Materials and Methods Appendix

Construction of hsnanog-pCS2+

Polymerase Chain Reaction amplifying human *NANOG*

pEP4 E02S CK2M EN2L (5 ng/µl)	1 µl
10X Buffer for KOD Hot Start DNA Polymerase	5 µl
dNTPs (2 mM each)	5 µl
$MgSO_4$ (25 mM)	3 µl
Primer 1 (10 µM)	2 µl
Primer 2 (10 µM)	2 µl
KOD (1U/ µl)	1 µl
H_2O	31 µl
Total	50 µl

Typical Cycling Programme

1 = 95 °C	2 min
2 = 95 °C	20 s
3= 61 °C	10 s
4= 70 °C	15 s
5= Go to 2, 25cycles	
6= 4 °C	for ever

Double Digestion of human *NANOG* and pCS2+ with Xho I and Xba I

	Fragment	Vector
DNA	24 µl	6.25 µl
10X NEBuffer 4	5 µl	5 µl
100X BSA	0.5 µl	0.5 µl
Xho I (20,000 U/ml)	2 µl	2 µl
Xba I (20,000 U/ml)	2 µl	2 µl
H_2O	16.5 µl	34.25 µl
Total	50 µl	50 µl

Incubate at 37 °C for 2 hours

Digestion product was purified following the QIAquick PCR Purification Protocol.

Human *NANOG* and pCS2+ ligation reaction

	Vector Only No ligase	Vector Only	Vector Insert
pCS2+Vector	6.5 µl	6.5 µl	6.5 µl
Insert	------	------	1 µl
2X Quick Ligase Buffer	10 µl	10 µl	10 µl
Quick T4 DNA Ligase	------	1 µl	1 µl
H_2O	3.5 µl	2.5 µl	1.5 µl
Total	20 ul	20 µl	20 µl

Incubate at room temperature for 5 minutes

Ligation products were used to transform competent *E coli* bacteria which were then spread on LB-agar plates containing ampicillin. Six colonies were picked from the plate spread with the ligation product of *NANOG* and pCS2+ and hsnanog-pCS2+ constructs were extracted from them. Xho I and Nco I were used to double digest the hsnanog-pCS2+ to confirm whether it was the correct construct.

Double Digestion of hsnanog-pCS2+ with Xho I and Nco I

hsnanog-pCS2+	1.5 µl
10X NEBuffer 4	2.5 µl
10X BSA	2.5 µl
Xho I	1 µl
Nco I	1 µl
H_2O	16.5 µl
Total	25 µl

Incubate at 37 °C for 2 hours

In vitro transcription of human *NANOG* mRNA

Construct hsnanog-pCS2+ was linearised by ASP718, and human *NANOG* mRNA was generated following a standard protocol of in vitro transcription.

Linearisation of hsnanog-pCS2+

hsnanog-pCS2+	12.5 µl
10XSuRE/Cut Buffer for Restriction Enzymes	5 µl
ASP718	2 µl
H_2O	30.5 µl
Total	50 µl

Incubate at 37 °C overnight

Human *NANOG* mRNA synthesis

Linearized hsnanog-pCS2+	6.1 µl
10X NEB RNA Pol Reaction Buffer	5 µl
DTT(100 mM)	5 µl
rNTPs(10mM A,C,U, 1mM G)	10 µl
Cap analogue (5mM)	5 µl
RNasin	2.5 µl
SP6 RNA polymerase	2.5 µl
H_2O	13.9 µl
Total	50 µl

Incubate at 37 °C for 30 minutes, add 2.5 µl of 5 mM GTP, incubate at 37 °C for 1 h 30 min, add 2 µl DNase I, incubate at 37 °C for 15 min, purify RNA through SPIN column.

Construction of mnanog-pcs2+

BstX I Digestion of pMXS-Nanog

pMXS-Nanog	1 µl
10X NEBuffer 3	1 µl
10X BSA	1 µl
BstX I (10,000 U/ml)	0.4 µl
H_2O	6.6 µl
Total	10 µl

Incubate at 37 °C for 2 hours

Polymerase Chain Reaction amplifying mouse *Nanog*

pMXS-Nanog	1 µl
10X Buffer for KOD Hot Start DNA Polymerase	5 µl
dNTPs (2 mM)	5 µl
$MgSO_4$ (25mM)	3 µl
Primer 1 (10 µM)	2 µl
Primer 2 (10 µM)	2 µl
KOD Hot Start DNA Polymerase (1U/ µl)	1 µl
H_2O	31 µl
Total	50 µl

Typical Cycling Programme

1 = 95 °C	2 min
2 = 95 °C	20 s
3= 61 °C	10 s
4= 70 °C	15 s
5= Go to 2, 25 cycles	
6= 4 °C	For ever

Double Digestion of mouse *Nanog* and pCS2+ with BamHI and Xho I

	mNanog	pCS2+
DNA	3 µl	8 µl
10X Buffer	2.5 µl	2.5 µl
10X BSA	2.5 µl	2.5 µl
BamHI	1 µl	1 µl
Xho I	1 µl	1 µl
H_2O	15 µl	10 µl
Total	25 µl	25 µl

Incubate at 37 °C for 2 hours.

