2 MATERIALS AND METHODS

In this section, all experiments employed in this study were explained in detail. Recipes for the solutions used in this study can be found in section 2.8. Primers for all the candidate promoters can be found in Appendix B.

2.1 Gene Reporter Assays

General scheme of this experiment is as summarized in Figure 2.1.

2.1.1 Primer Design

Primers to amplify candidate promoter regions were designed using the Primer3 program (Rozen and Skaletsky, 2000). Jilur Ghori at the Wellcome Trust Sanger Institute (WTSI) kindly wrote a program which adds different restriction enzyme sites at the end of each primer for directional cloning of the amplicons. Restriction enzyme pairs used for the inserts are;

- Sac I and BamH I
- BamH I and Nhe I
- Sac I and Hind III
- Hind III and Nhe I

Primers were synthesised in house or ordered via Sigma

(http://www.genxy.com/index.html). Each primer is provided with its optical density measurement at 260 nm (OD_{260}). The average molecular weight of each nucleotide is assumed as 330 ng. For a single stranded primer with length N, working dilutions were calculated according to the formula given below;

Volume of each primer (µl) = (Total amount of primer required (ng))/(330xNxOD_{260})

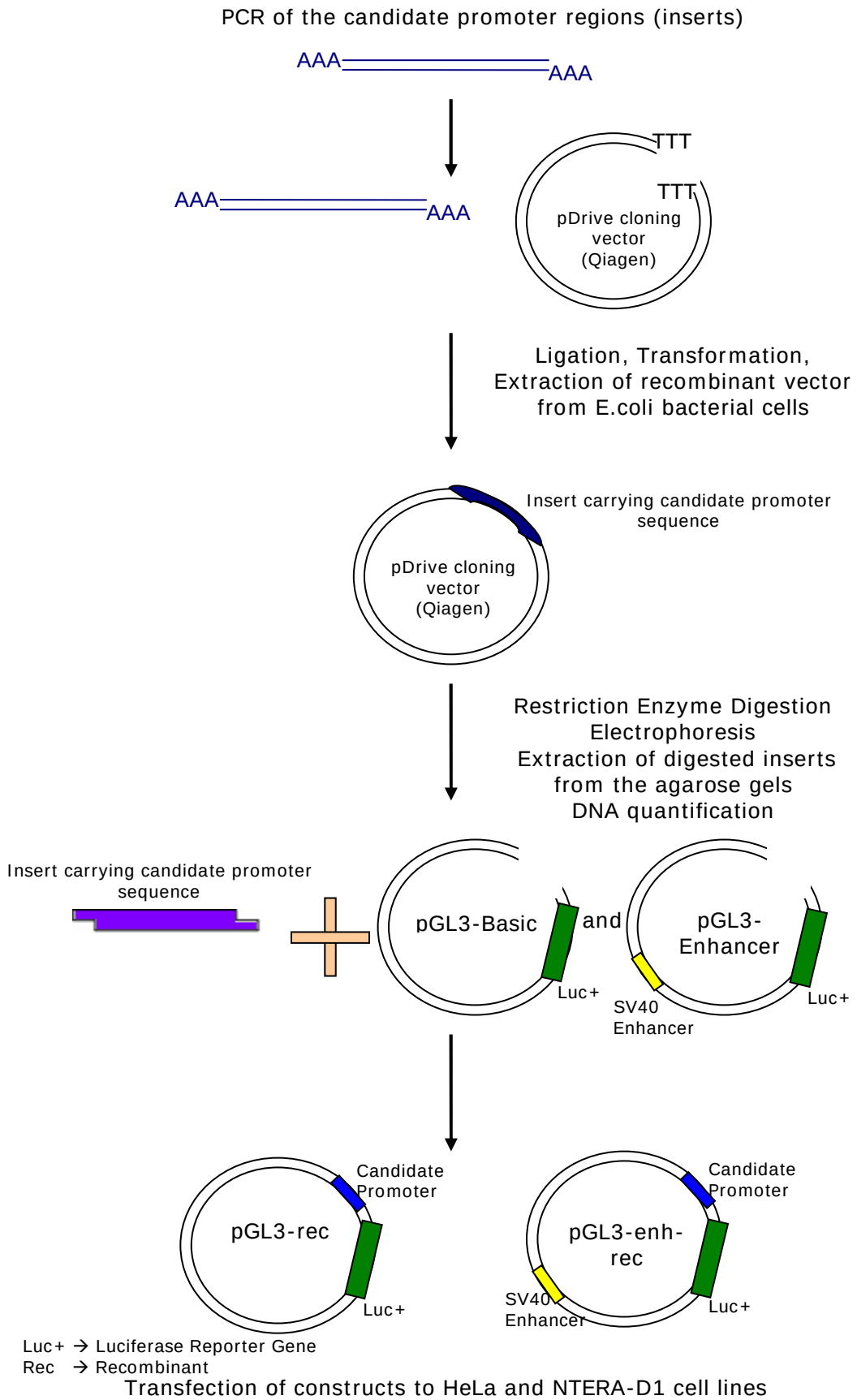

Figure 2.1 General Scheme of Cloning Procedure

Primers were stored at 4 ºC for short-term (less than one month) or –20°C for long term and working dilutions were prepared at 100 ng/µl for each primer with $T_{0.1}E$.

2.1.2 Sub-cloning

2.1.2.1 Polymerase Chain Reaction (PCR)

BD Titanium Taq Polymerase (Clontech, #639208) was used for all PCRs. The recipe for the reaction is given in Table 2.1;

Reagents	Amount (µl)
DD water	0
Sucrose Creosol Red	5
PCR buffer (10X)	1.5
10 mM dNTPs	0.6
Primer Mix (100 ng/µl each)	1.5
Human Genomic Template (30 ng/µl)	2.38
5 M Betaine (Sigma, #B0300)	3.9
Titanium Taq Polymerase (5 U/µl)	0.12
Total Volume	15

Table 2.1 Recipe for the PCR for amplification of candidate promoters.

CEPH sample NA12149A was used as the human genomic template to amplify sequences of interest.

The following PCR conditions were used for all reactions

1. 95 ºC for 2 min (Enzyme Activation)
2. 95 ºC for 30 sec (Melting)
3. 60 ºC for 30 sec (Annealing)
4. 72 ºC for 50 sec (Extension)

   Repeat step from 2 to 4 for 44 times
5. 72 ºC for 10 min (for extra addition of A-tail to products).

2.1.2.2 Electrophoresis

Electrophoresis was carried out in 1% agarose gel made in 1XTBE buffer with ethidium bromide (250 ng/µl). PCR reactions were loaded directly on the gels and run on 200 volts for 50 min. 100 bp DNA ladder (Invitrogen, #15628) was also loaded

onto the gel as a marker. DNA was visualised using a UV transilluminator and photographed digitally using LabWorks Image Acquisition and Analysis Software (UVP Bioimaging Systems).

2.1.2.3 TA cloning

pDrive cloning vector (Qiagen, #231122) was used for sub-cloning purposes. The map of this vector is shown in Figure 2.2.

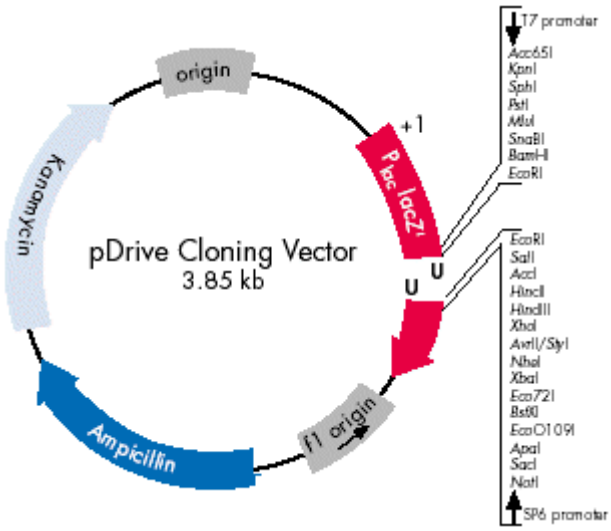

Figure 2.2 Map of pDrive TA cloning vector

T4 DNA Ligase kit (Roche, #481220) was used for ligations. The recipe for ligation reaction is as follows;

Reagents	Amount (µl)
PCR reaction containing the insert	1
pDrive vector (5ng/µl)	1
T4 Ligation Buffer (10X)	1
T4 Ligase (1 U/µl)	0.2
DD water	6.8

Table 2.2 Recipe for Ligation reaction for TA cloning

All ligation reactions were incubated overnight at 4 ºC.

