2: Materials & Methods

A new method to assess replication timing was developed by co-hybridisation of S and G1 phase DNA onto genomic clone microarrays. Initially an array covering 4.5 Mb of chromosome 22 was made to test this new method. The test array was constructed using tile path clones between CTA-415G2 and CTA-390B3 (approximately 17.5 – 21 Mb along the q arm of Chromosome 22). Subsequently a full tiling path was constructed using the methods optimised in the small tile path array. Replication Timing was assessed using the arrays made from chromosome 22 clones, an array sampling the whole genome a 1Mb intervals and a high density array sampling a region of Chromosome 22 (at a transition between a G light and G dark boundary) using high density PCR products.

Recipes for common buffers and reagents can be found in Appendix 1.

The strategy used for construction of a tiling path array is shown in Figure 2.1.

2.1 Construction of the 22 tile path array

2.1.1: Clone Selection and verification.

2.1.1.1: Clone selection

Clones were picked from the published 22q sequencing clones (Dunham et al 1999). In total 526 Chromosome 22 clones were picked (from the libraries detailed in Appendix 9b). Control clones were also picked from chromosome X (33 BACS and 62 PACS) to allow verification of copy number changes in male:female hybridisations; six *Drosophila* DNA clones acted as non-specific hybridisation controls.

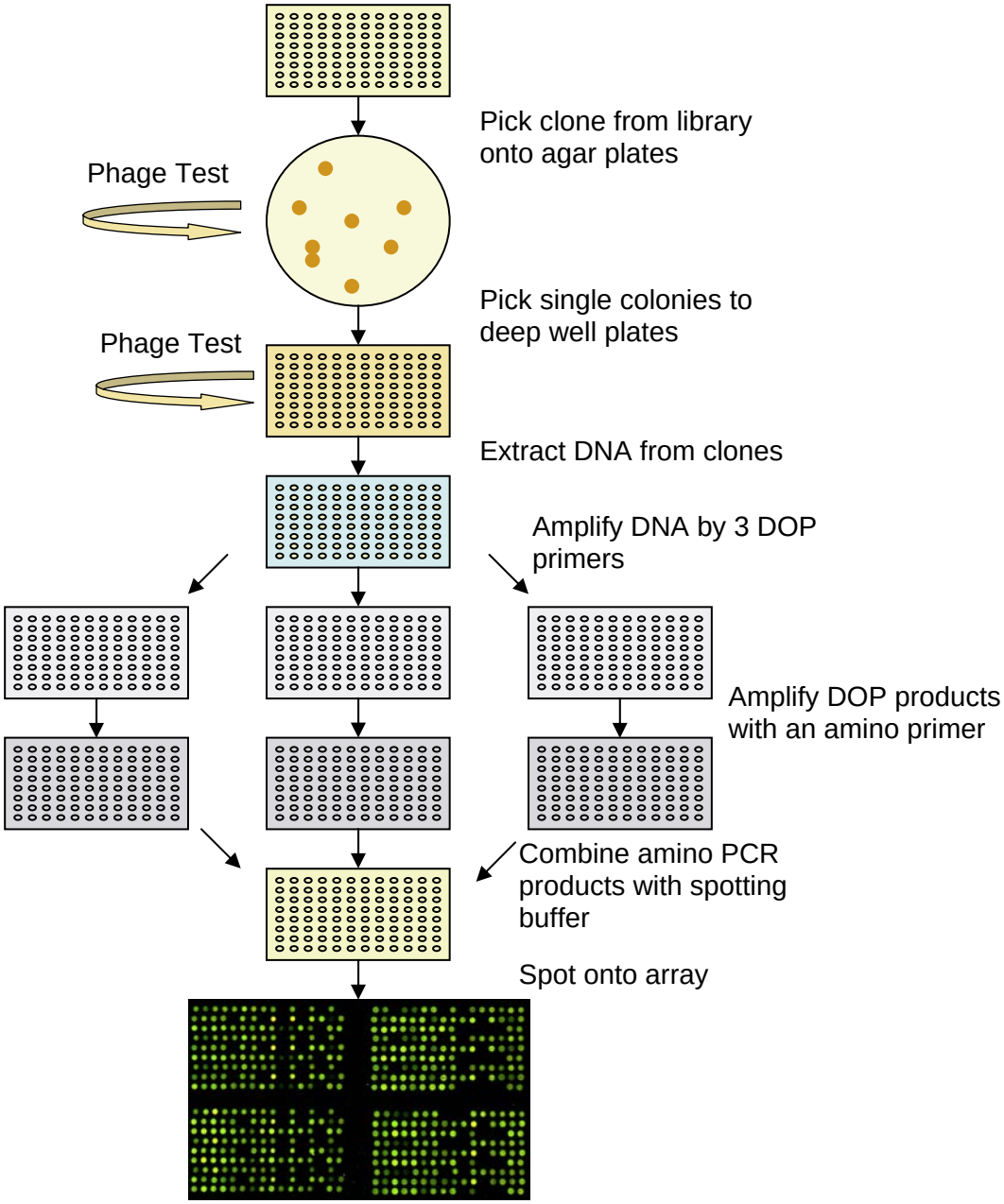

Figure 2.1: Flow diagram illustrating the construction of the tile path array

2.1.1.2: Preparation of glycerol clone stocks.

Clones were picked from libraries held at the Sanger Institute, or obtained as stabs from the University of Oklahoma and Research Genetics (Invitrogen). Clones were streaked onto LB Agar poured into Sterilin 10cm plates containing the appropriate antibiotic. Cosmids and PACs were streaked onto agar with a final kanamycin concentration of 30μg/ml. Fosmids and RP-11 BACs were picked onto agar with a final chloramphenicol concentration of 25μg/ml. CTA BACs (Appendix 9b) were

picked onto agar with a final chloramphenicol concentration of 12.5μg/ml. Clones were simultaneously streaked onto phage assay plates to test for bacteriophage infection. To prepare phage assay plates, LB agar containing 0.8% agarose was seeded with 2.25% DH10B phage-susceptible *E coli* grown in LB broth. This was poured onto a thin layer of LB agar, left to set and used on the same day. The clone plates and the phage assay plates were incubated overnight at 37^{o}C.

Phage assay plates were examined for DH10B growth, a clear lytic plaque indicating contamination of the corresponding clone with phage. If phage was found the whole agar plate was discarded and the remaining clones on the plate were re-streaked from the libraries.

Single colonies of clones that had passed phage testing were picked from the agar plates into deep well 96 well boxes (Costar) containing 1.5ml of LB broth containing 7.5% glycerol and the appropriate antibiotic for the clone picked (see above). Gas permeable plate sealers (Advanced Biotechnologies Ltd) were placed over the box. Clones were cultured at 37^{o}C for 16 hours, whilst shaking at 300rpm.

The deep well cultures were then tested for bacteriophage infection. A 96 pin hedgehog was dipped into a deep well plate before being stamped onto a phage assay plate. The hedgehog was rinsed in water, flamed with ethanol and left to cool in between each use. If phage was detected the whole 96 well plate was discarded and the remaining clones were repicked from the agar streaks. 200μl of culture from phage negative plates were alliquoted into 96 well flat bottom microtitre plates (Costar). The microtitre plates were frozen on dry ice and stored a -80^{o}C.

2.1.1.3: Clone Verification.

Initial clone verification was by HindIII digest fingerprinting. Bands were compared with a virtual digest performed on the published accession sequence for that clone. When the virtual fingerprint did not produce sufficient bands for reliable verification PCR was performed using primers designed to sequence tagged sites within the clone.

2.1.1.3.1: DNA preparation of Bacterial clones – Micro Prep.

Colonies were stamped from a glycerol stock (produced as described in 2.1.1.2) into a 96 deep well box (Costar) containing TY media and the appropriate antibiotic (as above) using a 96 pin hedgehog. The clones were cultured at 37^{o}C for 16 hours, shaking at 300 rpm.

