CHAPTER 2 Construction of hsnanog-pCS2+ and mnanog-pCS2+ and in vitro transcription of human *NANOG* and mouse *Nanog* mRNA

2.1 Introduction

Among various approaches that can be used to introduce mammalian *NANOG*/*Nanog* into zebrafish embryos, microinjecting *NANOG*/*Nanog* mRNA into the one-cell stage embryo is an efficient and convenient method to study the effects of exogenous *NANOG*/*Nanog* during the developmental time course and is therefore used in this thesis. In order to generate human *NANOG* and mouse *Nanog* mRNA by in vitro transcription, we subcloned human *NANOG* and mouse *Nanog* cDNA into the pCS2+ vector. Vectors pCS2 and pSP64T are generally used for in vitro transcription to generate stably functional mRNAs with 5'- termini GpppG cap and 3'-termini poly-A signal (Krieg and Melton, 1984; Melton et al., 1984). The pCS2+ vector contains a SP6 promoter, multiple cloning sites, and a poly-A signal, allowing RNA polymerase to synthesize messenger RNA of specific DNA template (Krieg and Melton, 1984; Melton et al., 1984; Wu and Alwine, 2004). In this chapter, human *NANOG* cDNA was amplified from the pEP4 E02S CK2M EN2L plasmid (Yu et al., 2009) and mouse *Nanog* cDNA was amplified from the pMXs-Nanog plasmid (Takahashi and Yamanaka, 2006). They were subcloned into the pCS2+ vector to produce constructs of hsnanog-pCS2+ and mnanog-pCS2+, which were subsequently transcribed *in vitro* to synthesize human *NANOG* and mouse *Nanog* mRNA.

2.2 Materials

Special Equipment

NanoDrop	ND-1000 Spectrophotomer
Electrocoating bath	Apollo Instrumentation
Peltier Thermal Cycler	MJ Research
Incubator	
Heat block	

Plasmids

Plasmids carrying human *NANOG* and mouse *Nanog* cDNA

Addgene plasmid 20924: pEP4 E02S CK2M EN2L (Yu et al., 2009)

Addgene plasmid 13354: pMXs-Nanog (Takahashi and Yamanaka, 2006)

The pEP4 E02S CK2M EN2L plasmid and a pMXs-Nanog colony plate were obtained from Wei Wang (Team 18, Wellcome Trust Sanger Institute).

pCS2+

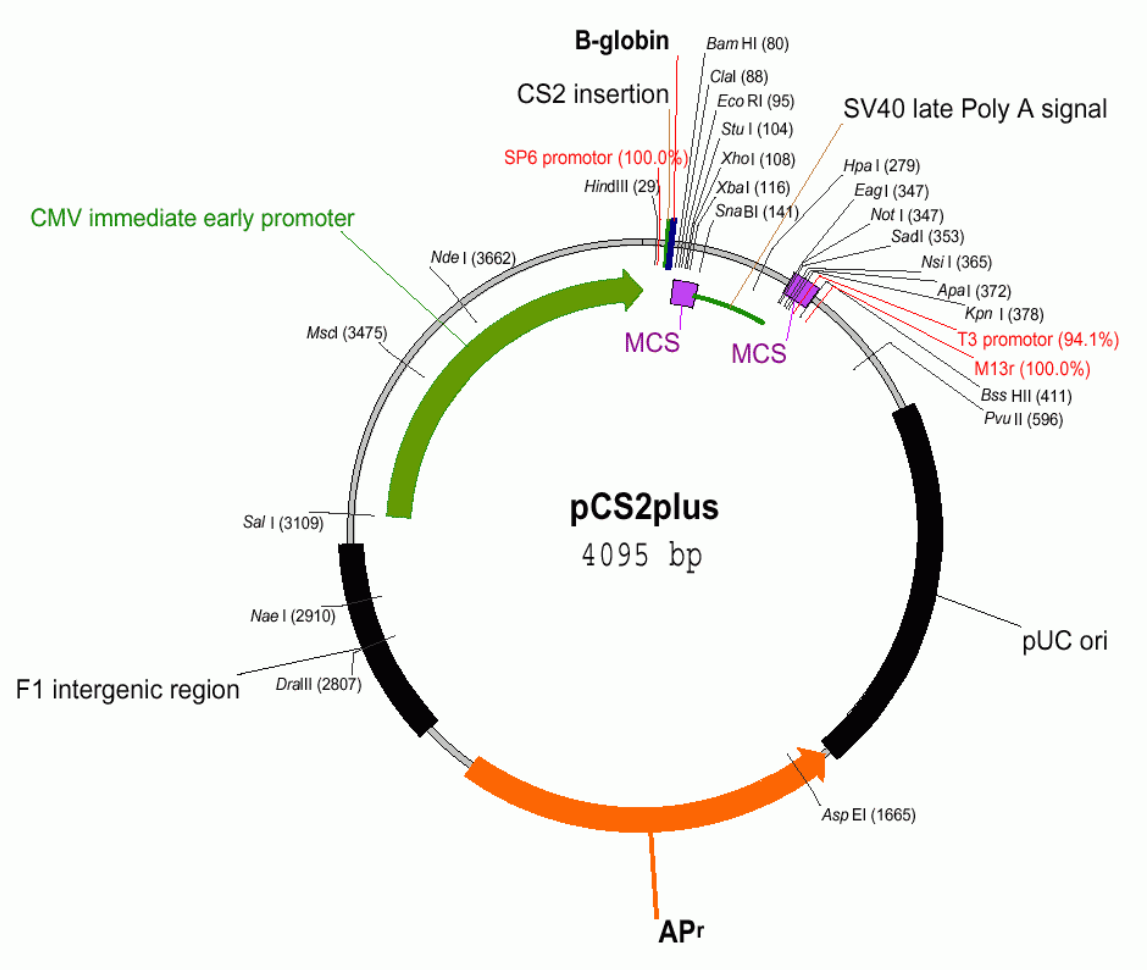

Figure 3. The pCS2+ Vector (www.imagenes-bio.de/info/vectors/pCS2plus.gif)

