Chapter 2

Materials and Methods

Materials

2.1. Composition of Solutions

Note: Only sterile filtered HPLC water was used to prepare solutions.

1 X SET

- 10 mM Tris-Cl pH 7.5
- 100 mM NaCl
- 1 mM EDTA

10 X PCR buffer

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

10 mM dNTP mix for PCR

- 10 mM each dNTP (dCTP, dGTP, dATP, dTTP)

4 X Spotting buffer

- 1 M sodium phosphate buffer pH 8.5
- 0.001% sarkosyl

Lysis Buffer for DNA preps

- 50 mM glucose
- 10 mM EDTA
- 25 mM Tris pH 8.0

10 X dNTP mix used in DNA labeling

(a) The following mix was used in the labeling reactions that were used with array hybridisations set up manually.

- 0.5 mM dCTP
- 2 mM each of dGTP, dTTP and dATP

(b) The following mix was used in the labeling reactions that were used with array hybridisations set-up using Tecan

- 1 mM dCTP
- 2 mM each of dGTP, dTTP and dATP

Hyb 3 Buffer

- 50% formamide (Fluka)
- 10% dextran sulphate
- 0.1% Tween 20 (BDH)
- 2 X SSC
- 10 mM Tris pH 7.4

Tecan-hyb Buffer

- 50% formamide (Fluka)
- 5% dextran sulphate
- 0.1% Tween 20 (BDH)
- 2 X SSC
- 10 mM Tris pH 7.4

PBS/0.05% Tween 20 (Hyb wash solution 1)

PBS/0.05% Tween 20 for washing the arrays was prepared by dissolving the following salts in one litre of HPLC water

- 7.33 g NaCl
- 2.36 g Na_2HPO_4
- 1.52 g $NaH_2PO_4H_2O$
- 500 µl Tween 20

Cell lysis buffer (CLB)

- 10 mM Tris-HCl pH 8.0
- 10 mM NaCl
- 0.2% Igepal
- 10mM Sodium butyrate
- 50 µg/ml PMSF
- 1 µg/ml Leupeptin

Nuclei lysis buffer (NLB)

- 50 mM Tris-HCl pH 8.1
- 10 mM EDTA
- 1% SDS
- 10 mM Sodium butyrate
- 50 µg/ml PMSF
- 1 µg/ml Leupeptin

IP dilution buffer (IPDB)

- 20 mM Tris-HCl pH 8.1
- 150 mM NaCl
- 2 mM EDTA
- 1% Triton X-100
- 0.01% SDS
- 10 mM Sodium butyrate
- 50 µg/ml PMSF
- 1 µg/ml Leupeptin

IP wash buffer 1 (IPWB1)

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

IP wash buffer 2 (IPWB2)

- 10 mM Tris-HCl pH 8.1
- 250 mM LiCl
- 1 mM EDTA
- 1% Igepal
- 1% Deoxycholic acid

IP elution buffer (IPEB)

- 100 mM $NaHCO_3$

- 1% SDS

TE (pH 8.0)

- 10 mM Tris base (pH 8.0)
- 1 mM EDTA

1 X PBS (for ChIP assays)

1 X PBS used for washing the cells in ChIP assay was prepared by dissolving the following salts in 1 litre of HPLC water and the pH was adjusted to 7.4

- 8 g NaCl
- 0.2 g KCl
- 1.44 g Na_2PO_4
- 0.24 g KH_2PO_4

2.2. Reagents

Antibodies

- a complete list of all the antibodies with the company name and catalogue numbers, used for ChIP assays in this study, is included in chapter 5
- Avidin-Texas Red (Molecular Probes)
- Goat anti-mouse FITC (Sigma)
- Mouse anti-digoxigenin FITC (Sigma)

Enzymes

- Proteinase K, ≥ 20 units/mg (GibcoBRL)
- RNase A, ≥ 50 Kunitz units/mg (ICN Biochemicals)
- Gold AmpliTaq, 5 units/µl (Perkin Elmer-Cetus)
- Taq polymerase, 5 units/µl (Perkin Elmer-Cetus)
- Klenow fragment, 40 units/µl (Invitrogen)
- DNase I, 80 Kunitz units/ml (Sigma)
- DNA polymerase I, 10 units/µl (Sigma)

Fluorophores

- Cy3-dUTP (Amersham)
- Cy5-dUTP (Amersham)

- Biotin-16-dUTP (Boehringer)
- Digoxigenin-11-dUTP (Boehringer)

Primer pairs

- Lists and sequences of human and mouse primer pairs used to construct the SCL array or perform real-time PCR are provided in Appendices 1, 2, 3, 4 and 5. The appendix numbers are referenced in the relevant sections of this thesis.

Other reagents

- Human C_ot 1 DNA (Invitrogen)
- Mouse C_ot 1 DNA (Invitrogen)
- Herring sperm DNA (Sigma)
- Trizol (GibcoBRL)

2.3. Cells and Cell lines

Human haematopoietic cell lines i.e. K562 (Lozzio and Lozzio 1977), Jurkat (Schneider et al. 1977), HL-60 (Collins et al. 1977) and HPB-ALL (Morikawa et al. 1978) were gifts from Dr. Tony Green, The Department of Haematology, Cambridge.

Mouse haematopoietic cell line 416B (Dexter et al. 1979) was also a gift from Dr. Tony Green, The Department of Haematology, Cambridge.

Mouse E14 embryonic stem cell line (Hooper et al. 1987) was obtained from Dr. Peri Tate, The Sanger Institute, Cambridge.

The normal human male and normal human female lymphoblastoid cell lines “HRC 575” and “HRC 160” respectively were kindly provided by Dr. Nigel Carter, The Sanger Institute, Cambridge.

2.4. Bacterial clones

The human PAC RP1-18D14 and BAC RP11-332M15 clones used for PCR amplifications for the array construction and FISH analysis were identified using the ENSEMBL Cytoview (www.ensembl.org/Homo_sapiens/cytoview). Similarly, the mouse BAC clones, RP23-242O20, RP23-246H17 and RP23-32K12, used for the array construction were identified using the ENSEMBL Cytoview (www.ensembl.org/Mus_musculus/cytoview). The human PAC clone (RP1-18D14) was a gift from Dr. Tony Green, The Department of Haematology, Cambridge. The other human BAC clone as well the mouse BAC clones were picked individually from the clone

archives established at the Sanger Institute. All the clones were phage tested and subsequently prepped.

Methods

2.5. Tissue Culture

2.5.1. Culturing of all Cell lines

All cell lines were cultured in suspension in 50 ml of media (Sigma) with the appropriate amount of fetal bovine serum (GibcoBRL) and other supplements (Sigma) in 75 cm^2 tissue culture flasks with vented caps (Corning). Table 2.1 provides the information about the media and supplements used for each cell line. Note: Mouse E14 ES cells were cultured by Dr. Peri Tate (The Sanger Institute, Cambridge).

Cell line	Media	Fetal bovine serum	Supplements
K562	DMEM	9% v/v	1% penicillin-streptomycin solution
Jurkat	RPMI 1640	9% v/v	1% penicillin-streptomycin solution, 2 mM L-glutamine
HL60	RPMI 1640	9% v/v	1% penicillin-streptomycin solution, 2 mM L-glutamine
HPB-ALL	RPMI 1640	16% v/v	1% penicillin-streptomycin solution, 2 mM L-glutamine
416B	RPMI 1640	16% v/v	1% penicillin-streptomycin solution, 2 mM L-glutamine
HRC 575	RPMI 1640	16% v/v	1% penicillin-streptomycin solution, 2 mM L-glutamine
HRC 160	RPMI 1640	16% v/v	1% penicillin-streptomycin solution, 2 mM L-glutamine

Table 2.1: List of all the cell lines used for the study presented in this thesis. Note: All cell types were cultured under 5% CO_2 at 37°C.