250µl of the resulting cultures was alliquoted into a 96 well round-bottom microtitre plate (Corning). Plates were spun at 938g for 4 minutes. 25µl of GTE solution (50mM glucose, 10mM EDTA, 5mM Tris pH8) was added to resuspend each pellet in the individual wells. 25µl of 2mM NaOH/1% SDS was added to each well and the plate incubated for five minutes at room temperature. 25µl of 3M KOAc was added and incubated for five minutes at room temperature. The contents of each well were transferred into a 0.2µm costar 96 well filter-bottom plate. This was placed on top of a 96 well round bottom plate, each well containing 100ml of isopropanol and spun at 938g for 2 minutes.

The filter-bottom plate was discarded and the isopropanol plate was left at room temperature for 30 minutes. It was then spun at 1536g for 20 minutes, the supernatant was discarded and the plate inverted on tissue to dry the pellet. 100µl of 70% ethanol was added to each well and the plate spun at 1536g for ten minutes. The supernatant was discarded and the ethanol wash repeated. Again the supernatant was discarded and the pellet dried until transparent. The pellet was then resuspended in 5µl of T0.1E containing 50ng RNAse.

2.1.1.3.2: HindIII digest

A HindIII digestion mix was prepared and 4 μl was added to each well containing DNA. The plate was pulse centrifuged to 100g and the plate incubated at 37^{o}C for 2 hours. The plate was again pulse centrifuged to 100g and the digest reaction was terminated by the addition of 2μl of orangeG solution to each well. The plate was pulse centrifuged to 100g and 1μl of the digestion mix was run on a 1% agarose gel. The gel was loaded with a Promega marker lane every 5 wells. The gel was then run at 90 volts for 15 hours at 4^{o}C.

The gel was stained with Vista green for 45 minutes at room temperature, whilst shaking and the gel was washed in distilled water and scanned on a Typhoon 8600 scanner (Molecular Dynamics).

2.1.1.3.3: Analysis of Fingerprint gels

Gels were quantified using the image analysis program Image 3.10b. Lanes were first defined using the marker lanes which allows for gels that have not run straight. Individual bands were then analysed manually to define the presence and absence of every band (The intensities and definition of the band was studied in conjunction with the neighbouring bands to differentiate bands from background.) The marker lanes were co-aligned to produce an output image with the size of the sample represented by the position of the band.

The band positions obtained from the fingerprint gels were compared to virtual digests carried out on the sequenced portions of each clones. Although the HindIII digest was carried out on the whole clone the virtual digest was only carried out on the sequenced portion of the clone. It was therefore expected that the HindIII digest would contain more bands than the virtual digest. Each clone was manually examined against the virtual digest and called as pass or fail, by comparing the number and position of the bands in the actual and virtual profiles.

2.1.1.3.4: Verification of clones by detection of a Sequence Tagged Site by the Polymerase Chain Reaction (STS PCR)

Clones that required verification by STS PCR testing were streaked on LB Agar supplemented with the required antibiotic. Agar plates were incubated overnight at 37^{o}C. A single colony was taken from each plate and transferred to 100µl of T0.1E. 5µl of this was then taken to use as a template in the STS PCR.

The STS PCR was carried out in a volume of 15µl. To each well the following reagents were added. 1.5µl of 10x PCR Buffer (67mM $MgCl_2$, 670mM Tris-Cl, 167mM $(NH_4)_2SO_4$), 1.5µl of dNTP's (final conc. 5mM each), 0.5µl bovine serum albumin (5mg/ml – Sigma), 0.2µl β-mercaptoethanol (0.72M), 0.12µl Taq Polymerase (5units/ml), 0.75µl primer pair mix (Forward and Reverse primers both present at 100ng/ml), 5.425µl of T0.1E/cresol red/sucrose solution (T0.1E, sucrose (28% w/v), cresol red (0.008% w/v)). 5µl of the colony dissolved in T0.1E was added to each well as a template. Genomic DNA (5ng) was used as a positive control and water was used as a negative control.

Thermal cycling was performed using the following conditions. A single denatration step at 94^{o}C for five minutes was performed. 34 cycles were completed using 94^{o}C for 30 seconds, 58^{o}C for 30 seconds and 72^{o}C for 30 seconds. This was followed by a final elongation at 72^{o}C for five minutes.

After the PCR, 7.5µl of the reaction mixture was run on a 2.5% agarose gel made with 1xTBE and stained with ethidium bromide. When the gel was studied on a UV transiluminator the presence of a band at the anticipated size confirmed the success of the PCR and confirmed the correct clone was present in the template.

Of the 526 chromosome 22 clones tested, 470 passed verification and were included on the array. 467 of these were obtained directly from golden path sequencing clones, while the remaining 3 were identified by BAC end sequence and positioned on the chromosome 22 sequence.

2.1.2: Construction of the array from the Chromosome 22 clone set

DNA was prepared as described in 2.1.1.3.1. The DNA was dissolved in 195µl of T0.1E to give a final DNA concentration of 1ng/µl. This was amplified by three different degenerate oligonucleotide (DOP) primers. The fact the DOP primers anneal frequently to the clone DNA sequence ensures the whole clone is represented. It also provides a target for a second 'amino' primer on the 5' end of the product, which enables further amplification of the DNA and attachment of a covalently bound amino group to the PCR product. This in turn allowed covalent attachment of the DNA to the microarray slides.

2.1.2.1: DOP PCR amplification of clone DNA.

DNA was amplified using three different Degenrate Oligonucleotide PCR (DOP) primers. The primers were synthesised by Oswell DNA service and their sequence is as follows,

DOP 1: CCGACTCGAGNNNNNNCTAGAA
DOP 2: CCGACTCGAGNNNNNNTAGGAG
DOP 3: CCGACTCGAGNNNNNNTTCTAG

DOP-PCR was performed in 1x TAPS 2 buffer, 0.25% W1, 0.25mM dATP, 0.25mM dCTP, 0.25mM dTTP, 0.25mM dGTP, 2.5U AmpliTaq polymerase (Perkin-Elmer) 5µl of DNA prepared in protocol 2.1.1.3.1. was used as a template in a final reaction volume of 50µl.

The reactions were carried out on PTC-225 Tetrad thermocyclers (MJ Research). The DNA was denatured at 94^{o}C for three minutes followed by 10 cycles of 94^{o}C for 90 seconds, 30^{o}C for 150 seconds, ramping at 0.1^{o}C per second to 72^{o}C, then 72^{o}C for 180 seconds. This was followed by a further 30 cycles of PCR, 94^{o}C for 60 seconds, 62^{o}C for 90 seconds and 72^{o}C for 120 seconds. A final extension step at 72^{o}C for 480 seconds finished the reaction. Three different PCR's utilising the 3 different DOP primers (DOP 1, 2 and 3) were performed for each separate clone DNA sample.

5ml of each product was run on a 2.5% agarose gel made with 1xTBE containing ethidium bromide, to confirm the success of the PCR reaction.

2.1.2.2: Secondary DNA amplification using an amino-linked primer.

A secondary PCR is performed using the DOP PCR products as a template. This reaction utilised a 5' amine-modified primer. The primer was designed so that the 10 bases at the 3' end matched the 10 bases at the 5' end of the DOP primers. The primer sequence was GGAAACAGCCCGACTCGAG. The reaction was carried out in 1x Amino-linking buffer, 0.25mM dATP, 0.25mM dCTP, 0.25mM dTTP, 0.25mM dGTP, amino-linking primer (10ng/µl) and 1% AmpiTaq polymerase (Perkin-Elmer). 2µl of DOP amplified DNA prepared as in 2.1.2.1 as a template. The reaction was carried out in a volume of 60µl.

The PCR's were carried out on PTC-225 Tetrad thermocyclers (MJ Research). After initial denaturation at 95^{o}C for 600 seconds, 35 cycles were performed as follows, 95^{o}C for 60 seconds, 60^{o}C for 90 seconds and 72^{o}C for 420 seconds. This was followed by a final extension at 72^{o}C for 600 seconds.