Recombinant Constructs

hsnanog-pCS2+

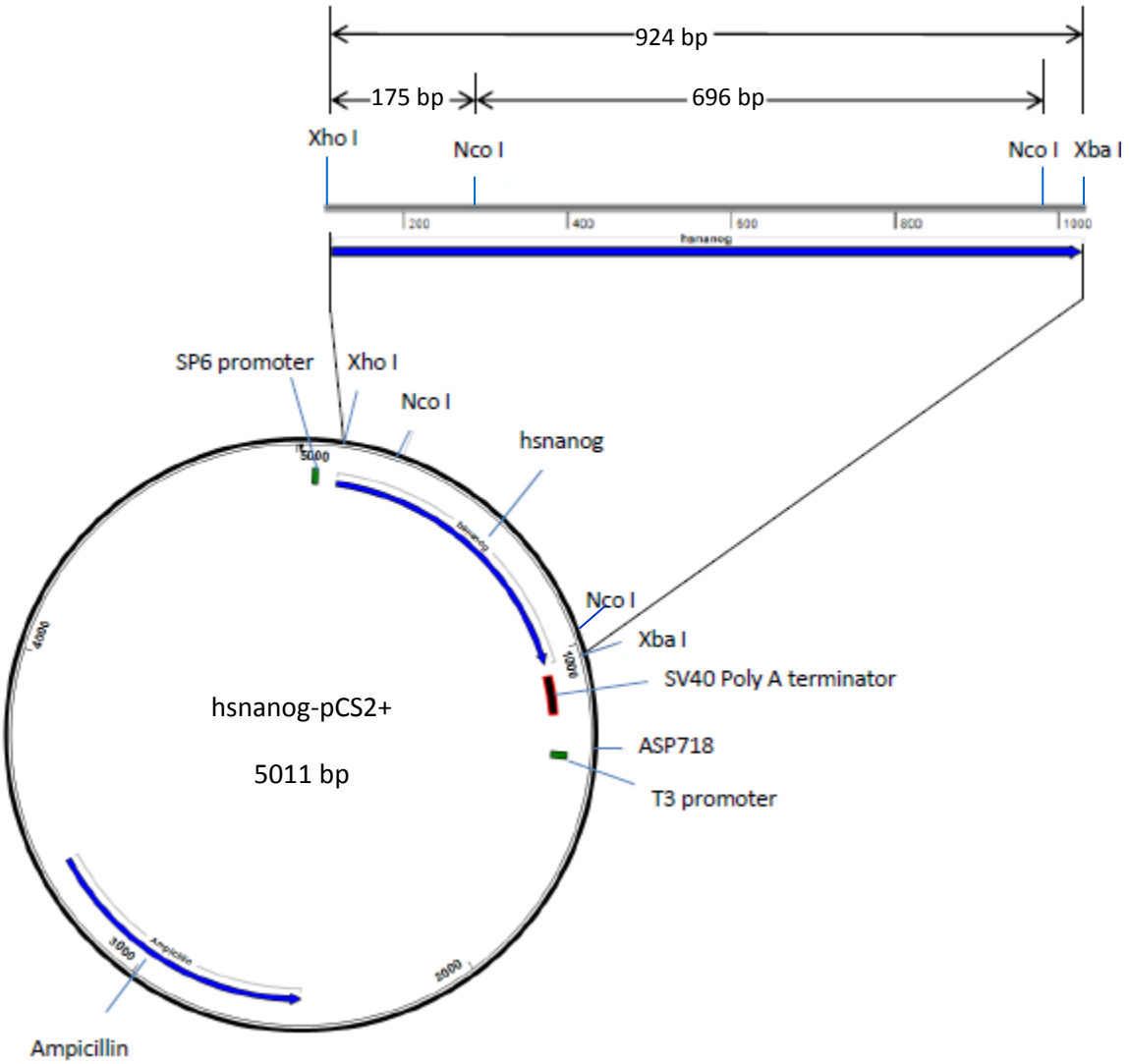

Figure 4. Recombinant construct hsnanog-pCS2+. Between restriction sites of Xho I and Xba I is the human *NANOG* cDNA insert, which is about 900 bp. Double Digestion of hsnanog-pCS2+ with Xho I and Nco I (New England Biolabs) generates three fragments, which are of approximately170 bp, 690 bp and 4,200 bp.