2.1.2.4 Transformation

25 µl of JM109 E.coli competent cells (Promega, #L2001) were incubated with 2 µl of ligation reaction on ice for 10 min. The cells were then incubated at 42 ºC for 50

sec and put on ice immediately for 2 min. 475 µl of SOC medium were added to the cells and incubated for 2 h at 37 ºC on a shaker at 300 rpm. Cells were spun at 4000 rpm (1600xg) for 5 min and resuspended in 100 µl of SOC medium. All of the transformed cells were spread onto LB-agar plates containing 50 µg/ml ampicillin and incubated for 14-16 h at 37 ºC. Then, 3 colonies from each plate were picked to check for the presence of the recombinant vector by colony PCR.

2.1.2.5 Colony PCR

The colony of interest was picked in 100 µl of DD water and diluted in 1:3 with DD water. It is heated to 95 ºC for 5 min for colony lysis. 1 µl of the colony pick solution was used for PCR. AmpliTaq Gold (Applied Biosciences, #N808055) DNA polymerase was used for the amplification whose recipe is given in Table 2.3.

Reagents	Amount (µl)
DD water	0
Sucrose Creosol Red	5
NEB PCR buffer (10X)	1.5
10 mM dNTPs	0.6
Primer Mix (100 ng/µl each)	1.5
Colony Lysate	2.38
5 M Betaine (Sigma, B0300)	2
AmpliTaq Gold Polymerase (1 U/µl)	0.12
Total Volume	15

Table 2.3 Recipe for colony PCR

PCR amplification steps were as follows;

1. 95 ºC for 1 min
2. 95 ºC for 20 sec
3. 60 ºC for 30 sec
4. 72 ºC for 50 sec

Repeat step from 2 to 4 for 34 times

PCR reactions were then loaded onto 2% agarose gel and run for 50 min at 100 V to check the insert amplification. Colonies which carries the corresponding recombinant vector were picked and inoculated in 10 ml of LB growth medium and incubated at 37

ºC on a shaker for about 16 h. 1 ml of bacterial culture is mixed with 500 µl of 50 % glycerol and stored at -70 ºC. 3 ml of bacterial culture was used for the extraction of the recombinant vector according to the mini-prep protocol described in section 2.1.2.6.

2.1.2.6 Mini-prep protocol using Qiaprep Spin Miniprep Kit

The mini-preps were prepared using QIAprep Spin Miniprep Kit (QIAGEN, #27104) and all centrifugations were performed using a standard table-top micro-centrifuge.

2.1.2.7 Restriction Enzyme Digestion

pDrive recombinant vectors were digested with appropriate restriction enzymes to cut the insert (section 2.1.1). All enzymes are ordered from NEB Life Sciences. The recipe for the digestion reaction is shown in Table 2.4. Promega Buffer E was used for all reactions.

Reagents	Amount (µl)	Final amounts
Restriction Enzyme I	0.5-2	10 Units
Restriction Enzyme II	0.5-2	10 Units
Promega Buffer E (10X)	4	1X
BSA	0.4	0.1 µg/µl
pDrive recombinant vector	30	~ 5 µg
DD water	up to 40	n/a
Total Volume	40	n/a

Table 2.4 Recipe for Restriction Enzyme Digestion

The reactions were incubated at 37 ºC for 3 h. Then, 8 µl of Sucrose/Creosol Red was added to the digests. They were then loaded to 1 % agarose gel and run for 3 h at 100 V. The inserts were cut from the gel and extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, #28704) according to protocol described below.

2.1.2.8 Extraction of DNA from Agarose Gel

All centrifugation steps were performed at 13,000 rpm on a Eppendorf desktop microfuge and all buffers were provided with the kit. The extraction procedure is given in step by step fashion below;

- Add 450 µl of Buffer QG
- Incubate @ 50 ºC for 10 min or until the gel slice has completely dissolved
- Check that the colour of the mixture is still yellow (If it is orange or violet, add 10 µl 3 M sodium acetate at pH 5.2 and mix)
- Apply the sample to the QIAquick column and centrifuge for 1 min. Discard flow-through
- Add 500 µl of Buffer QG and centrifuge for 1 min. Discard flow-through.
- Pipette out the remaining drops of yellow liquid from the top of the column.
- To wash, add 750 µl Buffer PE and centrifuge for 1 min. Discard flow-through
- Add 750 µl Buffer PE, leave for 10 min and centrifuge for 1 min
- Discard flow-through and centrifuge for 1 min again
- Place the column into a clean 1.5 ml microfuge tube
- To elute DNA, add 30 µl Buffer EB to the centre of the column's membrane, let it stand for 15-30 min. Centrifuge for 1 min.

2.1.3 Cloning inserts into Gene Reporter Vectors

2.1.3.1 Gene Reporter Vectors

pGL3 vectors from Promega were used as reporter vectors. Candidate promoters were cloned into pGL3-basic (#E1751) and pGL3-enhancer (#E1771) vectors. pGL3-promoter (#E1761) and pGL3-control vector (#E1741) were used as positive controls. pRL-SV40 vector (#E2231) was used as the internal control vector. The maps of these vectors can be found in the figures from 2.3 to 2.7.

