD) that is responsible for DNA binding, dimerization, nuclear translocation and interaction with the IκB proteins. The IκB proteins, including IκBα, β and ε, bind to NF-κB via ankyrin repeats and block its nuclear import and, thereby, its transcriptional activity (Zhang, Adams et al. 2002).

Generally, NF-κB dimerization is the classical p50-p65 heterodimer which binds on the 5'-GGGANNYYCCC-3' consensus sequence (Shankar, Cheng et al. 2005) to regulate gene expression. NF-κB can regulate many gene expressions, such as cytokines/chemokines, cell adhesion molecules, acute phase proteins, and cell-surface receptors, regulators of apoptosis and transcription factors.

Recently, many cases found aberrant genes NF-κB and activity in cancers. The next section will discuss the relationship of NF-κB and cancers.

1.5.2 NF-κB and cancer

The NF-κB family might behave as tumorigenic transcription factors was first put forward upon the cloning of the p50/p105 subunit (Ghosh, Gifford et al. 1990; Kieran, Blank et al. 1990) and analyzed its sequence. Sequence analysis revealed remarkable homology for over 300 amino acids at the amino-terminal end to the oncogene, v-rel. The v-rel is a potent transforming oncogene from the avian reticuloendotheliosis virus (Gilmore 1999).

In many cancers, aberrant activation and nuclear localization of NF-κB is actually quite frequent but most often results from defects in the pathways regulating NF-κB (Mayo and Baldwin 2000; Hammer, Mijakovic et al. 2006). IκB kinase (IKK) can inhibit IκB resulting in enhancing NF-κB activation (Mayo and Baldwin 2000; Hammer, Mijakovic et al. 2006). Some oncogenesis are correlated with the levels of IkBα and IkBβ proteins and coincided with the activation of IKK that govern the destruction of IkB factors (Rayet and Gelinas 1999). Other ways, the loss of negative feedback mechanisms, which inhibit the NF-κB response, can result in its aberrant activity. An example of these is the cylindromatosis (CYLD) tumor suppressor gene, which is associated with familial cylindromatosis (tumors of skin appendages). Losses of CYLD can lead to NF-κB activation (Wilkinson 2003) and oncogenesis. In addition, the

microenvironment of a solid tumor frequently contains high levels of inflammatory cytokines and/or hypoxic conditions, which both stimulate nuclear translocation of NF-κB (Mayo and Baldwin 2000; Hammer, Mijakovic et al. 2006).

The constitutive activation of NF-κB also appears to have a role in cell proliferation. NF-κB prevent Hodgkin's lymphoma cells from undergoing apoptosis under stress conditions (Bargou, Emmerich et al. 1997). It was further shown that growth factors such as epithelial growth factor (Siegfried and Cedar) and platelet-derived growth factor induce proliferation of tumor cells through activation of NF-κB (Romashkova and Makarov 1999). NF-κB signaling was also shown to promote pheochromocytoma 12 cells survivals by nerve growth factor ligand (Foehr, Lin et al. 2000).

Recently, NF-κB has the pro-survival and anti-apoptotic functions (Shishodia and Aggarwal 2002). Several gene products that negatively regulate apoptosis in tumor cells, including inhibitor of apoptosis proteins (IAPs) 1 and 2, X-linked IAP, cellular Fas-associated death domain-like interleukin-1β converting enzyme-like inhibitory protein (cFLIP), were shown to be controlled by NF-κB activation (Shishodia and Aggarwal 2002).

The production of angiogeneic factors, such as endothelial growth factor (VEGF) and Interleukin-8 (IL-8) has been shown to be regulating by NF-κB activation. NF-κB expression was associated with VEGF expression and microvessel density in human colorectal cancer (Chilov, Kukk et al. 1997). IL-8 also activate by NF-κB. Bombesin (BBS)-like peptide treated

PC-3 cell stimulated an NF-κB dependent migration of human umbilical vascular endothelial cells in vitro by activating VEGF and IL-8(Levine, Lucci et al. 2003). These findings suggest that increased expression of NF-κB contributes to tumor angiogenesis in cancer **(Table 1)**.

1.5.3 Cancer therapy by NF-κB

NF-κB is an important role in cancer development, which involves promotion, angiogenesis, and metastasis. Therefore, several strategies have been used to block the activation of NF-κB, and a wide range of compounds, such as IKK inhibitors, inhibitory peptides and decoy oligodeoxynucleotide, have been found to block various steps leading to NF-κB activation.

(1) Inhibitors of IKK Block NF-κB Activation

IκBα phosphorylation is a critical step in NF-κB activation by IKK, some compounds that block this phosphorylation to prevent IκBα's degradation. Like 4-hydroxy-2-nonenal, a lipid peroxidation product, inhibits activation of NF-κB in the human colorectal carcinoma cell line (RKO) and human lung carcinoma cell line (H1299) by directly blocking IKK (Ji, Kozak et al. 2001). The other compound, SC-514, is an IKK-2 inhibitor. IKK is a multisubunit complex that contains two catalytic subunits, IKK-1 and IKK-2, and the regulatory subunit IKKγ (Hammer, Mijakovic et al. 2006).Therefore, when SC-514 inhibits IKK-2，leading to

decreased level of IκBα phosphorylation/degradation and diminished p65 translocation into the nucleus in these cells at maximal kinase inhibition (Kishore, Sommers et al. 2003).

(1) Inhibitory peptides

The NF-κB related peptides are used to cross the cell membrane and block the nuclear localization of the NF-κB complex. For example, NBD peptide that selectively blocks the association of IKKγ with the rest of the IKK complex had been shown to inhibit NF-κB activation (May, D'Acquisto et al. 2000). In addition, SN-50 and o, o'-bismyristoyl thiamine disulfide can block NF-κB complex from the cytoplasm to the nucleus in the normal import machinery (Pieper and Riaz ul 1997).

(2) Decoy oligodeoxynucleotide (decoy ODNs)

The synthetic dsDNA with TFBSs can bind the transcription factors and block activation of genes, resulting in an effective therapy for treating diseases. The synthetic dsDNA are called "decoy oligodeoxynucleotide" (decoy ODNs) (Morishita, Higaki et al. 1998). Recently, the decoy ODNs targeted NF-κB to decrease gene expression and treat many diseases, such as cancer cachexia.

Cancer cachexia characterize by anemia, weight loss and progressive tissue wasting. It is a common complication of malignancy and is found in more than 60% of patients with malignant disease (Dewys, Begg et al. 1980). NF-κB can cause muscle degeneration in the tumor animal model.

NF-κB decoy ODNs was used to cure successfully colon26-induced cachexia in mice (Kawamura, Morishita et al. 1999).

Because NF-κB in tumor cells has aberrant activity, maybe the creation of NF-κB based promoter is efficient expression in tumor cells. However, NF-κB alone maybe is weaker efficiency of gene expression. Many transcription factors can coordinate with NF-κB to enhance gene expression. So that, I used other transcription factors coordinated with NF-κB and enhanced transgene expression. The next section will focus on Sp1, CREB, and MEF-2 and discuss the relationship of them, NF-κB, and cancers.

1.6 Simian-virus-40-protein 1 (Sp1)

Simian-virus-40-protein-1 (Sp1) was the first transcription factor identified and cloned, and shown to be a sequence-specific DNA-binding protein that activated a broad and diverse spectrum of mammalian and viral genes (Dynan and Tjian 1983; Dynan and Tjian 1985; Briggs, Kadonaga et al. 1986) .Sp1 can regulate cell proliferation and cell survival gene in tumor cell (Safe and Abdelrahim 2005). In addition to transcription factor, Sp1 is also a major role in cancer. Interestingly, some promoters can enhance gene expression by interaction between NF-κB and Sp1. The next section will focus on relationship of Sp1 and NF-κB or cancers respectively.

1.6.1 Sp1

Sp1 was the first mammalian transcription factor to be cloned and identified (Dynan and Tjian 1983). It binds to GC-rich sequences (Gidoni, Kadonaga et al. 1985; Imataka, Sogawa et al. 1992), including GC-boxes and CACCC boxes (also called GT box). Early studies indicated that Sp1 was responsible for recruiting TATA-binding protein (Pugh and Tjian 1991; Zenzie-Gregory, Khachi et al. 1993) and Fixing the transcriptional start site (Jolliff, Li et al. 1991; Schaefer, Brachwitz et al. 2004) at TATAA-less promoters. Sp1-sites are also found in the promoters of many housekeeping genes (Dynan and Tjian 1985). According to the above, Sp1 acts as a basal transcription factor and that Sp1 sites represent constitutive promoter elements that support basal transcription at these promoters. Sp1 can interact with NF-κB to improve gene expression, and affect tumor cells development.

1.6.2 Sp1 and NF-κB

Some activity of promoters is correlation between Sp1 and NF-κB. For example, mucin-6 (MUC6) promoter is upregulated by Sp1 and NF-κB (Sakai, Jinawath et al. 2005). Mucins are large extracellular proteins as molecular barrier at the epithelial surface and engage in morphogenetic signal transduction. Alterations in mucin expression or glycosylation accompany the development of cancer and influence cellular growth, differentiation, transformation, adhesion, invasion and immune surveillance (Hollingsworth and Swanson 2004). *Sakai H. et al.* analyzed MUC6 promoter, which have a putative NF-κB consensus sequence at -173 to

-164bp, and putative Sp family binding sites at -530 to -521 and -847 to -838bp (Sakai, Jinawath et al. 2005). They deleted two Sp-1 binding sites, or one NF-κB binding site and two Sp-1 binding sites, gene expression decrease 20% or 6.60% than wild-type MUC6 promoter respectively. The other case, the NMDA receptor 1 subunit (NR1) promoter (related to neuronal differentiation and plasticity) has NF-κB site can interacts with Sp1 to enhance gene expression (Magari, Rivera et al. 1997) **(Table 1)**.

1.6.3 Sp1 and cancer

Sp1 can regulate some tumor –related factors in tumor development. The major tumor –related factors are in angiogenesis and cell growth and cycle progression way. The explanation was in the following article.

(1) Angiogenesis：

Tumor growth and spread need the newborn blood vessel to provide the extra nutrient and supply the new cell growth. Tumors will grow to about 1mm diameter in the absence of new capillaries but further expansion requires the production of angiogenic growth factor by the cancer cells (Triezenberg, Kingsbury et al. 1988) . Initiation of angiogenesis is due to increased tumor secretion of mitogenic growth factors such as fibroblast growth factor (FGF) and VEGF (Datta, Rubin et al. 1992; Finkenzeller, Sparacio et al. 1997). Sp1 mediated angiogenesis through induction of VEGF activity. Analysis of the VEGF promoter demonstrated that four proximal Sp1 binding sites between -109 to -61 contributed to constitutive gene expression (Datta, Rubin et al. 1992).

(2) Cell growth and cycle progression：

Sp1 also plays a role in regulating several genes in tumor cells associated with cell growth and cycle progression. Such as cyclin D1 (Castro-Rivera, Samudio et al. 2001) and E2F1 (Walton, Wang et al. 1998), Sp1 can enhance their expression to stimulate proliferation and DNA synthesis in breast cancer cells.

1.7 cAMP response-element binding protein (CREB)

cAMP response-element binding protein (CREB) has been found to mediate transcriptional responses to a variety of growth factor and stress signals. CREB regulate many gene expressions. Genome-wide studies put the number of putative CREB target genes at about 5000, or nearly one-quarter of the human genome. When CREB or related factors whose aberrant expression is often associated with certain cancers (Conkright and Montminy 2005). In this section will discuss the relationship between CREB and cancer, and coordination between NF-κB and CREB.

1.7.1 CREB

CREB is a member of the CREB/ATF-1 (activating transcription factor 1)/CREM (CRE modulator) transcription factor family that mediates cyclic AMP (cAMP), growth factor-dependent, and calcium-dependent

gene expression through the cAMP response element (Shankar, Cheng et al. 2005). CREB is a 43-kDa basic/leucine zipper (bZIP) transcription factor that is expressed at the RNA level in most tissues. CREB binds to the consensus octanucleotide CRE element (5'-TGANNTCA-3') as a homodimer and heterodimers in conjunction with other members of the CREB/ATF superfamily of transcription factors (Shaywitz and Greenberg 1999). In resting cells, CREB exists in an unphosphorylated state that is transcriptionally inactive but can bind to DNA. Upon cell activation, CREB becomes phosphorylated, which induces its transcriptional activity by promoting its interaction with the 256-kDa-coactivator protein CREB binding protein (CBP). CBP serves as a molecular bridge that allows CREB to recruit and stabilize the RNA polymerase II complex at the TATA box, leading to switch certain genes on or off.

1.7.2 CREB and NF-κB

The activity of many inducible transcription factors, such as NF-κB, is regulated through their association with cellular coactivators (Shenkar, Yum et al. 2001). Interaction with the coactivator, CBP, appears to be necessary to optimize the transcriptional activity of NF-κB. The interaction of the p65 subunit of NF-κB with CBP involves the KIX region of CBP, which is the same region responsible for binding the transcriptionally active serine-133-phosphorylated form of CREB (Sheppard, Phelps et al. 1998; Zhong, Voll et al. 1998) In human germline Iγ1 (GL Iγ1) promoter, NF-κB interacts with CREB to enhance gene expression. The Human GL Iγ1 promoter has NF-κB binding sites and CREB sites, two factors were

proved that they seemed to interact with each other directly or indirectly by electrophoretic mobility shift assays (EMSA) (Dryer and Covey 2005).

1.7.3 CREB and cancer

A potential role for the CREB family in cellular transformation was first appreciated in clear-cell sarcomas of soft tissues (CCSST) (Zucman, Delattre et al. 1993). CCSST is an unusual malignancy of adolescents and young adults that typically arises in the deep soft tissues of the lower extremities close to tendon, fascia, and aponeuroses (Segal, Pavlidis et al. 2003). CCSST is typified by a chromosomal t(12;22)(q13;q12) translocation resulting in a fusion between the Ewing sarcoma gene (EWSR1) and activating transcription factor 1 (ATF1) (Schaefer, Brachwitz et al. 2004). The EWS–ATF1 can enhance expression of numerous CREB target genes by functioning as a strong activator. Indeed, disrupting EWS–ATF1 activity appears sufficient to block cell proliferation and promote cell apoptosis (Olsen and Hinrichs 2001; Schaefer, Brachwitz et al. 2004).

Viral onco-proteins, such as hepatitis B virus and human T-cell leukemia virus (HTLV-1) tax also influence CREB activity in their efforts to promote cellular transformation (Williams and Andrisani 1995; Yin, Paulssen et al. 1995). Based on this evidence, CREB will appear to cooperate with other factors, either in the context of a fusion protein or as part of a complex with an oncoprotein, to induce transformation. But whether CREB alone is capable of promoting tumorigenesis remained unclear (Conkright and Montminy 2005).

1.8 Myocyte-specific enhancer binding factor-2 (MEF-2)

Myocyte-specific enhancer binding factor-2 (MEF-2) was first described as a muscle-enriched transcription factor that bound to an A/T-rich DNA sequence in the control regions of numerous muscle specific genes (Black and Olson 1998). However, it soon became apparent that MEF2, while highly expressed in muscle cells, was also expressed at high levels in neurons and at lower levels in a wide range of cell types.

1.8.1 MEF-2

There are four vertebrate *MEF2* genes, *MEF2A*, *-B*, *-C* and *-D*, which are expressed in distinct, but overlapping, patterns during embryogenesis, and in adult tissues. MEF2 proteins have a MADS domain at their N-termini (The conserved domain was recognized after the first four members of the family, which were MCM1, AGAMOUS, DEFICIENS and serum response factor. The name MADS was constructed form the "initials" of these four "founders".). This domain mediates dimerization and binding to the DNA sequence 5'-CTA$(A/T)_4$TAG/A-3'. An adjacent MEF-2-specific domain influences DNA-binding affinity and cofactor interactions, and the C-terminal regions of MEF-2 proteins are required for transcriptional activation. Similar to other MADS domain proteins, MEF-2

factors associate with a variety of transcriptional cofactors to control specific sets of downstream target genes. MEF-2 proteins are especially sensitive to calcium signals, which act through multiple posttranslational mechanisms to modulate the transcriptional activity of these proteins (McKinsey, Zhang et al. 2002).

1.8.2 MEF-2 and NF-κB

Human Aβ H-J-J locus (encoding *Aspartyl-β-Hydroxylase*, *Junctin*, and *Junctate*) has MEF-2 binding site and NF-κB binding site(Feriotto, Finotti et al. 2005). Mutation of NF-κB binding site or MEF-2 binding site decreases gene expression **(Table 1)**. MEF-2 also interact Sp1 (Grayson, Bassel-Duby et al. 1998). In myoglobin promoter and muscle creatine kinase gene enhancers could find Sp1 and MEF-2 in DNA binding complexes by immunological detection in vitro.

1.8.3 MEF-2 and cancer

MEF-2 may be used as a sensitive marker of for the diagnosis of malignant or neoplastic counterparts (Mechtersheimer, Staudter et al. 1991; Kodama, Hirotani et al. 2002; Yamazaki 2004). MEF-2 is important in the regulation of cellular differentiation of sarcomatous cells. In the present study, the pulmonary related tumor cells expressed MEF-2 and other functionally significant molecules for their differentiation.

1.9 Summary

The creation of NF-κB binding site promoters maybe enhances transgene expression in tumor cell. But these promoters are weaker than other tumor specific promoters possibly. Sp1, MEF-2 and CREB interacts NF-κB to improve transgene expression. In addition, they relate to cancers. Therefore, the above four TFBSs assemble randomly to create NF-κB based promoter library to specific and efficient expression in tumor cells. These promoters have ubiquitous, tissue specific and tumor specific promoters. These promoters' sequences maybe have specific combinations, we can use them to study transcriptional mechanism in tumor cells and design novel drugs. They can apply to detect tumor cells sites in vivo and improve therapy for cancer. Maybe these methods also apply in other diseases to design related promoters.

Chapter 2 Materials and Methods

2.1 Materials

2.1.1 Primers

Name	Ordination	Sequence (5'→3')
NF-κB site	3'	ACCCCTGAAAGGCGAGATCT
	5'	TGGGGACTTTCCGCTCTAGA
Sp-1 site	3'	CACCCCCGCCCCGC
	5'	GTGGGGGCGGGGCG
CRE site	3'	TCGGACTGCAGTCTC
	5'	AGCCTGACGTCAGAG
MEF-2 site	3'	GCGAGATTTTTATTGAGGG
	5'	CGCTCTAAAAATAACTCCC
Adaptor (HindIII cutting site)	3'	GGCTTTCGAATATC
	5'	CCGAAAGCTTATAG

2.1.2 Cell lines

B16F10 (mouse melanoma, ATCCR Number: CRL-6475)

293 (human kidney cell, ATCCR Number: CRL-1573)

A549 (human lung carcinoma cell, ATCCR Number: CCL-185)

Balb/3T3 (mouse embryo fibroblast, ATCCR Number: CCL-163)

DBTRG-05MG (human glioblastoma, ATCCR Number: CRL-2020)

HepG2 (human hepatocellular carcinoma, ATCCR Number: HB-8065)

2.1.3 Bacterial strains

Escherichia coli DH5α: (Invitrogen, USA)

2.1.4 Plasmids

Plasmid	Description	Source
pNF-κB-hrGFP	NF-κB binding site	Stratagege
pCRE-hrGFP	CRE binding site	Stratagege
pAp-1-hrGFP	Ap-1 binding site	Stratagege
pNFAT-hrGFP	NFAT binding site	Stratagege
pARE-hrGFP	AmpR binding sites	From Dr Liao Lab
pAsRed2-N1	Have red fluorescent protein	B.D
pCMV-hrGFP	Have green fluorescent protein	From Dr Liao Lab
B2a	CMV promoter combine with anti-phOx protein	From Dr Liao
B16.4	pOSI-T combine with anti-phOx protein, but no eukaryotic promoter	From Dr Liao Lab

2.1.5 Chemicals, Enzymes, and reagents

Chemical	**Source**	**Catalog number**	**Application**
100bp DNA ladder	Protech	M1-100T	DNA electrophoresis

1kb DNA ladder	Protech	M1-1KB	DNA electrophoresis
4-Ethoxymethyene-2-phenyl-2-oxazolin-5-one (phOx)	SIGMA	E-0753	Detect gene expression and competitor material
Acetic acid	SHOWA	0101-3160	buffer
Agar	AMRESCO	J637	Bacterial culture
Agarose	MDBio	929049	DNA electrophoresis
Albumin bovine Fraction V (BSA)	MP	160069	Detect gene expression
Ampicillin	AMRESCO	0339	Bacterial culture
ATP	EPICENTRE	L0805	DNA ligation
$CaCl_2$	J.T.Baker	1332-01	Preparation of competent cell
Dulbecco's Modified Eagle's Medium	SIGMA	D5648-10L	Cell culture
Dimethyl sulfoxide (DMSO)	MP	196055	Frozen cell reagent
EDC	Fluka	WA10358	Buffer
EDTA	Tedia	ER-0531	Cell passage
Ethanol	SIGMA	E7023	DNA extraction
Fetal Bovine Serum	Biological industries	04-001-1A	Cell culture

Fluorescein isothiocyanate (FITC)	SIGMA	043K5322	Detect gene expression
Glycine	AMRESCO	0167-1kg	Competitor material
Glycerol	SHOWA	0706-0150	buffer
HindIII (Restriction enzyme)	MBI Fermentas	ER0501	Cloning
HCl	scharlau	AC0741	Buffer
Isopropanol	C-Echo	PH-3101	DNA extraction
Kanamycin	MDBio	226039	Antibiotics
KCl	SHOWA	1630-5160	Buffer
NaCl	AMRESCO	0241-1KG	Buffer
$NaHCO_3$	SHOWA	1925-4250	Buffer
Na_2HPO_4	scharlau	SO 0337	Buffer
NaH_2PO_4	SHOWA	1932-8250	Buffer
NaN_3	SHOWA	1924-6150	Buffer
NaOH	SHOWA	1943-0150	Buffer
$MgCl_2$	SHOWA	1301-7260	Buffer
Potassium Acetate (KOAc)	SHOWA	SN-2430E	Buffer
Propium iodide (PI)	SIGMA	P4170	Cell live/dead staining
Polyethylenimine (PEI)	Aldrich	40,872-7	transfection

Penicillin-Streptomycin Amphotericin B Solution (PSA)	Biological industries	03-033-1B	Cell culture medium
Restriction enzyme buffer R	MBI Fermentas	ER0501	Cloning
Sephadex G-25 beads	Amersham	17-0033-02	Purification of protein
Sodium dodecylsulfat	Fluka	632862	Buffer
T4 Polynucleotide Kinase	NEB	M0201S	Cloning
T4 Kinase Buffer	MBI Fermentas	M0201S	Cloning
T4 ligase (2U)	EPICENTRE	L0805H	Cloning
T4 ligase (10U)	EPICENTRE	L0805H	Cloning
T4 ligase buffer	EPICENTRE	L0805H	Cloning
Tanbead USPIO-101 (magnetic bead, with amine group)	TAN Bead	USNNI931231A	magnetic bead separation system
Tris base	MD bio	101-77-86-1	Buffer
Tri-HCl	MP	816124	Buffer
Trypsin	GIBCO	27250-018	Cell passage
Tryptone	Pronadisa	1612.00	Bacterial culture
Trypan blue stain	GIBCO	0759	Cell staining
Yeast extract	Pronadisa	1702.00	Bacterial culture

2.1.6 Kits

Kit	Source	Catalog number	Application
Gene-Spin™ 1-4-3 DNA extraction kit	Protech	PT-DNA14 3XL-V2	DNA extraction, clean-up
NucleoBond PC100	Macherey-Nagel	740573	DNA extraction, Midi preparation
Coomassie Plus™ Kit	PIERCE	23236	Detect protein concentration

2.1.8 Buffers and chemical reagents

2.1.8.1 For cell culture

- **1X PBS (pH7.4)**
  137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4 i
- **EDTA-trysin**
  2.5g trypsin, 0.1 M EDTA (pH8.0) in 1L 1X PBS, pH=7.4, 0.2 μm filtered
- **Versene**
- 0.2 g EDTA in 1L 1x PBS

2.1.8.2 For molecular biology

- **50X TAE buffer**

  48.4 g Tris base, 0.5 M EDTA (pH8.0) 20 ml, 11.42 ml acetic acid added double distilled water (ddH_2O) to 200 ml

- **Solution I (pH8.0)**

  50 mM Tris-HCl (pH=8.0); 10 mM EDTA (pH= 8.0); RNase A (stock: 100 μg/ml, work: 10mg/ml)

  Per 100ml: add 5ml 1M Tris-HCl and 10ml 0.1M EDTA in ddH_2O, adjust total volume to 100ml, autoclave, and add 1ml RNase A. Solution I store at 4°C.