mnanog-pCS2+

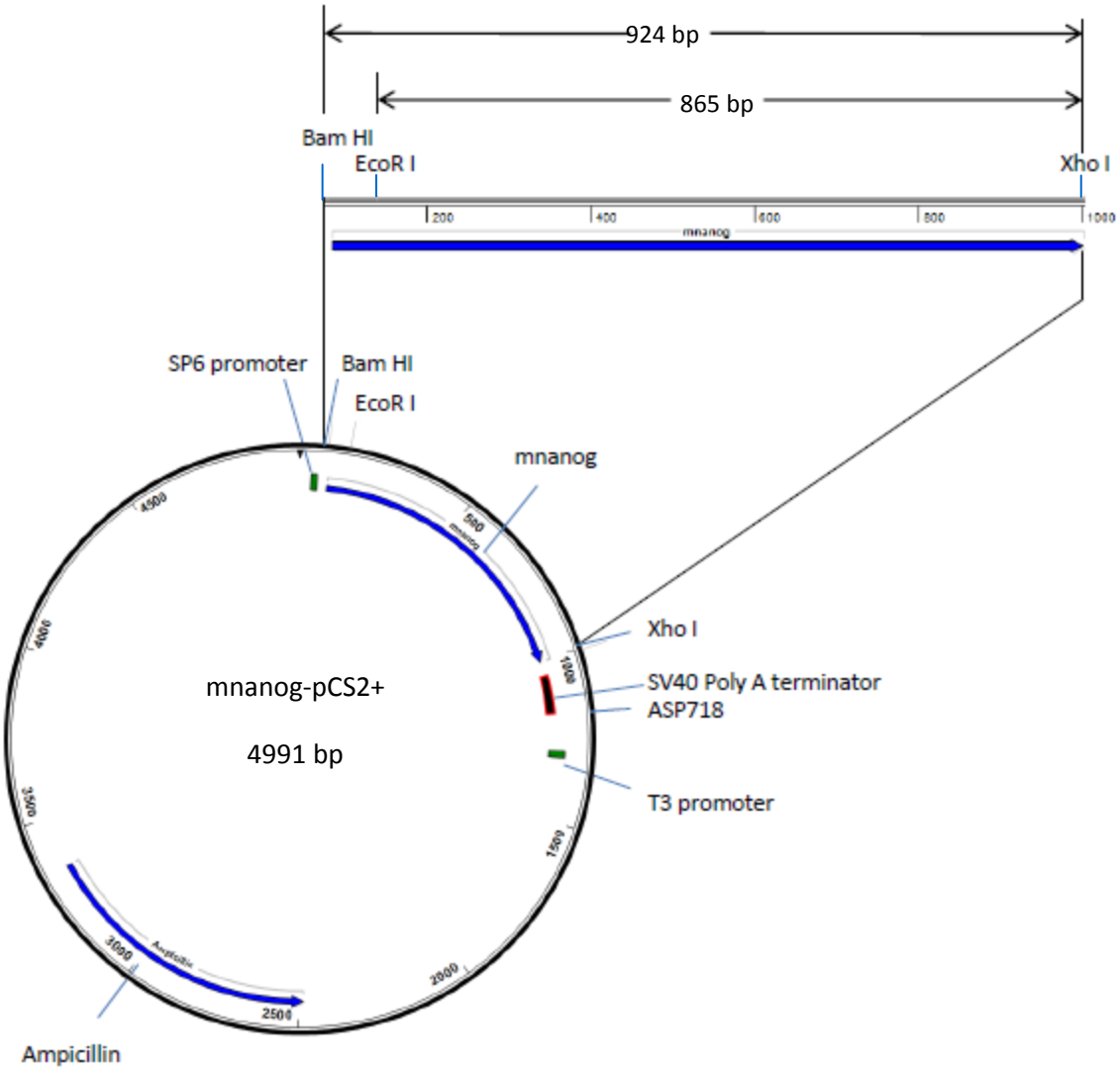

Figure 5. Recombinant construct mnanog-pCS2+. Between restriction sites of Bam HI and Xho I is the mouse *Nanog* cDNA insert, which is about 900 bp. Double Digestion of mnanog-pCS2+ with EcoR I and Xho I (New England Biolabs) generates two fragment, one of about 860 bp and the other of 4,100 bp.

Primers

Cloning Primers

Human *NANOG* forward primer 5’ GTCTCGAGATGAGTGTGGATCCAGCTTGTCC 3’

Human *NANOG* reverse primer 5’ CATCTAGATCACACGTCTTCAGGTTGCATG 3’

Mouse *Nanog* forward primer 5’ ATGGATCCATGAGTGTGGGTCTTCCTGGTCC 3’

Mouse *Nanog* reverse primer 5’CACTCGAGTCATATTTCACCTGGTGGAGTC 3’

Sequencing Primers

Sequencing Primers of hsnanog-pCS2+

Forward primer Sp6 promoter 5’ ATTTAGGTGACACTAT 3’

Reverse primer T3 promoter 5’ GGGAAATCACTCCCAATTAAC 3’

Sequencing Primers of mnanog-pCS2+

Forward primers Sp6 promoter 5’ ATTTAGGTGACACTAT 3’

5' GCTGACAAGGGCCCTGAGGAG 3'

5' ACGGCCAGCCTTGGAATGCTG 3'

Reverse primers T3 promoter 5’ GGGAAATCACTCCCAATTAAC 3’

5'CATTCCAAGGCTGGCCGTTCC 3'

Kits, enzymes and chemicals

KOD Hot Start PCR Kit	Novagen
QIAquick PCR Purification Kit	Qiagen
QIAquick Gel Extraction Kit	Qiagen
QIAprep Miniprep Kit	Qiagen
Restriction enzymes BstX I, Xho I, Xba I, Nco I, Bam HI, and EcoR I	NEB
Restriction enzyme ASP718	Roche
Quick T4 DNA Ligase	NEB
T4 DNA Ligase	NEB
SP6 RNAPolymerase	NEB
DNase I (RNase-free)	Roche
NEBuffer 3, NEBuffer 4 andNEB EcoR I Buffer	NEB
2X Quick Ligase Buffer	NEB
10X Ligase Buffer	NEB
SURE/Cut Buffer for Restriction Enzyme ASP718	Roche
10X NEB RNA Pol reaction Buffer	NEB
100X BSA	NEB
RNase Inhibitor	NEB
DTT	Promega

rATP, rCTP, rUTP, rGTP and Cap analogue	Promega
Ultra Pure™ Agarose	Invitrogen
DNA marker HyperLadder I and HyperLadder IV	Bioline
Ethidium bromide 10 mg/ml	Sigma
Ampicillin	
LB	
TAE	