The digestion product was examined by gel electrophoresis, the target bands were cut out and target DNA was purified from gel slices following the QIAquick Gel Extraction Protocol.

Mouse *NANOG* and pCS2+ ligation reaction

	Vector Only	Vector Insert
pCS2+Vector	8.5 µl	8.5 µl
Insert	------	8.5 µl
10X T4 Ligase Buffer	2 µl	2 µl
T4 DNA Ligase	1 µl	1 µl
H_2O	8.5 µl	------
Total	20 ul	20 µl

Incubate at 16 °C overnight

Ligation products were used to transform competent *E coli* bacteria. Transformed *E.coli* bacteria were spread on LB-Agar plates containing ampicillin. Ten colonies were picked from the plate spread with the ligation product of *Nanog* and pCS2+ and mnanog-pCS2+ constructs were extracted from them. EcoR I and Xho I were used to digest the mnanog-pCS2+ to confirm whether it was the correct construct.

Double Digestion of hsnanog-pCS2+ with EcoR I and Xho I

mnanog-pCS2+	1 µl
10X NEB EcoRI Buffer	2 µl
10X BSA	2 µl
EcoR I	2 µl
Xho I	2 µl
H_2O	11 µl
Total	20 µl

Incubate at 37 °C for 2 hours

In vitro transcription of mouse *Nanog* mRNA

Construct mnanog-pCS2+ was linearised by ASP718. And mouse *Nanog* mRNA was generated following a standard protocol of in vitro transcription.

Linearisation of mnanog-pCS2+

mnanog-pCS2+	8.2 µl
10X SuRE/Cut Buffer B	5 µl
ASP718	3 µl
H_2O	33.8 µl
Total	50 µl

Incubate at 37 °C overnight

Mouse *Nanog* mRNA synthesis

Linearised mnanog-pCS2+	8 µl
10X RNA Pol Reaction Buffer	5 µl
DTT (100 mM)	5 µl
rNTPs (10mM A,C,U, 1Mm G)	10 µl
Cap analogue (5mM)	5 µl
RNasin	2.5 µl
RNA polymerase	2.5 µl
H_2O	12 µl
Total	50 µl

Incubate at 37 °C for 30 minutes, add 2.5 µl of 5 mM GTP, incubate at 37 °C for 1 h 30 min, add 2 µl DNase I, incubate at 37 °C for 15 min, purify RNA through SPIN column.

Total RNA extraction and reverse transcription

Total RNA extraction from embryos

Collect embryos in eppendorfs, remove as much liquid as possible and freeze on dry ice. Store at -80 °C. Add 500 µl TRIZOL and homogenise embryos (using eppendorf pestle). Incubate at 65 °C for 10 minutes.

Spin phase lock tubes at 13 krpm for 1 min. transfer homogenate to Phase-lock tubes. Add 100 µl chloroform, shake (do not vortex), and spin at 12 krpm for 10 min.

Transfer upper aqueous phase to new tube. Add 300 µl isopropanol and mix by inversion. Incubate at room temperature for 10 min. Spin at 13 krpm for 10 min at 4 °C. Discard supernatant, add 70% ethanol, and vortex briefly. Spin at 13 krpm for 5 min for 4 °C. Discard supernatant, air dry for 5 min, resuspend in 88 µl ddH_2O.

Add 10 µl DNase Buffer 10X and 2 µl DNase I (RNase-free). Incubate at 37 °C for 1 hour. Take 2 µl and run on 1% gel to check quality of RNA.

Add 102 µl ddH_2O. Add 200 µl Phenol:Chloroform:isoamyl alcohol, vortex and spin at 13 krpm for 5 min. Transfer aqueous phase to new tube, add 200 µl isopropanol and mix by inversion. Spin at 13 krpm for 10 min at 4 °C. Discard supernatant, add 200 µl 70% ethanol and spin at 13 krpm for 5 min. Discard supernatant, air dry for 5 min, and resuspend in 20 µl ddH_2O. Determine yield by spectrophotometer.

Reverse transcription

Mix together the following:

RNA (1 µg)

Random primer (250 ng)

H_2O

Total 12 µl

Incubate at 65 °C for 10 min. Transfer tube straight onto ice and leave for 2 min.

Add 5X First strand buffer 4 µl

DTT (100 mM) 2 µl

dNTPs (10 mM) 1 µl

Incubate at 25 °C for 2 min. Add 1 µl SuperScriptII Reverse Transcriptase and mix. Incubate at 25 °C for 10 min. Incubate at 42 °C for 50 min. Incubate at 70 °C for 10 min. Make up to 100 µl with H_2O and use 1 µl for PCRs.

PCR reaction to amplify *beta-actin* from cDNA

cDNA	1 µl
10X Buffer for Hot Start Polymerase	1 µl
dNTPs (2 mM)	0.2 µl
$MgSO_4$ (25 mM)	0.2 µl
Primers (10 µM)	0.4 µl
KOD Hot Start Polymerase(1U/ µl)	1 µl
H_2O	5.4 µl
Total	10 µl

Thermal Cycling Program

1 = 94 °C	120 s
2 = 94 °C	20 s
3= 60 °C	20 s
4= 72 °C	30 s
5= Go to 2, 35cycles	
6= 4°C	for ever