- **Solution II**

  0.2 M NaOH, 1% (w/v) SDS

  Per 100 ml: add 0.8 g NaOH and 10 ml 10% SDS in DDH2O, adjust total volume to 100ml

- **Solution III（pH5.1）**

  2.8M KOAc

  Per 100 ml: add 27.48 g KOAc in ddH_2O, adjust pH to 5.1 by glacial acetic acid, and then adjust total volume to 100 ml

2.1.8.3 For cell staining

- **BSA-phOx-FITC**

  The preparation of 100 mg BSA dissolved in 10 ml, 0.1 M sodium bicarbonate solution (stock concentration is 1 M, pH=9), 100 mg phOx dissolved in 100 ml DMSO, and 100 mg FITC dissolved in 10 ml DMSO. 326 μl phOx and 584 μl FITC added in 10 ml, 10 mg/ml BSA, and shook in the shaker at RT for 90 minutes (The molar ratio, BSA vs.

phOx vs. FITC =1:10:10, the overall process must avoid the light). 1 ml, 1 M glycine was added and shook at RT for 15 minutes in excess to stop the reaction. BSA-phOx-FITC was purified by G-25 column. The chemical concentration was detected by Coomassie Plus^{TM} kit (The kit major detect protein concentration. This solution included BSA, so that this kit detects the concentration). The equal volume BSA-phOx-FITC dissolved in the equal volume glycerol and stored at −20°C.

- **PB buffer (0.1M, pH7.4)**

  10.9 g Na_2HPO_4, 3.2g NaH_2PO_4 added ddH_2O until 1L, adjust the pH of buffer to 7.4

- **phOx solution**

  100 mg phOx dissolved in 1ml DMSO

- **phOx-bead (phOx:bead=1:10, excess reaction)**

  100 µl magnetic beads, 5.43 µl phOx (1µg/µl) and 15 µg EDC mixed each other at 37°C for 4 hours. The PB buffer was added in this solution and purified by BD IMag™ Cell Separation System.

- **phOx-glycine (phOx:bead=1:10, excess reaction)**

  The preparation of glycine solution (10 mg glycine dissolved in 1ml 0.1 M sodium bicarbonate), get 1 ml glycine solution added in 289.6 µl phOx (100mg/ml) at 37°C for 4 hours

- **Staining buffer**

  1% BSA , 0.05% NaN_3 in 1x PBS

2.1.9 Media

- **LB (Luria-Bertani) broth**

  1% tryptone, 0.5% yeast extract, 1% NaCl

- **LB (Luria-Bertani)/Ampicillin broth**

  1% tryptone, 0.5% yeast extract, 1% NaCl, 50μg/ml ampicillin

- **LB (Luria-Bertani)/Ampicillin agar**

  1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50μg/ml ampicillin

- **SOB media**

  Per 1 L SOB media: 20 g Tryptone, 5 g Yeast Extracts, 0.5g NaCl and 0.186 g KCl (Before used, added 2.5 mL 2 M $MgCl_2$ 500 mL)

- **DMEM medium**

  13.35 g DMEM power, 3.7 g sodium bicarbonate in a glass cup, added ddH_2O until 1L, adjust the pH of medium to 7.3

- **Growth medium with DMEM**

  10% FBS, 1% PSA in DMEM medium

- **5 mM polyethylenimine (PEI) Stock solution**

  1.25 g PEI in 10 ml ddH_2O

2.1.10 Equipment

- -20℃ low temperature refrigerator (Frigidaire)
- 4℃ refrigerator (MINI KINGCON)
- -80℃ low temperature refrigerator (NUAIRE)
- Bench top orbital shaker 060 (LMS)

- Biophotometer DPU-414 (eppendorf)
- Centrifuge 5415D (eppendorf)
- Centrifuge 5804 R (eppendorf)
- DNA electrophoresis unit Gel Mate 2000 (Toyobo)
- Flow cytometer, FACSarray (BD)
- Flow cytometer, FACScan (BD)
- Heating block (FIRSTEK)
- Inverted research microscope, IX71 (Olympus)
- Laminar flow hood, Forma Class II, A 1284 (NSF)
- Microscope, CX31 (Olympus)
- Orbital Shaking incubator OS1500R (TKS)
- pH meter SP701 (Suntex)
- Thermal cycler (eppendorf)
- Uni-photo gel image system (EZ lab)
- Water bath (FIRSTECK)
- G-25 column (Bio-Rad, cat 7371532, USA)
- The MidiMACS™ Separation System **(**Miltenyi Biotec, USA**)**

2.2 Methods

2.2.1 Construction of NF-κB based promoter library

2.2.1.1 Preparation of inserts (NF-κB based promoter library)

In due to create NF-κB based promoter library, NF-κB, CRE, MEF-2

and Sp1 binding sites are constructional materials in this construction. First, two complementary oligonucleotides (100 μM) were annealed to yield short DNA fragments that included individual binding sites at 95°C hot water and cooling down to room temperature over 30 minutes. Next, these DNA fragments were phosphorylated for ligation, the reaction volumes as following:

Volume (μl)	DNA	kinase	ATP	Buffer	ddH_2O	Total
NF-κB site	3	1	1	1	4	10
CRE site	3	1	1	1	4	10
MEF-2 site	3	1	1	1	4	10
Sp-1 site	3	1	1	1	4	10
Adaptor (Hind III cutting site)	3	1	1	1	4	10

These samples incubated at 37°C for 30 minutes. And then, different combination of NF-κB, CRE, MEF-2 and Sp1 were ligated in a total DNA volume of 6.4 μl using different volume ratio for creating more many combinations. The different DNA volume ratio and condition of ligation reaction listed in the following table.

A. The different DNA volume ratio

Volume ratio					
Name / TFBSs	1: 1: 1: 1 (LL-1)	4: 1: 1: 1 (LL-2)	1: 4: 1: 1 (LL-3)	1: 1: 4: 1 (LL-4)	1: 1: 1: 4 (LL-5)
NF-κB site	1.6 μl	3.55 μl	0.95 μl	0.95 μl	0.95 μl
CRE site	1.6 μl	0.95 μl	3.55 μl	0.95 μl	0.95 μl
MEF-2 site	1.6 μl	0.95 μl	0.95 μl	3.55 μl	0.95 μl
Sp-1 site	1.6 μl	0.95 μl	0.95 μl	0.95 μl	3.55 μl
Total volume	6.4 μl	6.4 μl	6.4 μl	6.4 μl	6.4 μl

P.S.: Each tube (L-1~L-5) the total DNA volume is fixed.

B. The condition of ligation reaction

DNA (LL1~LL5)	10 μM ATP	ddH_2O	T4 ligase Buffer	T4 ligase (2U)	Total
6.4 μl	1 μl	0.6 μl	1 μl	1 μl	10 μl

The ligation reaction incubated at room temperature (RT) overnight. These ligation products mixed together and ligated themselves (the product name: LL-6). The condition of ligation reaction listed in the following table.

DNA (LL-6)	10 μM ATP	ddH_2O	T4 ligase Buffer	T4 ligase (2U)	Total
50 μl	8 μl	6 μl	8 μl	8 μl	80 μl

The ligation reaction incubated at RT overnight, and extracted DNA (the section 2.2.2) to condense volume. After the DNA extraction, DNA concentration was 114.8 ng/μl (total 3903.2 ng). Next, these DNA

fragments were combined with other elements (Hind III cutting sites, H'-1) by T4 ligase (2U). The condition of ligation reaction listed in the following table.

LL-6	H'-1	10 μM ATP	ddH_2O	Buffer	T4 ligase (2U)	Total
34 μl	56 μl	13 μl	1 μl	13 μl	13 μl	130 μl

P.S.: The molar ratio between LL6 and H'-1 were 1:10.

The product was cleaned up by DNA extraction (the section 2.2.2) and DNA concentration was 105.3 ng/μl. After DNA extraction, the product was digested by Hind III restriction enzyme (MBI Fermentas, USA).

DNA (LL6+H'-1)	Hind III	ddH_2O	Buffer R	Total
30 μl/ about 3 μg	1.5 μl	4.5 μl	4 μl	40 μl

P.S.: Hind III activity is 100% in Buffer R.

DNA (LL6+H'-1) concentration was 39.3 ng/μl. Next, these DNA fragments insert to vector with *anti-phOx eB7* (the section 2.2.1.3).

2.2.1.2 Preparation of vectors (B16.4) and digestion by HindIII

Preparation of Plasmid DNA (B16.4) was used by Midipreparation kit (the section 2.2.4.1). B16.4 plasmid added HindIII and then digested. The digested condition was listed as following.

DNA (B16.4)	HindIII	ddH_2O	Buffer R	Total
15 µl	10 µl	10 µl	65 µl	100 µl

The product was cleaned up by DNA extraction (the section 2.2.2) and DNA concentration was 477.8 ng/µl. After DNA extraction, the product was dephosphorylated to avoid self-ligation. The condition of dephosphorylated reaction listed in the following table.

DNA (B16.4)	Phosphatase	Buffer	ddH_2O	Total
18 µl/6 µg	6 µl	3 µl	3 µl	30 µl

P.S.: Before ligation, the product was must heat- inactive in 10 minutes.

The B16.4 plasmids that digested by HindIII was inserted NF-κB based promoter library (LL6+H'-1). This process illustrated in the next section.

2.2.1.3 NF-κB based promoter library insert to vectors (B16.4)

The ligation reaction incubated at RT overnight and condition was listed in the following table.

	Vector	Insect	Ligase (10U)	ddH_2O	ATP	Buffer	Total
V+L	2.5	0	1.5	8	1.5	1.5	15
1:5	2.5	2	1.5	6	1.5	1.5	15

P.S.: The molar ratio between B16.4 and LL6+H'-1were 1:5

The products transformed later into electroporation competent cells (the section 2.2.3.3), and colonies numbers were listed in the following table.

Ligation condition	Colonies number
V+L	100
1:5	2568

The total ligation products were transformed and incubated at 37°C for 8~12 hours in 100ml LB media (with kanamycin). No incubate for long times (>12 hours), because promoter library diversity maybe decrease.

2.2.2 DNA extraction (clean up)

The DNA digested or ligated by restriction enzyme or ligase was cleaned up by Gene-Spin™ 1-4-3 DNA extraction kit (Protech Taipei, Taiwan), following the commercial protocol. The DNA solution was spun at 13,000 rpm for 30 seconds in the spin column. The filtrate in the collection tube was discarded. 700 μl Washing solutions (Protech Co., Taipei, Taiwan) were added and the solution was spun for 1 minute at 13,000 rpm. This step was repeated twice. Then, the filtrate was discarded for 3 minutes at 13,000 rpm to remove residual trace of ethanol. The column was additionally incubated at 65°C for 5 minutes to evaporate ethanol. DNA was eluted by 30-50 μl ddH_2O in a new tube.

2.2.3 Transformation of *E. coli*

Generally, heat-shocked competent cells were transformed and amplified plasmids except for plasmids with promoter library. The diversity of plasmids with promoter library is important, so that electroporation competent cells were used to transform them [transform efficiency: heat-shock (10^6~10^7) < electroporation (10^8~10^9)].

2.2.3.1 Preparation of competent cell (heat shock)

One pick of *E. coli* was inoculated in 3 ml of LB broth and grew for 12 hours at 37°C with vigorous shaking (~225 rpm). 1 ml of the overnight culture was transferred into 100 ml LB broth and was then incubated at 37 °C with shaking (~225 rpm) until the OD_{600} was between 0.35~0.45. The culture was stored on ice for 10 minutes. The cells were recovered by centrifugation at 4100 rpm for 10 min and then resuspended in 30 ml ice-cold 0.1 M $CaCl_2$. The cells were pelleted by centrifugation at 4100 rpm for 10 minutes at 0°C. The pellet was resuspended in 2 ml 0.1 M $CaCl_2$ (containing 10% glycerol). The cells were dispensed at 100 µl per eppendorf tube and then were stored at -80°C.

2.2.3.2 Transformation of competent cell (heat shock)

Stored competent cells were thawed on ice. 1 µl~2 µl of plasmid DNA was mixed with 100 µl competent cells and was then stored on ice for 30 minutes. The mixture was incubated in a preheated 42°C heating block for 90 seconds and quickly thawed on ice for 2 minutes. Then 250 µl of LB broth was added to the cells. The culture was incubated at 37°C with shaking (~225 rpm) for 50 minutes. 100 µl of the culture was plated on the LB agar plate with 50 µg/ml ampicillin or 30 µg/ml kanamycin. The plate was inverted and then incubated at 37°C for 12~18 hours.

2.2.3.3 Preparation of competent cell by electroporation

A colony of *E. coli* was inoculated in 25 ml of LB media and grew for

20 hours at 37°C with vigorous sharking (~225 rpm). The overnight culture was transferred into 500ml SOB containing 2 M $MgCl_2$ and was then incubated at 37°C with shaking (~225 rpm) until the OD_{600} was between 0.35~0.45. The culture was stored on ice for 10 minutes. The cells were recovered by centrifugation at 2500 rpm for 15 minutes at 4°C and then resuspended was discarded. An appropriate amount of ddH_2O was added to resuspend cells. The step was repeated twice. The pellet was resuspended in 1 ml ddH_2O with 10% glycerol. The cells were dispensed at 20 μl per microtube and then were stored at -80°C.

2.2.3.4 Transformation of competent cell by electroporation

Stored competent cells were thawed on ice. 1 μl of plasmid DNA was mixed with 20 μl compement cells and transferred into a pre-cooled cuvette. Cells was then electroporated at 2.5 mF, 2.5 kV, and 200 Ω for 4~5 milliseconds. The mixture was immediately recovered in 1 ml LB, transferred to a test tube, and incubated at 37°C with agitation (~225 rpm) for 1 hour, 100 μl of the culture was plated on the LB agar plated or 100 ml both in 250 ml flask with 50 or 30 μg/ml kanamycin respectively. The plate or flask was incubated at 37°C.

2.2.4 Plasmid DNA extraction

2.2.4.1 Minipreparation

A single colony of *E. coli* was inoculated in 3 ml of LB broth (with

antibiotics) and grew overnight at 37°C with vigorous shaking (~225 rpm). 1~2 ml of the cells were recovered by centrifugation at 13,000 rpm for 1 minutes and then resuspended in 200 µl ice-cold Solution I buffer in a new tube. 250 µl Solution II buffer was added and mixed gently, then stood at RT for 10 minutes. 250 µl Solution III buffer was added to the mixture and mixed gently, then stood on ice for 10 minutes. Cells were spun at 13,000 rpm for 5 minutes at 4°C. The supernatant transferred to a fresh tube. And then an equal volume of phenol: chloroform (~700 µl) was added. The organic and aqueous phases were mixed by vortexing and then centrifuge the emulsion at 13000 rpm for 3 minutes at 4°C in a tube. The aqueous upper layer transferred to a fresh tube. DNA was precipitated from the supernatant by adding 0.7 (0.6~1) volumes of isopropanol at RT. the solution was mixed completely and then allowed the mixture to stand for 2 minutes at RT. The precipitated DNA was collected by centrifugation at 13000 rpm for 20 minutes at 4°C. The supernatant was removed by gentle aspiration. The tube stood in an inverted position on a paper towel to allow all of the fluid to drain away. 1 ml of 70% ethanol added to the pellet and inverted the closed tube several times. The DNA recovered by centrifugation at 13000 rpm for 5 minutes at 4°C in tube. The tube opened at RT until the ethanol has evaporated and no fluid was visible in the tube (5~10 minutes). The DNA dissolved in 50 µl ddH_2O and stored at -20°C.

2.2.4.2 Midipreparation

A single colony of *E. coli* was inoculated in 100 ml of LB broth (with antibiotics) and grew overnight at 37°C with vigorous shaking (~225 rpm).

The broth was centrifugated at 8,000 rpm at 4°C for 15 minutes. After supernatant was discarded, 4 ml buffer S1 (with RNase A) was added and then solution was vortexed. 4 ml buffer S2 was added to the suspension. The lysate was mixed gently by inverting the tube 6~8 times and incubated at RT for 2~3 minutes (max 5 minutes). **Do not vortex, as this will release contaminating chromosomal DNA from the cellular debris into the suspension.** The solution was added pre-cooled 4 ml buffer S3 (4°C) and inverted gently 6~8 times until a homogeneous suspension containing an off-white flocculate was formed. The suspension was incubated on ice for 5 minutes. A NucleoBond AX 100 Midi column was equilibrated with 2.5 ml buffer N2. The flow-through was emptied by gravity flow and discarded. The bacterial lysate was cleared by centrifugation at 12,000 rpm at 4°C. The lysate was then loaded onto the NucleoBond column, which was emptied by gravity flow. 10 ml buffer N3 was added to wash the column. This step was repeated once again. Plasmid was eluted with 5 ml of buffer N5 3.5 ml isopropanl was added to precipitate the eluted plasmid. The mixture was incubated on ice for 10 minutes and centrifuged at 13,000 rpm for 30 minutes at 4°C. 1 ml 70% ethanol was added to the pellet and stored at -20°C or the solution was centrifuged at 13,000 rpm for 5 minutes for further application. Last, the pellet was re-dissolved in 20 μl ddH_2O.

2.2.5 Cell culture and Subculture

2.2.5.1 Cell culture

Balb/3T3, 293, B16F10 and A549 cells were mainintained 90% DMEM, 10%FBS, 1%PSA medium and incubated at 37°C. These cells were subcultured 2~3 times in the week.

2.2.5.2 Subculture

Remove and discard culture medium. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed. Add 6.0 to 8.0 ml of growth medium and aspirate cells by gently pipetting. Add appropriate aliquots of the cell suspension to new culture vessels. These cells all were incubated at 37°C.

2.2.6 Polyethylenimine (PEI) transfection

Seeding 300,000 cells in 6-well 18hr before transfection (to make 50~70% confluency at the time of transfection). Immediately before transfection, cells were rinsed and supplemented with fresh serum-free culture medium (0.2 ml). The plasmid DNA (3~4 μg) and the desired amount of PEI were each diluted into 100 μl of 150 mM NaCl and vortexed. The different amounts of PEI used in different cell lines. After 5 minutes, add the PEI solution into plasmid DNA solutions **(Notice: not the reverse**

order), and then were vortexed. After 20 minutes, washed the well and dropped 200 μl Opti-MEM, then the transfection mixture was added to the cells, and then add 600μl Opti-MEM. After 18 hours incubation, 2 ml growth medium were added into each well. After 24~48 hours, the gene expression level was analyzed by flow cytometry.

2.2.7 Stain transfecants

Discard the culture medium at 48 hours after transfection. Rinse each well with 1ml PBS. Add 1ml Versene and incubate it at 37°C for 5 minutes. Add 1 ml DMEM into each well to harvest the transfectants. Centrifuge the cell mixture at 1500 rpm, at 4°C for 5 minutes. Discard the supernatant and suspend the pellet with 1ml staining buffer the cell into FACS tube. Centrifuge the mixture at 1500 rpm, at 4°C for 5 minutes. Discard the supernatant and put the tube upside down for 30 seconds. Add 1 μl BSA-phOx-FITC or 1 μl magnetic bead/ml staining buffer 1 hour. Wash the pellet with 1ml staining buffer twice. Analysis the partial cells by staining 1 μl BSA-phOx-FITC with flow cytometry. The others isolated by magnetic beads separation system combined with competing agents (phOx-glycine).

2.2.8 The isolation of different activity promoters by MBSCS

The transfecants to be separated is first magnetically labeled with 1 μl phOx-bead. After magnetic labeling, the transfecants are suspended in 3

mL PBS and passed through an LS Separation Column that is placed in the strong permanent magnet of the MidiMACS Separation Unit. The magnetizable column matrix serves to create a high-gradient magnetic field. The magnetically labeled cells are retained on the column and separated from the unlabeled cells that pass through until 3 ml solution is eluted. 3 ml competing agents (1 μl phOx-glycine dissolved in 3 ml PBS) were added. After removal of the column from the magnetic field, added 3 mL PBS in LS Separation Column and the retained fraction can be eluted.

2.2.9 Extraction of plasmids by Hirt method

The cells from magnetic beads separation were pelled by centrifugation and resuspended in125 μl of phosphate-buffered saline (PBS). An equal volume of 2X Hirt buffer was added and the solution was incubated at room temperature for 15min to allow the cell to lyse. After the addition of 62 μl of 5 M NaCl to give a final concertation of 1 M, the solution was placed at 4°C overnight. The samples were the centrifuged at 14000x g for 60 minutes at 4°C, and the suspentant was extracted with an equal volume of phenol-chloroform. The DNA was ethanol precipitated and resuspend in 10mM Tris-HCl, pH=7.4.