Once confluent, sub-culturing was carried out as follows:

1. 25 ml of fresh media was added to each flask and any clumps of cells were gently broken up using a syringe.
2. The culture was then distributed between three new 75 cm^2 flasks (Corning) and further 25 ml of fresh media was added to each flask, effecting a 1/3 dilution of the confluent starting culture.
3. The number of cells needed for chromatin immunoprecipitation (ChIP) experiments was quite high. Therefore, culture volumes to obtain the required number of cells per flask were suitably scaled up in 175 cm^2 culture flasks with vented caps (Corning).

2.5.2. Cell cryopreservation

For frozen storage, cells were pelleted at 259 g for 5 to 8 minutes, and resuspended at approximately $1X10^7$ cells/ml in 10% (v/v) DMSO in FBS (GibcoBRL). The resulting cell mixture was transferred into polypropylene cryotubes which were cooled overnight to -70°C. The cryotubes were then transferred to the gas phase of a liquid nitrogen vessel (approximately -180°C) for permanent storage. To reconstitute cultures, cells were thawed rapidly at 37°C, washed once with fresh media and finally resuspended in 10 ml of fresh media.

2.6. DNA Preparation

2.6.1. Preparation of Total Genomic DNA

Total genomic DNA was extracted from all the human cell lines listed in Table 2.1. Fresh cultures were grown in the appropriate media with added supplements (Table 2.1).

1. When confluent, the cells were spun down at 259 g for 5-8 minutes and the pellets were washed with PBS and spun down again.
2. The pellets were resuspended in 4.5 ml 1XSET, 250 µl of 10% SDS and 100 µl of 10 mg/ml Proteinase K (GibcoBRL) and the resulting solutions were incubated overnight at 37ºC.
3. 5 ml of Phenol (saturated with 0.1M Tris, Rathburn Chemicals) was added to each sample and the samples were rolled for 10 minutes at room temperature.
4. Samples were then centrifuged at 1620 g for 5 min at room temperature and the aqueous (top) layer was collected very gently using a P1000 tip (cut off at the tip to make a wider bore) and transferred to a fresh 50 ml Falcon tube. An equal volume of phenol/iso-amyl alcohol/chloroform (25:1:24, pH 6.6-8.0, BDH) was added to the aqueous layer.
5. 5 ml of 1XSET was added to the organic (bottom) layer and rolled for 10-15 minutes at room temperature.
6. The two layers were separated as before by centrifuging at 1620 g for 5 minutes at room temperature. The aqueous layer was again removed very gently using a cut-off P1000 tip and added to the aqueous layer collected in the earlier step containing the phenol/iso-amyl alcohol/chloroform.
7. The aqueous layer/isoamyl-alcohol/chloroform mixture was rolled for 10-15 minutes and then centrifuged at 1620 g for 5 minutes, both at room temperature. As in previous steps, the two layers were separated and the aqueous (top) layer was

added to a fresh 50 ml Falcon tube containing an equal volume of iso-amyl alcohol/chloroform (1:24) mixture. The organic (bottom) layer was discarded.

8. The aqueous layer/isoamyl alcohol/chloroform mixture was rolled for 10-15 minutes and then centrifuged at 1620 g for 5 minutes, both at room temperature. The aqueous layer was removed and placed in a fresh, empty 50 ml Falcon tube.
9. The DNA was precipitated by adding 1/10th the volume, i.e. 1 ml, of 3M NaAc (pH 6.0) and 2.5 X volume, i.e. 25 ml, absolute ethanol to the aqueous layer and incubated at –20ºC overnight.
10. The precipitated DNA was spooled out using sterile glass hooks and then dipped in 70% ethanol to rinse the DNA. The DNA was air dried on the hooks and then very gently dissolved in 400 µl 1XTE in 1.5 ml microfuges. The DNA was kept at 4ºC to dissolve. The concentration of the DNA samples was determined by measuring the optical density at 260 nm.

2.6.2. Preparation of DNA from BAC and PAC clones

DNA from human and mouse BAC, PAC clones was prepared by the following alkaline lysis “mini-prep” method.

1. All the clones (section 2.3) were grown in 10 ml cultures with 2 X TY media at 37ºC overnight supplemented with 25 µl of 10 mg/ml kanamycin (Sigma) for PAC and 10 µl chloramphenicol (Sigma) for the BACs.
2. The cultures were centrifuged the next day at 2080 g for 10 minutes at room temperature. The supernatant was poured off and 200 µl of lysis buffer was added to the pellets to lyse the cells.
3. The solution was transferred to 1.5 ml microfuges and 400 µl of freshly made 0.2M NaOH/1% SDS solution was added to each sample. The samples were incubated on ice for 10 minutes.
4. 300 µl of 3M NaAc (pH 5.2) was added to each sample and incubated on ice for 10-30 minutes.
5. The samples were centrifuged at 18000 g for 5 minutes at room temperature and the supernatant was transferred to fresh 1.5 ml microfuges and the precipitates were discarded. This step was repeated 3-4 times till the supernatant was clear.
6. 600 µl Isopropanol was added to the clear supernatant and incubated at –70ºC for 10 minutes.
7. The tubes were centrifuged at 18000 g for 5 minutes and the pellets were resuspended in 200 µl of 0.3M NaAc, pH 7.0.

8. 200 µl of water saturated phenol/chloroform[a] was added to the samples and vortexed briefly. Samples were centrifuged at 18000 g for 3 minutes. The aqueous (top) layer was collected in fresh 1.5 ml eppendorf tubes. This step was repeated twice.
9. The DNA was precipitated from the aqueous layer by adding 200 µl isopropanol and incubating the solutions at –70ºC for 10 minutes.
10. The solutions were centrifuged at 18000 g for 5 minutes and the pellets obtained were washed with 70% ice-cold ethanol.
11. The pellets were air dried at 37ºC and finally resuspended in 50 µl of T0.1E containing 200 µg/ml RNaseA. After a quick vortex and a quick centrifuge, the tubes were incubated at 55ºC for 15 minutes in a waterbath.
12. 1 µl of each sample was analyzed on a 1% agarose 1 X TBE gel and stained with ethidium bromide for visualization. The DNA samples were stored at –20ºC.

[a]Safety Note: All the steps with phenol/chloroform were performed in a fume cabinet.

2.6.3. Chromatin Immunoprecipitation (ChIP)

K562, Jurkat, HL60, HPB-ALL, 416B and ES cell line E14 were used for chromatin immunoprecipitation. Fresh cultures were grown (Table 2.1) for each cell line and cells were harvested for ChIP. Aliquots of cells used for each ChIP experiment were subjected to flow-sorting (Cytomation MoFlo High Performance Cell Sorter, Dako Cytomation) in parallel for cell-cycle analysis. For this, ~5 ml of cell culture was washed with 10 ml of PBS. The cells were then fixed in 5 ml of 70% ethanol.

Approximately 1 X 10^8 cells were harvested for each ChIP procedure which were then used to set-up ten immunoprecipitation (IP) conditions as described below.