5µl of each product was run on a 2.5% agarose gel as described previously to confirm the success of the PCR reaction.

2.1.2.3: Combining of amino products for spotting onto the array

For each clone DNA there were three different amino-linked DOP products. These were combined before spotting onto the array. 40µl of amino-linked DOP1, DOP2 and DOP3 product from the each clone DNA was alliquoted into the microtitre plate containing 39µl of 4x Spotting buffer (1M Sodium phosphate pH8.5, 0.001% sarkosyl). A 96 well 0.2µm costar filter plate was secured over a fresh 96 well microtitre plate. The contents of the first plate are transferred to the filter plate and it is centrifuged at 600g for five minutes. 15µl of the combined filtrate was transferred to 384 well plates in preparation for arraying.

2.1.3: Spotting of the Array

DNA from each clone was spotted in triplicate onto amine-binding glass microarray slides (3D link activated slides – Motorola) using a MicroGrid II array robot (Biorobotics). Slides were incubated in a humidity chamber with NaCl saturated water for 24-72 hours. The slides were then incubated in a 1% ammonium hydroxide solution for five minutes, followed by a five minute incubation in a 0.1% SDS solution. The DNA bound to the slides was denatured by incubation in distilled water at 95^{o}C for two minutes, before being plunged into ice cold water. Slides were dried by centrifugation at 150g for five minutes.

2.2: Construction of a High Resolution Arrays from PCR products.

2.2.1 Primer design

A high resolution array was constructed sampling at a resolution of 10Kb for a 4.5Mb region of 22q using approximately 500bp PCR products. The region chosen for analysis was between accession numbers AC005003 and AL079295 spanning bases 15398721 – 29982021 along the q arm of chromosome 22. In addition, overlapping 500bp products were designed to cover the central 200Kb of this region, allowing sampling at a very high resolution. This central 200Kb was positioned between 16495000-16695000 bp along the q arm of chromosome 22 and falls within the accession numbers Al021937-Z82246.

Primers were designed using the 'primer 3' program. The sequence chosen was repeat-masked and the primers blasted against the rest of the genome to ensure their target sequence was unique. The primer position was also weighted to be as close to the centre of each 10Kb region as possible. This allowed even sampling resolution. An amino linked tag sequence (5'-TGACCATG-3') was attached to the 5' end of the sense strand primer, to allow covalent binding to the slide. All primer sequences are in Appendix 2.

2.2.2. PCR amplification of 500bp products.

PCR reactions were performed using clone DNA as a template. The clone containing each primer was identified, picked and grown in 2xTY media containing the appropriate antibiotic as described in 2.1.1.2. The culture was then diluted 1:10 with sterile water for use as template. The PCR was performed in a 50µl reaction containing 12.5µl of the template in 1x amino-linking buffer, 0.05mM dATP, 0.05mM dCTP, 0.05mM dTTP, 0.05mM dGTP, 0.03125U/µl Taq polymerase (Perkin Elmer-Cetus), 5ng/µl sense-strand primer and 5ng/µl antisense-strand primer.

The reactions were carried out on PTC-225 Tetrad thermocyclers (MJ Research). The DNA was denatured at 94^{o}C for five minutes followed by a 30 cycles of 94^{o}C for 60 seconds, 62^{o}C for 90 seconds and 72^{o}C for 90 seconds. A final extension step at 72^{o}C for five minutes was performed. 5µl of the product was run on a 2.5% agarose gel. A successful PCR was denoted by a strong single band at approx 500bp. Reactions that were not successful at this stage were repeated using 10ng/µl genomic DNA as a template.

2.2.3 Preparation of products for spotting onto the array.

40µl of PCR product was aliquoted into the microtitre plate containing 13µl of 4x Spotting buffer (1M Sodium phosphate pH8.5, 0.001% sarkosyl). A 96 well 0.2µm costar filter plate was secured over a fresh 96 well microtitre plate. The contents of the first plate are transferred to the filter plate and were centrifuged at 600g for five minutes. 15µl of the combined filtrate was transferred to 384 well plates in preparation for arraying.

2.2.4 Spotting of arrays.

Arrays were spotted as described in section 2.1.3.

2.3: Acquisition of DNA for application to the array.

Several sources of DNA were used for array analysis. For array verification and male versus female studies total DNA was extracted from lymphoblastoid cell lines. For replication timing studies cells from cell lines were first sorted into G1 phase and S phase of the cell cycle before the DNA was extracted (2.3.2.3). Patient DNA was obtained from collaborators and control DNA used in these studies was a pool of 20 different male, or 20 different female DNAs obtained from ECACC (European Collection of Cell Cultures, UK). For Chromosome 22 add-in verification experiments individual chromosomes were flow sorted and the DNA extracted.

2.3.1: Extracting DNA from lymphoblastoid cell lines.

Five different lymphoblastoid cell lines (Table 2.1) were cultured and the DNA was extracted from logarithmically growing cell lines.

Table 2.1: Cell lines cultured for DNA extraction (names in brackets denote internal names)

Cell line name	**ECACC Number**	**Sex**
C0202-JAT (HRC 575)	94060845	Male
C0009-SAH (HRC 160)	93010702	Female
C0154-RA (HRC 193)	93012805	Male
C0020-RW (HRC 146)	92030511	Female
C0008-JH (HRC 159)	93010701	Female

2.3.1.1: Cell Culture of lymphoblastoid cell lines.

The Lymphoblastoid cell lines were cultured in RPMI 1640 media (Sigma) supplemented with 16% Foetal Bovine Serum (Gibco-BRL, Life Sciences), 2mM L-glutamine, 100units/ml penicillin and 10mg/ml streptomycin (all Sigma). They were incubated at 37^{o}C and split 1:2 every 3-4 days.

2.3.1.2: Extraction of DNA from lymphoblastoid cell line

From each culture, 5x10^{6} log phase cells were harvested and centrifuged at 300g for ten minutes. The cells were washed and resuspended in 0.5ml of Phosphate Buffered Saline (PBS). Genomic DNA was then extracted using a blood and cell culture mini kit (Quiagen) following the manufacture's instructions.

Briefly, cells were lysed by the addition of 0.5ml of lysis buffer (1.28M sucrose, 40mM Tris-Cl pH7.5, 20mM $MgCl_2$, 4% Triton X-100) and 1.5ml of ice-cold distilled water and incubation on ice for ten minutes. Lysed cells were centrifuged at 1300g for 15 minutes, the supernatant discarded and the pellet resuspended by vortexing in 0.25ml of lysis buffer and 0.75ml of ice-cold distilled water. The mix was centrifuged again for 15 minutes at 1300g and the supernatant discarded.

The nuclei were resuspended in 1ml of General lysis buffer (800mM guanidine HCl, 30mM Tris-Cl pH8.0, 30mM EDTA pH8.0, 5% Tween 20, 0.5% Triton X-100) by vortexing for 10-30 seconds. 25µl of Proteinase K (20mg/ml) stock solution was added and the nuclei incubated at 50^{o}C for 60 minutes.

Quiagen genomic tips (20/G) were equilibrated with 1ml of equilibration buffer (750mM NaCl, 50mM MOPS (3-(N-morpholino)propanesuphonic acid) pH7.0, 15% isopropanol, 0.15% Triton X-100). The buffer was allowed to flow through the tip by gravity. The prepared sample was vortexed for 10 seconds, the genomic tip placed over a 15ml falcon tube and the sample was applied to the resin in the genomic tip. The genomic tip was washed three times with 1ml of wash buffer (1.0M NaCl, 50mM MOPS pH7.0, 15% isopropanol), all solutions were allowed to move through the tip at 1g. The genomic tip was placed over a clean 15ml falcon tube and the DNA eluted

by the addition of 2x1ml of elution buffer (1.25M NaCl, 50mM Tris Cl pH8.5, 15% isopropanol). The eluted DNA was precipitated by the addition of 1.4ml of isopropanol and the falcon tube was inverted 10 times. The tube was then centrifuged at 5000g for 30 minutes and the supernatant removed. The DNA was resuspended in 100ul of T0.1E buffer.