Other materials include

CHROMA SPIN™ Columns	Clontech
One Shot TOP 10 Chemically Competent *E.coli*	Invitrogen
Amp Agar Plate	

Software

Lasergene (version 8, DNAStar)

2.3 Methods

Construction of hsnanog-pCS2+

Primers were designed to amplify human *NANOG* cDNA from pEP4 E02S CK2M EN2L (Yu et al., 2009) (Materials and Methods Appendix). The PCR product was purified following the QIAquick PCR Purification protocol. Human *NANOG* and pCS2+ were double digested with both Xho I and Xba I, and ligated, generating hsnanog-pCS2+ (Materials and Methods Appendix). Ligation product was used to transform chemically competent *E.coli* following a standard protocol of chemical transformation (One Shot TOP10 Chemically Competent *E.coli* guideline). Six hsnanog-pCS2+ colonies were picked and plasmids were extracted following a standard minipreparation protocol (QIAprep Miniprep Handbook). Diagnostic digest with Xho I and Nco I as well as sequencing were performed to confirm whether the recombinant constructs were correct.

In vitro transcription of human *NANOG* mRNA

Construct hsnanog-pCS2+ was linearized by ASP718 (Materials and Methods Appendix). Human *NANOG* mRNA was generated following a standard protocol of in vitro transcription (Materials and Methods Appendix).

Construction of mnanog-pCS2+

Plasmid pMXS-Nanog was extracted following a standard protocol of minipreparation (QIAprep Miniprep Handbook). Digestion with BstXI was performed to test whether the plasmid pMXS-Nanog was correct (Materials and Methods Appendix). PCR reaction was designed to amplify the mouse *Nanog* cDNA fragment from pMXS-Nanog (Takahashi and Yamanaka, 2006). Mouse *Nanog* and pCS2+ were double digested with both Bam HI and Xho I, ligated and mnanog-pCS2+ were generated (Materials and Methods Appendix). Ligation product was used to transform chemically competent *E.coli* following a standard protocol of chemical transformation (One Shot TOP10 Chemically Competent *E.coli* guideline). Ten mnanog-pCS2+ colonies were picked and plasmids were extracted following a standard protocol of minipreparation (QIAprep Miniprep Handbook). Diagnostic digest with EcoR I and Xho I as well as sequencing were performed to confirm whether the recombinant constructs were correct.

In vitro transcription of mouse *Nanog* mRNA

Construct mnanog-pCS2+ was linearized by ASP718. Mouse *Nanog* mRNA was generated following a standard protocol of in vitro transcription (Materials and Methods Appendix).

2.4 Results

Construction of hsnanog-pCS2+ and in vitro transcription of human *NANOG* mRNA

Correct hsnanog-pCS2+ constructs were made and human *NANOG* mRNA of good quality was generated. Figure 6 illustrates the human *NANOG* cDNA fragment of expected size, which is about 0.9 kb, amplified from pEP4 E02S CK2M EN2L (Yu et al., 2009). Human *NANOG* and pCS2+ were double digested with Xho I and Xba I (Figure 7). Concentration of double digestion products of human *NANOG* and pCS2+ were quantified to be 125.03 ng/µl and 14.19 ng/µl. Therefore the ratio of their volume in the ligation reaction system was decided to be 1: 6.5 (Materials and Methods Appendix). Six colonies were picked from the plate spread with transformed competent *E.coli*. Gel electrophoresis result shows that the six hsnanog-pCS2+ constructs are of good quality (Results Appendix, Figure S1). Double digestion results with Xho I and Nco I show that there are three bands of expected size, about 0.2 kb, 0.7 kb and 4.1 kb, suggesting that all the six hsnanog-pCS2+ clones have human *NANOG* cDNA within them (Figure 8). Sequencing results show that five of them are correct but the other one contain a mutation. Clone 3 was used to generate human *NANOG* mRNA in this chapter. Figure 9 illustrates complete linearization reaction and figure 10 shows RNA of good quality, with two bands of about 600 bp and 900 bp, which represent distribution of RNA conformations.

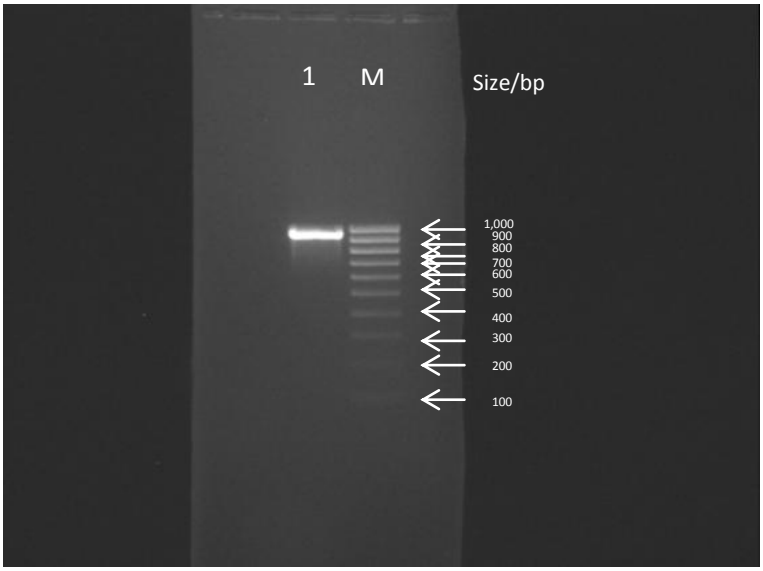

Figure 6. Gel electrophoresis of human *NANOG*

1, Human *NANOG* cDNA. M, Marker Hyperladder IV. In lane 1 is the human *NANOG* band of expected size, about 900 bp.