Chapter 3 Results

3.1. Polyethyleminie transfection assay

In order to establish the selectable system of promoter library, the transfection efficacy is important in this my experiment. DNA transfection technique was shown to deliver genes into various cell lines successfully, (Boussif, Zanta et al. 1996) its transfection efficiency is higher than other cationic polymers (Demeneix, Behr et al. 1998; Read, Singh et al. 2005).

In this experiment, Different doses of PEI/DNA complex were tried to transfect efficiently into various cell lines, including Balb/3T3 (murine fibroblast), 293 (human kidney cell), B16-F10 (murine melamona cell), A549 (human lung carcinoma cell), DBTRG-05MG (human glioblastoma cell) and HepG2 (human hepatocellular carcinoma cell) cell lines.

The efficiency rates of PEI transfection for Balb/3T3 and 293 cells were 64.95% and 60.92%, respectively **(Figure 7)**. For A549 and B16-F10 cells, the efficiency rates were 26.58% and 25.84%, respectively **(Figure 7, 8)** However, **(Figure 8)**, the efficiency rates only were 11.28% and 4.24% for the DBTRG-05MG and HepG2 cells, respectively. Therefore, PEI transfections were easy for Balb/3T3, 293, A549 and B16-F10 cell lines. PEI transfections were difficult for DBTRG-05MG and HepG2 cells.

Because PEI transfection for Balb/3T3, 293, A549 and B16-F10 cells were better transfection efficiency than HepG2 and DBTRG-05M, these

cells were selected as target cells . Furthermore, Balb/3T3 and 293 cells are belonging to immortal, non-tumorous. In contrast, A549 and B16-F10 cells are belonging to tumorous. They can be studied the genes expression between non-tumor and tumor cells.

3.2. Assay of NF-κB expression level in various cell lines

As previous description, NF-κB activities are higher in tumor cells than normal cells. Therefore, the NF-kB activities were monitored in Balb/3T3, 293, A549 and B16F10 cells to determine whether the difference exists in the selected target cell line.

In this experiment, pNF-κB–hrGFP vector was used to detect NF-κB activity in Balb/3T3, 293, A549 and B16F10 cell lines **(Figure 9, 10)**. Two plasmids were co-transfected into target cells: one plasmid containing the TFBS which drove the green fluorescence protein (*hrGFP*) gene; the other containing CMV promoter which drove the red fluorescence protein (*AsRed*). Co-transfection with pAsRed-N2 could avoid the difference in transfection efficiency at different experiment groups. Moreover, pARE-hrGFP containing ARE site (prokaryotic TFBSs) was as negative control vector to normalize the degree of transgene expression in different cell lines. The expression folds formula is described as below:

$$\textbf{Expression Fold} = \frac{\textbf{TFBSs-hrGFP}}{\textbf{CMV-AsRed X ARE-hrGFP}}$$

In Balb/3T3 and B16F10 cells, pNF-κB-hrGFP expression was 12-fold and 34-fold than pARE-hrGFP, 30-fold and 12-fold than

pAP-1-hrGFP **(Figure 9)**. In 293 and A549 cells, pNF-κB-hrGFP expression was 4-fold and 28-fold than pARE-hrGFP, 1.2-fold and 21-fold than pAP-1-hrGFP **(Figure 10)**. The NF-κB activities were lower in normal-like (293 and Balb/3T3) cells than tumor cells (A549 and B16F10). Interestingly, all cell lines had higher NF-κB activities than other transcription factors in this experiment **(Figure 9, 10)**.

3.3. Construction and selection of NF-κB based promoter library

Promoters' activity dependent to transcription factors and NF-κB has higher activity in tumor cells. Therefore, a new NF-κB based promoter library was created to select a new promoter sequence which can be overexpressed in tumor cell but not in normal cell.

3.3.1. Construction of NF-κB based promoter library

NF-κB based promoter library was created by randomly ligating oligonucleotides containing NF-κB, CRE, MEF-2 or Sp1 sites. First, two complementary oligonucleotides were synthesized for each individual binding sites, later they were annealed and phosphorylated to yield short DNA fragments (the section 2.2.1).

After oligonucleotides with different binding sites were ligated, cleaned up (the section 2.2.2) and then observed their sizes by DNA electrophoresis. The sizes of DNA fragments were between 100 bp and 300

bp after ligation **(Figure 11)**. An average size of joint DNA fragments was "200" bp. These primers' were about "20" bp sizes before ligation. Therefore, the promoter library had 1,048,576 ($4^{200/20}$= 4 ^{10}=1,048,576) variety of combinations probably. Adaptors with Hind III restriction enzyme site were added to ligate the end s of promoter fragments and then they were inserted to B16.4 vector (with anti-phOx gene). After ligation, the ligation efficiency was determined by transfomation. The results showed that the negative control (the cutting vector alone treated with T4 ligase) and my sample had about 100 and 2568 colonies respectively (the section 2.2.1.3). However, total ligation products only got 1/50 volume to transfom into *E. coli* on the plate (Ttotal 15 μl ligation product get 2μl to transform into *E .coli* by electroporation, and then total 1000 μl transformable volume got 100μl to spread on the plate. Therefore, total ligation products only got 1/50 volume transfom into *E. coli* on the plate.). Therefore, there are 123,400 varieties in promoter library actually.

3.3.2. Selection of NF-κB based promoter library

The expression of NF-κB based promoter library in Balb/3T3 and A549 cell lines were selected by MBSCS to high-activity promoters respectively. Balb/3T3 is normal-like cell line, and A549 is lung carcinoma cell line. NF-κB based promoter library transfected into normal and tumor cells to compare difference of gene expression and find tumor specific promoters.

3.3.2.1.Selection of NF-κB based promoter library in Balb/3T3

Original NF-κB based promoter library was transfected into Balb/3T3 and analyzed gene expression by flow cytometry **(Figure 12)**. Fluorescent expression of B2a plasmid (with CMV promoter and anti-phOx-eB7) was 19.48% and NF-κB based promoter library with anti-phOx-eB7 was 7.062%. Supposition, CMV promoter is certain to express. Different cell types, staining and transfection efficacy causes the fluorescence expression not arrived at 100%. When the fluorescent intensity (FLH-1) exceeds 10^1 values that gene is turned on.

How have many activities promoters in my established promoter library? The ratio was calculated by the following formula **(Figure 12)**:

In original promoters library, the ratio of active promoters

$$\text{ratio} = \frac{\text{fluorescent intensity of promoter library} - \text{negative control}}{\text{fluorescent intensity of CMV promoter - negative control}} = 7.062\text{-}3.48/19.48\text{-}3.48$$

$$= 22.38\%$$

In overall promoters, active promoters were 22.38% approximately. In another word, total 123,400 varieties kinds promoters had gene expression only were 27626 varieties.

In order to further analyzed promoter library activities, the observation of gene expression in original promoter library must did first. Under the fluorescent intensity (FLH-1) exceeds 10^1 values, most cell counts lain on FLH-1=10^1~3 x 10^1 region (M2). In FLH-1=3x 10^1~10^2 region (M3), cell counts were fewer than M2 region. However, in FLH-1 >10^2 region (M4),

cell counts were almost zero. According to the distribution (cell counts and fluorescent intensity) of gene expression in original NF-κB based promoter library, fluorescent intensity (FL1-H $>10^1$) was divided into three groups **(Figure 13)**:

(1) Low fluorescent intensity, FL1-H $=10^1 \sim 3 \times 10^1$

This ratio (M2) was 5.63% in overall situation. Under the overall expression ratio (M1), it occupied 79.75% (M2/M1).

(2) Medium fluorescence intensity, FL1-H $=3 \times 10^1 \sim 10^2$

The ratio (M3) was 0.77% in overall situation. Under the overall expression ratio (M1), it occupied 10.91 % (M3/M1).

(3) High fluorescence intensity, FL1-H $>10^2$

The ratio (M4) was 0.66%. Under the overall expression ratio (M1), it occupied 9.35% (M4/M1).

In order to distinguish the cells with high, medium or low expressive promoters, transfectants were isolated by the modified magnetic beads separation method. In the separation of processing, the competitor (glycine-phOx) was added to further exclude low expressive cells and remain the high-expressive cells by surface expression of anti-phOx molecule to competing with the competitor. After MBSCS separation, B2a and origin NF-κB promoter library vectors were transfected into Balb/3T3 cell and its gene expressive rates were 35.84% and 3.1.32%, respectively **(Figure 14)**.

In the same way, the ratio of activities promoter in isolated promoter

library was calculate by the following formula:

In isolated promoters library (after elution), the ratio of active promoters

$$\text{Ratio} = \frac{\text{fluorescent intensity of islated promoter library - negative control}}{\text{fluorescent intensity of B2a - negative control}} = 85.967\%$$

After isolation of promoter library (after elution), its' overall gene expression that compare to original library are increase or decrease by the following formula to calculate expression times.

$$\textbf{Expression times} = \frac{\text{First transfection, expression level of B2a}}{\text{Second transfection, expression level of B2a}} \times \frac{\text{Isolated promoter library (After elution)}}{\text{Original promoter library}}$$

$$= 3.2\text{-fold}$$

Because transfection and staining twice had different efficiency, CMV promoter activity divides by each other to remove from this interference. The isolated promoter library was 3.2-fold than origin. Such the isolated promoter library had higher gene expression than origin. In the preliminary experiment, this strategy seems to be practicable.

The comparison between original and isolated promoter library in the low, medium or high fluorescence intensity ratios were able to analyze

further changes of fluorescence intensity. According to prior way, isolated promoter activity could divide into three groups **(Figure 15)**:

(1) Low fluorescent intensity, FL1-H =10^1~3 x 10^1

This ratio (M2) was 25.50% in overall situation. Under the overall expression ratio (M1), it occupied 81.42% (M2/M1).

(2) Medium fluorescence intensity, FL1-H =3x 10^1~10^2

The ratio (M3) was 5.27 % in overall situation. Under the overall expression ratio (M1), it occupied 16.83 % (M3/M1).

(3) High fluorescence intensity, FL1-H >10^2

The ratio (M4) was 0.66%. Under the overall expression ratio (M1), it occupied 0.64% (M4/M1).

The comparison between original **(Figure 13)** and isolated (**Figure 15**) promoter library activities, two results were shown and analyzed by histogram **(Figure 16)**. In the low fluorescent intensity region, the ratio of isolated library (M2/M1) was increased 1.59% than original. And in medium fluorescent intensity region, the ratio of isolated library (M3/M1) increased 5.91 % than original.

However, gene expression rate decreased 8.71% in the high fluorescence intensity region. This cause knew unclearly. But total gene expression is enhancement (3.2-fold) after MBSCS. Therefore, MBSCS is the effective method that can exclude no and low expression of promoters.

MBSCS could divide three procedures. The first procedure is washing, the second procedure is competing and the third procedure is eluting **(Figure 5)**. In order to further analyze three procedures of MBSCS **(Figure**

17). The changes of three different fluorescent intensity regions were analyzed by flow cytometry. In three procedures, washing procedure of gene expression in low fluorescent intensity was higher level (83.51%) than competing (81.42%) or eluting (80.92%). However, in medium or high fluorescent intensity was lower level (16.59 or 0.54%) than competing (18.26 or 18.26 %) or eluting (18.98 or 0.64%), but they were no significant **(Figure 18)**.

After isolation, the ratio of medium/high-active promoters in overall promoters was calculated by following formula.

The ratio of "medium/high-active" promoters in overall promoters

$$= \frac{\text{Gene expression of medium/high-active promoter- negetive control}}{\text{Gene expression of B2a - negetive control}} = 5.69\%$$

If pick 100 colonies and purify their DNA after isolated plasmids transformed into *E. coil*, there will be 5.69 colonies with medium / high-active promoters among 100 colonies possibly. On the basis of Laboratory manpower and select probability, picked 120 colonies purified their DNA by minipreparation; maybe there will be 6.82 colonies with medium/high-active promoters. These plasmids transfected into Balb/3T3 and their gene expressions were analyzed by flow cytometry **(Figure 21)**. There were 25 plasmids by selecting and they divided into three groups simply as following.

High expression (100~50% Gate, proportion of gene expression):

B-2 (53.26%), B-8 (51.02%), B-25 (94.73%), B-26 (96.66%), B-27

(83.76%), B-28 (63.92%), B-29 (92.35%), B-31 (62.21%) and B-35 (62.28%)

Medium expression (50%~10% Gate, proportion of gene expression):

B-1 (25.65%), B-15 (28.03%), B-19 (41.6%), B-50 (15.48%), B-59 (22.08%)

Low expression (10%~ Gate, proportion of gene expression):

B-86 (8.95%), B-90 (1.48%), B-96 (2.91%), B-102 (3.16%)

Because these plasmids were purified by mimipreparation, DNA solutions involved chemical compounds or RNA probably. The purity of DNA solutions is important in transfection. Therefore, these select plasmids were purified further by midipreparation to analyze gene expression in different cells and find the tumor specific promoters.

3.3.2.2. Selection of NFκB-based promoter library in A549

In the same select isolated Balb/3T3 promoters' way, NF-κB based promoter library was transfected into A549 cell and selected by MBSCS **(Figure 19)**. The observation of gene expression in three procedures **(Figure 20)**, washing procedure of gene expression in low fluorescent intensity is higher level (92.83%) than competing (85.44%), but is lower than eluting (96.46%). In medium fluorescent intensity, washing (7.17%) is lower level than competing (14.91 %) but is higher level than eluted (3.11%). Interestingly, in high fluorescent intensity, eluting (0.61%) is higher level than washing (0.32%) or competing (0.22%). These results were no significant **(Figure 20)**.

After MBSCS separation, eluted cell pellets were collected, extracted

plasmids and then transform into *E. coil*. Picked 120 colonies and purified plasmids by mimipreparation to analyze their gene expression. These plasmids transfected into A549 and their gene expressions were analyzed by flow cytometry **(Figure 22)**. 12 pieces of plasmids were selected by their gene expression and divided into from three groups simply as following:

High expression (100~50% Gate):

A-2 (40.25%), A-10 (61.45%)

Medium expression (50%~10% Gate):

A-5 (25.1%), A-6 (27.98%), A-8 (17.12%), A-9(19.11%), A-11 (28.65%)., A-12 (18.42%)

Low expression (10%~ Gate):

A-23 (4.54%), A-107 (6.48%), A-120 (2.05%), A-141 (2.78)

These select plasmids were purified further by midipreparation to analyze gene expression in different cells and find the tumor specific promoters.

3.4. To compare with gene expressions of differential NF-κB based promoters in differential cell lines

3.4.1. Gene expressions of differential NF-κB based promoters from Balb/3T3 selection

Selectable plasmids from Balb/3T3 were transfected by PEI into B16F10, 293, Balb3T3 and A549 cells. Gene expression levels in four cells

were ordered and compared tumor with normal-like cells in each experiment **(Table 2)**. B-2 promoter in Balb/3T3 and 293 cells are lower activities than other promoters, but gene expression in A549 and B16F10 cells are higher than other promoters. B-31, B-35, B-28 and B-51 are ubiquitous promoters. B-28 is medium expression than other promoters; B-31, B-35 and B-51 are high expression than other promoters in these cells.

3.4.2. Gene expressions of differential NF-κB-based promoters from A549 selected

The same way of Balb/3T3, gene expression levels in four cells were ordered and compared tumor with normal-like cells in each experiment. Maybe A-2 and A-111 vector is a ubiquitous promoter, A-29 in A549 high expression than other cells **(Table 3)**.

3.5. DNA sequence of NF-κB-based promoters and TFBSs

Sequence of B-51 has been verified completely by autosequencing, but the complete information of sequence for B-2, B-6, B-31 and B-28 vectors have not been got yet. Because the length of these sequences is so long (over 1 kb), these sequences have only got their partial sequences (about 200 bp). There are one NF-κB site, two CREB sites, one Sp1 site

and four MEF-2 sites in the sequence of B-51 vector. B-51 promoter has high gene expression in Balb/3T3 and 293 cells than A549 and B16F10 cells **(Table 3)**. The partly sequences of B-2, B-6, B-31, B-28 promoters showed that they have different elements in their sequences **(Figure 23-24)**.

B-2 promoter has two NF-κB sites and CREB sites; B-6 promoter has one CREB site, three Sp1 sites and two MEF-2 sites. Both promoters are high activities of gene expression in tumor cells (A549 and B16F10) but low activities in non-tumorigenic cells (293 and Balb/3T3 cells). B-31 promoter has two Sp1 sites and one MEF-2 site; promoter is ubiquitous and high active for expression in four cell lines. B-28 promoter has one Sp1 sites and one MEF-2 site and is highly active for gene expression in Balb/3T3 cells.

In addition, I also selected and gained high expression promoters from A549 cells transfected with NF-κB based library, the results showed A-2 and A-111 are active in all cell lines. Because their insert sequences are so long (over 1kb) or certain unknown problem, these sequences cannot be obtained. Therefore, these promoters are not discussed.

Chapter 4 Discussion

In this study, the modified magnetic beads separation system (MBSCS) used to isolate lower gene expression and medium / high expression of promoters. Magnetic beads separation system does not separate high-expression and low-expression on cell surface. The low expressive population in cells transfected with promoter library occupies the most proportion of transfectants, it strongly interferes the selection for the promoter with high expression activity. Therefore, I hoped to exclude the promoters with low expression activities when the promoter library is selected with MBSCS. The competitor (glycine-phOx) was designed and used to decrease low-expression of promoters. By competing with glycine-phOx, low expressive transfectants would catch the competitor and be released from magnetic beads separation system. Thus, the low expressive transfectants could be excluded and increase the proportion of high expressive transfectants, later the promoter with high expression activity would be easier to identify.

Without competitor, the low fluorescence intensity ratio in total fluorescence intensity is about 79.83%, medium fluorescence intensity is 10.92% after selection in Balb/3T3 cell, and competitor treatment raises the medium fluorescence intensity to 16.83%. Furthermore, I analyzed the fluorescent intensities of transfectants after washing, competing and eluting procedures, the results showed that the proportions for medium

fluorescent intensity is 16.59% after washing, 18.26% after competing and 18.98% after eluting in BalB/3T3 cell **(Figure 18)**. In other cell, A549, medium fluorescent intensity is 7.17% after washing and 14.19% after competing. However, after eluting, medium fluorescent intensity is only 3.11%. Interesting, high fluorescent intensity is 0.61 after eluting, and is higher expression than washing (0.32%) or competing (0.22%) **(Figure 20)**.

According to these data, this system seems to be practicable to enhance the proportions for medium fluorescent intensity. However, the decrease of lower expression is not significant. Maybe it is due to the amounts of competitor that is not enough. The results may be improved by adding more competitors or decrease cell numbers.

Ubiquitiously or specifically high expressive promoters both were isolated from the promoter library; such as B-2 promoter is overexpressive in tumor cells (A549 and B16F10 cells). B-6 promoter is A549-specific promoter. How many NF-κB binding site in those sequences is not clear (because their complete sequences are not available) but partial sequence results showed they contain several predicted TFBSs (NF-κB, Sp1, CREB or MEF-2) in their sequences **(Figure 24)**. B-2 promoter sequence had two NF-κB binding sites which is conformable to previous results that NF-κB activities are more active in tumor than normal cells **(Figure 9-10)**. The activities of NF-κB in tumor cells usually are aberrant (Rayet and Gelinas 1999). In addition, NF-κB has higher affinity to DNA than other transcription factors. Many researches indicate that the dimers of NF-κB have high affinity for NF-κB binding sites (from 10^{13} to 10^{10} M) in comparison with most transcription factors

(whose affinity for cognate sites is generally close to 10^9 M) (Urban and Baeuerle 1990; Chen-Park, Huang et al. 2002; Bosisio, Marazzi et al. 2006), and can generate very stable complexes (half-life is about 45 minutes) on DNA (Zabel and Baeuerle 1990).

However, B-6 promoter may not had the NF-κB site and it is high expressive in A549 cells. Because B-6 promoter's complete sequence is not available yet, therefore the distribution of TFBSs in B-6 promoter is still unclearly. Promoter library were isolated by MBSCS and then individual plasmid was selected by transfecting each single plasmid into target cell to precisely determine its expressive activity. In transfection, one cell could gain more than one plasmid that may be the season why the plasmids with low expressive activities would be isolated after MBSCS. Although MBSCS still has some defects for efficiently selecting high expressive promoter, the B-51 and B-35 promoters are isolated and they are high expression in Balb/3T3 cell. Therefore, MBSCS should be practicable.

As previous section **(Chapter 1)**, 3 different TFBSs can interact with NF-κB to strengthen gene expression. B51 promoter sequence is complete sequenced and the result showed that there are one NF-κB, one Sp1, two CREB and four MEF-2 binding sites in the sequence. The result indicated that these TFBSs can assemble to form a high expressive promoter as our prediction. Recently, NF-κB–DNA complexes are further stabilized by cooperative protein–protein interactions with other sequence-specific transcription factor and architectural proteins, giving rise to multimolecular protein–DNA complexes called enhanceosomes. In vitro the interferon beta

(IFN-β) enhanceosome (Maniatis, Falvo et al. 1998) remains completely stable for over 10 hours (Yie, Merika et al. 1999). In this experiment, the selection of three TFBSs (Sp1, CREB, MEF-2 binding sites) ligated NF-κB binding sites randomly to create novel promoters. These three factors are related to tumor development, survival and metastasis. In addition, these also correlate with NF-κB in regulatory gene expression. The creation of NF-κB based promoter library by ligating randomly NF-κB, Sp1, CREB, or MEF-2 binding sites can isolate high expression or specific promoter successfully in different cell lines **(Table 3)**.

Li et al. synthesized the novel muscle promoters at 1999 (Li, Eastman et al. 1999) by random assembly of E-box, MEF-2, TEF-1, and SRE sites. They obtained high expression promoter in muscle by pick over hundreds of individual clones, and screening of for transcriptional activity in vitro and in vivo. However, they cannot control individual promoter by picking is high expression or not. Therefore, magnetic beads separation system is used in my experiment to improve this defect. In addition, *Li et al.* used known, muscle specific and high-expression transcription factor to create new muscle promoters. The probability of created high expression promoters is higher than my promoter library, because the selection of four transcription factors exists not only in tumor but also normal cells. That is to say, the high and tumor specific promoters are selected difficultly in my experiment.

However, tumor specific promoters (B-2, B-6) and ubiquitous promoters (B-31) were isolated and selected respectively by magnetic beads separation system combining with the competitor system (MBSCS)

in my experiment. MBSCS is able to select high or specific promoters easily. This strategy not only let us understand the context of high efficient promoter in tumor cells, but also let us knows which factors in the tumor cells are important. This may take advantage of it to more thorough understanding about tumor cells, and find the better treatment for cancer.