The DNA concentration was measured using a TD-360 flurometer (Turner designs). The DNA sample was diluted 1:100 in Flurometer buffer (10mM Tris, 1mM EDTA, 0.2mM NaCl, Hoechst 33258 1μg/ml) and the DNA concentration measured against a 500ng/μl standard.

Table 2.2: Concentration of DNA extracted from cell lines

Cell line name	**DNA Conc. (ng/μl)**	**Amount in labelling reaction (μl)**
C0202-JAT (HRC 575)	195.5	2.30
C0009-SAH (HRC 160)	210.5	2.14
C0154-RA (HRC 193)	215.5	2.09
C0020-RW (HRC 146)	225.0	2.00
C0008-JH (HRC 159)	257.0	1.75

The DNA was then used in a Random prime labelling reaction (2.4.1)

2.3.2: Extracting DNA from sorted S phase and G phase nuclei.

Two different lymphoblastoid cell lines and one lymphoblastoid cell line with a Chromosome 17: Chromosome 22 translocation were cultured and the DNA extracted from sorted S and G phase nuclei.

Table 2.3: Cell lines cultured for S and G1 flow sorting (names in brackets denote internal names)

Cell line name	**ECACC Number**	**Cell type**
C0202-JAT (HRC 575)	94060845	Male Lymphoblastoid
C0009-SAH (HRC 160)	93010702	Female Lymphoblastoid
1274	N/A	t(17:22) Lymphoblastoid

2.3.2.1: Time course experiment to determine when the maximum number of cells were in S phase.

Prior to sorting, two time-course experiments were performed to assess the best time interval between splitting and harvesting lymphoblastoid and fibroblastoid cell lines to obtain the maximum number of cells in S phase when harvesting.

A 1ml sample was taken from the lymphoblastoid cell culture every two hours (during working hours) for 76 hours after subculture 1:2. Each sample was harvested as described in 2.3.2.3

2.3.2.2: Harvest of Cell for sorting into S and G1 phase.

Lymphoblastoid cell lines (HRC 575, HRC 160, & 1274) were sub-cultured 1:2 into 4 x 75cm^3 flasks and incubated for 26 hours and harvested by centrifugation at 300g for five minutes. HRC 575 Cells were washed in 5ml of PBS and spun at 300g for five minutes and the pellet resuspended in 75mM KCl and incubated room temperature for fifteen minutes. The cells were resuspended in PAB at a concentration of $7x10^6$/ml before sorting. Cells were stained with Hoechst 33258 at a concentration of 2μg/ml before incubation at room temperature for five minutes.

The other lymphoblastoid cell lines (HRC 160 & 1274) were washed in 5ml of PBS and centrifuged at 300g for five minutes. The cell pellet was resuspended in 0.5ml of PBS and 4.5ml of 70% ethanol. Samples were stored at 4^oC. Just before sorting the cells were centrifuged at 300g for 10 minutes and resuspended at a concentration of $3x10^6$ per ml in Trisodium Citrate buffer (1% Trisodium Citrate, 1% Triton X-100, 0.5mM Tris, 3.75μl/ml spermine). After incubation at 4^oC for ten minutes the cells were stained with Hoechst 33258 at a concentration of 2μg/ml before incubation at room temperature for five minutes.

2.3.2.3: Sorting of the nuclei.

G1 and S phase cells were sorted by a Coulter-Elite flow cytometer (HRC 575 & HRC 160) or a Mo-Flo flow cytometer (1274 and HRC 160 fractions)

Nuclei were sorted using gates in the positions indicated in Table 2.4 and Figure 2.2.

Table 2.4: Table to indicate where gates are positioned on cell profiles (Figure 2.2).

Cell line Sorted	**Profile Showing Gates**
HRC 575 (G1 and S phase)	A
HRC 575 (G1, 4 fractions of S phase & G2/M)	B
HRC 160 (G1 and S phase)	C
HRC 160 (G1, 4 fractions of S phase & G2/M)	D
1274 (t17:22) (G1 and S phase)	E

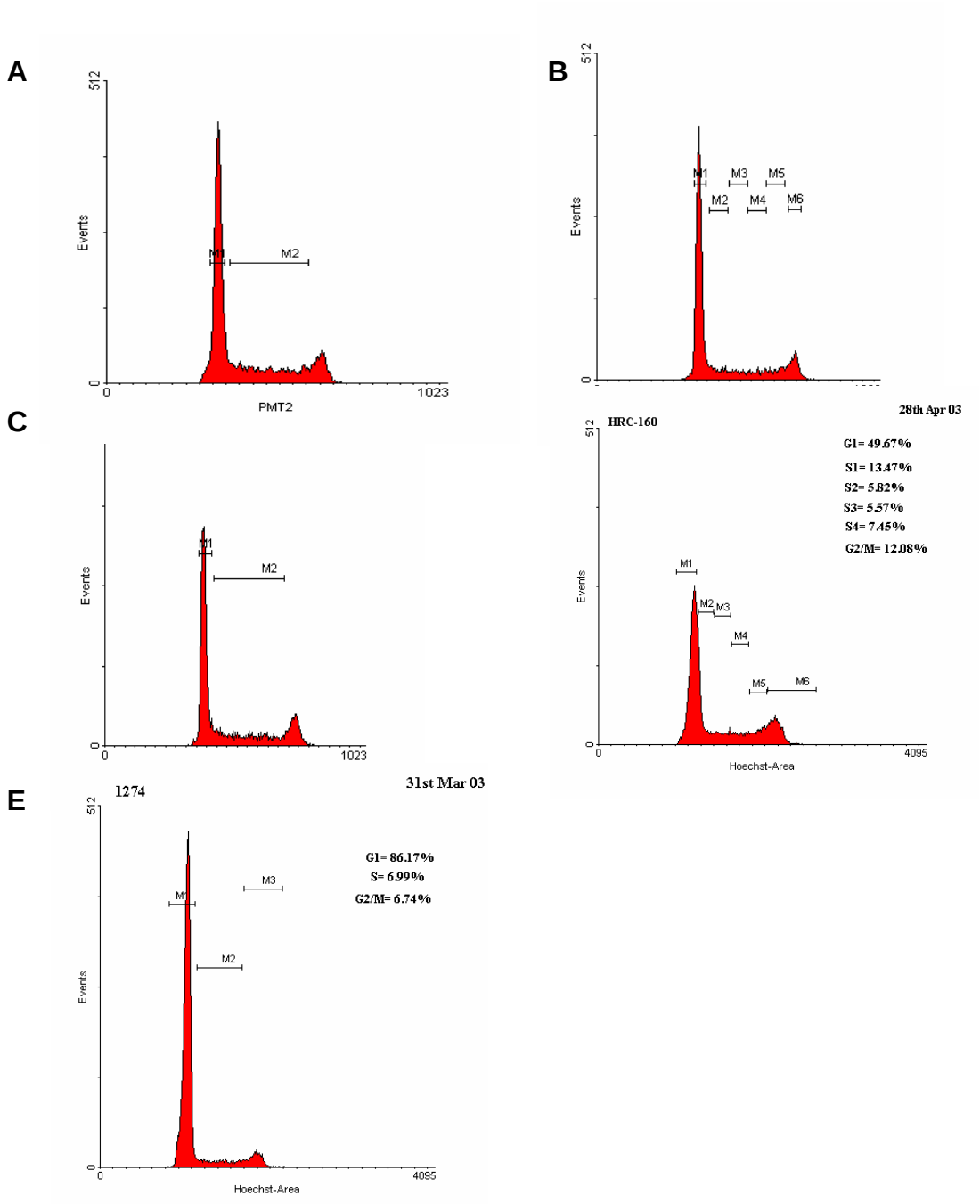

Figure 2.2: Flow sorter profiles and gate positions of sorted cell lines.