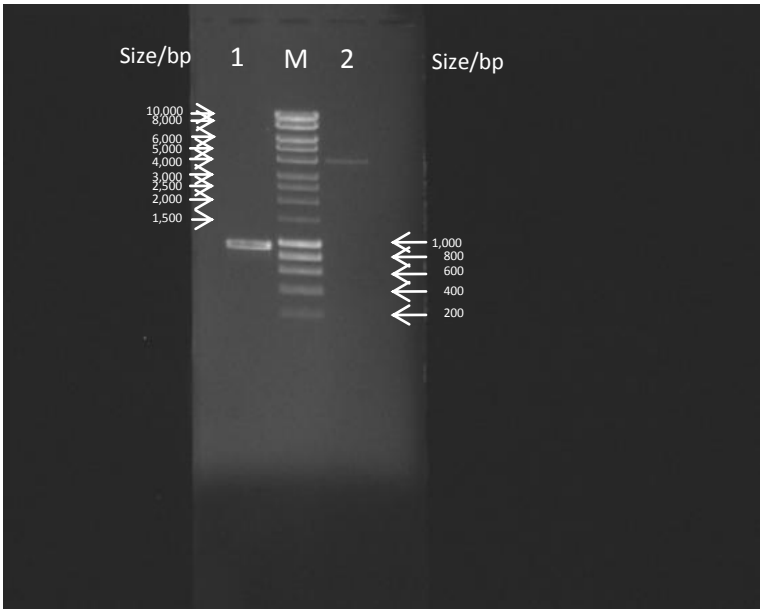

Figure 7. Human *NANOG* and pCS2+ double digested with Xho I and Xba I

1, Human *NANOG* double digested with Xho I and Xba I.

2, pCS2+ double digested with Xho I and Xba I. M, Marker Hyperladder I.

In lane 1 is the band of human *NANOG* double digested with Xho I and Xba I. Band size is about 900 bp. In lane 2 is the band of pCS2+ double digested with Xho I and Xba I. Band size is about 4,000 bp which is expected for linearized pCS2+.

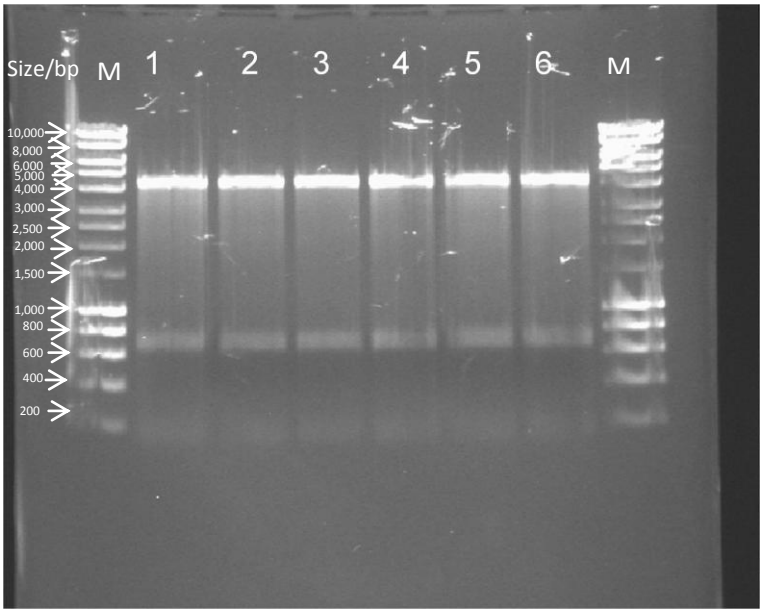

Figure 8. hsnanog-pCS2+ double digested with Xho I and Nco I

1-6, Double digestion of hsnanog-pCS2+ 1-6 with Xho I and Nco I. M, Marker Hyperladder I. There are three bands in each lane. The size of the three bands is about, 0.2 kb, 0.7 kb and 4 kb respectively, which is expected for double digestion of hsnanog-pCS2+ constructs (Figure 5) with Xho I and Nco I, and confirms that all these recombinant constructs contain human *NANOG* inserts.