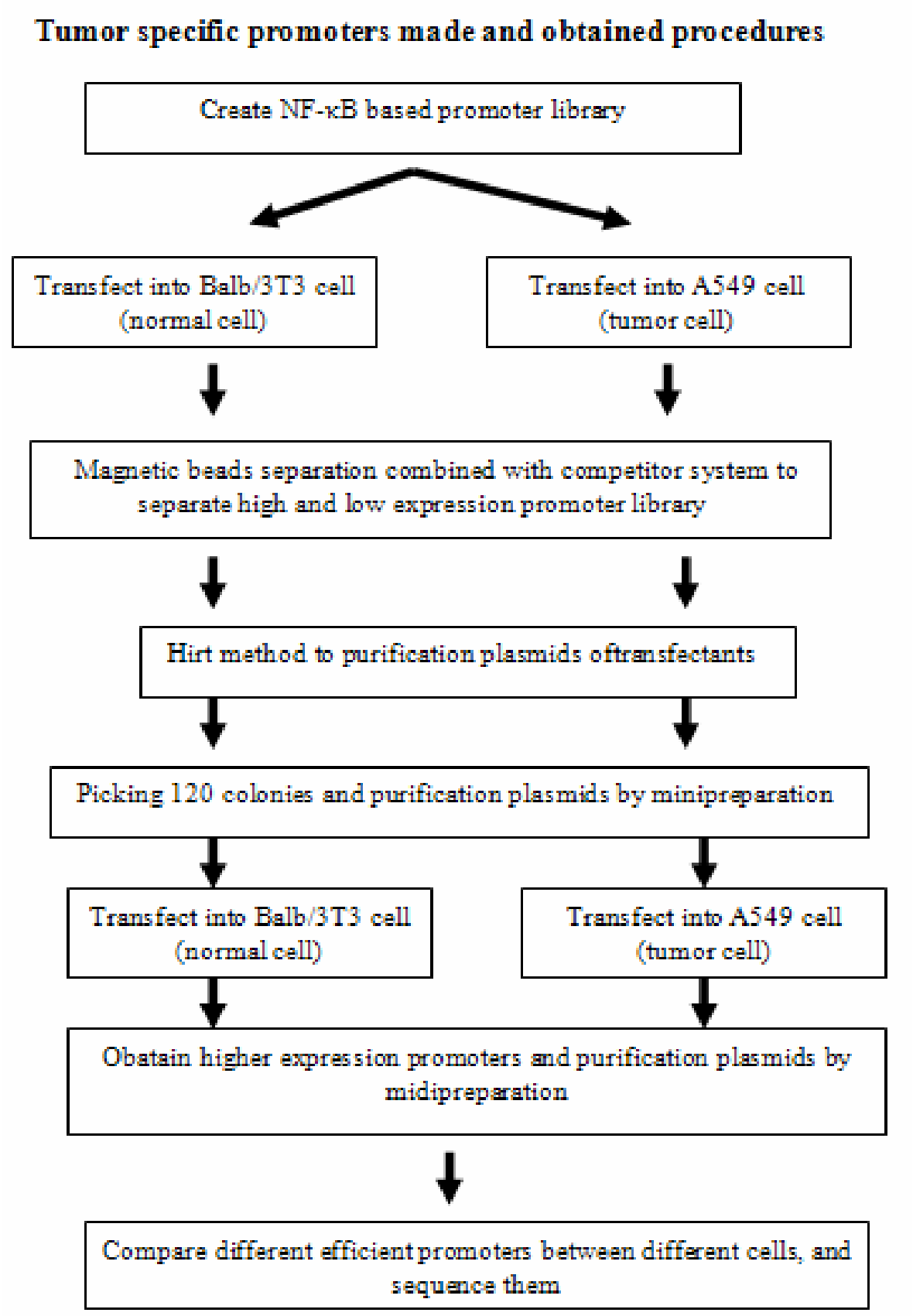


Figure 1 Tumor specific promoters made and obtained procedures

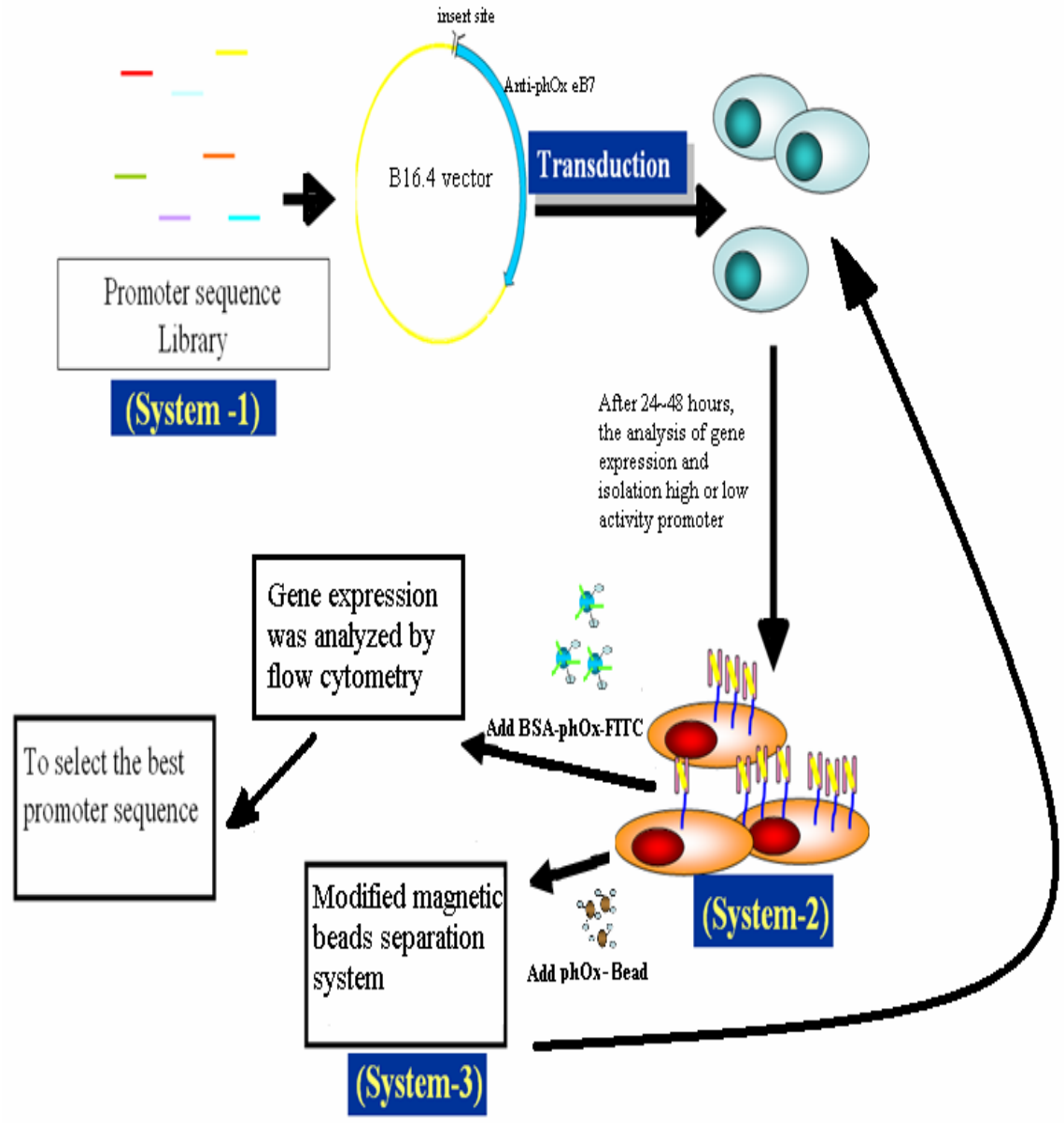


Figure 2 The scheme of construction and screening promoter library

The construction and screening of promoter library used three systems in overall procedure. System-1, NF-κB based promoter library was constructed by promoter shuffling system. System-2, reporter gene system can detect gene expression by phOx-bead. System-3, High active promoters were selected by modified magnetic beads separation system.

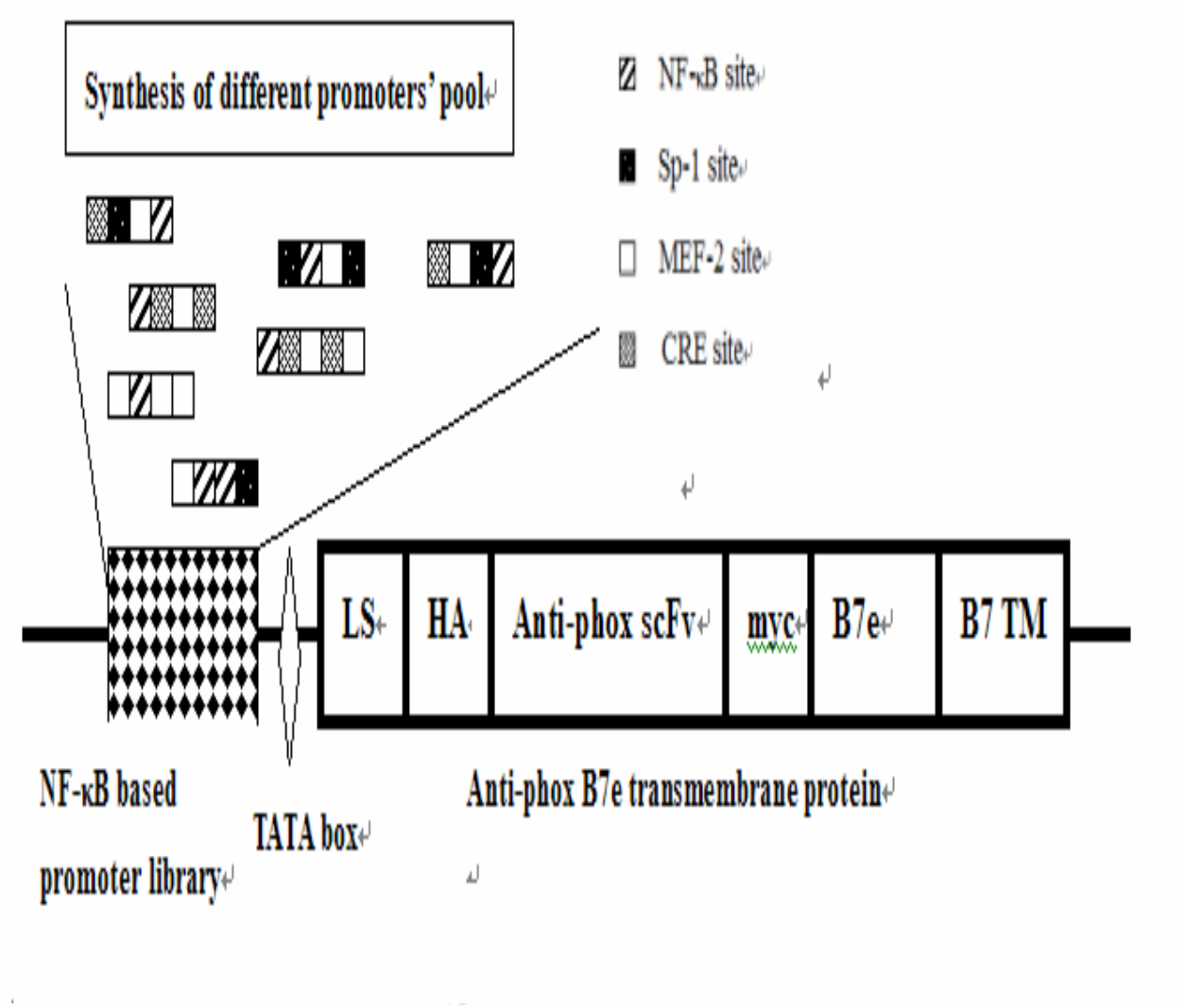


Figure 3 System-1: The construction of NF-κB based promoter library by promoter shuffling system

Random assembly used four different binding sites to create promoters. First, four different TFBSs assembled and then ligated HidIII cutting site to form large DNA fragments. After the formation of large DNA fragments, they and B16.4 vector (involving with anti-phOx protein) were cutted by HindIII. Finally, they were inserted into B16.4 vector.

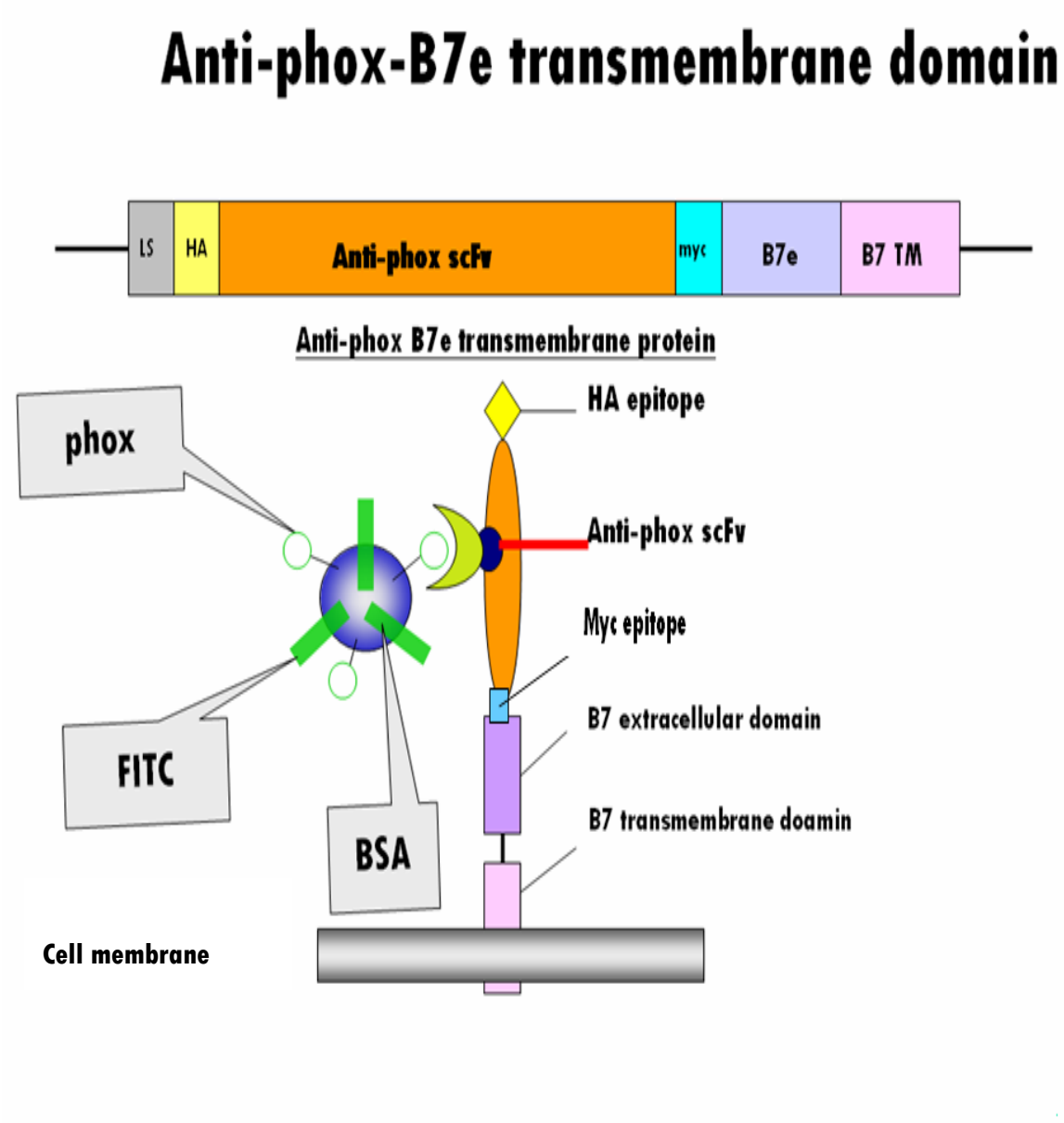


Figure 4 System-2: Reporter gene system

The construction of anti-phOx-B7e transmembrane domain was recognized by phOx derivative. Myc and HA epitope also were recognized by their related antibodies. These epitope can help us to know the reporter gene product is full or partial. Expression level of B7 transmembrane protein is higher than other transmembrane proteins. That is more sensitive to detect and assay gene expression (Schaefer, Brachwitz et al. 2004).

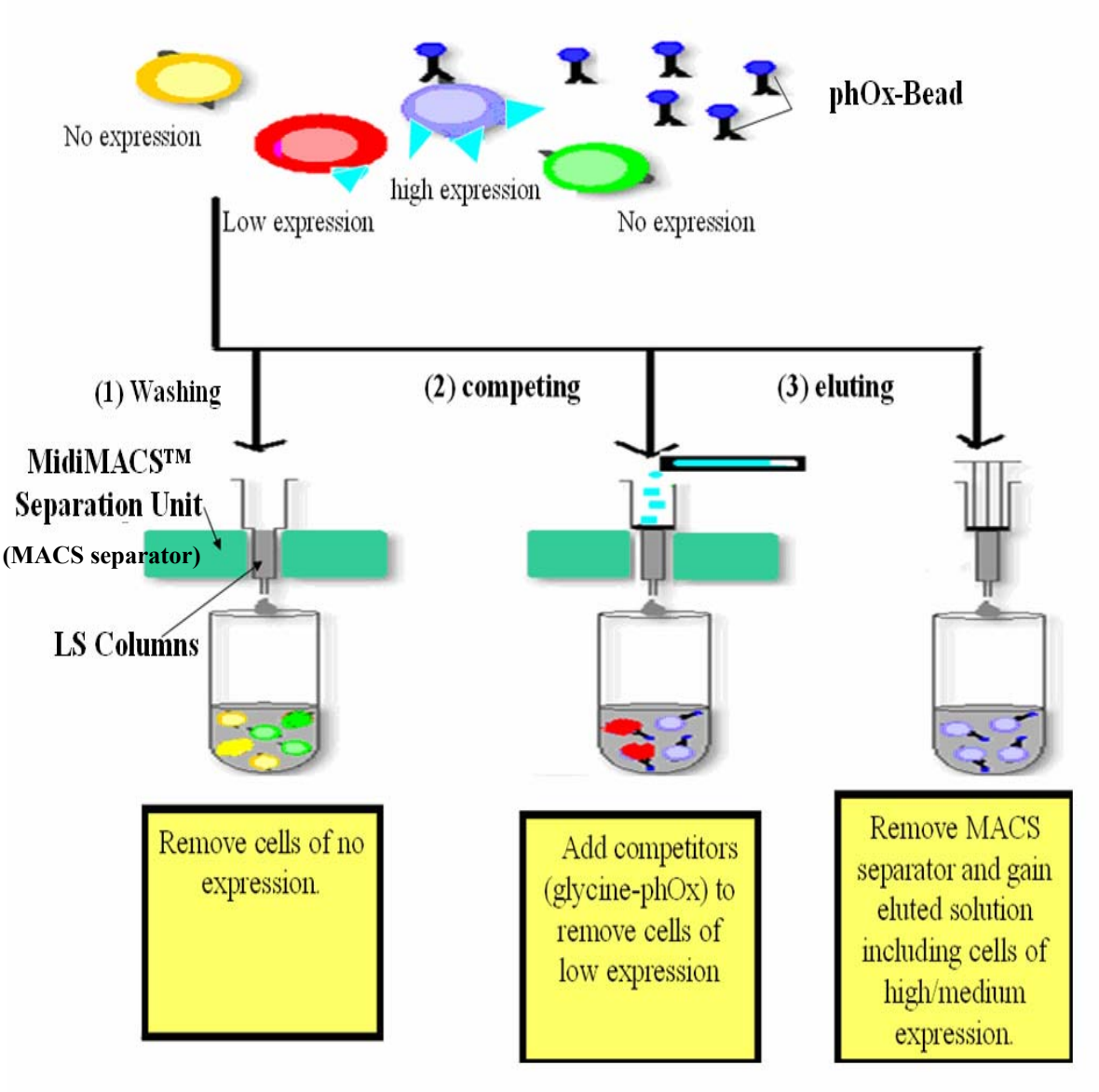


Figure 5 System-3: Modified magnetic beads separation system

The traditional magnetic bead separation system combines with the competitor (glycine phOx). The three procedures are (1) Washing procedure. The first transfectants passed magnetic column directly, and plasmids from these transfectants were collected. (2) Competing procedure. Adding the competitor to washing low expression transfectants, plasmids from these transfectants were collected. (3) Eluting procedure, MACS separator was removed and then residual transfectants was eluted and collected to purify plasmids.

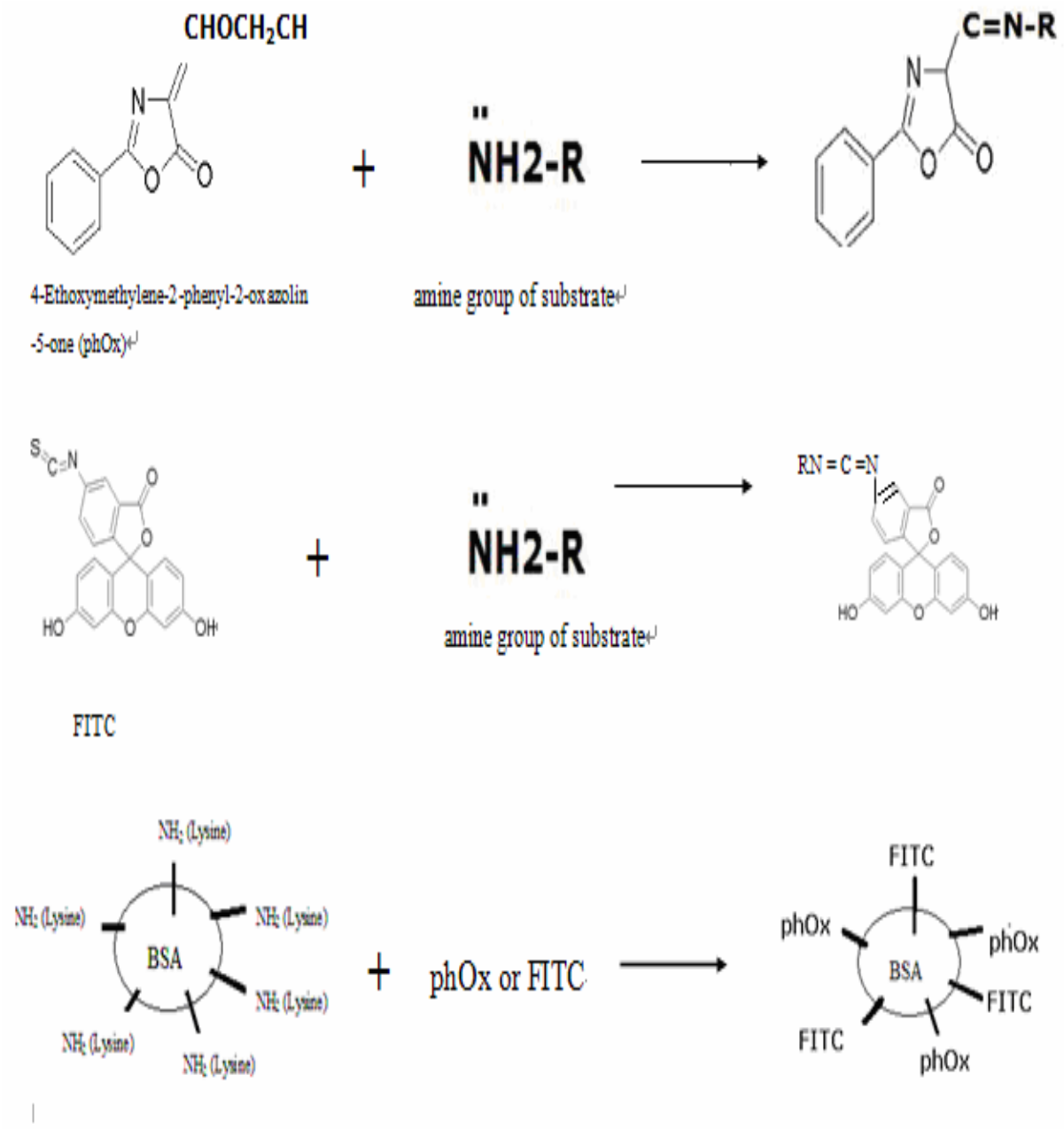


Figure 6 The modification of phOx and FITC

phOx and FITC are modified by substrates of amino group, BSA involve 67 Lysine residues (Lysine has amine group on side chain), so that can attack phOx and FITC.