Cells were sorted into Sheath Buffer by their Hoechst intensity. To every 10^5 sorted nuclei, 25mM EDTA/1% sodium lauroyl sarcosine solution and 20µg/ml proteinase K (Gibco-BRL, Life Sciences) was added and the sample incubated overnight at 42^{o}C. The proteinase K was inactivated by the addition of 0.04mg/ml of Phenylmethylsulfonyl fluoride (Sigma) and incubation at room temperature for 40 minutes. The DNA was precipitated by the addition of 20µl 5M NaCl, 1µl of non-

fluorescent pellet paint (Novagen) and 1ml of absolute ethanol and incubated overnight at –20^{o}C. The precipitate was spun at 7700g for 15 minutes to pellet the DNA. The pellet was washed with 500µl of 70% ethanol then centrifuged at 7700g for 7 minutes. The pellet was resuspended in TE pH 8.0 and the DNA concentration was measured on the TD-360 flurometer as described previously and 1µl of DNA was run on a 1% agarose/TBE gel stained with ethidium bromide (Figure 2.3).

The DNA prepared above was used as the input for the random prime labelling reaction

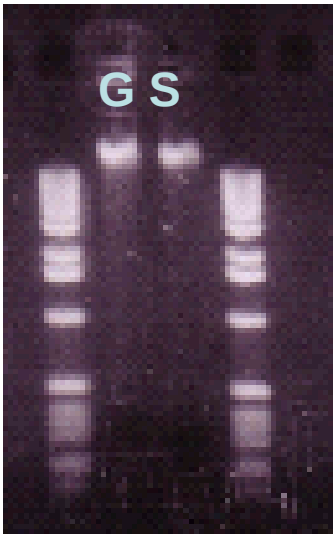

Figure 2.3: Purified S and G1 phase DNA run between two 1Kb markers.

2.3.3: Extraction of DNA from sorted Chromosomes

DNA was extracted from sorted chromosome 22s in the same way as DNA was extracted from nuclei. This is described in section 2.3.2

2.3.4: Male and Female control Pools.

Pools of 20 normal male DNA and 20 normal Female DNA were used as controls in the microdeletion studies. DNA was obtained from Human Random Collection at ECACC. The sex of each DNA was verified by PCR using chromosome Y specific primers. Each control DNA was made by pooling 20µg of DNA from each of 20

individuals and diluting the mixed DNAs to a concentration of 200ng/μl in distilled water.

2.3.5: Obtaining DNA for Microdeletion studies.

DNA from five patients with DiGeorge syndrome was obtained from collaborators at the Department of Clinical Genetics at Addenbrookes Hospital, Cambridge.

DNA from six patients with a DiGeorge-like phenotype, but that did not show a DiGeorge deletion on Chromosome 22 using standard fluorescence *in situ* hybridisation (FISH) probes was obtained by collaboration with the Molecular Medicine Unit, ICH.

2.4: Labelling of DNA and application to the array

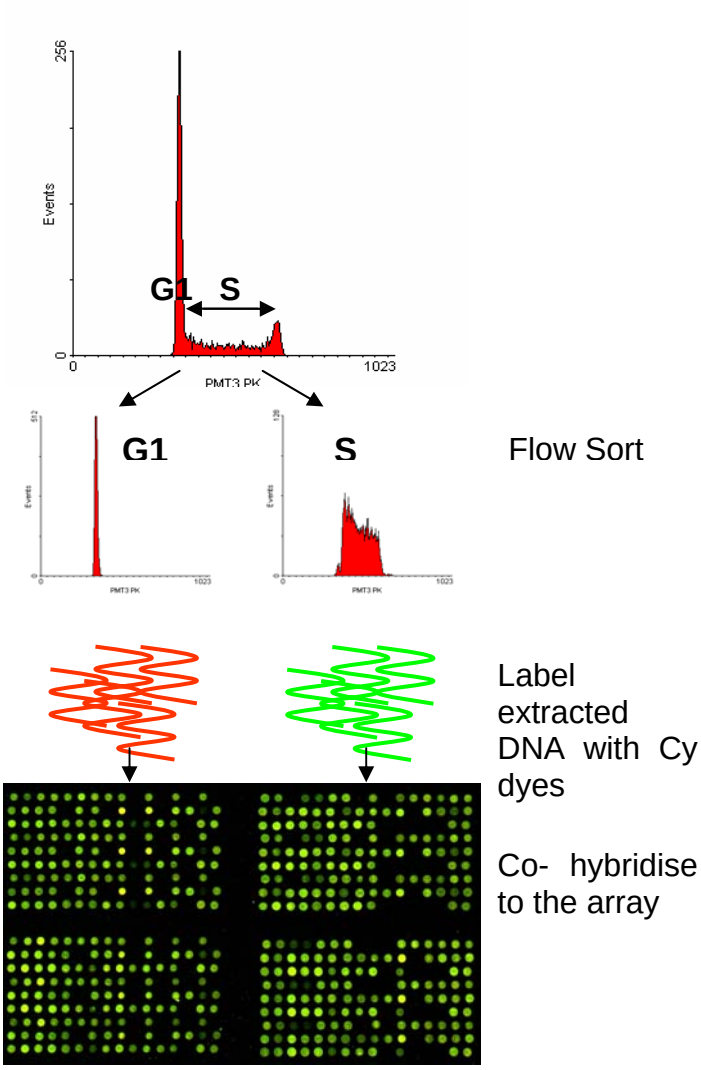

Figure 2.4: Flow diagram illustrating how DNA was applied to the constructed array.

The stages involved in the flow sorting, DNA labelling and hybridisation to the array is shown in Figure 2.4.

2.4.1: Labelling of DNA

450ng of each DNA sample was labelled using Cy-dye modified dCTP (for arrays less than 6cm^2). The DNA was added to water to give a final volume of 66µl. 60µl of random priming solution (Invitrogen – bioprime labelling kit) was added and the DNA denatured at 100^{o}C for 10 minutes. The DNA and primer mix was plunged into ice and 15 µl of 10x dNTP mix (2mM dATP, 2mM dGTP, 2mM dTTP and 0.5mM

dCTP in TE buffer), 6µl of 1M Cy3-dCTP or Cy5-dCTP (NEN Life Sciences) and 3µl of Klenow fragment (Invitrogen – bioprime labelling kit). The final reaction volume of 150µl was incubated at 37^{o}C overnight in the dark to prevent bleaching of the Cy dye.

The reaction was quenched by the addition of 15µl stop buffer (Invitrogen – bioprime labelling kit) and unincorporated nucleotides were removed by passage of the reaction mix through microspin G50 columns (Pharmacia). Columns were prepared following the manufacturers instructions and 55µl of the reaction mix was then loaded to the sloped surface of each of 3 spin columns, the columns placed in an empty 1.5ml eppendorfs to collect the labelled DNA and centrifuged at 735g for two minutes. Identical samples were pooled and 5µl was run on a 2.5% agarose gel made with TBE buffer and ethidium bromide. A smear indicated a successful labelling reaction (Figure 2.5).

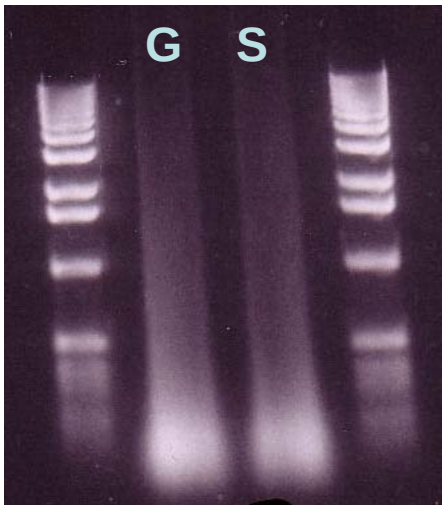

Figure 2.5: Labelled S and G1 phase DNA

2.4.2: Precipitation of Pre-hybridisaion and hybridisation DNA

For a 6cm^2 array, 180µl of Cy3 labelled DNA, 180µl of Cy5 labelled DNA, 135µl of human Cot1 DNA (Roche), 54µl of 3M sodium acetate pH 5.2 and 1000µl of 100% ethanol were added to a 1.5ml eppendorf tube for the hybridisation mix. To a separate 1.5ml eppendorf tube, a pre-hybridisation solution was prepared containing 80µl Herring sperm DNA (10mg/ml Sigma), 135µl human Cot1 DNA, 24µl of 3M sodium

acetate pH 5.2 and 900µl of 100% ethanol was added. The DNA was precipitated at –70^{o}C for 30 minutes or –20^{o}C overnight.