Fixation

1. The cells were collected by centrifuging at 259 g for 8 minutes at room temperature and resuspended in 50 ml of serum free media in a glass flask.
2. DNA-protein and protein-protein interactions were cross-linked by adding formaldehyde (37%, BDH AnalaR). 500 µl, 1010 µl or 1355 µl formaldehyde was added drop-wise to a final concentration of 0.37%, 0.75% for histone modifications and 1% for transcription factors respectively.
3. The cross-linking was carried out at room temperature with constant but gentle stirring for 10 minutes (for histone modifications) or 15 minutes (for transcription factors).
4. 3.15 ml, 3.41 ml or 3.425 ml (for 0.37%, 0.75% or 1% formaldehyde concentration respectively) of ice-cold 2M glycine was added to a final concentration of 0.125M with

constant but gentle stirring for 5 minutes at room temperature to stop the cross-linking reaction.

5. Cells were transferred to 50 ml falcon tubes and kept on ice whenever possible. The cells were pelleted by centrifuging at 259 g for 6-8 minutes at 4°C and washed with 1.5 ml of ice-cold PBS.
6. After washing, the cells were pelleted at 720 g at 4°C for 5 minutes and the supernatant was removed.

Cell and Nuclei Lysis

7. Cells were lysed by adding 1.5 X pellet volumes of ice-cold cell lysis buffer (CLB). The cell pellets were gently resuspended and incubated on ice for 10 minutes.
8. The nuclei were recovered by centrifuging the samples at 1125 g for 5 minutes at 4°C.
9. After carefully removing the supernatant, the nuclei were lysed by resuspending the pellet in 1.2 ml of nuclei lysis buffer (NLB) and incubating on ice for 10 minutes.

Sonication

10. 720 µl of IP dilution buffer (IPDB) was added and the samples were transferred to 5 ml glass falcon tubes (Falcon 2058).
11. The chromatin was sonicated to reduce the DNA length to an average size of 600 bp using the Sanyo/MES Soniprep sonicator. The tip of the probe was dipped to reach approximately halfway down the total level of the liquid sample and the tube was kept constantly on ice (Conditions for sonication like number of bursts, length of bursts and power setting depend on the sonicator tip used). The settings used for the sonicator were:
    - Amplitude: 14 microns
    - Number of bursts: 8
    - Length of bursts: 30 seconds

The samples were allowed to cool on ice for 1 minute between each pulse (5 µl of the sheared chromatin was run on an agarose gel to check sonication, see step 32).

12. The sonicated chromatin was transferred to 2 ml microfuge tubes and spun down at 18000 g for 10 minutes at 4ºC.

Immunoprecipitation

13. The supernatant was transferred to a 15 ml falcon tube and 4.1 ml of IP dilution buffer was added[a].

14. The chromatin was precleared by adding 100 µl of normal rabbit IgG (Upstate Biotechnology). The samples were incubated for I hour at 4°C on a rotating wheel.
15. 200 µl of homogeneous protein G-agarose suspension (Roche) was added to the precleared chromatin and the samples were incubated for 3-5 hours at 4°C on a rotating wheel.
16. The samples were centrifuged at 1620 g for 2 minutes at 4°C to pellet the protein G-agarose beads and the supernatant was used to set up various immunoprecipitation (IP) conditions in 2 ml microfuge tubes. An aliquot of 270 µl of chromatin was stored at -20°C to be used as input sample for array hybridisations and real-time PCR. An NLB:IPDB buffer at the ratio of 1:4 was prepared to set-up IP conditions as follows:
    - No chromatin – 1350 µl NLB:IPDB buffer
    - No antibody – 675 µl chromatin + 675 µl NLB:IPDB buffer
    - Normal Rabbit IgG – 675 µl chromatin + 675 µl NLB:IPDB buffer + 10 µl rabbit IgG (Upstate Biotechnology)
    - Test IP conditions – 675 µl chromatin + 675 µl NLB:IPDB buffer + 5-20 µg* of test antibody

(*5-20 µg for antibodies raised against histone modifications and 10 µg for the antibodies raised against specific transcription factors). A complete list of antibodies that were tested for this study is provided in chapter 5.

17. The samples were incubated at 4°C overnight on a rotating wheel.
18. The samples were centrifuged at 18000 g for 5 minutes at 4ºC and the lysate/Ab samples were transferred to fresh 2 ml microfuge tubes. 100 µl of homogeneous protein G-agarose suspension was added to each sample and the samples were incubated at 4°C for at least 3 hours on a rotating wheel.
19. The samples were centrifuged at 6800 g for 30 seconds at 4°C to pellet the protein G-agarose beads.
20. The supernatant was removed and the protein G-agarose beads were carefully washed. For each wash, the wash buffer was added, the samples were vortexed briefly, were centrifuged at 6800 g for 2 minutes at 4°C and left to stand on ice for 1 minute before removing the supernatant. The washes were carried out in the following sequence:
    a) The beads were washed twice with 750 µl of cold IP wash buffer 1. The beads were transferred to a 1.5 ml microfuge tube after the first wash.
    b) The beads were washed once with 750 µl of cold IP wash buffer 2.

c) The beads were washed twice with 750 µl of cold TE pH 8.0.

Elution

21. DNA-protein-antibody complexes were eluted from the protein G-agarose beads by adding 225 µl of IP elution buffer (IPEB). The bead pellets were resuspended in IPEB, briefly vortexed and centrifuged at 6800 g for 2 minutes at room temperature.
22. The supernatant was collected in fresh 1.5 ml microfuge tubes. The bead pellets in the original tubes were resuspended in 225 µl of IPEB again, briefly vortexed and centrifuged at 6800 g for 2 minutes. Both the elutions were combined in the same tube.

Reversal of cross-links

23. The reversal of cross-links step was carried out on the Input sample which was stored at -20°C previously. 0.1 µl of RNase A (10 mg/ml, 50 Kunitz units/mg[*], ICN Biochemicals) and 16.2 µl of 5M NaCl (to the final concentration of 0.3 M) was added to the Input DNA sample.
24. Similarly, 0.2 µl of RNase A (10 mg/ml, 50 Kunitz units/mg[*]) and 27 µl of 5M NaCl (to a final concentration of 0.3 M) was added to each of the IP test samples. All the samples including the Input DNA sample were incubated at 65ºC for 6 hours to reverse the cross-links.
25. 9 µl of Proteinase K (10 mg/ml, 20 U/mg, GibcoBRL) was added to each sample and incubated at 45ºC overnight[b].

Extraction of DNA

26. 2 µl of yeast tRNA (5 mg/ml, Invitrogen) was added to each sample just before adding 250 µl of phenol (Sigma) and 250 µl of chloroform[c].
27. The samples were vortexed and centrifuged at 18000 g for 5 minutes at room temperature. The aqueous layer (top layer) was collected in fresh 1.5 ml microfuge tubes and 500 µl of chloroform was added to each sample.
28. The samples were vortexed and centrifuged at 18000 g for 5 minutes at room temperature. The aqueous layer was transferred to a fresh 2.0 ml microfuge tubes.
29. 5 µg of glycogen (5 mg/ml, Roche), 1 µl of yeast tRNA (5 mg/ml, Invitrogen) and 50 µl of 3M NaAc (pH 5.2) was added to each sample and mixed well. The DNA was precipitated with 1375 µl of 100% ethanol and incubating at –70ºC for 30 minutes (or -20°C overnight).
30. The samples were centrifuged at 20800 g for 20 minutes at 4ºC. The DNA pellets were washed with 500 µl of ice-cold 70% ethanol and air-dried for 10-15 minutes.

31. The DNA pellets of the IP samples were resuspended in 50 µl of sterile filtered HPLC water and 100 µl for the Input DNA samples.
32. 5 µl of each sample was run on a 1% agarose 1XTBE gel and visualised with ethidium bromide to check DNA size. Samples were stored at –20°C.