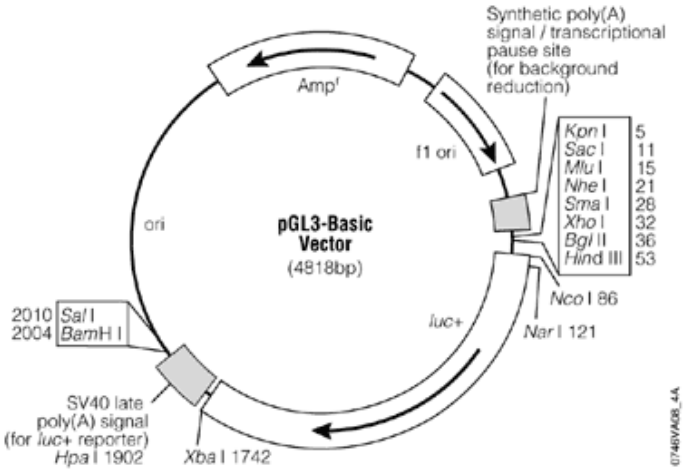

Figure 2.3 pGL3-basic vector map

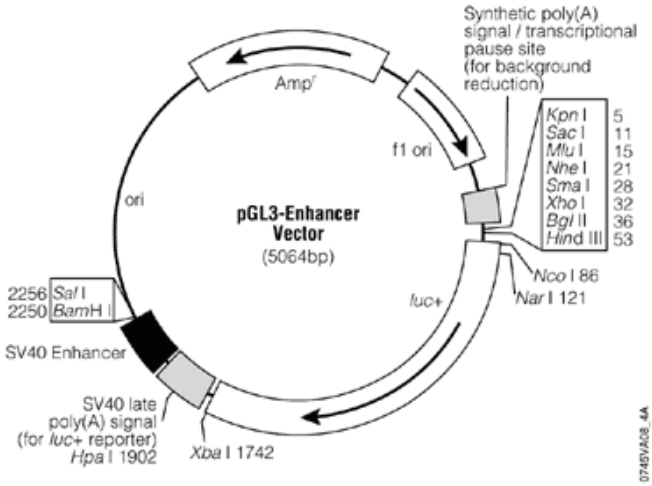

Figure 2.4 pGL3-enhancer vector map

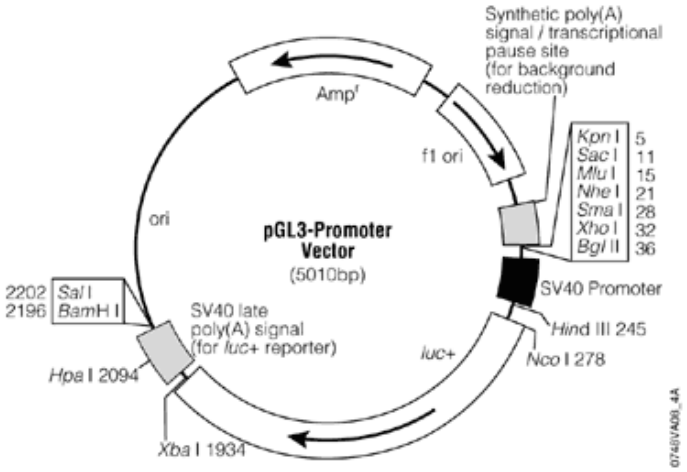

Figure 2.5 pGL3-promoter vector map

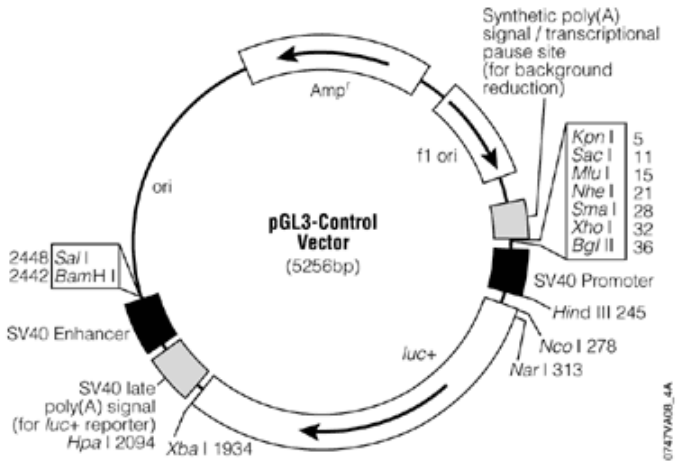

Figure 2.6 pGL3-control vector map

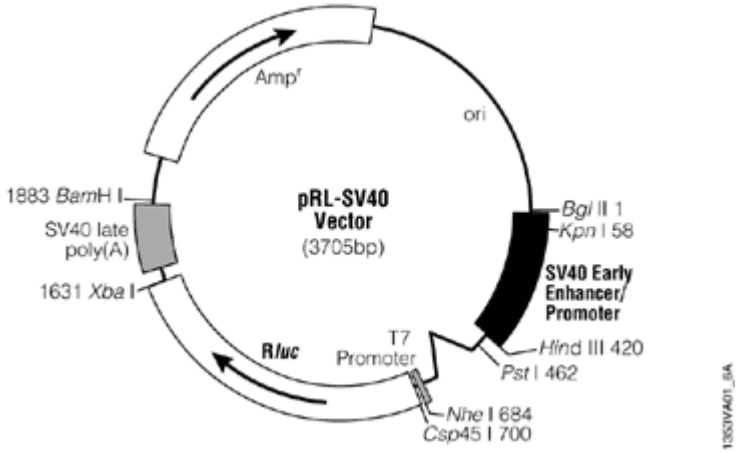

Figure 2.7 PRL-SV40 vector map

2.1.3.2 Preparation of the cloning vectors

pGL3-basic and pGL3-enhancer vectors were digested with the appropriate enzymes (Table 2.5) sequentially.

Insert Digestion	Corresponding vector digestion
Bam HI and Nhe I	Bgl II and Nhe I
Sac I and Bam HI	Sac I and BamH I
Sac I and Hind III	Sac I and Hind III
Hind III and Nhe I	Hind III and Nhe I

Table 2.5 Restriction Enzyme Pair for the digestion of vectors

The digestion reaction set up is shown in Table 2.6. The reaction was incubated at 37 ºC for 3 h.

Reagents	Amount (µl)	Final amounts
Restriction Enzyme	0.5-2	10 Units
NEB Buffer (10X)	10	1X
BSA	1	0.1 µg/µl
Vector	variable	10 µg
DD water	up to 100	n/a
Total Volume	100	n/a

Table 2.6 Restriction enzyme digestions of cloning vectors

The digest is ethanol-precipitated using Pellet Paint (Novagen, #69049) as follows; 2 µl of Pellet Paint, 0.1 volume of 3 M sodium acetate at pH 5.2 and 2 volumes of absolute ethanol were added to the sample, mixed and incubated for 2 min at room temperature. Samples were spun at 14,000 rpm on a desktop microfuge for 5 min and washed with 500 µl of 70% ethanol by spinning 5 min at 13,000 rpm. Pellets were air-dried for 2 min and resuspended in 30 µl $T_{0.1}E$.

Samples were then digested with the second enzyme as described above, ethanol precipitated using Pellet Paint, and resuspended in 30 µl $T_{0.1}E$. 10 µl of Sucrose/Creosol Red was added to samples which were loaded to 1 % agarose gel and run for 6 h at 50 V to eliminate undigested vector band. The cut vector band was extracted from the gel using QIAquick gel extraction kit as described in 2.1.2.8. The digested vectors were quantified using NanoDrop spectrophotometer (NanoDrop Technologies) and dephosphorylated as described below.

Shrimp Alkaline Phosphatase (USB, #70092Y) was used for dephosphorylation of double digested vectors. For every 0.62 µg of pGL3-basic and 0.59 µg of pGL3-enhancer vectors, 1 unit of SAP was used to dephosphorylate 1 pmol of DNA termini. It is assumed that 1.0 µg of 3 kb plasmid contains 1.0 pmole of DNA termini. Reaction set up was listed in Table 2.7.

Reagents	Amount (µl)
Double digested vector	30
10X Reaction Buffer	1
SAP	variable (1 U per 1 picomole of DNA termini)
DD water	up to 50

Table 2.7 Reaction set up for vector dephosphorylation

Reactions were incubated at 37 ºC for 1 hour. The enzyme was heat-inactivated at 65 ºC for 15 min. The dephosphorylated vector was purified using phenol/chloroform three times as described in section 2.1.3.3. Then, 0.1 volumes of 3 M Sodium Acetate at pH 5.2 and 2 volumes of absolute ethanol were added to the purified sample and incubated for 1 hour at -70 ºC. The sample was spun at 13,000 rpm at 4 ºC for 15 min to precipitate DNA. Then, it was washed with 500 µl of 70 % Ethanol by centrifuging for 5 min at 13,000 rpm. The pellet is air-dried for 10 min and resuspended in 30 µl of $T_{0.1}E$.

2.1.3.3 Phenol – chloroform purification of DNA

Equal volume of phenol:chloroform:isoamyl alcohol (25:4:1) (Invitrogen, #15593031) was added to the sample, mixed well and spun at 13,000 rpm on a microfuge for 5 min. The aqueous layer (bottom layer) was then transferred to a new tube.

2.1.4 Ligations

Roche Rapid DNA Ligation Kit (Roche Applied Sciences, #11635379001) was used to generate recombinants vectors carrying candidate promoters. 10 ng of vector (pGL3-basic or pGL3-enhancer) was used for each ligation reaction. Amount of insert required to set up ligation reaction was calculated according to equation below;

$$\textbf{Amount of insert} = \frac{\textbf{Size of insert (bp)}}{\textbf{Size of vector (bp)}} \times (\textbf{Amount of vector}) \times \mathbf{3}$$

Insert and vector were mixed and the volume is completed up to 10 µl by 1X DNA dilution buffer. 10 µl of 2X DNA ligation buffer and 1 µl of (1 U) of T4 DNA ligase

were added and the reaction was incubated for 30 min at room temperature. 2 µl of the ligation reaction was used to transform E. coli cells according to protocol described in section 2.1.2.4. On the next day, 3 colonies from each plate were checked by colony-PCR using the protocol described in section 2.1.2.5. Colonies which carries the recombinant vector were picked and inoculated in 10 ml of LB growth medium and incubated at 37 ºC on a shaker for about 16 h. For long-term storage, 1 ml of bacterial culture is mixed with 500 µl of 50 % glycerol and stored at -70 ºC. For plasmid extraction, 2 ml of bacterial culture was used and extraction was performed according to the mini-prep protocol described in section 2.1.2.6. The mini-preps were quantified using NanoDrop spectrophotometer.