2.4.3: Application of the DNA to the array.

To the arrays produced as described in 2.1.3. a rubber cement wall was placed around the area of the array to create a well. When this first layer was dry a second layer was applied and left to dry.

The precipitated labelled and pre-hybridisation DNA mixes were centrifuged at 7700g for 15 minutes. The supernatant was tipped off and 500µl of 80% ethanol was added to wash the pellet. The samples were centrifuged at 7700g for 7 minutes and the supernatant was removed.

Hybridisation buffer was preheated to 70^{o}C. 160µl of the hybridisation buffer was added to the Herring sperm/Cot 1 DNA and the pellet resuspended (pre-hybridisation solution). The Cy labelled/Cot 1 DNA was resuspended in 60µl of hybridisation buffer and 6µl of yeast tRNA (100µg/µl, Invitrogen) added (hybridisation solution). The DNA/hybridisation buffer mixes were denatured at 70^{o}C for 10 minutes.

The pre-hybridisation solution was applied to the array in the centre of the rubber cement well. Care was taken to ensure that the pre-hybridisation solution covered the entire slide surface enclosed by rubber cement. The array was incubated with the pre-hybridisation solution in a square humidity chamber containing 3MM paper (Whatman) saturated with 2xSSC and 40% formamide and placed in an oven on a platform rocking at 5rpm at 37^{o}C for 60 minutes. The hybridisation solution was placed at 37^{o}C for 60 minutes in the dark.

After the pre-hybridisation incubation, as much pre-hybridisation solution as possible was removed from the slide. The hybridisation solution was then applied to the array and care was taken that the hybridisation solution was distributed over the area enclosed by the rubber cement. The slide was placed into a second small slide size hybridisation chamber containing 3mm paper (Whatmann) saturated with 2xSSC and

20% formamide. The chamber was sealed with parafilm and placed in an oven at 37^{o}C for 48 hours rocking at 5rpm.

2.4.4: Washing the array.

The slides were removed from the hybridisation chamber and the rubber cement wall was carefully removed using forceps. The slides were placed into a Hellendahl jar (Raymond Lamb) containing PBS/0.05% Tween 20 (BDH). The slides were then transferred into a trough containing PBS/0.05% Tween 20 and placed on a platform rocking at 70rpm for 10 minutes. The slides were transferred to a trough containing 50% formamide/2xSSC and placed in an oven at 42^{o}C whilst rocking for 30 minutes. The slides were transferred back into a trough containing fresh PBS/0.05% Tween 20 and washed for 10 minutes, rocking at 70rpm. The slides were dried by centrifugation at 150g for 5 minutes and stored in the dark before scanning.

2.5: Scanning and analysis of the array

2.5.1: Scanning of the slides

Slides were scanned on an Axon 4000B scanner (Axon instruments). The photon multiplier tube (PMT) levels used for detection were adjusted and tailored for each individual array. Arrays were analysed using Genepix 4.0. Software (Axon instruments). A scanned array image can be seen in Figure 2.6. Single image tiff files from Genepix 4.0 were imported into the 'Spot' analysis program (Jain, Tokuyasu et al. 2002) for analysis.

Figure 2.6: The 22q tile path array

2.5.2: Analysis of the slide

2.5.2.1: Description of the 1Mb and 22Tile path analysis programs

For analysis of the arrays, the Cy3 and Cy5 intensities were normalised. to adjust for any imbalance in the scanning of the flurochromes. For analysis of the replication

timing arrays the S phase ratios were scaled. The scaling factor was obtained from the S phase profile (Figure 2.2) and was determined by the fraction through S phase at which 50% of the DNA had replicated. The scaling factor was applied to the analysis of the co-hybridisation of S and G1 phase DNA performed on the 1Mb Chip. The average replication time of individual chromosomes was then used to normalise individual tile path arrays. The scaling factors for each array are shown in Table 2.5.

The 1Mb tiling path array was analysed using an Excel spreadsheet in the following way;

- Spots with less than twice the intensity of the average *Drosophila* spot intensity in either channel were rejected. Only spots with over twice the *Drosophila* background ratio in both channels were accepted and passed through to the next stage of analysis.
- A raw ratio of intensities was calculated by dividing the test (S phase) intensity by the reference (G1 phase) intensity.
- The raw ratios were normalised by dividing each individual ratio by the median of all ratios (for autosomes on the 1Mb array).
- The ratios were multiplied by the appropriate scaling factor to determine replication timing as shown in Table 2.5. This distributed all the ratios between 1 and 2.2.
- The median of the duplicates representing each locus was calculated.
- Spots that deviated more than 5% from this median are rejected and omitted from further analysis.
- The ratio taken for each locus was the median of accepted spots.
- The ratio of each locus was plotted against chromosome position.
- The average replication time of each chromosome was calculated to be used for the normalisation of individual tiling path arrays.

A second Excel spreadsheet was used to analyse the tiling path arrays;

- Spots with over twice the *Drosophila* background ratio in both channels were accepted and passed through to the next stage of analysis.

- A raw ratio of intensities was calculated by dividing the test (S phase) intensity by the reference (G1 phase) intensity.
- The raw ratios were normalised by dividing each individual ratio by the mean of all ratios (for the chromosome 22 clones only).
- The ratios were multiplied by the appropriate scaling factor to determine replication timing as shown in Table 2.5 (1.75 for replication timing arrays).
- The median of the triplicates representing each locus was calculated.
- Spots that deviate more than 5% from this median were rejected.
- If two or more spots are within 5% of the median, their average was taken as the final ratio at that locus. If two of more spots are not within 5% of the median all spots representing that locus are rejected.
- The ratio of each locus was plotted against chromosome position.

For the chromosome 22 tile path array ratios were then plotted on a graph against position of the midpoint of the clone on chromosome 22. The length and midpoints of clones were obtained from clones with end sequences by mapping the ends back against the published chromosome 22 sequence (Dunham, Shimizu et al. 1999). For clones where end sequences were unavailable the midpoint of the accessioned sequence was used.

2.5.2.2. Analysis of replication timing on arrays.

The analysis of replication timing experiments utilising the whole genome at a 1Mb resolution and a 22 tile path array are described in section 2.5.2.1 and normalised using the values reported in Table 2.5.

Tile path arrays for chromosome 6 and chromosome 1, produced in collaboration with our laboratory, were used to assess replication timing. The chromosome 1 array was constructed by Simon Gregory and Rachel Cooper, members of the chromosome 1 mapping group at the Sanger Institute. Replication timing experiments on the chromosome 1 tile path array were normalised to the average replication timing for chromosome 1 reported by the 1Mb resolution array i.e. 1.52. Array analysis was performed using a program written by Carol Scott at the Sanger Institute, available at http://intweb.sanger.ac.uk/cgi-bin/humace/1mbsetends.cgi.

The chromosome 6 tile path array was constructed in collaboration with Koichi Ichimura at Dept. of Pathology, University of Cambridge. The replication timing experiments performed on the chromosome 6 tile path array were normalised to 1.44. Analysis was performed using a spreadsheet provided by Koichi.