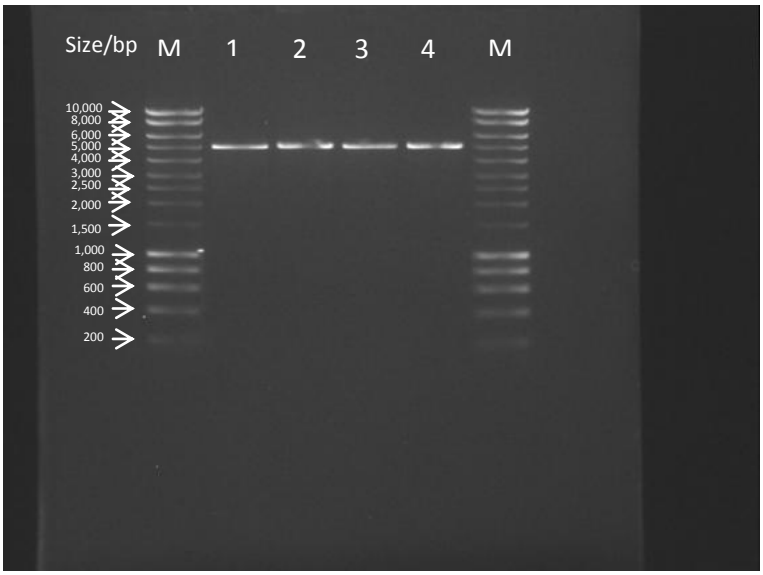

Figure 9. Linearization of hsnanog-pCS2+

1-4, hsnanog-pCS2+ construct 3 linearized by ASP718. M, Marker Hyperladder I. The size of linearised hsnanog-pCS2+ is about 5 kb. The clear single band indicates complete linearization.

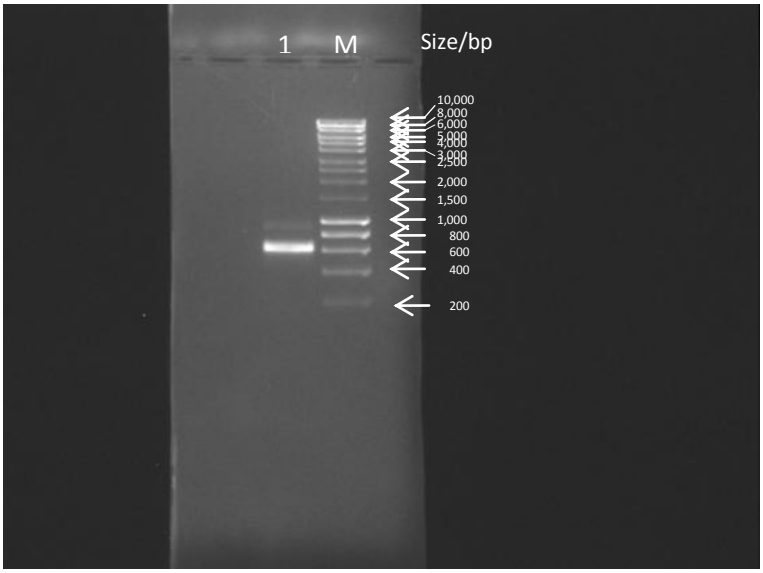

Figure 10. Human *NANOG* mRNA

1, Human *NANOG* mRNA. M, Marker Hyperladder I. In lane 1 there are two bands. The size of the brighter one is about 600 bp. The size of the other one, which is faint, is about 900 bp.

Construction of mnanog-pCS2+ **and in vitro transcription of mouse *Nanog* mRNA**

Correct mnanog-pCS2+ constructs were made and mouse *Nanog* mRNA of good quality was generated. Mouse *Nanog* cDNA of expected size, which is about 900 bp, amplified from pMXs-Nanog (Takahashi and Yamanaka, 2006) is illustrated in figure 11. Figure 12 shows that double digest reaction works well, with one band of about 900 bp for the double digested mouse *Nanog*, and a band of about 4,000 bp as well as a smear of approximately 200 bp for digested pCS2+. The concentration of double digestion products of mouse *Nanog* and pCS2+ were quantified to be 21.69 ng/µl and 12.20 ng/µl. Therefore the ratio of their volume in ligation reaction system was decided to be 1: 1 (Materials and Methods Appendix). Ten colonies were picked from the plate spread with *E.coli* bacteria transformed with ligation product. Gel electrophoresis result shows that these ten mnanog-pCS2+ constructs are of good quality (Results Appendix, Figure S2 and Figure S3). EcoR I and Xho I double digestion result shows that all of them are correct ones because they are cut into two bands, of about 850 bp and 4,000 bp and sequencing results also confirm that clones 1,2,4,6-9 have correct target sequences (Figure 13, Figure 14). Figure 15 and figure 16 show that mnanog-pCS2+ is linearized and mouse *Nanog* mRNA of good quality is generated, with two bands of about 800 bp and 1,000 bp.

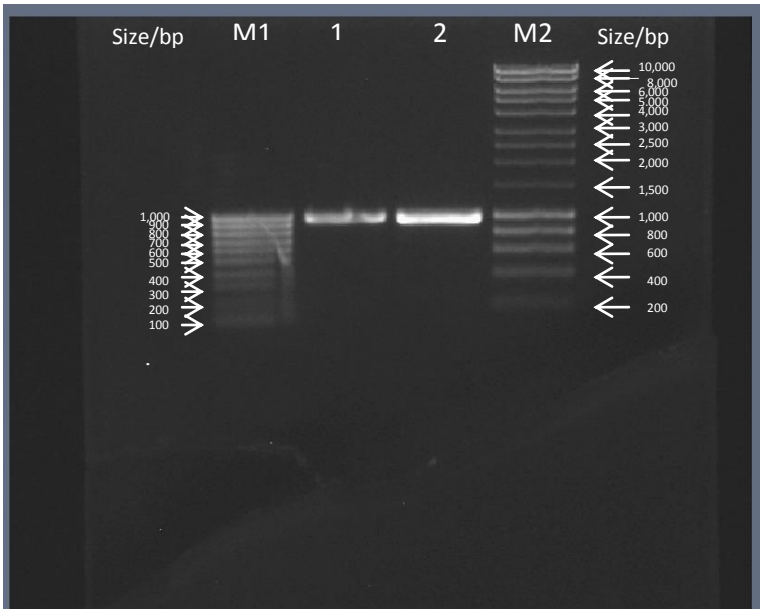

Figure 11. Gel electrophoresis of mouse *Nanog*

M1, Marker Hyperladder IV. M2, Marker Hyperladder I. 1-2, Mouse *Nanog.* In lane 1 and lane 2 is the mouse *Nanog* band of about 900 bp. In lane M1 is marker hyperladder IV. In lane M2 is marker hyperladder I.