2.1.5 Transfections

Human adherent cell lines (HeLa S3 and NTERA-D1) were transfected using QIAGEN Effectene Transfection Reagent (QIAGEN, #301425) with recombinant reporter vectors. The transfection was performed in 48-well plate format, with the reagents provided with the kit, according to the protocol described below;

- The day before transfection, seed $2x10^4$ cells per well in a 48-well plate (Corning, #3548) in 200 µl appropriate growth medium containing serum and antibiotics.
- Incubate the cells under their normal growth conditions (at 37 °C and 5% CO_2). The wells should be 40–80% confluent on the day of transfection.
- The day of transfection, dilute 150 ng DNA and 50 ng of internal control vector DNA (pRLSV-40) with the DNA-condensation buffer, Buffer EC, to a total volume of 50 µl. Add 1.2 µl Enhancer and incubate at room temperature for 2-5 min.
- Dilute 4 µl of Effectene Reagent in 50 µl of Buffer EC and add to the DNA-Enhancer mixture. Mix by pipetting up and down 5 times
- Incubate the samples for 5–10 min at room temperature to allow transfection-complex formation.
- While complex formation takes place, gently aspirate the growth medium from the plate, and wash cells once with 200 µl PBS. Add 100 µl fresh growth medium to the cells.
- Add 150 µl growth medium to the wells containing the transfection complexes. Mix by pipetting up and down twice, and immediately add the

transfection complexes drop-wise onto the cells in the 48-well dishes. Gently swirl the dish to ensure uniform distribution of the transfection complexes.

- Incubate the cells with the transfection complexes under their normal growth conditions for 48 h for expression of the transfected gene. Assay the cells for expression of the transfected gene using a luminometer.

2.1.6 Dual Luciferase Reporter Assays

A dual luciferase assay (Promega, #E1910) was used to measure the luciferase and renilla activity within the transfected cells. Two days after transfection, growth media was removed and cells were washed with 100 µl of 1XPBS. Then, cells were incubated in 100 µl of 1X Passive Cell Lysis Buffer for 20 min at room temperature for cell lysis. 20 µl of the lysate were transferred to luminometer plates (Greiner Bio-One Inc, #655083) and they are ready for assay. Luciferase assays were performed with MicroLumat Plus LB 96V luminometer (Berthold Technologies). First, 50 µl of Luciferase Reagent II was injected to the lysate and luciferase activity was measured for 10 sec. Then, 50 µl of *Stop and Glo*® reagent was injected to the lysate to stop the luciferase activity and catalyse the renilla reaction, incubated for 1.6 sec and then renilla activity was measured for 10 sec.

2.2 Cell Culture

2.2.1 HeLa S3 cell line

HeLa S3, human cervical carcinoma cell line, was kindly provided by Team 76 (Cancer Genome Project) at the Sanger Institute. This adherent cell line was grown in high glucose (5 g/l) DMEM (Gibco, #41966029) supplemented by 10 % FBS (Gibco, #10108165) and 1X antibiotic/antimycotic solution (Gibco, #10378016) in an humidified incubator at 37 ºC and 5 % CO_2. Cells were spun at 1200 rpm (250 g) for 5 min and resuspended in appropriate amount of complete growth media. Cell were trypsinized every 3-4 days (section 2.2.3) and $4x10^4$ cells were seeded per cm^2 of flask area. Doubling time of this cell line is ~ 1.5 days.

2.2.2 NTERA-2 clone D1 cell line

NTERA-2 clone D1 (NTERA-D1), human Caucasian pluripotent embryonal carcinoma cell line was purchased from European Collection of Cell Cultures (ECAC) (ECAC, #93021013). This adherent cell line was grown in high glucose (5 g/l) DMEM (Gibco, #41966029) supplemented by 10 % FBS and 1X antibiotic/antimycotic solution in an humidified incubator at 37 ºC and 5 % CO_2. Cells were spun at 1200 rpm (250 g) for 5 min and resuspended in appropriate amount of complete growth media. Cells were trypsinized every 3-4 days (section 2.2.3)and $8x10^4$ cells seeded per cm^2 of flask area and Doubling time of this cell line is about 3 days.

2.2.3 Trypsinizing Cells

When the cells were 90-100% confluent, they were taken from the incubator, the growth media was discarded and appropriate amount of 1XPBS (3 ml per 25 cm^2 flask area) was added onto cells to remove the residual of FBS. Cells were washed twice by 1XPBS by rocking the flasks for 5 sec and the solution was discarded. Then, appropriate amount of Trypsin/EDTA solution (Gibco, #15240096) (2 ml per 25 cm^2 flask area) was added and the cells were incubated for 5-6 min in the 37 ºC incubator for detachment. Then 1 ml of complete growth media was added to trypsinized cells to inhibit trypsin. Cell were counted and seeded at the required density to the cell flasks or plates.

2.2.4 Freezing the Cells

The cells which are in their early passage (at most 3 passages) were trypsinized and counted. Then, 2 million cells was resuspended in 1 ml of 95 % FBS/5% DMSO solution and put in 1 ml cryogenic vials and stored at -70 ºC for one day. Vials were transferred to the liquid nitrogen tank (-180 ºC) the next day for long-term storage.

2.2.5 Thawing the Cells

Cells were taken from the liquid nitrogen tank and rapidly thaw in a water-bath at 37 ºC. Cells were transferred to a 15 ml eppendorf tube and 7 ml of complete growth media was added onto cells. Then, cells were spun at 1200 rpm (250 g) for 5 min, the media was removed and cells were resuspended in 5 ml of 1XPBS completely and spun again at 1200 rpm (250 g) for 5 min. 1XPBS was discarded and cells were resuspended in 10 ml of complete growth media and seeded onto 25 cm^2 flasks.

2.3 Affymetrix Expression Arrays

2.3.1 Isolation of Total RNA from cells

RNeasy™ Mini Kit (QIAGEN, #74104) was used to isolate total RNA from HeLa S3 and NTERA-2 clone-D1 cells according to the manufacturer's instructions. The total RNA yields and quality from both cell lines are given in Table 2.8 below.

Cell Type	Number of cells	Amount of total RNA (µg)	A_{260}/A_{280}	A_{260}/A_{230}
HeLa S3	$4x10^6$	32.2	2.12	2.10
NTERA-2 clone D1	$4x10^6$	61.5	2.13	2.17

Table 2.8 Total RNA yields and quality from both cell lines

2.3.2 Checking the quality of the RNA

The quality of RNA samples were checked by running on 1 % agarose gel in 1X MOPS (3-(N-morpholino) propanesulfonic acid) buffer. Agarose gel was prepared as described here; 0.4 g of agarose was weighed, 8 ml of 5XMOPS buffer and DEPC-treated water up to 40 ml was added. The mixture was boiled in the microwave oven until melted. The solution was cooled for 2-3 min before adding 1 ml of 38% FA in a fume hood. A small gel tank was cleaned with RNaseZap® (Ambion, #9780) and the gel solution was poured to the tank and let to solidify for 30 min.

2 µl of RNA loading buffer was added to the samples and they are incubated at 65 ºC for 10 min to denature any RNA secondary structure. The samples were put immediately placed on ice for 2 min, short-spun to be ready for loading to the gel. RNA ladder (Promega, #G3191) was used to assess to quality of RNA extraction.

2.3.3 Preparation of RNA for hybridization to Affymetrix Expression Arrays

GeneChip™ Human Genome U133A 2.0 Expression Analysis Array (Affymetrix, #900466) was used to determine the gene expression profile of both cell lines. One Cycle Target Labelling and Control Reagents Kit (Affymetrix, #900493) which includes all the reagents and materials was used for the preparation of the total RNA to the arrays. The preparation steps of total RNA content of the cells for the hybridization to arrays are schematically represented in Figure 2.8. The critical specifications of GeneChip™ Human Genome U133A 2.0 Expression Analysis Array are given in Table 2.9.