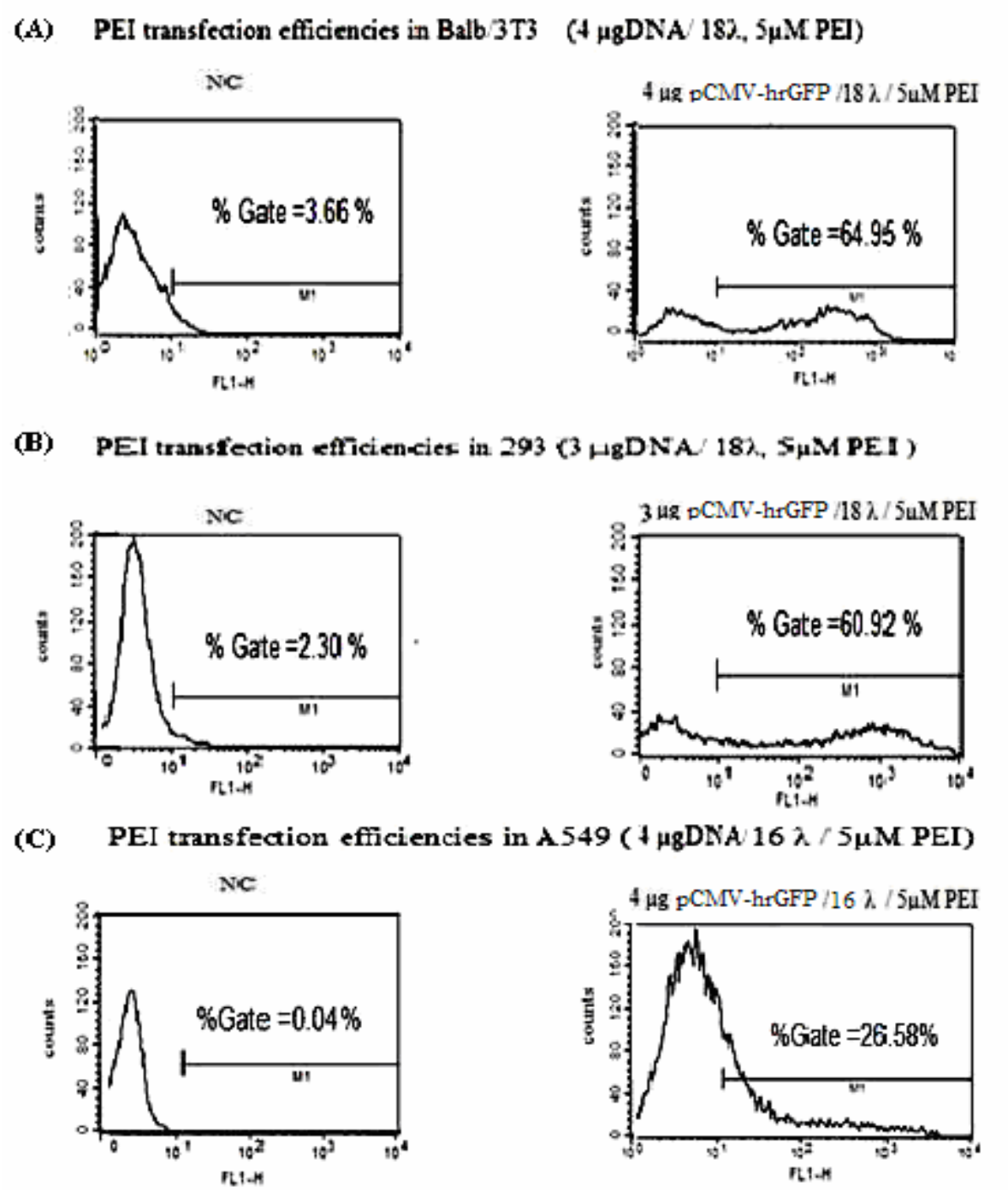


Figure 7 PEI transfection efficiency in Balb/3T3, 293 and A549 cells

(A) Transfection efficiency by using PEI in Balb/3T3 cell. PEI mixed pCMV-hrGFP (4 μg DNA, 18 μl/5 μM PEI) to transfect in Balb/3T3 cell, the transfection efficiency is 60.29 %. **(B) Transfection efficiency by using PEI in 293 cell**. PEI mixed pCMV-hrGFP (3 μg DNA, 18 μl/5 μM PEI) to transfect in 293 cell, the transfection efficiency is 58.62 %. **(C) Transfection efficiency by using PEI in A549 cell**. PEI mixed pCMV-hrGFP (4 μg DNA, 16 μl/5 μM PEI) to transfect in A549 cell, the transfection efficiency is 26.58 %.

Transfection efficiency = %Gate NC-% Gate pCMV-hrGFP NC is no transfection

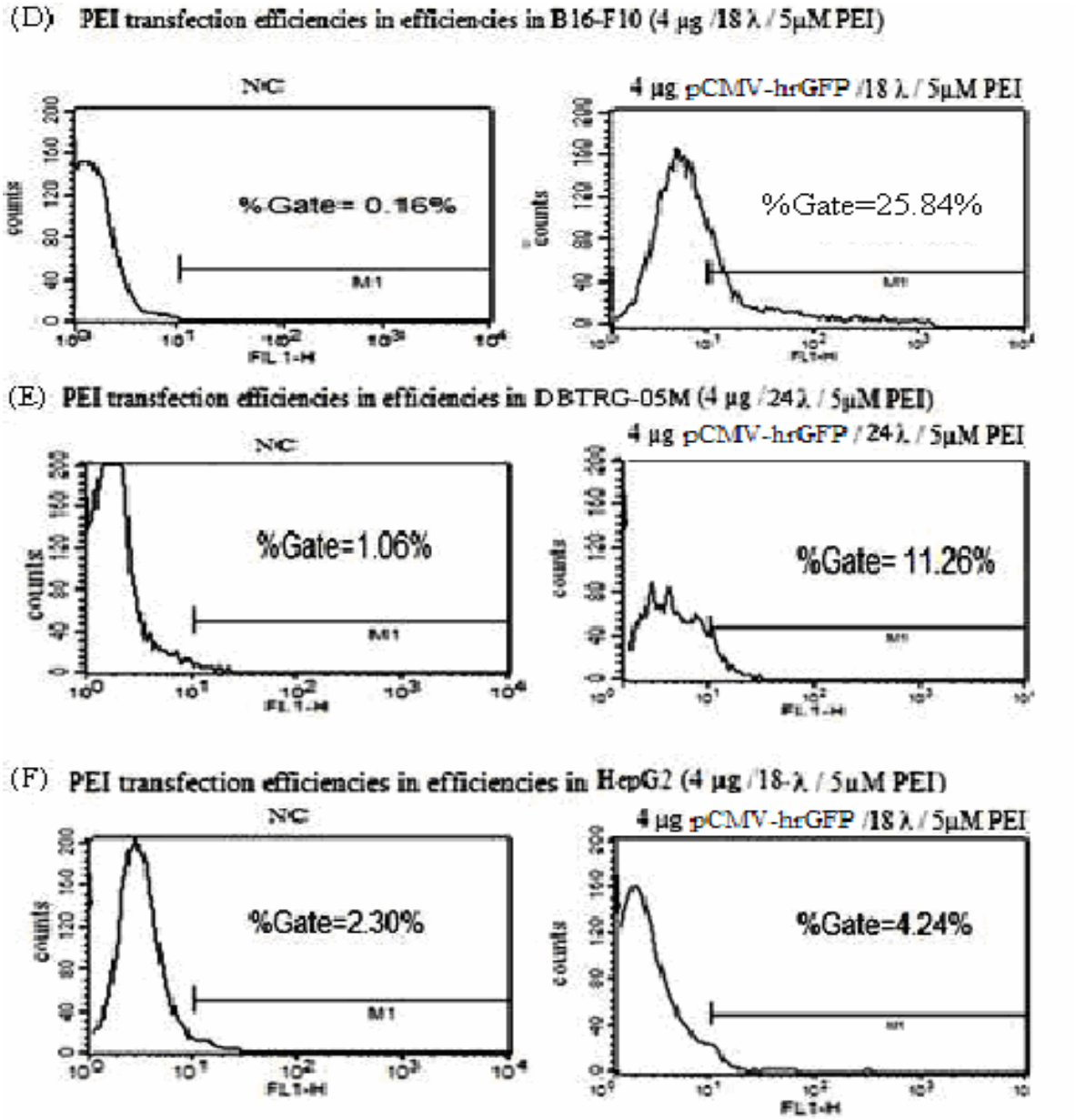


Figure 8 PEI transfection efficiency in DBTRG-05M, B16-F10 and HepG2 cells

(D) Transfection efficiency by using PEI in B16F10 cell. PEI mixed pCMV-hrGFP (4 μg DNA, 18 μl/5 μM PEI) to transfect in B16-F10 cell, the transfection efficiency is 25.68 %. **(E) Transfection efficiency by using PEI in DBTRG-05M cell**.PEI mixed pCMV-hrGFP (4 μg DNA, 24 μl/5 μM PEI) to transfect in DBTRG-05M cell, the transfection efficiency is 10.20 %. **(F) Transfection efficiency by using PEI in HepG2 cell**. PEI mixed pCMV-hrGFP (4 μg DNA, 18 μl/5 μM PEI) to transfect inHepG2 cell, the transfection efficiency is 1.94 %.

Transfection efficiency = %Gate NC-% Gate pCMV-hrGFP NC is no transfection

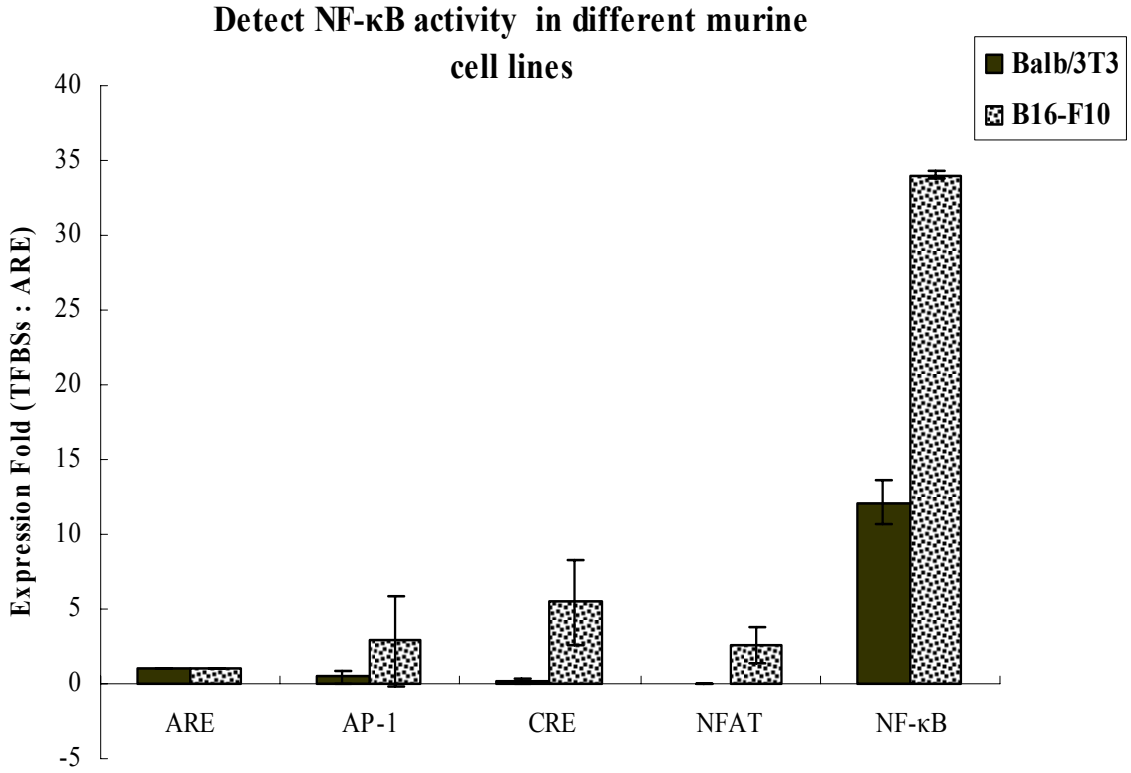


Figure 9 To detect NF-κB activity in Balb/3T3 and B16-F10 cells

Four eukaryotic TFBSs (AP-1, CRE, NFAT, and NF-κB) and one prokaryotic TFBS (ARE) detect them activities. ARE site is as control group for transfection efficiency, because eukaryotic cells have no factors can bind on it and then express gene. pNF-κB-hrGFP and other vectors of expression fold were calculate by the following formura:

$$\text{Expression fold} = \frac{\text{TFBSs-hrGFP}}{\text{CMV-AsRed X ARE-hrGFP}}$$

Under expression fold of pARE-hrGFP is 1, to compare Balb/3T3 with B16F10 in gene expression of different plasmids.

P.S.: Co-transfection weight ratio: TFBSs-hrGFP: AsRed= 3:1

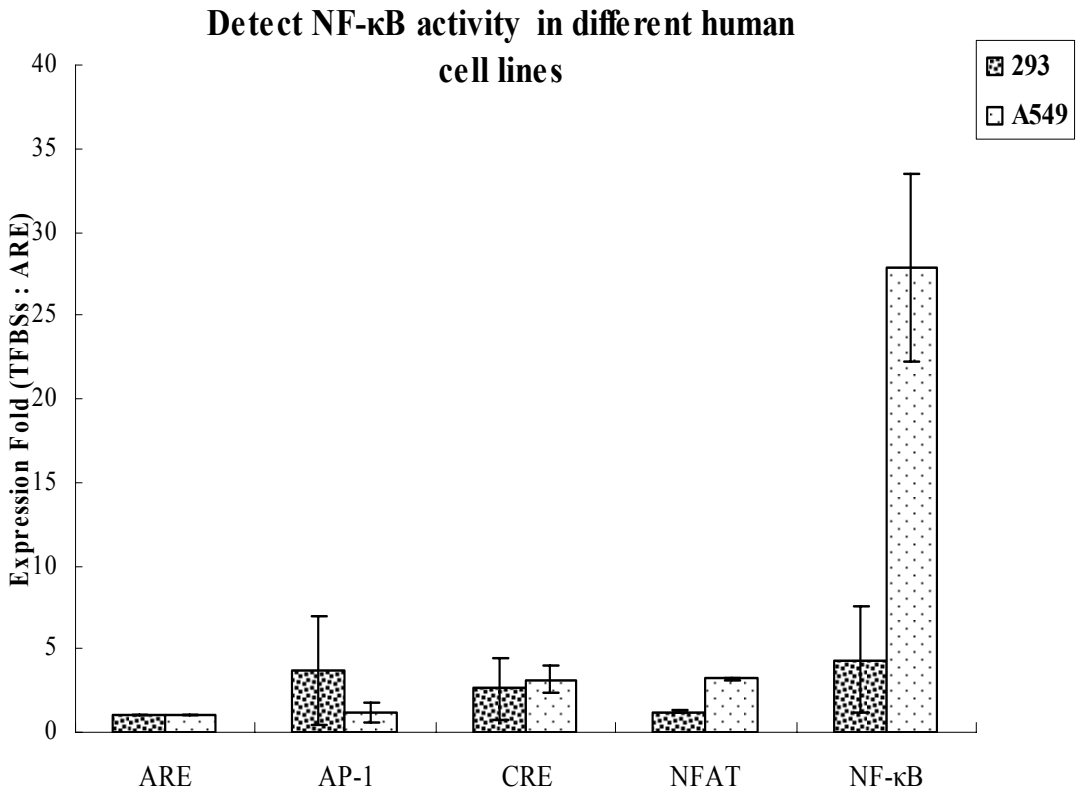


Figure 10 To detect NF-κB activity in 293 and A549 cells

Four eukaryotic TFBSs (AP-1, CRE, NFAT, and NF-κB) and one prokaryotic TFBS (ARE) detect them activities. ARE site is as control group for transfection efficiency, because eukaryotic cells have no factors can bind on it and then express gene. pNF-κB-hrGFP and other vectors of expression fold were calculate by the following formura:

$$\text{Expression fold} = \frac{\text{TFBSs-hrGFP}}{\text{CMV-AsRed X ARE-hrGFP}}$$

Under expression fold of pARE-hrGFP is 1, to compare A549 with 293 in gene expression of different plasmids.

P.S.: Co-transfection weight ratio: TFBSs-hrGFP: AsRed= 3:1

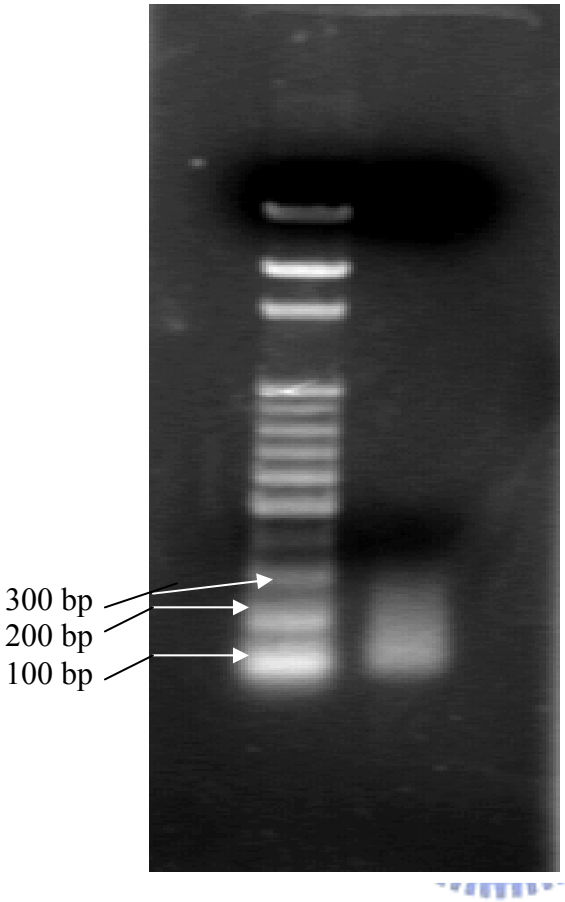


Figure 11 The joint size of short binding sites assembling

After random assembly of NF-κB, CRE, MEF-2 and Sp-1 sites, cleaned up and observed by DNA electrophoresis.