[a]The sheared chromatin can be snap frozen in liquid nitrogen at this stage and the frozen samples should be stored at -70°C. When needed, the samples should be thawed on ice and the experiment carried on as per the protocol.

[b]The samples can be stored at -20°C after the step no. 22. When needed the samples can be thawed at room temperature and the DNA extracted as per the protocol.

[c]Safety Note – The phenol/chloroform steps were carried out in a fume cabinet.

*The amount of enzyme causing the hydrolysis of RNA at a rate such that k (velocity constant) equals unity at 25°C and pH 5.0.

2.7. Construction of the SCL Genomic Tiling Path Microarray

2.7.1. Generation of Human and Mouse SCL Genomic Tiling Path Amplicons

A. Primer Design

1. Primers pairs used to amplify PCR products for the human and mouse SCL tiling array were designed from the relevant genomic sequence for the human and mouse SCL. All the sequences were first analysed for their repetitive content using RepeatMasker. Primers were then designed using the masked sequences and Oligo 6 Software (Molecular Biology, Insights) and Primer3 software and website (Whitehead Institute, http//Frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky 2000).
2. Primer pair sequences were compared against the entire human or mouse genome sequence using e-PCR (Schuler 1997). The primer sequences for human and mouse amplicons are shown in Appendices 1 and 2 respectively.

B. First Round Amplification

1. PCR reactions were set up in 96 well thin-walled Thermowell plates (Costar, 6511) by mixing the following on ice:

1 µl	DNA template (50 ng/µl)
7 µl	1mM dNTP
3.2 µl	10Xtaq Gold buffer II
3.2 µl	DMSO

2.1 µl	$MgCl_2$
0.2 µl	5 U/µl Gold AmpliTaq
1 µl	Forward primer with 5' adaptor sequence
1 µl	Reverse primer
13.3 µl	Sterile ddH_2O
32.0 µl	

2. The cycling conditions for the reaction were as follows: 95ºC for 8 min; then 35 cycles of: 95ºC for 30 sec, 56ºC for 45 sec, 72ºC for 45 sec; followed by 72ºC for 10 min.
3. The PCR products were analyzed on 2.5% agarose 1 X TBE slab gels and stained with ethidium bromide for visualisation.
4. Depending on the intensity of the band, 1-4 µl of the first round PCR products were transferred to new 96-well plates in 100 µl TE. These served as the source of template for the second round reaction and were stored at –20ºC.

C. First Round Amplification (alternative method)

First round PCR was also performed using DNA from bacterial PAC and BAC clones as follows:

5. PCR reactions were set up in 96 well thin-walled Thermowell plates (Costar, 6511) by mixing the following on ice:

5 µl	DNA Template (1/100 dilution of miniprep DNA)
2 µl	10 X PCR buffer
1 µl	10mM dNTP mix
0.5 µl	gene-specific primer (sense) with 5' adaptor sequence
0.5 µl	gene-specific primer (antisense)
0.125 µl	5 U/µl Taq polymerase (Perkin Elmer-Cetus)
4 µl	5M Betaine (Sigma)
6.875 µl	Sterile ddH_2O
20.0 µl	

6. The following thermal cyclic conditions were used with varying number of cycles: 95ºC for 5 min; then 30/35 cycles of: 95ºC for 1 min, 60/55ºC for 1 min 30 sec, 72ºC for 1 min 30 sec; followed by 72ºC for 5 min.
7. The PCR products were analyzed on 2.5% agarose 1 X TBE slab gels and stained with ethidium bromide for visualization.

8. The single gel bands were picked up as gel stabs using P20 filter tips and transferred to deep well plates and sterile water was added to each and left overnight at 4ºC for the DNA to diffuse into the water. This DNA was used as a template for the second round PCR and was stored at –20ºC.

D. Second Round Amplification

1. PCR reactions were set up in 96 well thin-walled Thermowell plates (Costar, 6511) by mixing the following on ice:

15 µl	DNA template as prepared in the first round
6 µl	10 X PCR buffer
3 µl	10mM dNTP mix
1.5 µl	5'-aminolink universal primer (sense) 200 ng/µl
1.5 µl	Reverse primer 20 µM (antisense)
0.375 µl	5 U/µl Taq polymerase (Perkin Elmer-Cetus)
32.625 µl	Sterile ddH_2O
60.0 µl	

2. The thermal cyclic conditions used were: 95ºC for 5 min; then 25 cycles of 95ºC for 1 min, 45ºC for 1 min 30 sec, 72ºC for 1 min 30 sec; followed by 72ºC for 5 min.
3. The PCR products were analyzed on 2.5% agarose 1 X TBE slab gels and stained with ethidium bromide for visualization.
4. 50 µl of the PCR products were filtered through into new 96 well plates (Falcon, Microtest U-Bottom Tissue culture plates) along with 15 µl of 4 X Spotting buffer and stored at –20ºC until required for array printing.

2.7.2. Microarray Slide Printing and Processing

1. The DNA elements were arrayed onto amino binding slides (Motorola) at 20-25ºC, 40-50% relative humidity using the MicroGrid II arrayer (Biorobotics/Apogent Discoveries).
2. The array elements were spotted in a 16 block format with spots in quadruplicate or duplicate in the first and second generation arrays respectively.
3. The slides were transferred into a microscope slide rack and placed in a humid chamber (NaCl saturated with water in an air-tight container) and incubated for 24-72 hours at room temperature.
4. The slides were removed from the humid chamber and immersed in a 1% (w/v) solution of ammonium hydroxide and incubated for 5 minutes with gentle shaking.

5. The slides were then transferred to a solution of 0.1% (w/v) sodium dodecyl sulphate and incubated for 5 minutes with gentle shaking.
6. The slides were briefly rinsed in Milli-Q ddH_2O (Miili-Q plus 185 purification system) at room temperature and then placed in 95ºC Milli-Q ddH_2O for 2 minutes to completely denature the bound DNA elements.
7. The slides were transferred to ice-cold Milli-Q ddH_2O and then briefly rinsed two times in Milli-Q ddH_2O at room temperature.
8. The slides were dried by spinning at 180 g for 5 minutes.
9. The slides were stored in a slide box and kept at room temperature in a cool dry place until used.

2.8. Hybridisation of the array

2.8.1. Random Labeling of DNA samples

The SCL genomic tiling path array hybridisations using total genomic DNA and/or DNA obtained by chromatin immunoprecipitation were set up either manually or using the Tecan HS 4800 hybridisation station (an automated hyb-station). The array-CGH hybridisations using the 1 Mb BAC genomic microarrays were set up using Tecan automated hyb-station. For both hybridisation set-ups, the DNA was labeled using BioPrime Random Labeling Kit (Invitrogen) but with different labeling reactions as described below.

A. Labeling method used with manual hybridisation set-up

1. The following reagents were mixed on ice in a 1.5 ml microfuge:
    - 40 µl 2.5 X Random primer solution
    - x µl DNA *
    - (44-x) µl sterile H_2O

* The DNA amount that was labeled was different for each sample. For example, 600 ng of total genomic DNA was labeled per reaction for array-CGH hybridisations whereas the amount of DNA labeled in the ChIP-chip hybridisations was ~200 ng.

2. This mixture was heated at 100°C for 10 minutes to denature the DNA and then snap-chilled on ice. The following reagents were added to the tube on ice:
    - 10 µl 10 X dNTP mix (a)
    - 4 µl 1 mM Cy3/Cy5 labeled dCTP (1 mM Cy3-dCTP, 1 mM Cy5-dCTP, Amersham)
    - 2 µl Klenow fragment (40 U/µl)

The final volume per labeling reaction was 100 µl.