Critical Specifications of Human Genome U133A 2.0 Expression Analysis Array	
Feature Size	11 µm
Probe Pairs/Sequence	11
Hybridization Controls Poly-A Controls Normalization Controls Housekeeping/Control Genes	bioB, bioC, bioD, cre dap, lys, phe, thr 100 probe sets GAPDH, beta-Actin, ISGF-3 (STAT1)
Array Format	49
Fluidics Protocol	EukGE-WS2v5
Hybridization Volume	200 µl
Library Files	HG-U133 Plus 2.0

Table 2.9 Critical Specifications of Human Genome U133A 2.0 Expression Analysis Array

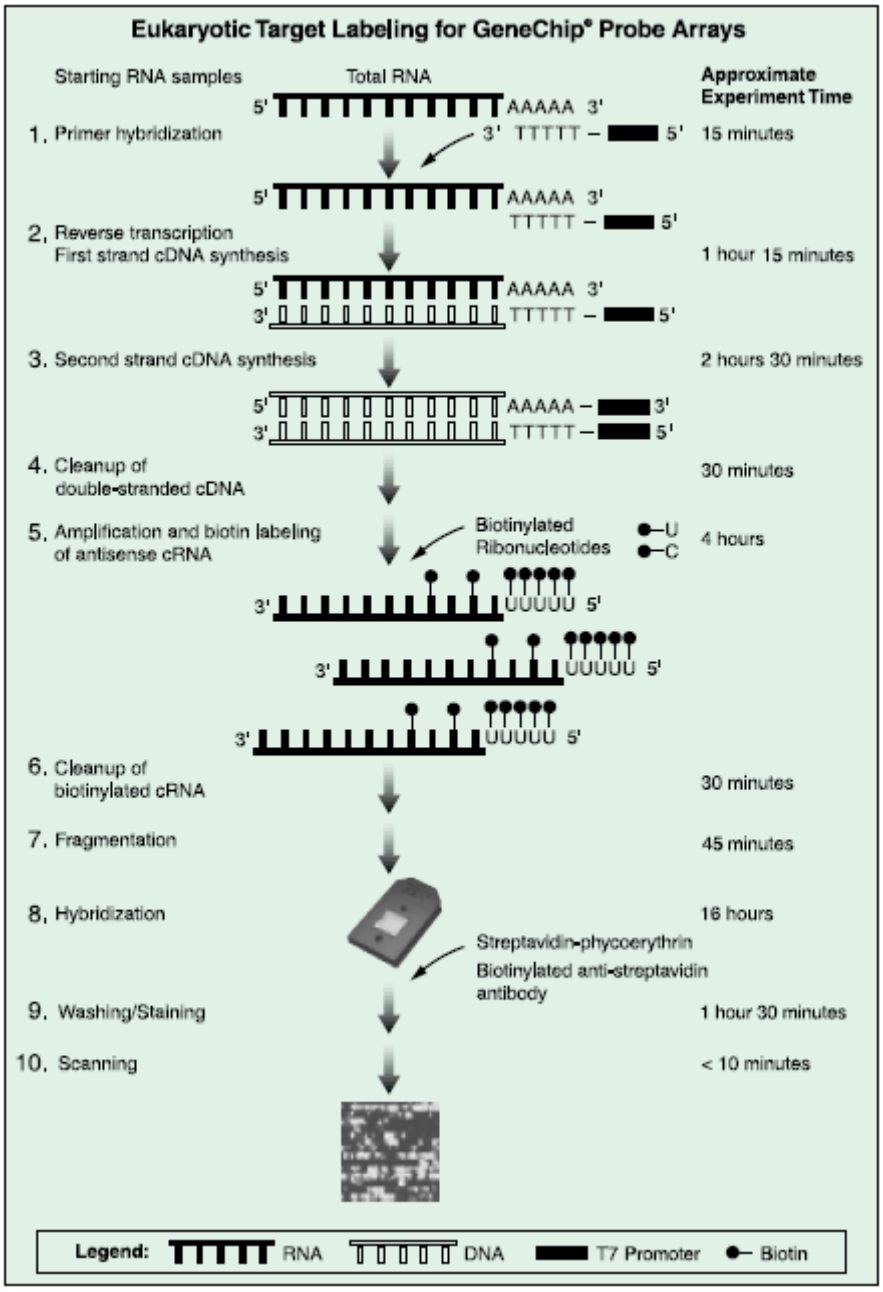

Figure 2.8 Schematic Representation of eukaryotic RNA labelling assay for expression profiling using GeneChip™ expression arrays

2.3.3.1 First Strand cDNA synthesis

Total RNA is first reverse transcribed using SuperScript II reverse transcriptase and T7-Oligo(dT) Promoter Primer in the first strand cDNA synthesis reaction according to the manufacturer's instructions

(http://www.affymetrix.com/support/technical/manual/expression_manual.affx). For reverse transcription reaction, 5 µg of total RNA was used. In order to monitor the

labelling efficiency independent from the quality of the starting total RNA, four *B. subtilis* polyadenylated transcripts (lys, phe, thr, dap) were added to the reaction at known concentrations (set by the manufacturer). Each GeneChiP™ array contains probes for these transcripts and their signal will be accepted as reference to evaluate the labelling efficiency of the sample.

2.3.3.2 Second Strand Synthesis

After the first strand synthesis, RNase H is added to the reaction to digest the RNA from RNA:cDNA hybrid. Then, E. coli DNA ligase and E.coli DNA Polymerase I were added for second strand cDNA synthesis. All reactions were set up according to the manufacturer's instructions

(http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

2.3.3.3 Cleanup of Double Stranded cDNA

Double Stranded cDNA was cleaned up using the cDNA Cleanup Spin Columns according to the manufacturer's instructions

(http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

2.3.3.4 Synthesis of Biotin Labelled cRNA

Double stranded cDNA containing T7-promoter sequence is the template for the synthesis of antisense cRNA in the presence of four natural ribonucleotides and one biotin-conjugated nucleotide analogue for labelling. The reaction was set up according to the manufacturer's instruction

(http://www.affymetrix.com/support/technical/manual/expression_manual.affx). The labelling reaction was incubated for 16 h at 37 ºC.

2.3.3.5 Cleanup of Biotin Labelled cRNA

Biotin labelled cRNA was cleaned up using the cRNA Cleanup Spin Columns according to the manufacturer's instructions

(http://www.affymetrix.com/support/technical/manual/expression_manual.affx).

2.3.4 Fragmentation of Biotin Labelled cRNA

Biotin Labelled cRNA was quantified using NanoDrop spectrophotometer and 20 µg cRNA was break down to 35 to 200 base fragments by metal-induced hydrolysis. For fragmentation, 8 µl of 5X Fragmentation Buffer was added to 20 µg of biotin labelled cRNA and the volume is completed up to 40 µl with RNase-free water. The reaction was incubated for 94 ºC for 35 min and then immediately placed on ice. The fragmentation efficiency was checked by running 2 µl of the reaction on an RNA gel as described in 2.3.2.

2.3.5 Eukaryotic Target Hybridization

The following hybridization cocktail was prepared for 49 Format Array as described in Table 2.10.

Fragmented cRNA	15 µg
Control Oligonucleotide B2 (3 nM)	5 µl
20X Eukaryotic Hybridization Controls (bioB, bioC, bioD,cre)	15 µl
Herring Sperm DNA (10 mg/ml) (Promega D1811)	3 µl
BSA (5 mg/ml) (Invitrogen, #15561-020)	3 µl
2X Hybridization Buffer	150 µl
DMSO (Sigma, #D5879)	30 µl
DD water	to a final volume of 300 µl

Table 2.10 Recipe for the hybridization cocktail

The probe array was equilibrated to room temperature before use. Hybridization cocktail was heated to 99 ºC for 5 min and incubated at 45 ºC for 5 min. Meanwhile, the probe array was wet by filling it with 200 µl of 1X Hybridization Buffer and

incubated at 45 ºC for 10 min with rotation. The hybridization cocktail was spun at maximum speed on a table-top centrifuge for 5 min to remove any insoluble material and transferred to a new tube avoiding any insoluble matter at the bottom of the tube. After 10 min of incubation of the probe array at 45 ºC with rotation, the buffer was removed from the array and the probe array was filled with 200 µl of hybridization cocktail and placed in hybridization oven at 45 ºC and incubated for 16 h while rotating 60 rpm.

2.3.6 Washing and Staining of the probe array

After 16 h of hybridization, the hybridization cocktail was removed from the probe array and the probe array is filled with 200 µl of Non-stringent wash buffer A. The probe array was washed and stained using Affymetrix Fluidics Station 450 according to the manufacturer's instructions

(http://www.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). The program named EUkGE-WS2v4_450 was used for washing and staining of the array.

2.3.7 Array Scanning

After the probe array washed and stained, it was scanned using Affymetrix Scanner controlled by Affymetrix Microarray Suite (GCOS). A quality control report was generated for each array scanned to assess the efficiency of the experiment.