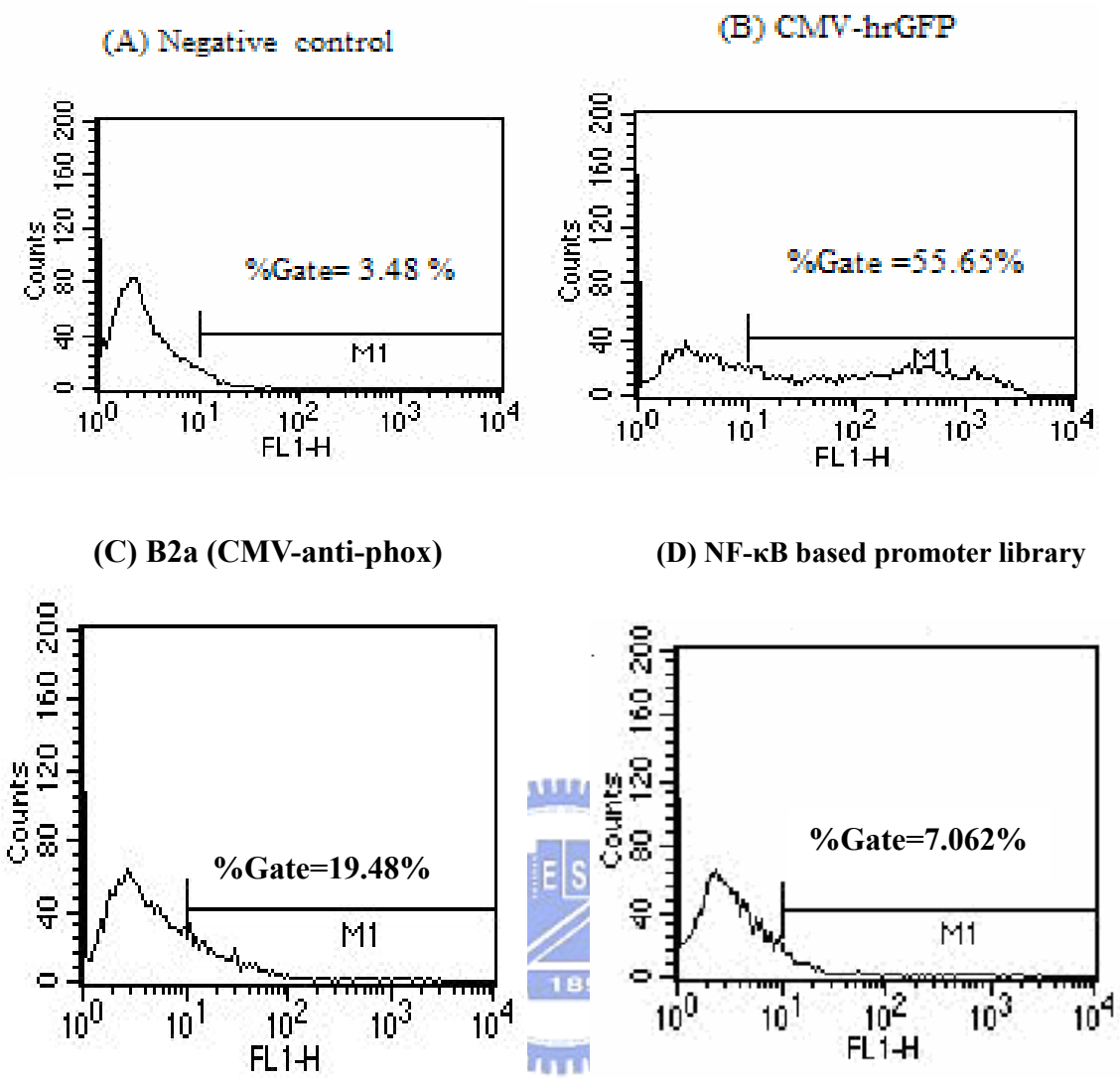
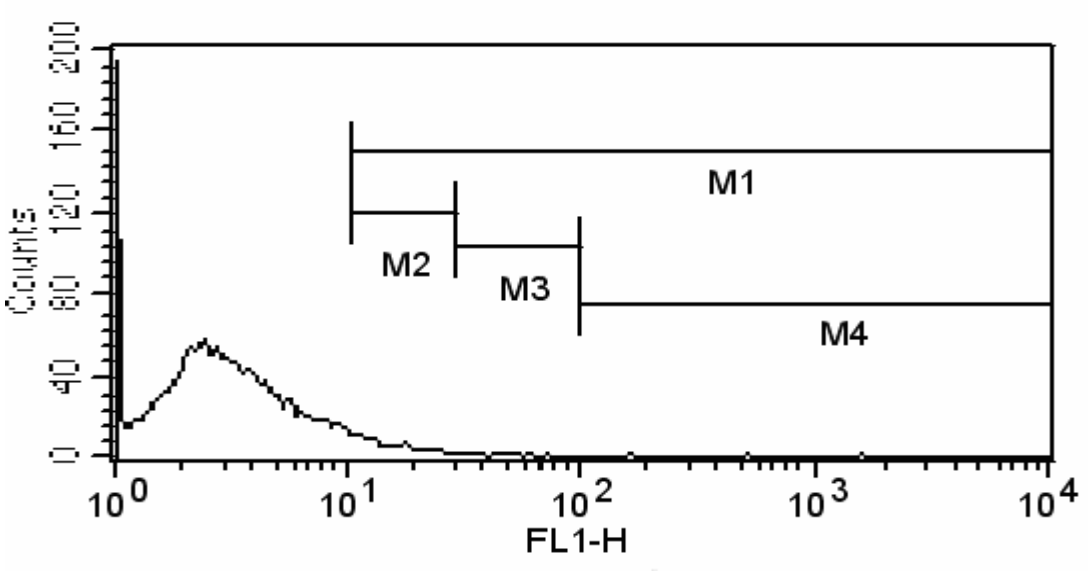


Figure 12 Analysis gene expression of original promoter library

(A) Negative control, no transfection. **(B) pCMV-hrGFP**, positive control. Be able to know the transfection efficacy. **(C) B2a**, positive control. Be able to know stain BSA-phOx-FITC is success or not. **(D) Original Promoter library**, no treatment or separation.

Gene expression of original promoter library



NF-κB based promoter library

Marker	Events	% Gated	% Tota	Mean	Mx/M1 (%.X=2~4)
All	8430	100.00	36.37	8.17	
M1	595	7.06	2.57	72.27	
M2	475	5.63	2.05	14.73	79.75
M3	65	0.77	0.28	48.75	10.91
M4	56	0.66	0.24	588.07	9.35

In overall regions, cell counts ratio in M2, M3, and M4.

In overall FL1-H >10^1 regions, cell counts ratio in M2, M3, and M4.

Figure 13 Analysis of original promoter library in different fluorescence intensity

The fluorescent region (M1) was divided to three groups: M2 (FLH-1<$3x10^1$) is low expression region. M3 (FLH-1=$3x10^1$ ~ $1x10^2$) is medium expression region. M4 (FLH-1>$1x10^2$) is high expression region. To analyze different regions cell ratio in overall expression region (M1).

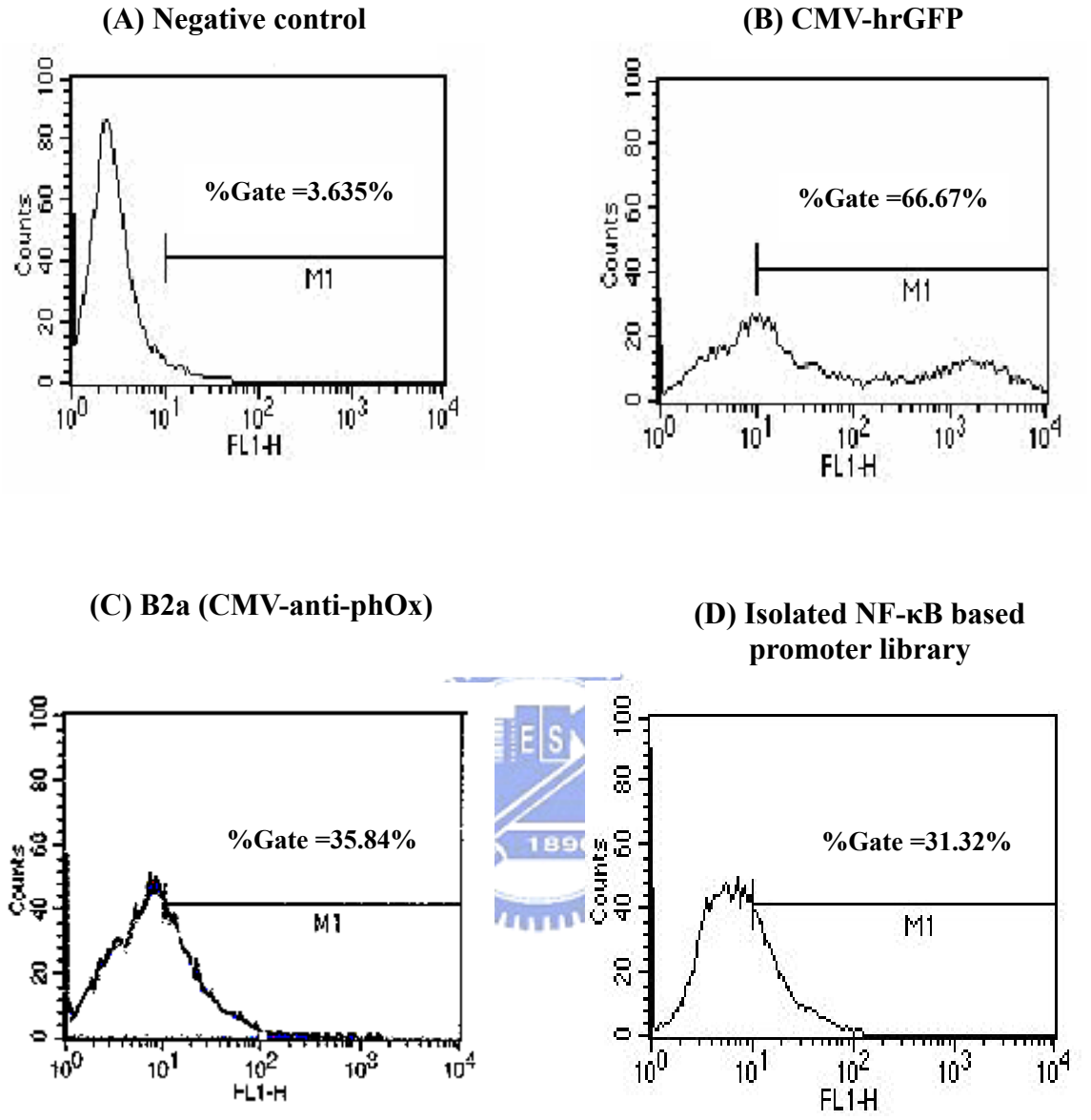
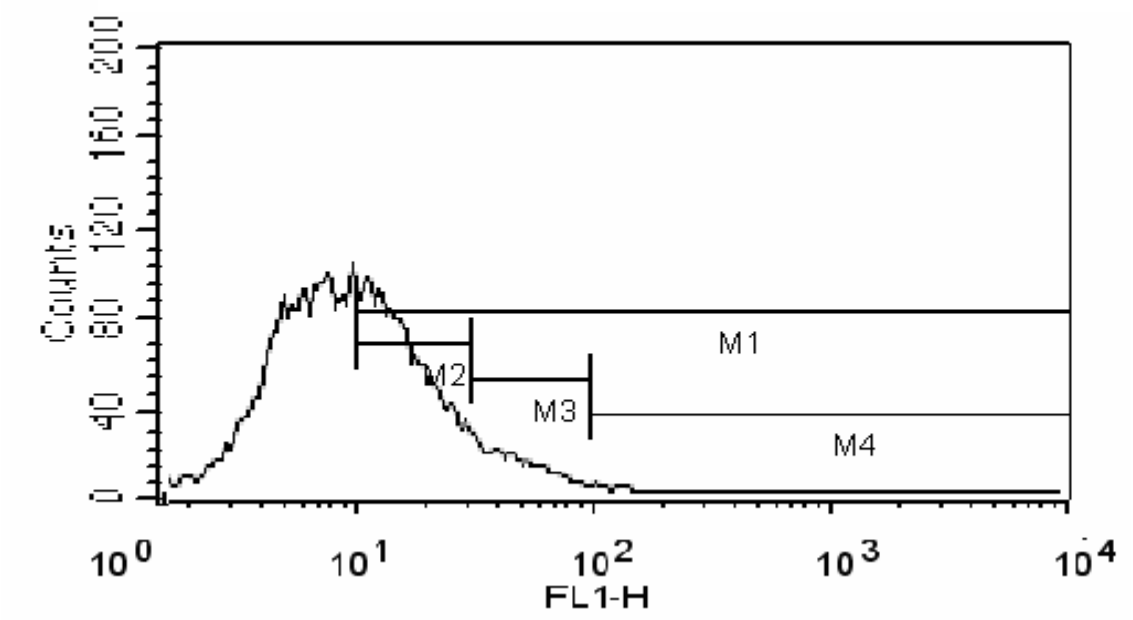


Figure 14 Analysis gene expression of isolated promoter library

(A) Negative control, no transfection. **(B) pCMV-hrGFP**, positive control. Be able to know our transfection efficicay. **(C) B2a**, positive control. Be able to know stain BSA-phOx-FITC is success or not. **(D) Isolated promoter library**, after magnetic beads separation.

Gene expression of isolated NF-κB based promoter library



Isolated NF-κB based promoter library

Marker	Events	% Gated	% Total	Mean	Mx/M1 (%,X=2~4)
All	9538	100.00	68.01	10.27	
M1	2987	31.32	21.30	21.34	———
M2	2432	25.50	17.34	15.79	———
M3	503	5.27	3.59	45.14	81.42
M4	19	0.20	0.14	130.16	16.83
					0.64

In overall regions, cell counts ratio in M2, M3, and M4.

In overall FL1-H >10^1 regions, cell counts ratio in M2, M3, and M4.

Figure 15 Analysis of isolated promoter library in different fluorescence intensity

The fluorescent region (M1) was divided to three groups: M2 (FLH-1<$3x10^1$) is low expression region. M3 (FLH-1=$3x10^1$ ~ $1x10^2$) is medium expression region. M4 (FLH-1>$1x10^2$) is high expression region. To analyze different regions cell ratio in overall expression region (M1).

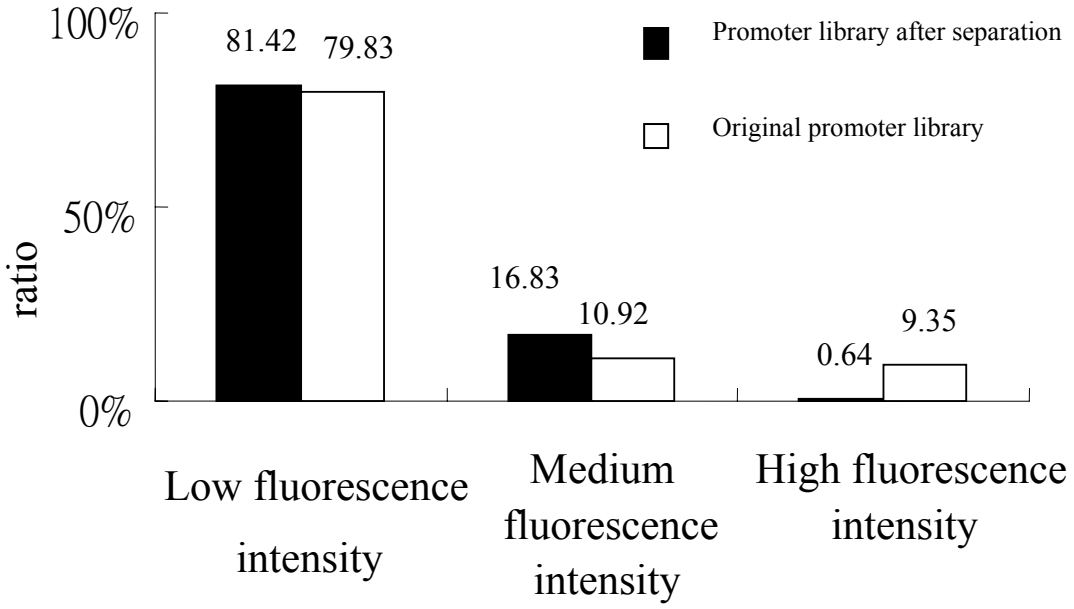


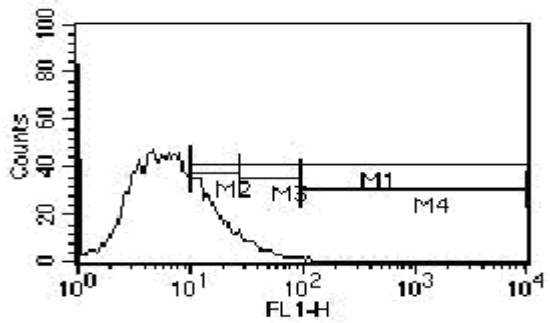
Figure 16 The comparison of between isolated and original promoters in the different fluorescence intensity

The comparison between isolated and original promoters had activities by calculating cell number ratio in different fluorescence intensity regions.

$$\text{Ratio} = \frac{\text{Cell number in different regions}}{\text{Cell number in overall expression region}}$$

Low fluorescence intensity, ratio=M2/M1; Medium fluorescence intensity, ratio=M3/M1; High fluorescence intensity, ratio=M4/M1

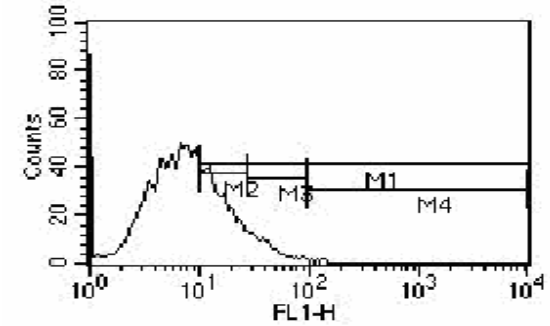
(A) Washing procedure



(A) Washing procedure

Marker	Events	% Gated	% Total	Mean
All	9404	100.00	61.11	9.65
M1	2772	29.48	18.01	20.66
M2	2315	▶ 24.62	15.04	15.58
M3	460	▶ 4.89	2.99	41.67
M4	15	▶ 0.16	0.10	167.49

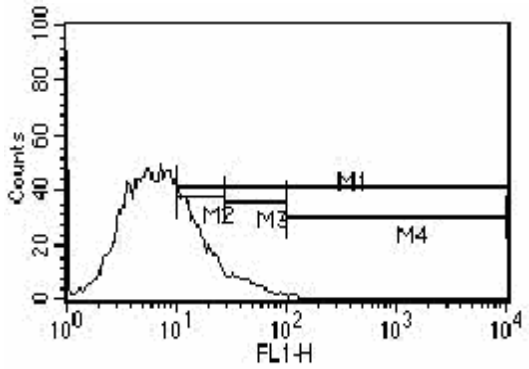
(B) Competing procedure



(B) Competing procedure

Marker	Events	% Gated	% Total	Mean
All	9468	100.00	66.19	11.41
M1	3440	36.33	24.05	21.91
M2	2801	▶ 29.58	19.58	15.73
M3	628	▶ 6.63	4.39	41.72
M4	29	▶ 0.31	0.20	193.60

(C) Eluting procedure



(C) Eluting procedure

Marker	Events	% Gated	% Total	Mean
All	9538	100.00	68.01	10.27
M1	2987	31.32	21.30	21.34
M2	2417	▶ 25.34	17.23	15.39
M3	567	▶ 5.94	4.04	43.39
M4	19	▶ 0.20	0.14	130.16

Figure 17 Three procedures of gene expression by MBSCS in Balb/3T3 cell

(A) Washing procedure gene expression. Plasmids from the first transfectants pass magnetic column directly were transfected into Balb/3T3 again. **(B) Competing procedure of gene expression.** Plasmids from washing transfectants by the competitor were transfected intoBalb/3T3 again. **(C) Eluting procedure of gene expression.** After removing magnetic column, plasmids were collected to transfect intoBalb/3T3 again. Three procedures in different fluorescent intensity regions (low, medium and high, see the definitions at p55-56) were analyzed and compared gene expression.

▶ The ratio of low (M2), medium (M3) and high (M4) expression regions to overall expression (FL1-H>10^1, M1)

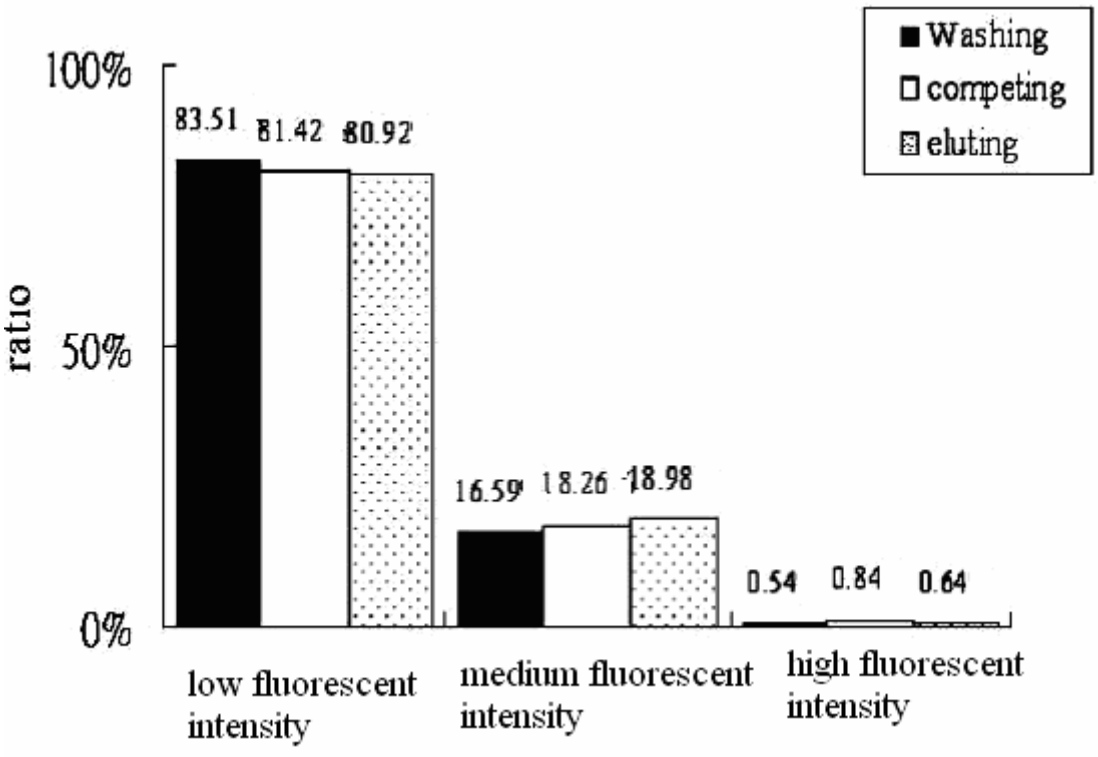


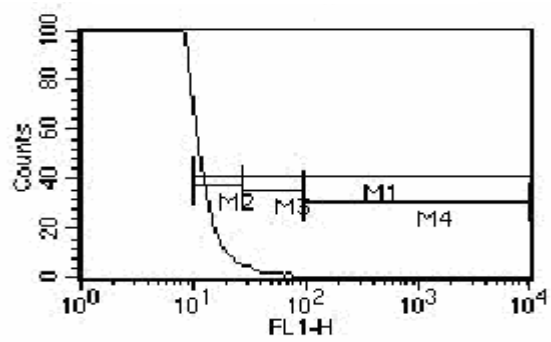
Figure 18 To compare three procedures of gene expression in different fluorescent regions in Balb/3T3 cell

The comparison between isolated promoters had activities by calculating cell number ratio in different fluorescence intensity regions.

$$\text{Ratio} = \frac{\text{Cell number in different regions}}{\text{Cell number in overall expression region}}$$

Low fluorescence intensity, ratio=M2/M1; Medium fluorescence intensity,ratio=M3/M1; High fluorescence intensity, ratio=M4/M1

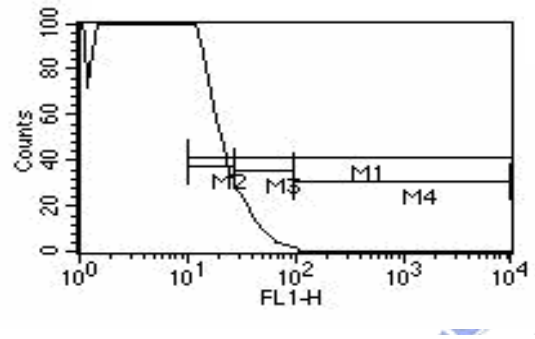
(A) Washing procedure



(A) Washing procedure

Marker	Events	% Gated	% Total	Mean
All	56470	100.00	10.24	3.67
M1	2483	4.40	0.45	16.14
M2	2305	▶ 4.08	0.42	13.81
M3	178	▶ 0.32	0.03	41.86
M4	8	▶ 0.01	0.00	136.40

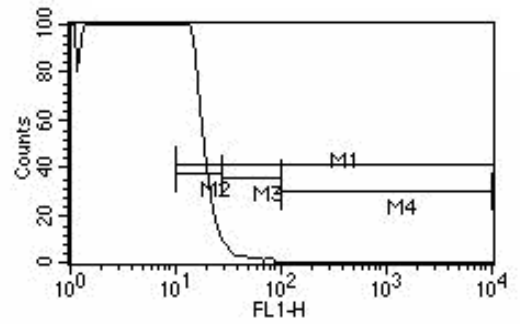
(B) Competing procedure



(B) Competing procedure

Marker	Events	% Gated	% Total	Mean
All	51096	100.00	7.03	6.34
M1	8730	17.09	1.20	19.50
M2	7459	▶ 14.60	1.03	15.45
M3	1302	▶ 2.55	0.18	40.10
M4	19	▶ 0.04	0.00	226.65

(C) Eluting procedure



(C) Eluting procedure

Marker	Events	% Gated	% Total	Mean
All	63037	100.00	14.03	5.34
M1	7738	12.28	1.72	17.11
M2	7467	▶ 11.85	1.66	14.05
M3	241	▶ 0.38	0.05	40.67
M4	47	▶ 0.07	0.01	386.24

Figure 19 Three procedures of gene expression by MBSCS in A549 cell

(A) Washing procedure gene expression. Plasmids from the first transfectants pass magnetic column directly were transfected into A549 again. **(B) Competing procedure.** Plasmids from washing transfectants by the competitor were transfected intoA549 again. **(C) Eluting procedure gene expression.** After removing magnetic column, plasmids were collected to transfect intoA549 again.