3. The reagents were mixed gently but thoroughly and incubated at 37°C overnight.
4. 10 µl stop buffer was added to the reaction mix to terminate the reaction.

B. Labeling method used with the hybridisation set-up using Tecan

1. The following reagents were mixed on ice in a 1.5 ml microfuge:
    - 60 µl 2.5 X Random primer solution
    - x µl DNA *
    - (70.5-x) µl sterile H_2O

* The DNA amount labeled was different for each sample. For example, 450 ng of total genomic DNA was labeled per reaction for array-CGH hybridisations whereas the amount of DNA labeled in the ChIP-chip hybridisations was ~200 ng.

2. This mixture was heated at 100°C for 10 minutes to denature the DNA and then snap-chilled on ice. The following reagents were added to the tubes on ice:
    - 15 µl 10 X dNTP mix (b)
    - 1.5 µl 1 mM Cy3/Cy5 labeled dCTP (1 mM Cy3-dCTP, 1 mM Cy5-dCTP, Amersham)
    - 3 µl Klenow fragment (40 U/µl)

The final volume per labeling reaction was 150 µl.

3. The reagents were mixed gently but thoroughly and incubated at 37°C overnight.
4. 15 µl stop buffer was added to the reaction mix to terminate the reaction.

Note: Only one labeling reaction was required for the SCL genomic tiling path array but two labeling reactions were used for the 1 Mb BAC genomic array.

2.8.2. Purification of labeled DNA Samples

The DNA samples labeled using the two labeling reactions described above were purified using the same protocol as described below.

1. Micro-spin G50 columns (Pharmacia Amersham) were used to remove the unlabeled nucleotides from the labeled DNA samples.
2. Two columns were used for each of the 100 µl labeling reactions and three columns were used for each of the 150 µl labeling reactions (100 µl and 150 µl being the final volumes of the labeling reactions).
3. The resin was resuspended in the columns by vortexing gently. The caps were loosened one-fourth and the bottom of the tubes snapped off. The columns were placed in 2.0 ml microfuge tubes and centrifuged at 1700 g for 1 minute.

4. 50 µl of sterile filtered HPLC water was applied to the resin-bed and the columns were centrifuged at 1700 g for 1 minute.
5. The columns were placed in fresh 1.5 ml microfuge tubes and the labeled DNA samples were carefully applied to the resin-bed. The columns were then centrifuged at 1700 g for 2 minutes.
6. The purified DNA samples were collected in the 1.5 ml microfuge tubes and the samples from the same labeled reaction were pooled together. The final volumes for the labeled DNA samples were approximately 120 µl (from 100 µl reactions) and 180 µl (from 150 µl reactions).
7. 5 µl of each labeled DNA was analyzed on a 1% agarose 1 X TBE gel and stained with ethidium bromide for visualization. The samples were used for hybridisation and stored in an opaque box at –20ºC.

2.8.3. Manual set-up of array hybridisations

The grid size of the SCL genomic tiling path array was 2 X 2 cm and the "open-well" method described below was used for the hybridisations that were set-up manually.

Preparation of the slide and the hybridisation chambers

1. The slide was prepared for hybridisation as follows:

   Based on the position of the spotted array grid on the slide, four layers of rubber cement were applied carefully around the grid to make a well. All the corners of the well were sealed properly to avoid leaking during hybridisation. The rubber cement was allowed to dry completely before setting up the hybridisations.
2. Two humidified slide chambers were prepared for the pre-hybridisation and hybridisation steps. The pre-hybridisation chamber was made from a square petri-dish and two cocktails sticks were glued to the floor of the dish. 2 X SSC/40% formamide (Fluka) solution was applied to Whatman paper strips lining the floor of the slide chamber.
3. Similarly, hybridisation chambers were prepared by lining the top and bottom of a slide mailer (R. A. Lamb) with whatman paper strips soaked in 2 X SSC/20% formamide (Fluka) to keep it humid.

Preparation of pre-hybridisation and hybridisation solutions

4. Two 2.0 ml microfuge tubes were set up for each slide - one with the pre-hybridisation mix and the second with the hybridisation mix. The following reagents were mixed together on ice and kept in the dark as much as possible:

Tube 1 (Pre-hybridisation mix)

- 400 µg Herring sperm DNA (10 mg/ml, Sigma)
- 67.5 µl Human Cot 1 DNA (Invitrogen)
- 25 µl 3M NaAc (pH 5.2)
- 800 µl 100% ice-cold ethanol

Tube 2 (Hybridisation mix)

- 120 µl Cy3-labeled DNA
- 120 µl Cy5-labeled DNA
- 90 µl Human Cot 1 DNA (Invitrogen)
- 35 µl 3M NaAc (pH 5.2)
- 1100 µl 100% ice-cold ethanol

5. The DNA samples in both reactions were precipitated by incubating at –70ºC for 30 minutes (or -20°C overnight). The DNA samples were centrifuged at 18000 g for 20 minutes at room temperature. The pellets were washed with 500 µl of ice-cold 80% ethanol and air dried.
6. The pre-hybridisation DNA pellet was resuspended in 75 µl of Hyb-3 buffer and the hybridisation DNA pellet in 30µl of Hyb-3 buffer and 3 µl of yeast tRNA (100 µg/µl, Invitrogen). Heating the solutions at 70°C helps to resuspend the pellets properly.

Array hybridisation and washing

7. Both the pre-hybridisation and the hybridisation DNA mixes were denatured for 10 minutes at 100ºC and quickly centrifuged.
8. The hybridisation mix was snap-chilled on ice and then kept at 37ºC for an hour for pre-annealing.
9. The denatured herring sperm/Cot 1 pre-hybridisation mix was applied very carefully to the slide covering the whole array area.
10. The slide was transferred to the humidified slide chamber (see step 2 above) and incubated at 37ºC in a rocking incubator at 5 rpm for an hour.
11. After the incubation, the slide was removed from the slide chamber and held at an angle to let the pre-hybridisation solution run into a corner to remove as much solution as possible.
12. The hybridisation mix was applied very carefully covering the array area and made sure that there were no bubbles within the array area.

13. The slide was transferred into a humidified slide chamber (see step 2 above) and the slide chamber was tightly sealed with parafilm to minimize evaporation and thus avoid the slide from drying out during incubation.
14. The slide was incubated for 48 hours at 37ºC in a gently rocking incubator (5 rpm).
15. The slide was removed from the incubator and the rubber cement was removed very carefully.
16. The slide was given a quick rinse in PBS/0.05% Tween 20 (BDH) to remove excess hybridisation solution.
17. The slide was first washed in PBS/0.05% Tween 20 (BDH) for 10 minutes at room temperature on a shaking platform.
18. The second wash was performed in 50% Formamide/2 X SSC at 42ºC for 30 minutes in a rocking incubator (maximum speed).
19. The third and final wash was performed in PBS/0.05% Tween 20 for 10 minutes at room temperature on a shaking platform.
20. The slides were then centrifuged at 180 g for 5 minutes to dry and were ready to be scanned. The slides were always stored in a light-proof slide box.