2.4 Construction of Tilepath Arrays

In order to generate a high density single nucleotide polymorphism (SNP) map of human chromosome 20, chromosome sequence was sheared into small fragments of size around 2 kb (Spencer et al., 2006). These DNA fragments were then cloned into pUC18 vector, amplified and re-sequenced for finding SNPs, and they were stored in

$T_{0.1}E$ -70 ˚C for further use. For our study, we picked around 1800 of those fragments that will cover 3.5 Mb region on human chromosome 20q12-13.2. These fragments were amplified with pUC18 primers where the forward primer contained an amino group linked to sixth carbon atom at the 5' terminal to enable them for printing onto a microarray. The amino-linked primer were ordered from Operon (www.operon.com).

The PCR amplification protocol was given in Table 2.11. Fragment that are picked were diluted 1:10 with TE buffer for use in PCR.

Reagent	Amount (µl)
DNA Template (1:10 diluted)	1
10X CHIP PCR Buffer	6
10 mM dNTPs	3
5' amino linked forward primer (100 ng/µl)	0.75
Reverse primer (100 ng/µl)	0.75
AmpliTaq Gold Polymerase	0.3
DD water	48.2

Table 2.11. PCR recipe for amplification of DNA fragments to be spotted on the array.

PCR amplification steps are as follows;

1. 95 ˚C for 5 min
2. 95 ˚C for 1 min
3. 60 ˚C for 1 min
4. 72 ˚C for 4 min

   Repeat steps 2-4 for 30 times
5. 72 ˚C for 5 min

Quality and size of the PCR reactions were checked by running 2 µl of the reactions on 1 % agarose gels with ethidium bromide at 100 volts for 2 h.

Then, PCR reactions (~55 µl) were transferred to filter plates (pore size of 0.65 µm) (Millipore, #MSDV6550), 15 µl of 4X Spotting Buffer were added and centrifuged at 2000 rpm for 10 min into 96-well plates. Centrifuged samples were now ready for spotting onto microarrays or alternatively they can be kept at -20 ˚C until further use. Samples were sent to Microarray Facility at WTSI for printing. Each fragment had

three replicates on each array. In total, 162 arrays were printed in 4 batches and less than 5% of the spots were unusable on each batch.

2.5 Chromatin Immunoprecipitation (ChIP)

The experimental protocol is kindly provided by Vetrie Lab at Sanger Institute and some modifications have been introduced. ChIP experiments were performed on HeLa and NTERA-D1 cell lines.

2.5.1 Cell Harvesting

HeLa or NTERA-D1 cell lines were grown in 500 cm^2 cell culture dishes (Corning, #431110) under their normal growth conditions until they reach 100 % confluency. Growth media was removed and cells were washed with 25 ml of 1XPBS to be ready for chromatin fixation.

2.5.2 Chromatin Fixation

Fixation conditions vary depending on the antibody used in further steps of the experiment. Table 2.12 lists the fixation conditions depending on the antibody. Dimethyl Adipimidate.2HCl (DMA) (Pierce, #20660), Disuccinimidyl Glutarate (DSG) (Pierce, cat. no.20593) and Disuccinimidyl Suberate (DSS) (Fluka, #80424) which cross-links the proteins in the cells were used to increase cross-linking efficiency. Formaldehyde (FA) solutions were prepared from 38% stock solution (BDH Analar, #101135B) in serum-free growth media and supplemented by 1 mM $MgCl_2$ to keep cells adherent during fixation.

Antibodies recognising	First Fixation Solution	Duration (min)	Second Fixation Solution	Duration (min)
Modified histones	0.37 % FA	15	-	-
CTCF	1% FA	15		
RNA polymerase II	1 % FA	60	-	-
	10 mM DMA	45	1% FA	15
	10 mM DMA	60	1% FA	15
	2 mM DSS	52.5	1 % FA	15
	2 mM DSG	52.5	1 % FA	15

Table 2.12 Chromatin Fixation Conditions

For fixation, 50 ml of fixation solution (see Table 2.12) was added onto the cells in 500 cm^2 culture dishes and incubated at room temperature on orbital shaker. For the sequential fixation, cells were first incubated with 50 ml of first fixation solution (see Table 2.12) on an orbital shaker. The solution was discarded and 50 ml of 1 % formaldehyde solution was added onto the cells for further 15 min incubation on orbital shaker. Then, 3.3 ml of 2 M Glycine solution was added (0.125 mM of final concentration) to the plates and incubated for 5 min on orbital shaker to stop the fixation reaction. The solution was discarded and cells were washed with 20 ml of 1X PBS by rocking the plate for 5 sec.

One protease tablet (Roche Applied Sciences, # 11697498001) was dissolved in 20 ml of ice-cold 1XPBS. 2 ml of ice-cold 1XPBS supplemented by proteases were added onto plates and cells were scraped from the plates using a universal plate lid. One plate of HeLa cells or two plates of NTERA-D1 cells (~150 million cells in total) were collected to one 15 ml eppendorf tube.

Cells were spun at 2000 rpm (825 g) for 6 min ºC, the supernatant discarded. The cell pellet was fully resuspended in 1.5 ml of ice-cold 1XPBS supplemented by proteases and spun again at 2000 rpm (825 g) for 5 min at 4 ºC. Now, the cells are ready for lysis and chromatin extraction.

2.5.3 Cell Lysis and extraction of chromatin

Cells were fully resuspended in 3 ml of Cell Lysis Buffer by gently pipetting up and down and incubated for 10 min on ice for complete lysis. Then, the lysate was centrifuged down at 3200 rpm for 5 min at 4 ºC to pellet nuclei. The supernatant was carefully discarded and the pellet were fully resuspended in 1.2 ml of Nuclei Lysis Buffer by gently pipetting up and down and incubated for 10 min on ice. After incubation, 720 µl Dilution Buffer, the sample was mixed gently and transferred to a 5 ml falcon tube. Now, the samples are ready for sonication.

2.5.4 Sonication

An MSE Sanyo Soniprep 150 sonicator was used to sonicate the chromatin samples. Sonication was performed to fragment the chromatin between 300 – 600 bp fragments. Higher amplitude settings were used for samples fixed with either 1% formaldehyde or other protein cross-linking chemicals. The sonication conditions were listed in Table 2.13.

Cell Type	HeLa S3		NTERA-D1	
Fixation Conditions	**0.37% FA**	**1% FA**	**0.37% FA**	**1% FA**
Amplitude (microns)	14	15.2 (maximum)	14	15.2 (maximum)
Number of Bursts	8	8	6	6
Length of bursts (sec)	30	30	30	30

Table 2.13 Sonication Conditions

The sonication probe (Exponential microprobe, 3 mm in diameter) was placed approximately 1-2 cm below the surface of the sample. Sample was kept on ice-water bath at all times during sonication. Also, one min break was allowed between each burst to prevent over-heating of the sample.

After sonication, the lysate were transferred to a 2 ml eppendorf tube and centrifuged for 10 min at 13,000 rpm at 4 ºC to remove insoluble cellular debris. Then, the lysate was transferred to a 15 ml falcon tube and 4.1 ml Dilution Buffer was added to bring the ratio of Nuclei Lysis Buffer to Dilution Buffer to 4:1. At this point, chromatin is

ready for immunoprecipitation. For long term storage, the chromatin was flash-frozen by keeping the samples for 5 min in dry ice and stored in -70 ºC until further use.

2.5.5 Antibodies

Antibodies used in this study are listed in Table 2.14.