Analysis of the other types of array experiment was carried out using the same spreadsheet analysis as used for the replication timing arrays, described in section 2.5.2.1. For arrays assessing microdeletions, comparing cell lines or detecting breakpoints no scaling factor was used. For other arrays a scaling factor were applied as appropriate. (Table 2.5)

Table 2.5: Scaling factors for Microarray experiments

Array Experiment	Scaling Factor Applied
Replication Timing on 1Mb Array	1.44
Replication Timing on Chr 22 TP Array	1.75
Replication Timing on Chr 1 TP Array	1.52
Replication Timing on Chr 6 TP Array	1.44
Replication Timing on Chr 22 of t(17:22)	1.75

2.5.2.3: Analysis of chromosome 22 copy number change on the Chromosome 22 tile path arrays.

To verify that the chromosome 22 clones on the tile path array report the correct copy number changes, a series of experiments was performed adding a different amount of chromosome 22 into self:self hybridisations. To achieve this, copies of chromosome 22 were flow sorted to separate them from the rest of the genome. The DNA from the chromosomes was extracted as described in section 2.3.2.4. Four separate hybridisation experiments were performed as summarised in Table 2.6.

Table 2.6: Chromosome 22 add in experiments performed

Experiment	Cy3 labelled DNA	Cy5 labelled DNA
1	Genomic DNA	Genomic DNA
2	Genomic DNA + 1 copy of chromosome 22	Genomic DNA
3	Genomic DNA + 2 copies of chromosome 22	Genomic DNA
4	Genomic DNA + 4 copies of chromosome 22	Genomic DNA

Arrays were normalised on only the X clones to give a test:reference ratio of 1:1

2.5.3.4 Analysis of arrays reporting copy number change when different S phase fractions are hybridised onto the array.

S phase was sorted into four equal fractions based on the DNA content of the nuclei. Gates were placed on the cell cycle profile as shown in Figure 2.2b and nuclei into each of the S phase fractions, denoted S1, S2, S3 and S4 were sorted. G1 and G2/M nuclei were also sorted. DNA was extracted from the sorted nuclei as described in section 2.3. DNA from each fraction was hybridised against the G1 phase DNA. The arrays were normalised using the cell cycle profile by calculating the median value of each fraction as a proportion of total S phase.

The median value for each S phase fraction was expressed as a proportion of G1 to provide a scaling Figure between 1 and 2. These are summarised in Table 2.7.

Table 2.7: Scaling factor applied for the S phase fraction experiments

Array Hybridisation	Scaling Factor
G1:G1	1.00
S1:G1	1.17
S2:G1	1.36
S3:G1	1.53
S4:G1	1.71
G2/M:G1	2.00

2.5.3.5: Analysis of microdeletion studies.

All ratios obtained from arrays used in microdeletion studies were normalised so the average test:reference ratio was 1:1.

2.5.3.6: Analysis of DNA immunoprecipitated using antibodies against histone acetylation.

DNA from the lymphoblastoid cell line HRC 575 was assayed for histone acetylation on chromosome 22. This was done in collaboration with the Microarrays and Transcriptional Control group at the Sanger Institute. The chromatin immunoprecipitation was performed by Pawendeep Dhami using either of two antibodies, one for histone H3 acetylation and one for histone H4 acetylation. The Histone H3 antibody used was Anti-acetyl-Histone H3 (Upstate, USA). It is a rabbit polyclonal IgG antibody that recognises and is specific for acetylated human H3 of approx. 17kDa. The Histone H4 antibody used was Anti-acetyl-Histone H4, ChIP grade (Upstate, USA). It is extracted from rabbit antiserum. The antibody recognises acetylated histone proteins of aprox 10kDa. The antibody is known to cross react with acetylated histone H2B and may cross react with other acetylated proteins.

The arrays were normalised so that the average ratio of input DNA: immunoprecipitated DNA was 1:1.

2.6: Transcription analysis of a lymphoblastoid cell line.

2.6.1: Extraction of RNA from lymphoblastoid cell line.

Total RNA was extracted from the lymphoblastoid cell line HRC 575 using the Trizol purification method. Lymphoblastoid cells were cultured as described in 2.2.2.1 and harvested during the exponential stage of their growth. The cells were washed in PBS, quantified using a Haemocytometer and pelleted by centrifugation at 300g for 10 minutes. 1ml of Trizol (Gibco-BRL, Life Sciences) was added to every 10^7 cells and mixed thoroughly. The sample was incubated at room temperature for 5 minutes and

1ml samples were aliquoted into 2ml eppendorf tubes. 0.2ml of chloroform was added to each aliquot and mixed by vortexing at 15 seconds. Samples were incubated at room temperature for 3 minutes and centrifuged at 12000g for 15 minutes at 4^{o}C. The aqueous layer was transferred into a new 2ml eppendorf tube. 500µl of isopropanol was added and mixed by inversion. The sample was incubated at room temperature for 10 minutes and centrifuged at 12000g for 15 minutes at 4^{o}C. The supernatant was removed and the RNA pellet was washed with 1ml of 75% ethanol by centrifugation at 7500g for 5 minutes at 4^{o}C. The supernatant was removed and the pellet air dried. The pellet was resuspended in 50µl of HPLC water, 0.01% diethyl pyrocarbonate (DEPC) and incubated at 55^{o}C until the pellet was completely dissolved. The RNA was quantified using a spectrophotometer. 2µg was electrophoresed on a 1% agarose gel made up with TBE to assess the quality of the RNA. 1ml of 100% ethanol was added to the sample for storage at -70^{o}C.

2.6.2: Synthesis of cDNA

10µg of total RNA was incubated for 10 minutes at 65^{o}C with 100 pmol of a HPLC purified T7-(T)24 primer from the Superscript ds-cDNA Synthesis Kit (Gibco-BRL, Life Sciences). First strand buffer (1x concentration), dNTP's (10mM each) and DTT (final concentration 0.1M) was added to the RNA mix and incubated at 42^{o}C for 2 minutes. 200U/ml of superscript II reverse transcriptase was added, mixed and incubated at 42^{o}C for 1 hour. A second strand master mix was made (1x second strand buffer, dNTP's (10mM each) 10 U/µl *E. Coli* DNA Ligase, 10 U/µl *E. Coli* DNA Polymerase II, 10 U/µl *E. Coli* RNase H, made up to a reaction volume of 130µl with DEPC treated water). This was added to the first strand product, mixed thoroughly and incubated at 16^{o}C for 2 hours. 10 Units of T4 DNA Polymerase was added, the sample incubated at 16^{o}C for 5 minutes and the reaction quenched with EDTA pH8.0 (Final concentration 30mM).

Phase-lock tubes (eppendorf) were used to clean the cDNA. The phase lock tubes were prepared by centrifugation at 7700g for 30 seconds. An equal volume of room temperature buffer saturated phenol (65ml Alkalin buffer (10mM Tris HCl pH 8.0, 1mM EDTA) added to 1ml phenol:chloroform:isoamyl alcohol 25:24:1) was added to

the double stranded DNA and vortexed. The DNA mix was added to the phase-lock microfuge tube, spun at 7700g for 2 minutes and the aqueous top phase transferred to a fresh eppendorf tube. The DNA was precipitated in 0.5x vol 7.5M NH_4OAc, 4µl of glycogen (5 mg/ml) and 2.5x vol 100% ethanol. The cDNA was pelleted by centrifugation at 7700g for 20 minutes. The pellet was washed twice by the addition of 500µl of 80% ethanol to the pellet and centrifugation at 7700g for 5 minutes. The pellet was then air dried and resuspended in 12µl of DEPC treated water.

2.6.3: Production and labelling of cRNA and application to the array

Labelled cRNA was synthesised from the cDNA. Reagents from the Bioarray High Yield RNA transcript labelling Kit (Enzo Diagnostics) were used. The 12µl of ds-cDNA synthesised previously were used and the following reagents added; 10µl of DEPC treated water, 4µl 10x HY Reaction Buffer, 4µl Biotin-labelled ribonucleotides, 4µl DDT, 4µl RNase Inhibitor mix and 2µl T7 RNA Polymerase. This was mixed and incubated at 37^{o}C for 5 hours. The RNA was cleaned with the RNeasy mini kit (Qiagen). Samples with a yield greater than 40µg of cRNA were subsequently hybridised to Affymetrix U133 oligonucleotide arrays (Affymetrix). Hybridisation was at 45 °C for 16 hours.