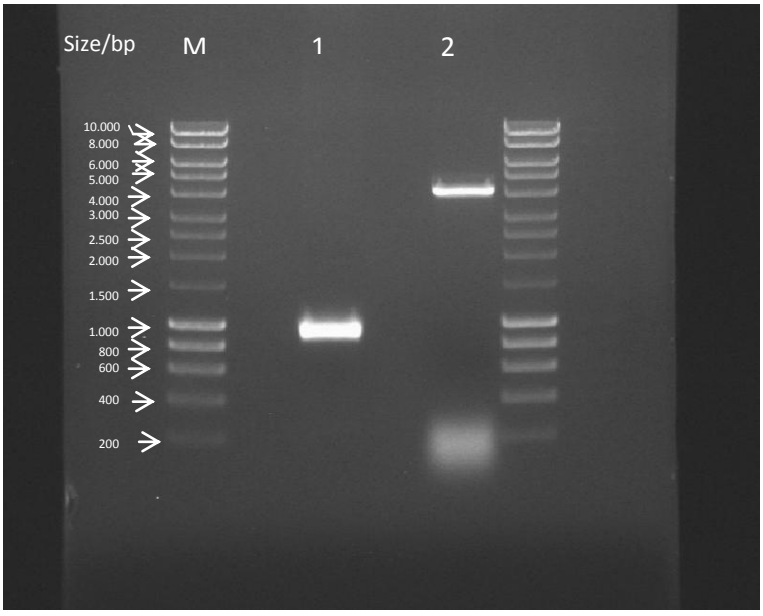

Figure 12. Mouse *Nanog* and pCS2+ double digested with Bam HI and Xho I

M, Hyperladder I. 1, Mouse *Nanog* cDNA double digested with Bam HI and Xho I. 2, pCS2+ double digested with Bam HI and Xho I . In lane 1 is a band of about 900 bp. It is mouse *Nanog* double digested with Bam HI and Xho I. In lane 2 are two bands, the bright single band of about 4,000 bp and the smear of approximately 200 bp, which is expected for the complete double digestion of pCS2+ by Bam HI and Xho I.

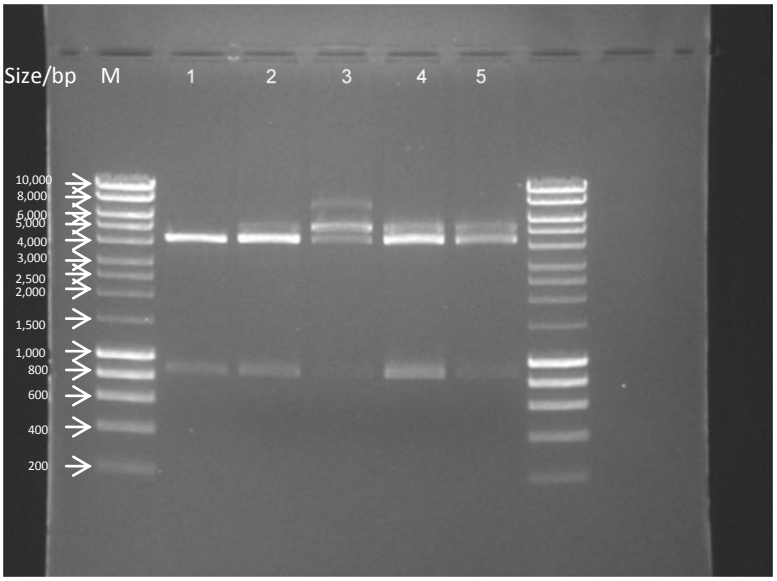

Figure 13. mnanog-pCS2+ 1-5 double digested with EcoR I and Xho I

M, Hyperladder I. 1-5, mnanog-pCS2+ constructs 1-5 double digested with EcoR I and Xho I. In lanes 1-5 are bands of mnanog-pCS2+ constructs 1-5 double digested with EcoR I and Xho I. Bands in all these lanes have the same pattern except those in lane 3. There are three bands in lanes 1, 2, 4 and 5. The size of two of them is about 850 bp and 4,000 bp, which is expected for double digestion products of mnanog-pCS2+ by EcoR I and Xho I and confirms that mnanog-pCS+ constructs 1,2,4 and 5 have inserts. The other band of about 5,000 bp probably represents uncut mnanog-pCS2+, which results from incomplete digestion reaction. In lane 3 there are four bands, including two expected bands of about 850 bp and 4,000 bp and the other two of about 5,000 bp and 7,500 bp, which probably owes to incomplete digestion.