Company	Cat. No.	Raised for	Modification	Residue	Raised in	Abbreviation
Santa Cruz Biotechnology	sc-15914 (C-20)	CTCF	-	-	Goat	CTCF
Abcam	ab7766	Histone 3	Di-methylated	Lysine 4	Rabbit	H3K4me2
Abcam	ab8580	Histone 3	Tri-methylated	Lysine 4	Rabbit	H3K4me3
Abcam	ab8895	Histone 3	Mono-methylated	Lysine 4	Rabbit	H3K4me
Abcam	ab9045	Histone 3	Di-methylated	Lysine 9	Rabbit	H3K9me2
Abcam	ab7312	Histone 3	Tri-methylated	Lysine 27	Rabbit	H3K27me3
Upstate	06-599	Histone 3	Acetylated	Lysine 9, 14	Rabbit	H3Ac
Upstate	06-866	Histone 4	Acetylated	Lysine 5, 8,12 and 16	Rabbit	H4Ac
Abcam	ab5131	PolII CTD	Phosphorylated	Serine 5	Rabbit	polII

Table 2.14 Antibodies used in this study

2.5.6 Pre-clearing of the Chromatin

To reduce the non-specific binding, chromatin was treated with the isotype of the antibody which will be later used for immunoprecipitation. 100 µg of either Rabbit IgG (Upstate, #12-370) or Goat IgG (Santa Cruz Biotechnology, #sc-3850) was added to the chromatin according to the antibody used for the immunoprecipitation and incubated for 1 hour in the cold room on a rotator. Then, 200 µl of homogenous Protein G Agarose Beads (Roche Applied Sciences, #1719-416) were added to the chromatin and incubated 3 h in the cold room on a rotator. After incubation, the sample was centrifuged at 3000 rpm for 2 min at 4 ºC to pellet the beads. The supernatant is transferred to a new tube and it is ready for immunoprecipitation. 270 µl of pre-cleared chromatin was taken as the input chromatin (complete DNA content of the cells) and stored in -20 ºC until further use.

2.5.7 Immunoprecipitation

For immunoprecipitation, 10 µg of antibody was incubated with 1080 (modified histones and CTCF) or 1350 µl (RNA polymerase II) of chromatin. The negative control antibody (the isotype of the corresponding antibody) was always included in the experiment to determine the background level. All antibody incubations were performed overnight (14-16 h) in the cold room on a rotator.

2.5.8 Addition of Protein G Agarose Beads to Antibody/Chromatin Mixture

Chromatin/Antibody complex was centrifuged at 13,000 rpm for 5 min at 4 ºC on a table-top centrifuge and supernatant was transferred to a new tube. 100 µl of fully resuspended Protein G Agarose Beads were added to each mixture and incubated 3 h in the cold room on a rotator. Then, the samples were centrifuged at 13,000 rpm for 20 sec at 4 ºC to pellet the beads, the supernatant was discarded and the beads are ready for washing.

2.5.9 Washing Antibody/Chromatin/Bead Complexes

This step is necessary to remove non-specific complexes and unbound protein and DNA molecules. The beads were washed with 750 µl of Wash Buffer I twice. For each wash, the sample was vortexed for 3 sec and centrifuged at 7500 rpm for 2 min at 4 ºC. The tubes were left undisturbed for one min before removing the supernatant at each wash. Then, the beads were washed once with 750 µl of Wash Buffer II and twice 750 µl TE at pH 8.0 as described above. Now, the antibody/chromatin complex is ready to be separated from the beads.

2.5.10 Elution of the Antibody/Chromatin Complexes

225 µl of Elution Buffer was added to the washed beads, vortexed for 3 sec and incubated for a min at room temperature. Then, the samples were vortexed again and

centrifuged at 7500 rpm for 2 min at room temperature. The supernatant was carefully transferred to a new tube. This step was repeated and elutions (containing antibody/chromatin complexes) were collected in the same tube.

2.5.11 Reversal of Cross-linking and Digestion of RNA

1 µl of RNase (ICN Biochemicals, #101076) from 2 mg/ml stock solution and 27 µl of 5 M NaCl (final concentration of 0.3 M) were added to the samples. Also, 1 µl of RNase and 16.2 µl of 5 M NaCl were added to input chromatin samples. Then, the samples were incubated at 65 ºC for 6 h to reverse the cross-linking.

2.5.12 Digestion of Proteins and Recovery of DNA

After reversal of cross-linking, 9 µl of Proteinase K (Gibco-BRL #25530-031) from 10 mg/ml stock solution were added to the samples and the samples were incubated at 45 ºC overnight to digest proteins.

To recover DNA, 2 µl of tRNA (Gibco-BRL 15401-029) from 5 mg/ml stock solution was added immediately before adding 500 µl of water saturated phenol (Rathburn Chemicals, cat. no RP3024). The sample was mixed well and centrifuged at 13,000 rpm on a table-top centrifuge for 5 min. The aqueous layer was transferred to a new tube and 500 µl of chloroform (Rathburn Chemicals, RH1009) was added. The sample mixed well and centrifuged at 13,000 rpm on a table-top centrifuge for 5 min and the aqueous layer was transferred to a new tube. 1 µl of tRNA, 5 µl glycogen (Roche Applied Sciences, #901 393), 50 µl 3 M Sodium Acetate and 1200 µl absolute ethanol were added to the sample and the sample is put at -70 ºC for 30 min for DNA precipitation. Then, the sample was centrifuged at 13,200 rpm for 20 min at 4 ºC to precipitate DNA. The DNA pellet was washed with 500 µl of 70 % ethanol by centrifugating at 13,000 rpm for 5 min at room temperature. The supernatant was

removed and DNA pellet was air-dried for 10 min. 100 µl or 50 µl of dd water were added to input chromatin or ChIP samples respectively.

5 µl of Sucrose/Creosol Red was added to 5 µl of each ChIP sample or 1 µl of input chromatin and the mixes were loaded on 1 % agarose gel and run for 2 h at 100 V to check the recovery of DNA.

2.6 Real-time PCR

2.6.1 Assessing ChIP efficiency

A Real-time PCR kit from Eurogentec (#RT-SN2X-03+WOUN) was used to assess the crosslinking efficiency or antibody performance. This kit provides a 2X reaction buffer which contains the PCR buffer, dNTPs, HotGoldStar DNA polymerase, MgCl2, SYBR© Green I, stabilizers and passive reference. The real-time PCR reactions are set up according to Table 2.15.

Reagents	Volume (µl)
2X Reaction Buffer	6.25
1.5 µM Primer Mix	2.5
DNA template	variable
DD water	up to 13
Total Volume	13.00

Table 2.15 Reaction set up for real time PCR with Eurogentec real-time PCR kit

There are 11 primer pairs designed to span promoter region of C20orf121 (-1946 bp to+393 bp; TSS is at +1) used for real-time PCR (see section Appendix B for primer list). ChIP sample and input chromatin were diluted in 1:10 and 1:20 respectively, and 2 µl of the diluted materials were used as templates for their corresponding reaction. Human genomic DNA (50 ng) was used as the positive control. The real-time PCRs are performed on ABI PRISM® 7700 Sequence Detection System in a 96-well plate format according to manufacturer's instructions. The real time PCR steps are as follows; incubation for 2 min at 50 ºC followed by 10 min incubation at 95 ºC, then

40 cycles of 15 sec incubation at 95 ºC and 1 min incubation at 60 ºC. Ct values and amplification curves were generated automatically by the ABI PRISM® 7700 software.

2.6.2 Validation of ChIP on chip enrichment

The real-time PCR kit mentioned in section 2.6.1 is used to validate the enrichments obtained from ChIP on chip experiments. Input chromatin is diluted in 1:40 for amplification. Then, the reactions were set up according to Table 2.15, 0.1 µl DNA template (ChIP material or 1:40 diluted input chromatin) was used. The real-time PCR steps and analysis are described in section 2.6.1. Primers that are used to validate ChIP on chip results were listed in Appendix B.

2.7 Preparation of ChIP samples for hybridization onto microarrays

2.7.1 Labelling of ChIP samples

ChIP'ed DNA and input chromatin were randomly labelled using Bioprime Labelling Kit (Invitrogen, cat.no. 18094-011). 40 µl of ChIP sample were mixed with 60 µl 2.5X random primers solution and make up to 130.5 µl with dd water. For each ChIP sample, 2 µl of input chromatin was mixed with 60 µl 2.5X random primers solution and make up to 130.5 µl with dd water. The samples were denatured at 100 ºC for 15 min and immediately cool on ice for 5 min.

15 µl of deoxynucleotides mix (2 mM dATP, 2mM dGTP, 2 mM dTTP and 1 mM dCTP) was added to the reaction. 1.5 µl of cyanine-3 labelled dCTP analogue (NEN Life Sciences, #NEL576) was added to the ChIP'ed DNA and 1.5 µl of cyanine-5 labelled dCTP analogue (NEN Life Science, #NEL577) was added to input chromatin reaction. Then, 3 µl of Klenow Fragment was added to both reactions and they were

incubated at 37 ºC overnight in the dark. After the incubation, 15 µl of Stop Buffer was added to the reactions.