2.8.4. Array hybridisation set-up using the Tecan hybridisation station

Tecan HS 4800 hybridisation station (automated hyb-station) was used to set-up array-CGH hybridisations (on SCL tiling path array and 1 Mb BAC genomic array) and some of the ChIP-chip hybridisations (on the SCL genomic tiling path array). Tecan is a fully automated hyb station where the microarray slides are loaded on the Tecan's slide holder and the hybridisation mix is agitated to ensure even hybridisation. The SCL array area was 2 X 2 cm and consequently the smaller chambers were used on the Tecan to set up the hybridisations. However, larger chambers were required for the 1 Mb BAC genomic arrays

Preparation of the hyb station

1. The slide holders and the slide chambers were carefully cleaned and the slides were loaded on to the slide holder.
2. The wash solutions were prepared (described in section 2.1) and poured into the wash bottles of the Tecan and the hyb station was primed to remove any air bubbles in the liquid channels.

Preparation of pre-hybridisation and hybridisation solutions

Note: The following volumes were doubled for the larger chambers for the 1 Mb BAC genomic arrays.

3. Two 2.0 ml microfuge tubes were set up for each slide - one with the pre-hybridisation mix and the second with the hybridisation mix. The following reagents were mixed together on ice and kept in the dark as much as possible:

Tube 1 (Pre-hybridisation mix)

- 40 µl Herring sperm DNA (10 mg/ml, Sigma)
- 67.5 µl Human Cot 1 DNA (Invitrogen)
- 12 µl 3M NaAc (pH 5.2)
- 300 µl 100% ice-cold ethanol

Tube 2 (Hybridisation mix)

- 180 µl Cy3-labeled DNA
- 180 µl Cy5-labeled DNA
- 135 µl Human Cot 1 DNA (Invitrogen)
- 55 µl 3M NaAc (pH 5.2)
- 1100 µl 100% ice-cold ethanol

4. The DNA samples in both reactions were precipitated by incubating at –70ºC for 30 minutes (or -20°C overnight). The DNA samples were centrifuged at 18000 g for 20 minutes at room temperature. The pellets were washed with 500 µl of ice-cold 80% ethanol and air dried.
5. The pre-hybridisation and hybridisation DNA pellets were resuspended in 120 µl of Tecan-hyb buffer each and 3 µl of yeast tRNA (10̲0̲ µg/µl, Invitrogen) was added to the hybridisation mix. Heating the solutions at 70ºC helps to resuspend the pellets properly.

Array hybridisation and washing

6. The pre-hybridisation and hybridisation solutions were denatured at 100°C for 10 minutes. The hybridisation solution was snap-chilled on ice and then pre-annealed at 37°C for 1 hour. The pre-hybridisation solution was kept at 70°C until applied to the slide.
7. After vortexing, using a displacement pipette, 100 µl of the pre-hybridisation solution was injected onto the slide very slowly and carefully to avoid any air bubbles.
8. The pre-hybridisation step was performed at 37°C for 1 hour. The slides were washed once with PBS/0.05% Tween and dried with short blasts of nitrogen gas.

9. 100 µl of the hybridisation solution was injected slowly onto the slide using a displacement pipette. The hybridisation step was performed at 37°C for 48 hours (the 1 Mb BAC array was hybridised for 24 hours).
10. The slide washing was carried out on the Tecan which was programmed to perform the washes in the sequence listed in Table 2.2.

Steps	Wash Solutions	Temperature	No. of Washes	Wash Duration	
				Wash time	Soak time
1	PBS/0.05% Tween	37°C	10	1 min	30 secs
2	0.1 X SSC	52°C	5	1 min	2 min
3	PBS/0.05% Tween	R/T	10	1 min	30 secs
4	HPLC water	R/T	2	30 secs	

Table 2.2: Wash steps for hybridisations performed on the Tecan. The solutions were prepared in advance (section 2.1) using HPLC water. R/T stands for room temperature.

11. The slides were dried on the Tecan with short blasts of nitrogen gas and stored in the dark until scanning.

2.8.5. Scanning and Data Analysis for ChIP-chip experiments

After the images were scanned, two softwares – Quantarray and Scanarray Express (Perkin Elmer) were used to analyse the scanned images as described below. The analysis output from both the softwares were analysed using an excel spreadsheet.

1. Cy3 and Cy5 images at 5 µm resolution were acquired using the Scanarray 4000 confocal laser-based scanner (Perkin-Elmer) using a laser power of 100% and a photo multiplier tube (PMT) value of between 80%-85%.
2. Fluorescent intensities of each spot on the array were quantitated using Quantarray (version 3.0) (Perkin-Elmer) using the fixed circle quantitation methods. Using this software, each spot has to be aligned manually to obtain the mean signal intensity values (intensity-background) for each spot. Data was normalized to the median ratio for each array element.
3. The other software, ScanArray Express (Perkin Elmer) was used to quantitate the fluorescent intensities of the spots using the fixed circle quantitation and the TOTAL normalization methods. This software can automatically locate the spot position on the scanned image of the array to obtain the signal intensity values. Mean intensity ratios (intensity-background) were reported for each spot representing an array element.

4. Further analysis of the ChIP-chip data was carried out in a Microsoft Excel spreadsheet in an identical manner irrespective of the software used to quantitate the signal intensities. In the spreadsheet, each array element was associated with its genomic sequence position information.
5. The array elements which did not perform reliably in the validation experiments or sequencing of the products were removed from the final data-set (as described in chapter 3). In total, 360 array elements in human and 411 array elements in mouse were used for the final analysis.
6. Mean ratios, standard deviations (SD) and coefficients of variation (CV) were calculated for the replicate spots representing each array element (quadruplicate spots in the 1st generation array and duplicate spots in the final array, described in chapter 3).
7. The data was visualized by plotting the mean ratios of all array elements along the Y-axis and the respective genomic positions along the X-axis (see chapters 4, 5 and Appendices 6 to 11 for the ChIP-chip profiles generated for various assays performed for this study).
8. The baseline levels of each plot was normalised to a value of one, so that all the experiments could be directly compared from this baseline value. This was done by determining the median ratio for each experiment and dividing all the ratios (obtained in that experiment) by this value.

2.9. Sequence Analysis

A. Comparative genomic sequence analysis of mouse and human SCL loci

1. The genomic sequences for the human and mouse SCL loci represented on the tiles were aligned using the mLAGAN web server (www.lagan.stanford.edu/lagan_web/index) (Brudno et al. 2003).
2. The alignment also included the corresponding rat locus, thus providing a three-species alignment which was of better quality than an alignment of only the human and mouse syntenic regions.
3. The sequence co-ordinates used for all three species were as follows: human (chr1: 47,262,288-47,518,922, NCBI build 35), mouse (chr4:113,872,305-114,130,000, NCBI build 34) and rat (chr5:135,282,000-135,540,000, RGSC 3.4).
4. Based on this alignment, sequence homology peaks between human and mouse were determined as percentage identity across 100 bp windows of sequence. Peaks

of non-coding sequence conservation were then associated with histone acetylation and methylation data (in chapters 4 and 6).

5. The mean sequence conservation of non-coding DNA for each array element was determined and the array elements were then grouped by level of conservation (in 5% intervals). From this, the average level of acetylation or methylation for each group was calculated.
6. Simple regression analysis was performed based on the acetylation levels of array elements and their percentage identity of non-coding sequence conservation in these groupings.

B. Local comparative genomic sequence alignments of SCL regulatory regions

Local comparative genomic sequence alignments of SCL regulatory regions found in human, chimp, mouse, rat and dog were obtained from the UCSC Genome Browser (http://www.genome.ucsc.edu/). These included sequences representing the human regulatory regions known as SCL -12, -7, p^{1a}, +3 and +51 (see chapters 4 and 7). The genomic coordinates of these sequences are shown in the relevant sections.

C. Identification of conserved transcription factor (TF) sites

TF binding sites were identified using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) (Heinemeyer et al. 1998) and TESS (http://www.cbil.upenn.edu/tess/) (Schug and Overton 1997) web servers.