Three procedures in different fluorescent intensity regions (low, medium and high, see the definitions at p55-56) were analyzed and compared gene expression.

▶ The ratio of low (M2), medium (M3) and high (M4) expression regions to overall expression (FL1-H>10^1, M1)

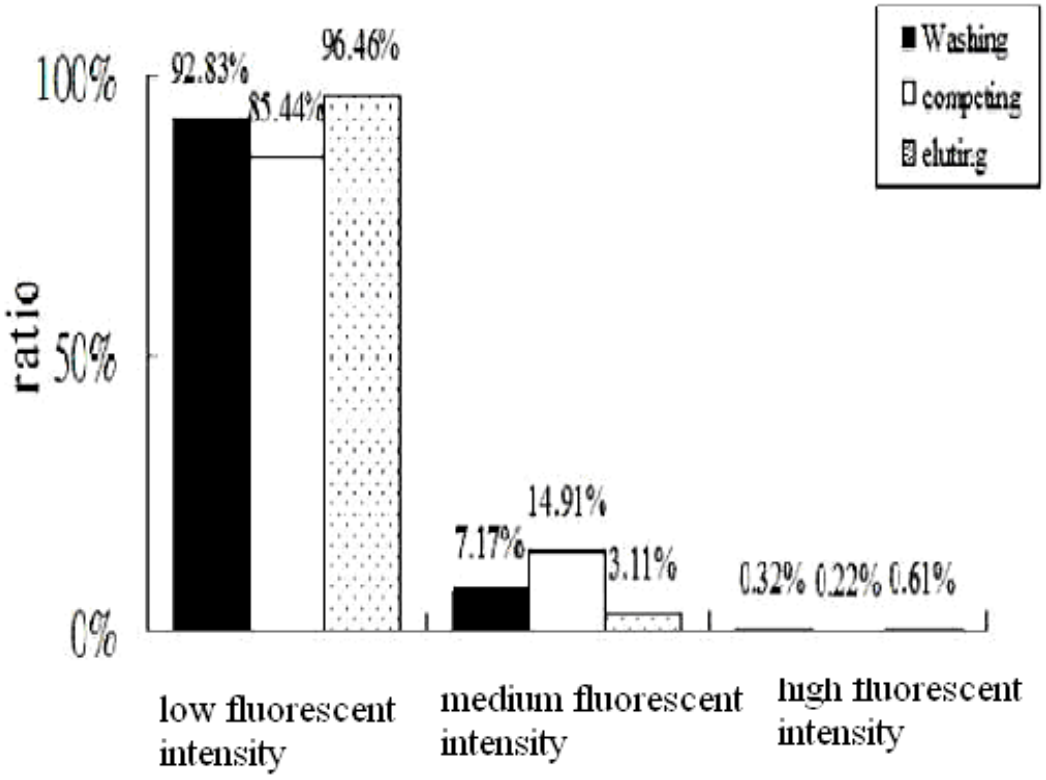


Figure 20 To compare three procedures of gene expression in different fluorescent regions in in A549 cell

We analyze and compare islated with original promoters by calculating cell number ratio in different fluorescence intensity regions.

$$\text{Ratio} = \frac{\text{Cell number in different regions}}{\text{Cell number in overall expression region}}$$

Low fluorescence intensity, ratio=M2/M1; Medium fluorescence intensity, ratio=M3/M1; High fluorescence intensity, ratio=M4/M1

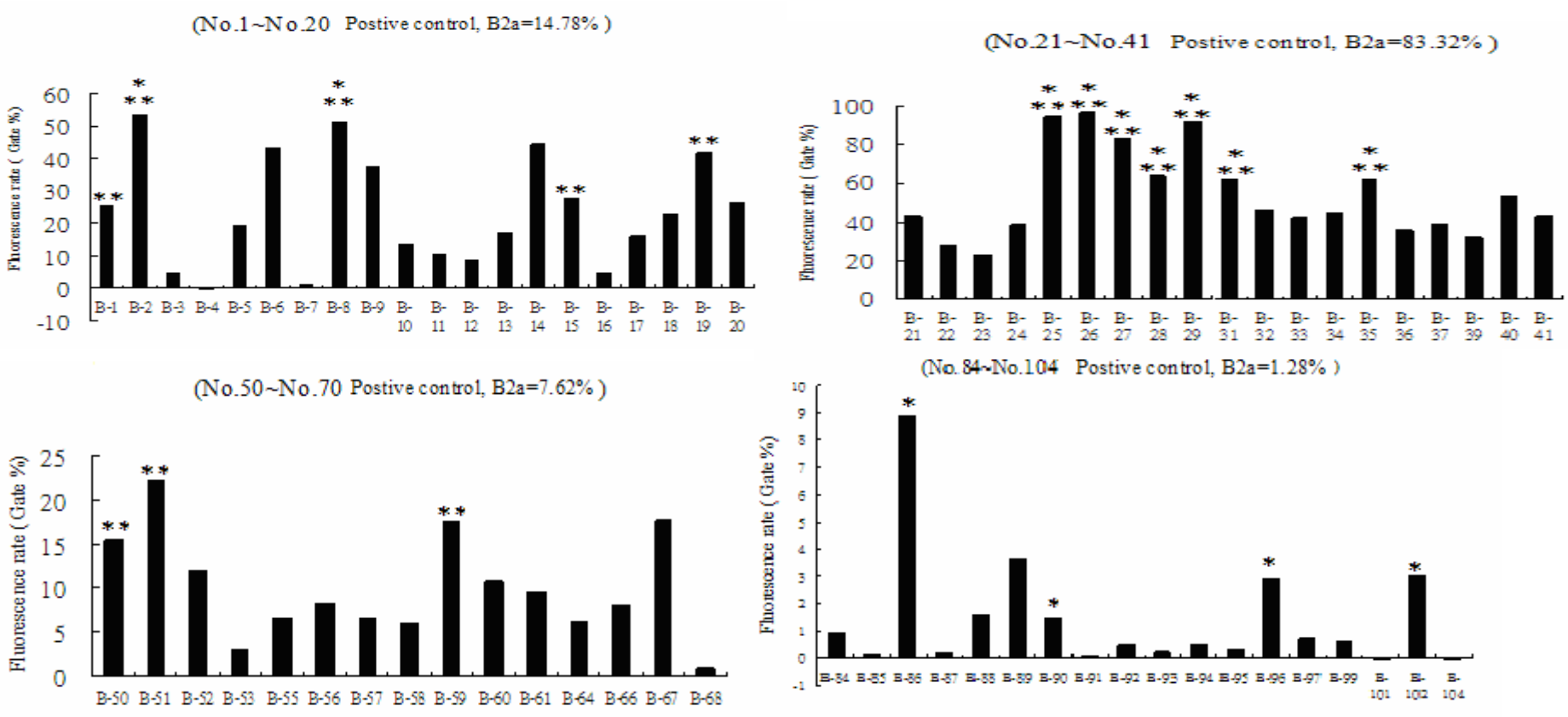


Figure 21 Primary select isolated promoter library from Balb/3T3 cell

＊＊＊ high expression promoters (50~100 % of gene expression) and my selection

＊＊ medium expression promoters (10~50% of gene expression) and my selection

＊ low expression promoters (~10% of gene expression) and my selection

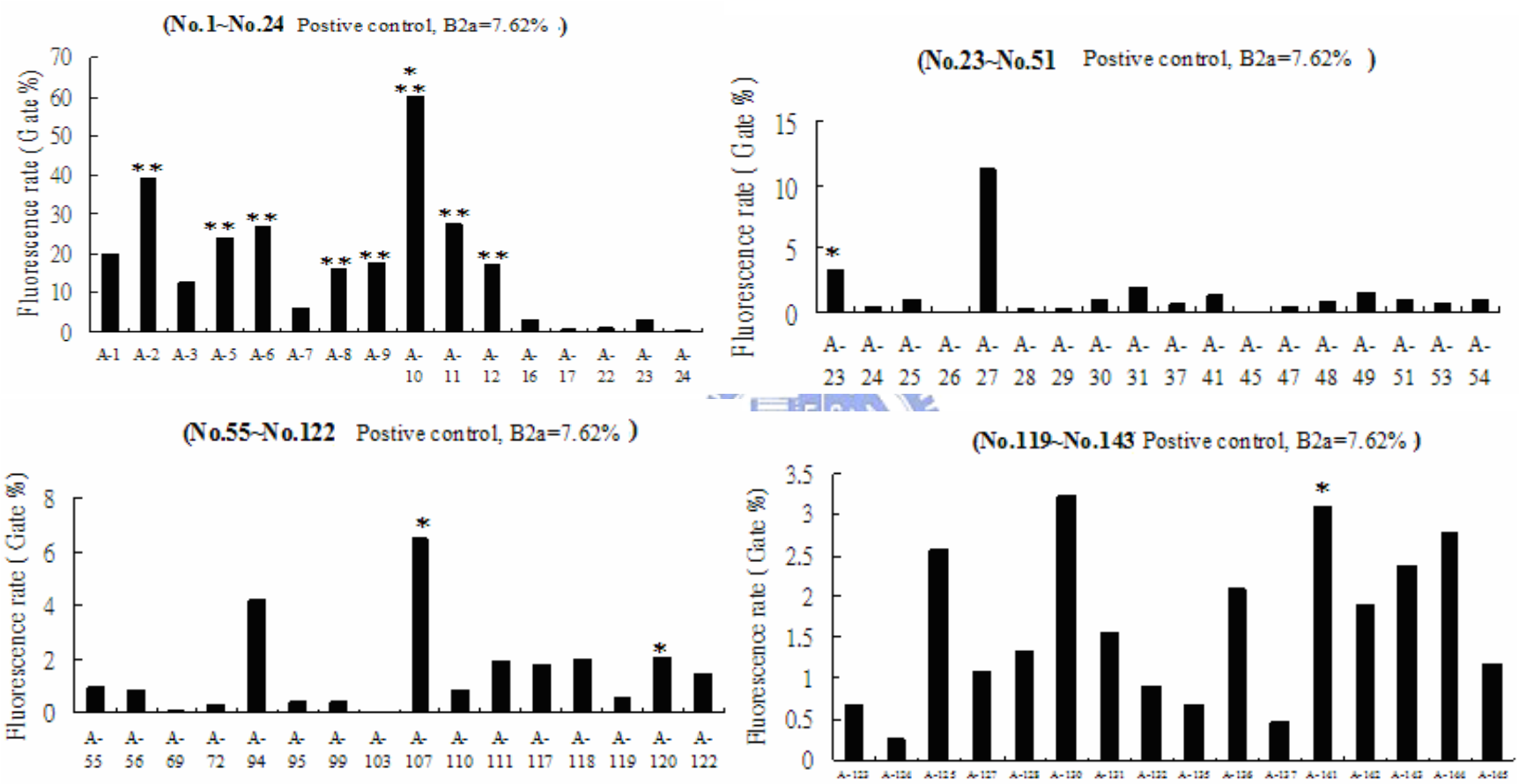


Figure 22 Primary select isolated promoter library from A549 cell

＊＊＊ high expression promoters (50~100 % of gene expression) and my selection

＊＊ medium expression promoters (10~50% of gene expression) and my selection

＊ low expression promoters (~10% of gene expression) and my selection

(A) Analysis of TFBSs in B-51 promoter by TFSEARCH web

```
 1 ACGCTCTAAA AATAACTCCC TGGGGACTTT CCGCTCTAGA CTCTGACGTC entry       score
                                        -------  M00041 CRE-BP 100.0
                                        -------  M00039 CREB   100.0
                                        <------  M00041 CRE-BP 100.0
                                        <------  M00039 CREB   100.0
    ---------------->                           M00006 MEF-2  98.6
                         ---------->            M00054 NF-kap 97.5
                         ---------->            M00053 c-Rel  95.9
                         ---------->            M00052 NF-kap 95.4
                        ----------->            M00208 NF-kap 95.1

51 AGGCTCGCCC CGCCCCCACA GCCTGACGTC AGAGGGGAGT TATTTTTAGA entry       score
   >                                            M00041 CRE-BP 100.0
   >                                            M00039 CREB   100.0
   -                                            M00041 CRE-BP 100.0
   -                                            M00039 CREB   100.0
                         -------->              M00041 CRE-BP 100.0
                         -------->              M00039 CREB   100.0
                         <--------              M00041 CRE-BP 100.0
                         <--------              M00039 CREB   100.0
                                  <-------------- M00006 MEF-2  98.6
                               ------->          M00083 MZF1   94.8
                                              - M00083 MZF1   94.8
       <----------                              M00008 Sp1    94.5

101 GCGGGGAGTT ATTTTTAGAG CGCGCTCTAA AAATAACTCC CCGAAAGCTT entry       score
   -                                            M00006 MEF-2  98.6
      <---------------                          M00006 MEF-2  98.6
                          ---------------->     M00006 MEF-2  98.6
   ------>                                      M00083 MZF1   94.8

151 GGTACCGAGC TCGGATCCAC TAGTAACGGC CGCCAGTGTG CTGGAATTCG entry       score
                          -------->             M00227 v-Myb  93.6

201 ACCCTCTACA CGCATGCAGA GGTAACCTAC ACGCATGCAG ATGGTCGA   entry       score
                                    ---------->  M00075 GATA-1 90.6
```

(http://www.cbrc.jp/research/db/TFSEARCH.html)

(B) The diagram of TFBSs in B-51 promoter

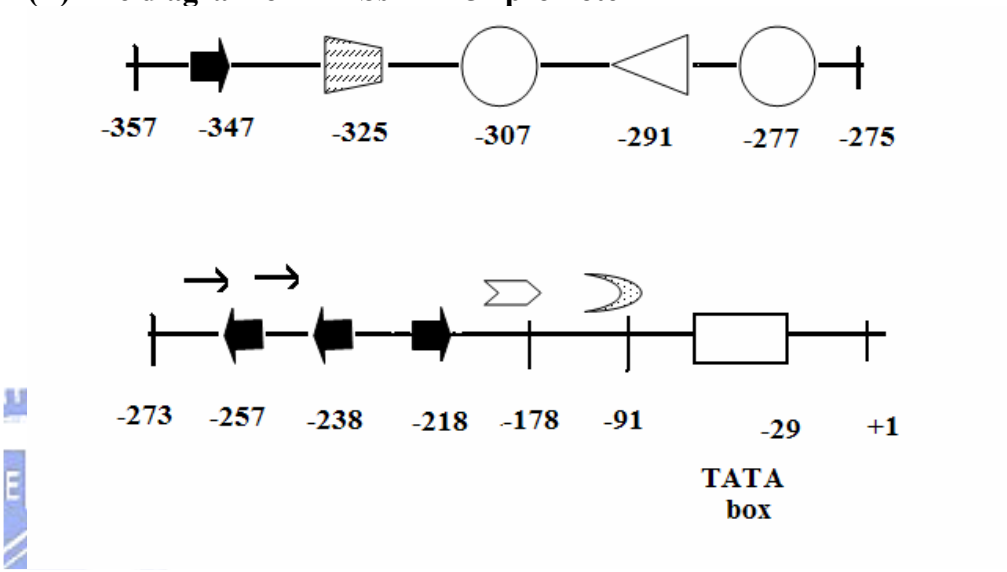


Figure 23 The full sequence of B-51 and its transcription factors binding sites

(A) Analysis of TFBSs in B-51 promoter by TFSEARCH web
(B) The diagram of TFBSs in B-51 promoter
B-51 promoter can find four MEF-2, one Sp1, one NF-κB and two CREB in my design In additation, it can found MZF-1 v-Myc and GATA -1

MEF-2 Sp1 CREB NF-κB
MZF-1 v-Myc GATA-1

B-2 vector

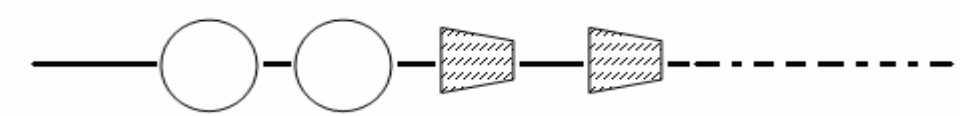
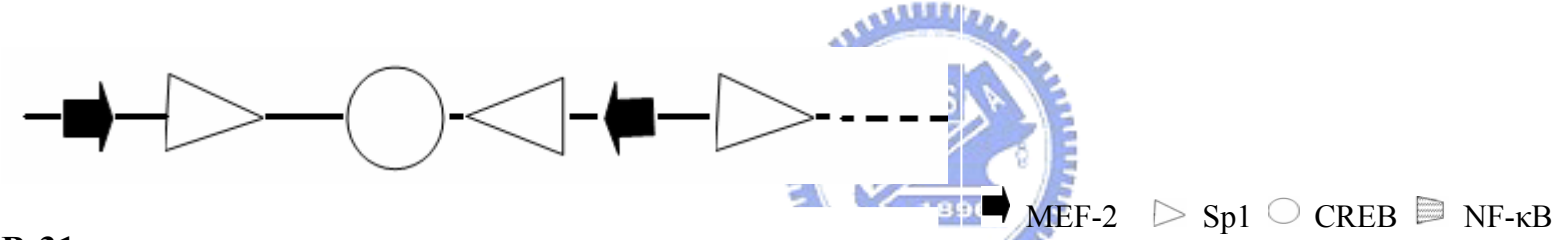
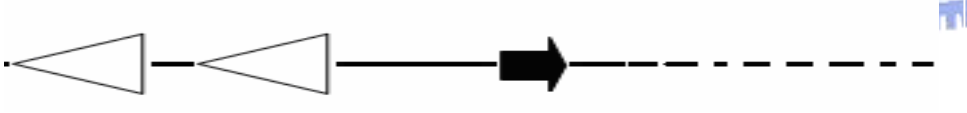


Figure 24 Partial sequences of B-2, B-6, b-35, B-28 and their transcription factors binding sites

B-6 vector



B-31



B-28

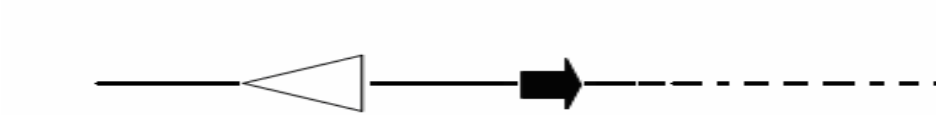


Table 1 The relationship between NF-κB and other transcription factors in regulatory gene expression

Promoter	Target TFBSs	Method	Gene Expression	Reference
MUC6 mucin promoter	One NF-κB site and two Sp1 sites	Deletion Mutation	no mutation : 100 % one SP-1 site mutation : 36% two SP-1 site mutation : 20% one NF-κB site and two SP1 sites mutation : 6.66 %	(Sakai, Jinawath et al. 2005)
NR1 promoter	One NF-κB site and one Sp1 site	Mutation and Transfect Sp1 vector	no mutation vs. no mutation+ Sp1 ----------enhance 3-fold mutation NF-κB site vs. mutation NF-κB site+ Sp1 ----------no effect mutation NF-κB site vs. mutation Sp1 site +transfect Sp1 ----------enhance 1.5-fold mutation NF-κB site and Sp1 site + Sp1 ----------enhance 0.8-fold	(Magari, Rivera et al. 1997)
IL-12 promoter include $(NF\text{-}\kappa B)_4$ sites	Fours NF-κB site	Transfect CBP vector	IL-12 promoter CBP 50 ng 1.4-fold 100 ng 1.8-fold 200 ng 1.7-fold	(Sheppard, Phelps et al. 1998)
Human Aβ H-J-J locus (encoding Aspartyl-β-Hydroxylase, Junctin, and Junctate) Putative TFBSs (-265 ~ +1 bp)	One NF-κB site and one MEF-2 site	Mutation	No mutation : 100 % Mutation E-box : 73.6 % MEF-2 box : 13.9 7%	(Feriotto, Finotti et al. 2005)

Table 2 Analysis gene expression of the promoters from primary select in Balb/3T3 cell

No.	Cell lines	Order	Mean	illustration
B-1	A549 / 293	16,10/ 17,22	19.87,20.74/27.31,13.42	
	B16F10 / Balb/3T3	22,5/22,9,18,16	18.45,18.07/14.07,12.61,31.02,11.08	
B-2	**A549 / 293**	**9,1/22,20**	**18.18,28.23/20.76,14.18**	tumor specific promoter
	B16F10 / Balb/3T3	**8,1/24,18,24,22**	**19.9,30.2/21.75,11.67,13.12,10.94**	
B-6	**A549 / 293**	**5,8/21,21**	**18.21,20.23/21.69,13.95**	A549 specific promoter
	B16F10 / Balb/3T3	**21,4/25,16,23,21**	**15.01,20.93/14.91,12.12,13.46,10.95**	
B-8	A549 / 293	15,2/nd,nd	14.85,28.65/nd,nd	
	B16F10 / Balb/3T3	nd,nd/23,7,21,19	nd,nd/15.51,14.83,16.37,11.54	
B-9	A549 / 293	13,4/20,19	15.32,27.16/21.9,28.12	
	B16F10 / Balb/3T3	20,9/21,4,25,15	14.9,13.16/16.45,17.56,22.85,12.9	
B-14	A549 / 293	18,7/18,18	13.6,19.86/23.75,32.91	
	B16F10 / Balb/3T3	23,24/18,22,19,14	15.52,12.73/42.56,20.5,20.51,10.97	
B-15	A549 / 293	21,3/19,17	15.21,28.3/22.17,45.17	
	B16F10 / Balb/3T3	24,3/20,12,20,24	14.31,17.85/31.68,12.94,20.17,12.54	
B-19	A549 / 293	2,9/16,16	23.46,20.1/44.44,71.86	
	B16F10 / Balb/3T3	16,2/19,13,17,20	15.48,15.63/56.19,14.38,26.18,11.15	
B-20	A549 / 293	14,15/nd,nd	28.19,19.84/nd,nd	