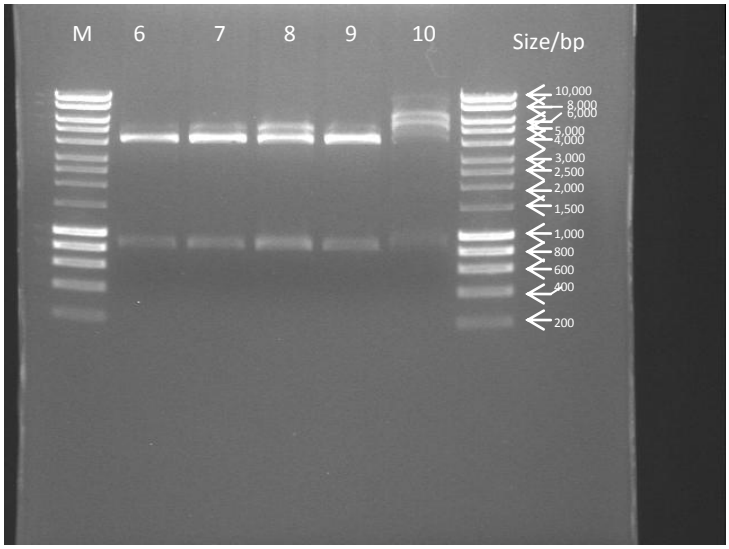

Figure 14. mnanog-pCS2+ 6-10 double digested with EcoR I and Xho I

M, Marker Hyperladder I. 6-10, mnanog-pCS2+ 6-10 double digested with EcoR I and Xho I. In lanes 6-10 are bands of double digestion of mnanog-pCS2+ 6-10 with EcoR I and Xho I. In lanes 6-9 digested mnanog-pCS2+ share the same band pattern with digested mnanog-pCS2+ 1, 2, 4 and 5 (Figure 13), three bands located at positions of 0.85 kb, 4 kb and 5 kb. In lane 10, there are five bands, two of which, of approximately 0.85 kb and 4 kb, are expected products of double digestion and the other three, of about 5 kb, 6.5 kb and 9 kb, are probably caused by incomplete digestion.

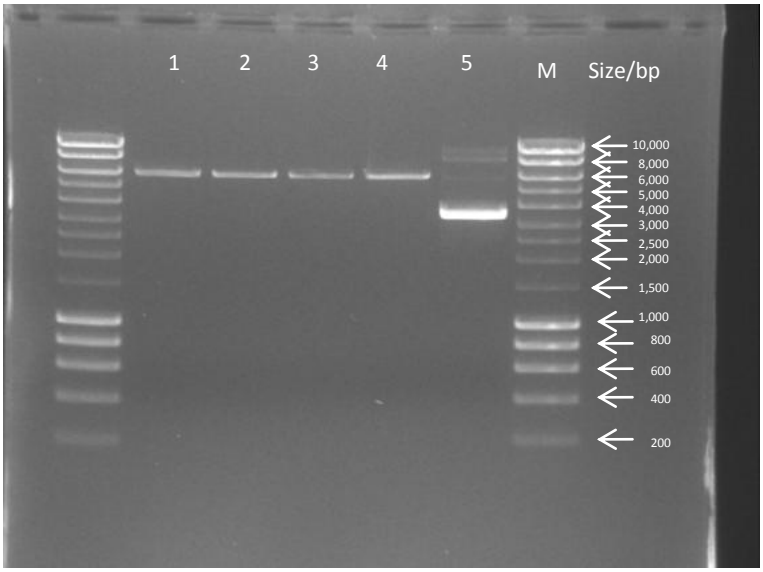

Figure 15. mnanog-pCS2+ ASP718

M, Marker Hyperladder I. 1-4: linearized mnanog-pCS2+. 5, uncut mnanog-pCS2+. In lanes 1-4 are the linearized mnanog-pCS2+ product which is a neat single band, demonstrating complete digestion compared with the bands of uncut mnanog-pCS2+ in lane 5.

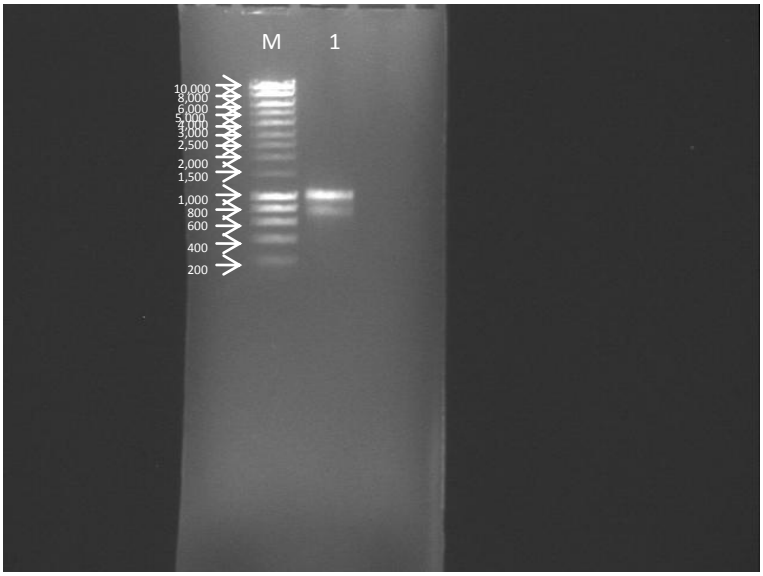

Figure 16. Mouse *Nanog* mRNA

M, Marker Hyperladder I. 1,Mouse *Nanog* mRNA. In lane one are bands of mouse *Nanog* mRNA. There are two bands, one of about 0.8 kb and the other of about 1 kb. The band pattern represents distribution of RNA conformations.