2.7.2 Removal of unlabelled nucleotides

Micro-spin G50 columns from Pharmacia Amersham (#275330-01) was used to remove unlabelled nucleotides. Since the capacity of each column is 50 µl, 3 columns were used for each sample. The resin in the columns was resuspended by gentle vortexing and the bottom closure of the columns was snapped off. The caps of the columns were loosened by one-quarter turn and the columns were placed in 1.5 ml eppendorf tubes. The columns were centrifuged at exactly 735xg for 1 min and water was discarded. 50 µl of HPLC purified water was applied to each column and centrifuged at 735xg for 1 min. Then, the columns were placed onto 2 ml eppendorf tubes and 50 µl of the labelling reactions was applied to the centre of the angled surface of the resin of the column and the columns were centrifuged for 2 min at 735xg. The columns were discarded and the flow-through samples were combined in the same tube. 5 µl of the samples were run on a 1 % agarose gel for 2 h at 100 V to check the labelling efficiency.

2.7.3 Competitive Hybridization of Labelled Samples onto microarrays

2.7.3.1 Preparation of the samples for the hybridization

The following reactions were prepared for each sample

Cy3 Labelled DNA	~180 ul
Cy5 Labelled DNA	~180 ul
Human Cot1 DNA	135 ul
3 M sodium acetate at pH 5.2	55 ul
Absolute Ethanol	1200 ul

The reactions were mixed gently, and put at -70 ºC for 1 hour to precipitate the DNA. Meanwhile, the hybridization buffer was heated in a 70 ºC heat block. The samples were spun at 13,000 rpm at 4 ºC for 15 min. The supernatant was removed and 500 µl

80 % ethanol was added to the samples and spun at 13,000 rpm for 5 min. The supernatant was removed and the samples re-spun at 13,000 rpm for 1 more min and residual ethanol was take off with a small tip. The pellets were dried for 10 min in the dark. 125 µl of hybridization buffer and 3 ul yeast tRNA were added to the pellet and the samples were left for 2-3 min in a 70 ºC heat block before resuspending the pellet.

The samples were denatured for 15 min at 100 ºC and immediately cool on ice for 5 min. They were pulse-spun and incubated 1 hour at 37 ºC before the hybridization onto arrays.

2.7.3.2 Hybridization

Tecan HS4800 Pro® hybridization station was used for hybridizations of the samples onto microarrays. The small TECAN chambers with dimensions 50.8 mm by 18 mm were used for hybridizations. 100 µl of the sample was injected onto chambers and hybridized for 45 h at 37 ºC in the dark. Then, the slides were washed and dried according to the protocol given in Table 2.16.

Type	Number of Runs	Solution	Wash Time (sec)	Soak Time (sec)	Temperature (ºC)
Wash	10	1XPBS/0.05% Tween20	60	30	37
Wash	5	0.1X SSC	60	120	52
Wash	10	1XPBS/0.05% Tween20	60	30	23
Wash	2	DD water (HPLC)	30	0	23
Drying	8	CO_2	30	-	23

Table 2.16. Slide washing and drying protocol on Tecan HS4800 Pro hybridization station.

2.7.4 Array Scanning

Slides were scanned using ScanArray Express HT microarray scanner (Perkin Elmer). Two laser of 633 and 543 nm wavelength were used to detect signal from Cy5 and Cy3 labelled molecules respectively. PMT gains used for Cy5 and Cy3 lasers were 65 and 67% respectively.

2.8 Solutions

$T_{0.1}E$

10 mM Tris-HCl (pH8.0)
0.1 mM EDTA

10X TBE

Add the following to 800 ml distilled water

- 108g Tris base (Sigma-Aldrich, #T3253)
- 55g Boric acid (Fisher, B/3800/53)
- 9.3g EDTA (NBS Bio, #0105)

Adjust volume to 1 L with additional distilled water

Dilute 1:10 to obtain 1XTBE agarose gel running buffer

SOC medium

2.0 g Bacto®-tryptone
0.5 g Bacto®-yeast extract
1 ml 1M NaCl
0.25 ml 1M KCl
1 ml Mg2+ stock (1M MgCl2 • 6H2O, 1M MgSO4 •7H2O), filter-sterilized
1 ml 2M glucose, filter-sterilized

- Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml distilled water
- Stir to dissolve
- Autoclave and cool to room temperature
- Add 2M Mg2+ stock and 2M glucose stock
- Filter the complete medium through a 0.2µm filter unit.
- Adjust pH to 7.0

LB Media

10 mg/ml Bacto®-tryptone
5 mg/ml Bacto®-yeast extract
10 mg/ml NaCl
Adjust pH to 7.4

LB-agar

(for LB-agar plates)
1 L of LB media
15 g Agar

Autoclave before use

LB growth medium

92.4 ml LB broth
7.5 ml 100% glycerol
0.1 ml 25 mg/ml chloroamphenicol

10X NEB PCR Buffer

For 100 ml;
8 g Tris
2.2 g $(NH_4)_2SO_4$
6.7 ml 1 M $MgCl_2$

Adjust pH to 8.8 and add DD water up to 100 ml. Filter sterilize before use.

Cresol red solution

0.1 g/l cresol red in $T_{0.1}E$

Sucrose/cresol red (1 litre of 40%)

400 g sucrose
0.1 g cresol red
Made up to 1000 ml with DD water

5X MOPS Buffer

0.2 M MOPS
50 mM Sodium Acetate
5 mM EDTA

Treated with DEPC by adding 1 ml of DEPC per litre of solution and incubate overnight, then autoclave.

2X RNA Loading Buffer

50% deionized formamide
1X MOPS Buffer
6.5% formaldehyde
5.4% saturated bromophenol dye
5.4% glycerol
15 ug/ml Ethidium Bromide

Cell Lysis Buffer (ChIP)

10 mM Tris-HCl pH 8.0
10 mM NaCl
0.2% NP40
10mM sodium butyrate

1 Roche Protease Tablet was added per 10 ml of solution

Nuclei Lysis Buffer (ChIP)

50 mM Tris-HCl pH 8.1
10 mM EDTA
1% SDS
10mM sodium butyrate

1 Roche Protease Tablet was added per 10 ml of solution

IP Dilution Buffer (ChIP)

20mM Tris-HCl pH 8.1
150 mM NaCl
2mM EDTA
1% Triton X-100
0.01% SDS
10mM sodium butyrate

1 Roche Protease Tablet was added per 10 ml of solution

IP Wash Buffer I (ChIP)

20 mM Tris-HCl pH 8.1
50 mM NaCl
2 mM EDTA
1% Triton X-100
0.1% SDS

IP Wash Buffer II (ChIP)

10 mM Tris-HCl pH 8.1
250 mM LiCl
1 mM EDTA
1% NP-40
1% deoxycholic acid

IP Elution Buffer (ChIP)

100 mM $NaHC0_3$
1% SDS

Hybridization Buffer (ChIP)

2x SSC
50% deionised formamide
10mM Tris-HCl pH7.4
5% dextran sulphate
0.1% Tween 20

Promega Buffer E (10X)

6 mM Tris-HCl
6 mM $MgCl_2$
100 mM NaCl
1 mM DTT
Adjust pH to 7.5 at 37 ºC.

10X PCR CHIP Buffer

500 mM KCl
50 mM Tris-HCl, pH 8.5
25 mM $MgCl_2$

4X Spotting Buffer

1 M NaH_2PO_4
Adjust pH to 8.5

Add 0.001% Sarkosyl

1X PBS at pH 7.4

(0.2 M phosphate + 1.5 M NaCl)

2.28 g NaH_2PO_4 (mw=120)
11.5 g Na_2HPO_4 (mw=141.96)
43.84 g NaCl
Bring final volume to 1 L with dd water
Adjust pH to 7.4

5X MOPS Buffer

0.2 M MOPS at pH 7.0
50 mM sodium acetate
5 mM EDTA

Autoclave before use

DEPC-treatment of DD water

1 L DD water
1 ml DEPC

Incubate overnight and autoclave before use.

2X RNA Loading Buffer

50% deionized formamide
5.4% saturated bromophenol/xylene
cyanol dyes
5.4% glycerol
15 ug/ml Ethidium Bromide
6.5% formaldehyde
1x MOPS