2.10. Statistical analysis

z-tests and simple regression analyses were performed using the Microsoft Excel macro (chapters 4 and 6).

2.11. Cluster analysis of histone modification data

1. Cluster and TreeView programs were used to functionally cluster and visualize the histone modification data (Eisen et al. 1998).
2. The ratios obtained in the ChIP-chip experiments were log transformed (i.e. all the data values, x, were replaced by $\log_2(x)$.
3. Hierarchical clustering was performed (Cluster by Eisen et al. 1998) by using “average linkage clustering” with the correlation (centred) similarity metric and no weightings calculated for the individual array elements.
4. The output files were then visualized in TreeView.

2.12. Real-time PCR

A. Primer design

1. Primer pairs for all the real-time PCR assays, performed for this study, were designed by using the Primer Express software version 2.0 (Applied Biosystems).
2. Primer pair sequences were compared against the entire human genome using e-PCR (Schuler 1997). The amplicons generated by these primer pairs were between 70 bp to 150 bp in length.
3. Standard curves were generated for the primer pairs used in the ChIP verification assays. From these, the PCR yields were calculated for each of the tested primer pairs.

The complete lists of all the primer pair sequences, used in the real-time PCR assays, are provided in Appendices 3, 4 and 5 and referenced in the relevant sections of this thesis.

B. Real-time PCR amplification

The chromatin immunoprecipitated (ChIP) DNA samples were used to set-up quantitative real-time PCR as follows:

1. The ChIP DNA samples were diluted to 1 in 10 dilution i.e. 5 µl of the sample was resuspended in 45 µl of sterile filtered HPLC water.
2. The SYBR green PCR was set-up in a 96-well plate (Applied Biosystems) in a 25 µl reaction, in duplicate or triplicate for each sample, by mixing the following reagents on ice:
    - 2.5 µl Water
    - 5 µl 1.5 µM forward and reverse primer mix
    - µl SYBR green PCR mix (Applied Biosystems)
    - 5 µl ChIP DNA samples
3. PCR was performed on a 7700 sequence detection system (Applied Biosystems).The following thermal cyclic conditions were used: 50ºC for 2 min; 95ºC for 10 min; then 40 cycles of: 95ºC for 15 sec and 60ºC for 1 min.
4. C_T values were extracted using Sequence Detector 1.7a (Applied Biosystems) with the same threshold and the ΔC_T values were determined as follows:

   $\Delta C_T = C_T$ input - C_T ChIP sample
5. Fold enrichments were calculated by using the following formula:

   Fold enrichment = $(1+\text{PCR yield})^{\Delta CT}$

Mean fold enrichments were calculated for each assay.

2.13. Procedures for fluorescence in-situ hybridisation (FISH)

2.13.1. Preparation of metaphase chromosomes

Fixed metaphase preparations were prepared from human haematopoietic cell lines K562, Jurkat, HL-60 and HPB-ALL by the following method:

1. Fresh cultures for each cell line were grown to confluency and sub-cultured to get approximately 50% confluent cultures and left overnight.
2. 40 µl of BrdU (3 mg/ml) was added to 20 ml of culture and mixed well. The culture was incubated for 3 hours at 37°C.
3. 20 µl ethidium bromide (10 mg/ml) was added to the 20 ml culture (final concentration: 10 µg/ml). At the same time, 40 µl colcemid (10 µg/ml) was also added to a final concentration of 0.02 µg/ml and the cells were incubated for 2 hours.
4. At the time of harvest, the contents of the culture flask were transferred to a 50 ml falcon tube and the cells were pelleted by centrifuging at 259 g for 8-10 minutes.
5. The supernatant was discarded to leave the pellet as dry as possible. The cell pellet was loosened gently but thoroughly by flicking the base of the tube.
6. 10 ml of pre-warmed (to 37°C) 75 mM KCl was added and the cells were gently resuspended to break any clumps of cells. The cell suspension was incubated at 37°C for 12-15 minutes.
7. 1 ml of fresh ice-cold 3:1 methanol:glacial acetic acid (BDH) fix was added to the cell-suspension and was immediately mixed thoroughly by gentle swirling. The cells were pelleted by centrifugation at 259 g for 10 minutes.
8. The supernatant was removed and the cell pellet was loosened thoroughly. 10 ml of fixative was added with constant but gentle mixing. The cell suspension was centrifuged at 259 g for 10 minutes.
9. The fixation step no. 8 was repeated a further 3 times and after the last centrifugation step, the supernatant was removed to leave the cell pellet as dry as possible.
10. The fixed cell pellet was resuspended in a small volume (1 to 2 ml) of 3:1 fix so that the solution remained cloudy.
11. The fixed metaphase preparation was assessed by dropping a small aliquot onto a microscope slide from a pipette tip. Metaphase spreads can be detected under phase contrast using a light microscope.
12. The fixed metaphase preparations for all cell lines were stored at -20°C until required.

2.13.2. Probe labelling by nick translation

PAC (RP1-18D14) and BAC (RP11-332M15) DNA probes for use in FISH were labeled by nick translation by the following method:

1. 1 µg of DNA (5 µl) was labeled in a 25 µl reaction by adding the following to a 1.5 ml microfuge tube on ice:
    - 2.5 µl 10 X nick translation buffer
    - 1.9 µl nick translation dNTP mix
    - 0.7 µl Biotin-16-dUTP or Digoxigenin-11-dUTP (Boehringer)
    - 0.5 µl DNase I (1 µg/ml working solution)
    - 1 µl DNA polymerase I (10 U/µl, Sigma)
    - 5 µl DNA (1 µg)
    - x µl sterile ddH_2O (to make the final volume to 25 µl)
2. The contents were mixed by lightly flicking the tube. The tube was spun microfuged briefly to bring down the solution.
3. The solution was incubated at 14°C for 90 minutes.
4. To stop the reaction, 2.5 µl of 0.5M EDTA was added to the tube. The contents were mixed thoroughly and the tube was transferred to ice.
5. 2.5 µl of the sample was analysed by electrophoresis on a 1% 1 X TBE agarose gel and visualized by staining with ethidium bromide.
6. Meanwhile, the labeled DNA sample was precipitated by adding 2.5 µl of 3M NaAc (pH 7.0) followed by 1 ml of ice-cold 100% ethanol. The solution was mixed well by inverting and incubated at -70°C for 30 minutes (or overnight at -20°C).
7. The DNA was pelleted by spinning in a microfuge at 18000 g for 10 minutes. The supernatant was discarded; the pellet was washed with 500 µl of ice-cold 70% ethanol and microfuged at 18000 g for 5 minutes.
8. The supernatant was removed to leave the pellet as dry as possible. The pellet was air-dried by keeping open tubes at 37°C for 25 to 30 minutes.
9. The DNA was resuspended in 10 µl of $T_{0.1}E$ buffer by flicking the tube and the DNA sample was stored at -20°C until required.

2.13.3. Metaphase spread slide preparation

Fixed metaphase spreads (of K562, Jurkat, HL-60 and HPB-ALL) were prepared on clean glass microscope slides by the following procedure:

1. The fixed metaphase suspension of a cell line was brought to room temperature (initially stored at -20°C).
2. The cell-suspension was mixed by gently flicking the tube or, if necessary, using a pasteur pipette.
3. Using a fine-tip pasteur pipette, a single drop of the cell-suspension was dropped onto a clean glass microscope slide kept on a horizontal surface, immediately followed with 1 drop of fresh 3:1 fix.
4. The slide was left to air-dry in the horizontal position. Once dry, the slide was examined for metaphase spreads under a phase contrast light microscope.
5. The area of spread cells on the slide was marked with a diamond pen.
6. The slides were fixed by incubating in fresh 3:1 fix at room temperature for 30-60 minutes.
7. After air-drying, the slides were dehydrated by passing through a fresh 70%, 70%, 90%, 90% and 100% ethanol series for 1 minute each.
8. After air-drying, the slides were incubated in acetone for 10 minutes at room temperature.
9. The slides were air-dried and stored in a sealed box containing desiccant until used (usually within the week they were made).