	B16F10 / Balb/3T3	12,7/17,21,3,17	16.91,18.12/178.55,13.09,147.45,12.21	
B-25	A549 / 293	1,24/11,9	24.18,14.85/84.17,335.15	
	B16-F10 / Balb/3T3	1,14/10,19,22,6	144.78,13.76/393.8,14.6,60.97,13.67	
B-26	A549 / 293	3,19/5,6	20.41,32.83/161.87,377.94	
	B16F10 / Balb/3T3	3,21/5,17,4,4	62.68,17.58/377.64,13.17,169.31,38.32	
B-27	A549 / 293	11,23/13,11	18.35,14.5/63.85,287.16	
	B16F10 / Balb/3T3	13,19/11,n.d,15,23	14.86,14.5/145.19,nd,36.1,13.52	
B-28	**A549 / 293**	**4,16/8,14**	**19.16,19.51/113.15,191.47**	Ubiquitous ,med ium expression promoter
	B16F10 / Balb/3T3	**6,20/16,n.d,6,12**	**24.17,35.97/286.06,nd,160.58,13.81**	
B-29	A549 / 293	10,17/10,13	19.78,17.47/94.13,244.35	
	B16F10 / Balb/3T3	18,18/6,23,12,25	14.42,12.61/158.49,16.42,67.35,14.28	
B-31	**A549 / 293**	**6,11/2,4**	**21.43,19.47/258.86,455.84**	Ubiquitous promoter
	B16F10 / Balb/3T3	**5,15/15,14,2,9**	**74.8,12.82/421.74,12.84,234.22,11.93,**	
B-35	**A549 / 293**	**7,6/1,7**	**19.07,21.33/275.47,369.07**	Ubiquitous promoter
	B16F10 / Balb/3T3	**2,6/4,20,1,2**	**22.51,15.54/625.3,12.51,223.92,151.43**	
B-46	A549 / 293	8,20/3,10	14.22,14.84/224.77,325.23	
	B16F10 / Balb/3T3	11,17/1,8,7,3	25.1,11.47/342.38,13.59,183.29,59.58	
B-47	A549 / 293	20,14/7,12	17.18,17.96/148.04,280.24	
	B16F10 / Balb/3T3	10,23/7,10,14,13	19.86,13.25/230.7,13.07,46.43,11.72	
B-50	A549 / 293	22,5/6,8	16.51,22.89/158.33,364.95	
	B16F10 / Balb/3T3	14,16/13,2,11,10	30.72,14.07/230.43,275.15,70.1,35.72	

B-51	**A549 / 293**	**19,12/9,2**	**14.94,18.24/96.64,509.75**	Ubiquitous promoter
	B16F10 / Balb/3T3	**7,13/9,1,8,11**	**28.24,12.99/514.73,367.85,111.53,17.98**	
B-59	A549 / 293	24,18/12,3	16.87,16.43/64.98,481.05	
	B16F10 / Balb/3T3	15,11/3,3,9,18	34.89,12.68/231.78,96.37,74.97,21.24	
B-86	A549 / 293	n.d,n.d/n.d,n.d	nd,nd/nd,nd	
	B16F10 / Balb/3T3	nd,nd//8,5,10,5	nd,nd/132.03,57.51,49.21,29.19	
B-90	A549 / 293	12,13/4,1	48.81,21.06/198.07,525.2	
	B16F10 / Balb/3T3	19,22/14,6,5,8	27.59,14/442.82,47.24,162.02,13.53	
B-96	A549 / 293	23,21/14,5	16.36,16.94/61.84,413.64	
	B16F10 / Balb/3T3	9,12/2,11,13,1	33.65,13.5/172.5,14,40.84,41.7	
B-102	A549 / 293	17,22/15,15	16.44,26.33/49.19,79.47	
	B16F10 / Balb/3T3	4,17,8,10/12,15,16,7	95.59,16.95,15.66,15.07/53.32,14.1,37.32,13.98	

This table shows promoters from Balb/3T3 selected gene expressive intensities. Gene expression intensities are in total mean level order. (n.d. = no detect, total mean= Gate x Mean)

Table 3 Analysis gene expression of the promoters from primary select in A549 cell

No.	Cell lines	order	Mean	illustration
A-2	**A549 / 293**	**2,1,9/10,5**	**44.49,16.42,59.44/650.97,1249.74**	Ubiquitous promoter
	B16F10 / Balb/3T3	**13,1/2,1**	**40.34,198.01/595.58,847.22**	
A-5	A549 / 293	13,10,2/12,14	17.2,29.98,19.34/381.44,520.5	
	B16F10 / Balb/3T3	8,3,8/12,12	35.32,15.26/129.19,145.18	
A-6	**A549 / 293**	**11,5,7/5,6**	**19.69,16.55,44.59/1517.46,982.04**	Ubiquitous promoter
	B16F10 / Balb/3T3	**11,3/3,4**	**39.23,105.82/129.19,335.88**	
A-8	A549 / 293	14,9,8/7,7	19.19,31.38,43.09/1376.1,950.31	
	B16F10 / Balb/3T3	12,4/9,7	36.08,67.26/295.41,155.87	
A-9	A549 / 293	12,3,4/14,3	19.79,15.71,16.48/54.85,1454.35	
	B16F10 / Balb/3T3	14,12/10,11	35.07,20.41/325.5,155.87	
A-10	A549 / 293	4,7,1/13,9	17.71,16.49,22.15/60.41,877.77	
	B16F10 / Balb/3T3	9,8/13,13	28.7,26.12/283.55,118.98	
A-11	A549 / 293	10,nd,6/6,8	19.05,nd,86.13/1454.42,889.89	
	B16F10 / Balb/3T3	2,7/8,6	28.9,31.35/324.05,250.86	
A-12	A549 / 293	8,6,nd/11,11	18.43,16.31,nd/445.65,796.78	
	B16F10 / Balb/3T3	1,14/11,14	21.08,9.87/27.57,54.94	
A-23	A549 / 293	5,nd,3/9,10	18.97,nd,88.55/1358.84,853.59	
	B16-F10 / Balb/3T3	3,9/7,9	26.52,24.71/250.2,184.67	

A-29	A549 / 293	9,4,11/4,10	18.47,15,97.46/2044.04,853.59	
	B16F10 / Balb/3T3	7,10/14,10	27.96,24.54/145.01,160.76	
A-107	A549 / 293	3,11,10/3,2	19.47,27.27,51.74/2519.82,1485.96	
	B16F10 / Balb/3T3	5,5/4,3	29.28,65.95/494.49,433.56	
A-111	**A549 / 293**	**7,12,13/2,4**	**27.75,58.31,179.86/2644.74,1381.51**	Ubiquitous promoter
	B16F10 / Balb/3T3	**10,6/1,2**	**39.92,32.24/550.32,480.47**	
A-120	A549 / 293	6,13,5/8,13	17.32,100.47,29.38/1363.28,777.35	
	B16F10 / Balb/3T3	6,2/6,5	35.26,122.36/349.23,270.74	

This table shows promoters from A549 selected gene expressive intensities. Gene expression intensities are in total mean level order. (n.d. = no detect, total mean= Gate x Mean)

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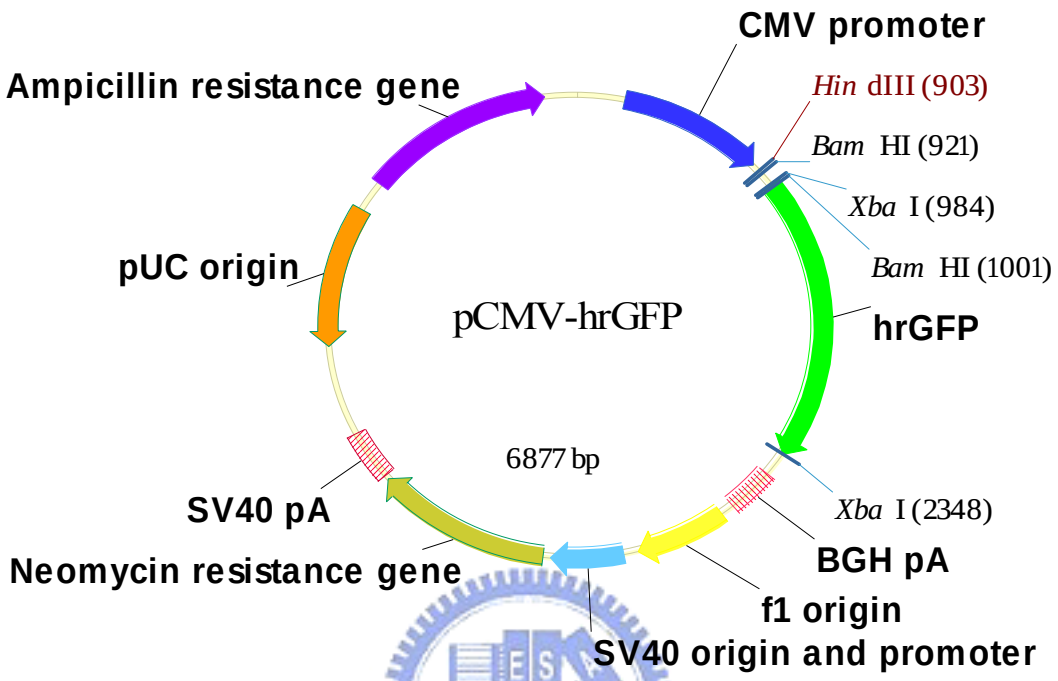
Appendix

A1. The map of pCMV-hrGFP



Plasmid backbone: pcDNA 3.1/V5-His-Topo
Bacterial resistance: Ampicillin (Invitrogen)
Promoter: CMV **Report gene:** hrGFP (PS: two TATA box, stop codon: before V5-His)
Sequencing Primer Locations:
•Forward primer : T7; pcDNA3.1-MCS-hrGFP
•Reverse primer : BGHR primer ; hrGFP-3' primer
•T7 / pcDNA3.1-MCS-hrGFP :
T7 5' primer: 5'-TAATACGACTCACTATAGGG-3'
pcDNA3.1-MCS-hrGFP 5' primer: 5'-CTC ATG AGC GGA TAC ATA TT-3'
•BGHR primer / hrGFP-3' primer:
BGH Reverse 3' primer: 5'-TAGAAGGCACAGTCGAGG-3'
hrGFP-3'(XbaI) 3' primer:
5'-AATCTAGAGAGTCCCGCTCAGAAGAACT-3'
Propagation in *E. coli*
• Suitable host strains:Top10 DH5α, HB101, and other general purpose strains.
• Selectable marker: plasmid confers resistance to ampicillin (100 mg/ml) to E. coli hosts.
• E. coli replication origin: pUC-derive origin
• Copy number: High
F1 origin: allow rescue of single-stranded DNA

A2. The sequence of pCMV-hrGFP

gacggatcgggagatctcccgatcccctatggtcgactctcagtacaatctgctctgatgccgcatagttaag
ccagtatctgctccctgcttgtgtgttggaggtcgctgagtagtgcgcgagcaaaatttaagctacaacaagg
caaggcttgaccgacaattgcatgaagaatctgcttagggttaggcgttttgcgctgcttcgcgatgtacggg
ccagatatacgcgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagccc
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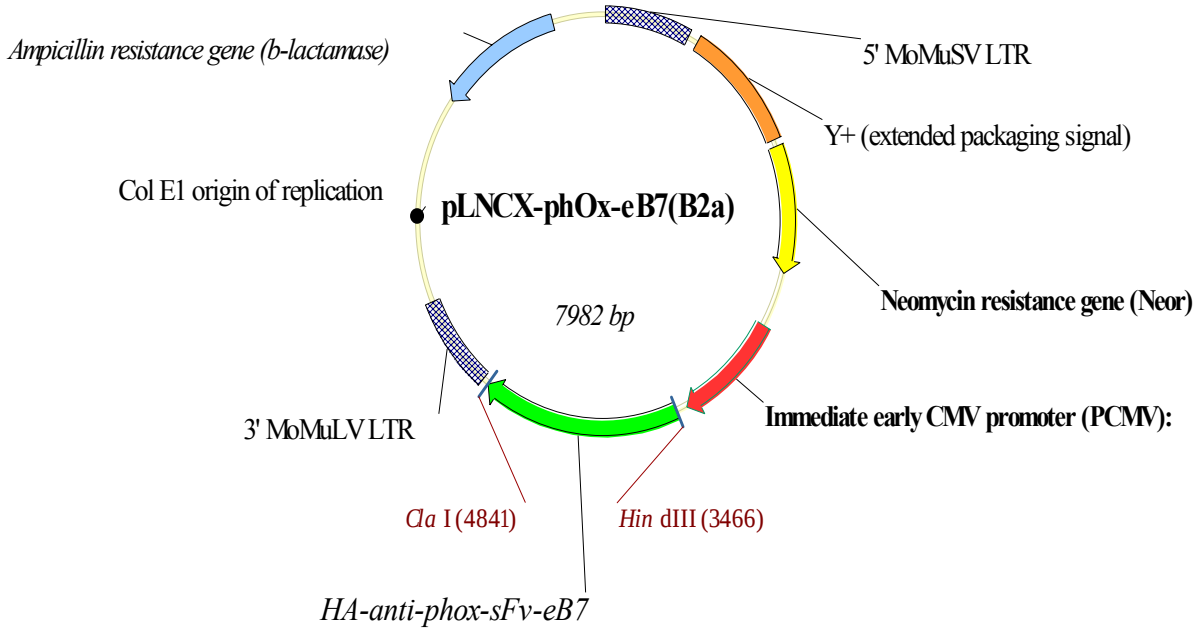
A3. The map of B2a



Plasmid backbone: pLNCX (BD) **Bacterial resistance:** Ampicillin

Promoter: CMV **Report gene:** anti-phox-eB7

Sequencing Primer Locations:

• pLNCX Seq/PCR Primers:

5' primer: 5'-AGCTCGTTTAGTGAACCGTCAGATCG-3'

3' primer: 5'-ACCTACAGGTGGGGTCTTTCATTCCC-3'

•phOx/B7:

5' primer: 5'-CCGCTCGAGATGGAGACAGACACACTC-3'

5' primer: 5'-TGCTCTAGACTAAAGGAAGACGGTCTG-3'

Propagation in E. coli

• Suitable host strains: DH5a, HB101, and other general purpose strains.

• Selectable marker: plasmid confers resistance to ampicillin (100 mg/ml) to E. coli hosts.

• E. coli replication origin: Col E1

• Copy number: low

Report gene: HA-anti-phox-eB7

Antibody Staining: (1)phOx-BSA-FITC (2)anti-HA (3)anti-B7

A4. The sequence of B2a

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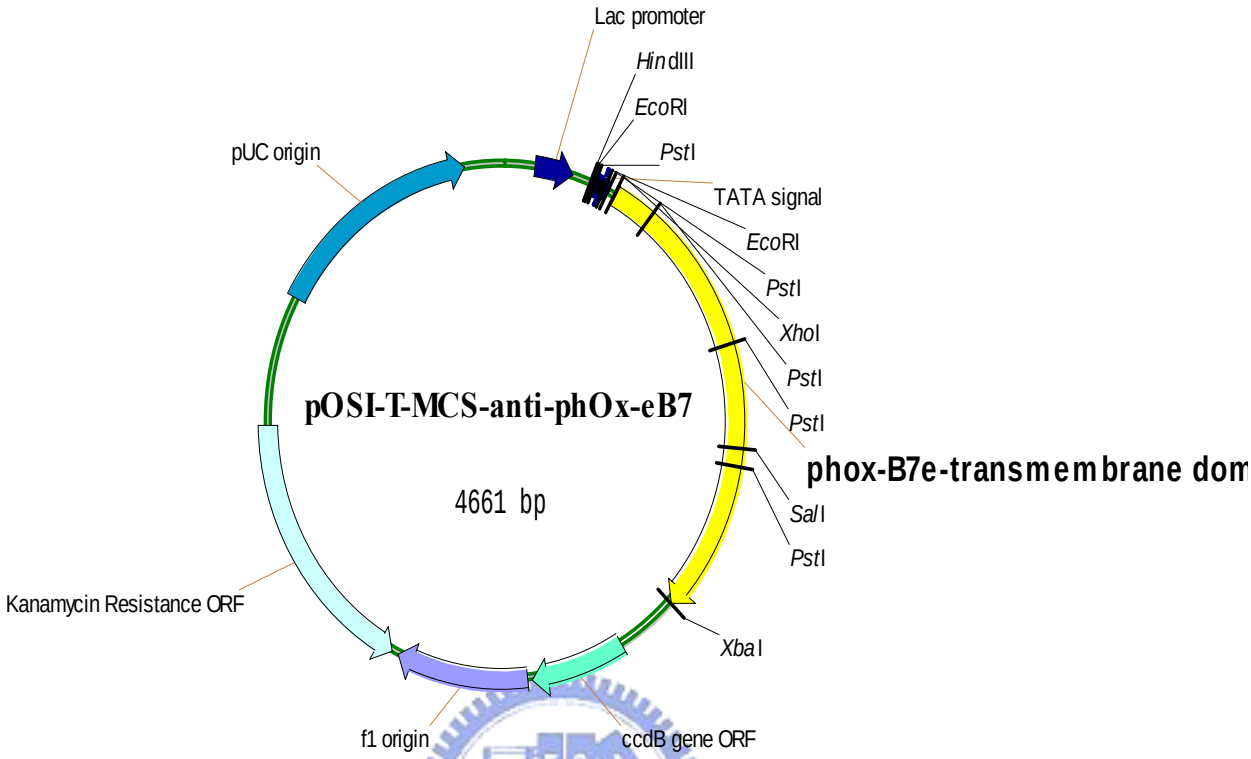
A5. The map of B16.4



Plasmid backbone: pOSI-T (Genemark) **Bacterial resistance:** kanamycin

Promoter: **Report gene:** anti-phox-eB7

Sequencing Primer Locations:

• pLNCX Seq/PCR Primers:

5' primer: 5'-AGCTCGTTTAGTGAACCGTCAGATCG-3'

3' primer: 5'-ACCTACAGGTGGGGTCTTTCATTCCC-3'

•phOx/B7:

5' primer: 5'-CCGCTCGAGATGGAGACAGACACACTC-3'

5' primer: 5'-TGCTCTAGACTAAAGGAAGACGGTCTG-3'

Propagation in E. coli

• Suitable host strains: DH5a, HB101, and other general purpose strains.

• Selectable marker: plasmid confers resistance to kanamycin (60 mg/ml) to E. coli hosts.

• E. coli replication origin: pUC

• Copy number: high

Report gene: HA-anti-phox-eB7

Antibody Staining: (1)phOx-BSA-FITC (2)anti-HA (3)anti-B7

A6. The sequence of B16.4

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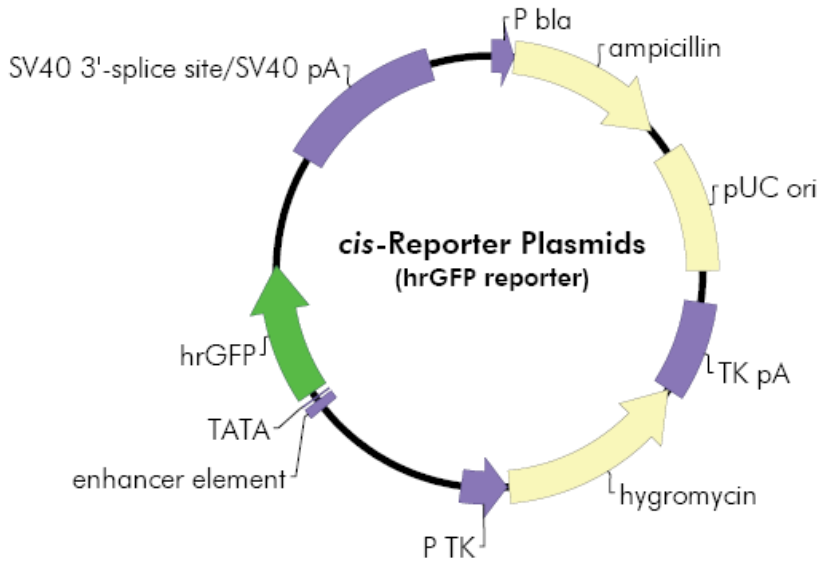
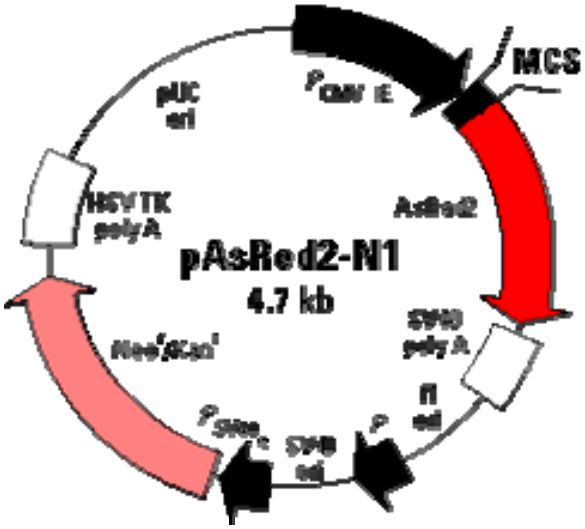
A7. The maps of pAP-1-hRGFP, pCRE-hrGFP, pNFAT-hrGFP and pNF-κB-hrGFP



cis-Reporter Plasmid	Enhancer element configuration	Enhancer element sequence
pAP-1-hrGFP	AP-1 (6×)	(**TGACTAA**)$_6$
pCRE-hrGFP	CRE (4×)	(AGCC**TGACGTCA**GAG)$_4$
pNFAT-hrGFP		(GGA**GGAAAAACTGTTTCA**TACAGAAGGCGT)$_4$
pNF-κB-hrGFP	NF-κB (5×)	(T**GGGGACTTTCC**GC)$_5$

http://www.stratagene.com/manuals/219073.pdf

A8. The map of pAsRed2-N1



http://www.clontech.com/clontech/techinfo/vectors/vectorsA-B/pAsRed2-n1.shtml