2.13.4. Hybridisation of metaphase spreads

Human PAC and BAC DNA probes were hybridised onto K562, Jurkat, HL-60 and HPB-ALL metaphase spreads in two-colour hybridisations by the following method:

1. A coplin jar of 70% formamide (70% formamide/2 X SSC) was pre-warmed to 65°C in a waterbath.
2. The following reagents were added to a 1.5 ml microfuge tube on ice:
    - 0.5 µl labeled DNA each from the two probes (30-50 ng)
    - 2 µl Human C_ot-1 DNA (Invitrogen)
    - 0.2 µl placental DNA
    - 9.8 µl Hyb-3 buffer
3. The contents of the tube were mixed thoroughly by vortexing and spun briefly in a microfuge to bring down the solution.
4. The probe mix was denatured by incubating at 65°C for 10 minutes.
5. The probe mix was then pre-annealed by incubating at 37°C for 20 minutes to 3 hours.

6. Meanwhile, the metaphase-spread slides were denatured in 70% formamide at 65°C for 1 minute 45 seconds (the time duration of slide denaturation is crucial to obtain good morphology chromosomes).
7. The denatured slides were quenched in 70% ice-cold ethanol for 1-2 minutes.
8. The slides were then dehydrated by passing through a fresh 70%, 70%, 90%, 90%, 100% ethanol series, for 1 minute in each jar. The slides were then air-dried.
9. The pre-annealed probe mix was pipetted onto the slide and the metaphase-spread spot was covered with a clean 22 X 22 mm coverslip.
10. The edges of the coverslip were sealed with rubber cement.
11. The slides were incubated overnight at 37°C in a humid atmosphere.

2.13.5. Detection of labeled probes

After hybridisation, biotin- and digoxigenin-labeled probes were detected immunochemically by the following three layer detection method carried out on an automated wash-station, Cadenza immunostainer (Shannon).

Pre-Cadenza steps:

1. 2 coplin jars of 2 X SSC and 2 coplin jars of 50% formamide (50% formamide/1 X SSC) were pre-warmed to 42°C in a waterbath.
2. The dried rubber cement sealing from the slides was removed by soaking the slides in 2 X SSC at room temperature.
3. After the coverslips came off the slides, the slides were passed through 4 stringency washes. The first wash was carried out by incubating the slides in 2 X SSC at 42°C for 5 minutes.
4. The slides were then washed twice by incubating in 50% formamide at 42° for 5 minutes each.
5. The slides were finally washed by incubating in 2 X SSC at 42°C for 5 minutes.

Cadenza washes:

6. The slides were then loaded very carefully onto the Cadenza.
7. The antibody solutions were prepared in blocking buffer and 100 µl of the prepared solution was used per slide.
8. Three layer antibody detection was performed on the Cadenza in the following order:
    - The first detection layer was 1:333 dilution of 1 mg/ml avidin conjugated to Texas Red (Molecular Probes) and 1:500 dilution of mouse anti-digoxigenin conjugated to FITC (Sigma).

- The second detection layer was 1:250 dilution of 1 mg/ml biotinylated anti-avidin (Vector Labs) and 1:250 dilution of goat anti-mouse FITC (Sigma).
- The third detection layer was 1:333 dilution of 1 mg/ml avidin conjugated to Texas Red (Molecular Probes).

9. Between detection layers, the slides were washed with 4 X SSC/0.05% Tween 20 (BDH).
10. The final wash on the Cadenza after the three layer detection was with blocking buffer.

Post-Cadenza steps:

11. The slides were taken off the Cadenza and rinsed briefly in 2 X SSC.
12. The slides were then stained for 2-3 minutes in 0.8 µg/ml 4,6-Diaminidine-2-phenylindole dichloride (DAPI, Boehringer) solution prepared in 2 X SSC.
13. After staining, the slides were again rinsed briefly in 2 X SSC.
14. The slides were then dehydrated by passing through a fresh 70%, 70%, 90%, 90% and 100% ethanol series allowing 1 minute in each coplin jar.
15. The slides were air-dried.
16. 20 µl of anti-fade mountant (Citiflour AF1) was applied to each slide and 20mm X 50mm clean coverslip was placed on the slide carefully to avoid air-bubbles.
17. The edges of the coverslip were sealed by applying clear nail-varnish. The slides were stored at 4°C.

2.13.6. Acquisition of FISH images

1. Slides were imaged using an Axioplan 2 microscope (Zeiss) with a CoolSNAP HQ camera (Photometrics) and narrow band pass filters (Chroma).
2. The slides were scanned to locate metaphase spreads at X20 magnification using a DAPI filter and probes signals were detected using a FITC and a Texas Red filter.
3. Metaphase spreads were captured at X100 magnification using SmartCapture 2 (Digital Scientific) imaging software.

2.14. RNA Extraction

Fresh cultures of cell lines K562, Jurkat, HL-60 and HPB-ALL were grown in the appropriate media (section 2.4.1). When the cultures were confluent, RNA was extracted from each of the cell line by the following procedure:

1. Cultured cells were counted using a haemocytometer (to get approximately $1X10^7$ cells). The cells were transferred to 50 ml falcon tubes and spun down by centrifuging at 259 g for 10 minutes.
2. The cell pellet was washed with 10 ml of PBS and the cells were spun down again by centrifuging at 259 g for 10 minutes.
3. 1 ml of Trizol reagent (GibcoBRL) was added per $1X10^7$ cells and mixed well and the sample was incubated at room temperature for 5 minutes.
4. 0.2 ml of chloroform (Sigma) was added per 1 ml of Trizol reagent used.
5. The sample was mixed vigorously for 15 seconds and incubated at room temperature for 2-3 minutes.
6. After incubation, the sample was centrifuged at 18000 g for 15 minutes at 2-8°C.
7. The aqueous phase (upper layer) of the centrifuged sample was transferred to a new 2 ml microfuge tube and 0.5 ml of isopropanol (BDH AnalaR) was added per 1 ml of the Trizol reagent used. The solutions were mixed by inverting the tube.
8. The sample was incubated at room temperature for 10 minutes and centrifuged at 18000 g for 15 minutes at 2-8°C. The RNA should now be visible as a pellet at the bottom of the tube.
9. The supernatant was removed very carefully and the pellet was washed once with 75% ethanol (BDH AnalaR). At least 1 ml of 75% ethanol was used per 1 ml of Trizol reagent used.
10. The sample was vortexed and centrifuged at 18000 g for 5 minutes at 2-8°C.
11. The supernatant was removed and the pellet was air-dried.
12. The pellet was resuspended in 50-100 µl DEPC water (0.1% diethyl pyrocarbonate dissolved in ddH_2O and autoclaved) and incubated at 55-60°C until the pellet was completely dissolved.
13. The total RNA extracted was quantitated using spectrophotometer.
14. The RNA quality was assessed by electrophoresis of 2 µg of the sample on 1% agarose 1 X TBE minigel (made up with DEPC water).
15. 3 X volumes of 100% ethanol (BDH AnalaR) was added to the aqueous sample and the samples were then stored at -70°C.