2.6.4: Washing and analysis of array.

Arrays were washed and stained with streptavidin-phycoerythrin (SAPE, Molecular Probes) before signal amplification was performed using a biotinylated anti-streptavidin antibody (Vector Laboratories) following the recommended Affymetrix protocol for high density chips. Scans were carried out on a GeneArray scanner (Agilent Technologies). The fluorescence intensities of scanned arrays were analysed with Affymetrix GeneChip software. The Affymetrix Microarray Suite 5.0 was used for the quantification of gene expression levels. Global scaling was applied to the data to adjust the average recorded intensity to a target intensity of 100. Quantification data was exported from Affymetrix Microarray Suite 5.0 into Excel for further analysis. Presence or absence of gene expression was determined by a ‘present’ call,

in any of the oligos representing a gene, as determined by Affymetrix Microarray Suite 5.0.

2.7: FISH analysis of DiGeorge and VDJ recombination regions.

Deletions detected on the array were verified by fluorescence *in situ* hybridisation (FISH). The clones detailed in Tables 2.8 and 2.9 were picked for analysis.

Table 2.8: Clones picked from the VDJ recombination region;

Clone Name	**Accession Number**
cN9C5	D87023
cN9G6	D87020
cN22A12	D86999
cN24A12	D86998
cN29D3	D86991
cN31F3	D87002
cN35B9	D87010
cN48A11	D87007
cN50D10	D87011
cN52F2	D87006
cN61E11	D87014
cN63E9	D87013
cN68D6	D87015
cN75C12	D87017
cN81C12	AP000360
cN84E4	D87021
cN92H4	D87024
cN102D1	D86994

Table 2.9: Clones picked from the DiGeorge Region;

Clone Name	**Accession Number**
519d21	Ac008079
995o6	Ac008132
cN61D6	D87012
56c	Ac000080
Bac32	Ac007050
49c12	Ac000079
98c4	Ac000092
52f6	Ac005500
Pn_5	Ac002472
83c5	Ac000087

2.7.1: Mini Prep of Bacterial clone DNA

Clones chosen for verification were cultured in 10ml of TY media containing the appropriate antibiotic (see 2.1.1.2). The culture was grown at 37^{o}C for 16 hours whilst shaking at 200rpm. The culture was centrifuged at 2,000g for 10 minutes to pellet the bacteria. The pellet was resuspended in 200µl of Lysis buffer (10mM EDTA, 250mM Tris pH8.0, 50mM glucose, made with sterilised distilled water) and incubated at room temperature for 10 minutes. 400µl of 4M NaOH, 1% SDS was added and the preparation was incubated on ice for 5 minutes before 300µl of 3M Sodium acetate pH5.2 was added followed by a further 10 minute incubation on ice. The preparation was centrifuged at 7700g for 5 minutes and the supernatant was transferred into a fresh 1.5ml eppendorf tube. This was repeated until a clear supernatant was obtained. 600µl of isopropanol was added and mixed gently with the supernatant, this was incubated at –70^{o}C for 10 minutes.

The eppendorf tube was spun at 7700g for 5 minutes, the supernatant tipped off and the pellet resuspended in 200µl of 0.3M Sodium acetate pH7. 200µl of phenol:chloroform:water was added, mixed by vortexing and the eppendorf tube was spun at 7700g for 3 minutes. 150µl of the top aqueous layer was transferred into a fresh 1.5ml eppendorf tube. A further 50µl of 0.3M Sodium acetate pH 7.0 was added to the original phenol:chloroform:water containing tube; this was mixed by vortexing and the tubes were centrifuged at 7700g for 2 minutes. 50µl of the aqueous top layer was pooled with the first 150µl; 200µl of isopropanol was added, mixed by inversion and incubated at –70^{o}C for 10 minutes. The tubes were centrifuged at 7700g for 5 minutes and the supernatant discarded. The pellet was washed in 500µl of ice-cold 70% ethanol and spun at 7700g for 2 minutes. The supernatant was removed, the pellet dried and resuspended in 50µl of T0.1E containing 200mg/ml of RNAase A and incubated at 55^{o}C for 15 minutes. 1µl of each DNA preparation was run on a 1% agarose gel and the DNA was quantified using a TD-360 flurometer as previously described.

2.7.2: Nick Translation

Nick translation was performed to label the DNA with a dUTP conjugated to either the hapten biotin-16-dUTP (Roche) or digoxigenin-11-dUTP (Roche). 1μg of DNA prepared as described in 2.7.1 was used as the input DNA to the reaction. A reaction mix was made up using the input DNA, 1xNT buffer (50mM TrisHCl pH7.5, 10mM $MgSO_4$, 0.1mM dithiothretitol, 50μg/ml Bovine serum albumin), 10mM dATP, 10mM dCTP, 10mM dGTP, 30mM hapten-dUTP, 0.02 units/ml DNA polymerase 1 and 1μl Deoxyribonuclease 1 (DNAse 1 - Sigma.)

Concentration and incubation time was determined by a DNAase 1 titration for each separate vector (cosmid, fosmid, BAC and PAC). DNAse 1 was diluted in 50% glycerol, 100mM TrisHCl pH7.5, 20mM $MgSO_4$, 0.2mM dithiothretitol, 100μg/ml Bovine serum albumin to a concentration of 1μg/ml. If PAC or BAC DNA was used as input DNA the nick translation was incubated at 14^{o}C for 70 minutes. If cosmid or fosmid DNA was used as input DNA the nick translation was incubated at 14^{o}C for 40 minutes.

The reaction was stopped by the addition of 2.5μl of 0.5M EDTA. 2.5μl of 3M Sodium acetate pH7 was also added. The DNA was precipitated by the addition of 1000μl of absolute ethanol and incubation at –70^{o}C for 30 minutes. The pellet was washed in 500μl of 80% ethanol and the pellet resuspended in 10μl of T0.1E. 1μl was run on a 1% agarose gel to confirm a product size between 200-500bp.

2.7.3: Metaphase spread preparation

Metaphase preparations were made from two lymphoblastoid cell lines, HRC 575 (male) and HRC 160 (female). The lymphoblastoid cell lines were cultured as described in 2.3.3.1. Twenty-four hours after sub-culturing, BromodeoxyUridine (Roche) was added to a final concentration of 15μg/ml. The culture was incubated at 37^{o}C for three hours. Ethidium Bromide (Sigma) was then added to a concentration of 10μg/ml and the culture incubated at 37^{o}C for a further 75 minutes. Colcemid (Gibco-BRL, Life Sciences) was added to a final concentration of 0.1μg/ml and the culture

incubated for 45 minutes. The culture was transferred to 50ml Falcon tubes, centrifuged at 300g for 10 minutes and the supernatant was removed leaving the pellet as dry as possible. The tube was flicked to loosen the pellet and 10ml of 75mM KCl (pre-warmed to 37^{o}C) was added to resuspend the pellet. The suspension was transferred to a 15ml Falcon tube and incubated at 37^{o}C for 10 minutes. Ice cold fixative was prepared (3 parts dried methanol: 1 part glacial acetic acid- BDH) and 3ml was added to the cell suspension and mixed by gentle swirling. The suspension was centrifuged at 300g for 10 minutes. The supernatant was removed and the pellet resuspended in 10ml of ice-cold fix. Centrifugation and resuspension in fix was repeated twice more. After a final centrifugation at 300g for ten minutes the pellet was resuspended in enough fix to give a desired density of nuclei.

2.7.4: Hybridisation to Metaphase spreads

Slides were sonicated in 2% Decon solution, washed in 96% ethanol and polished dry using lint free tissue. A drop of metaphase suspension was applied to each end of the slide and allowed to spread and air dry. The slides were placed in a jar of fix (3 parts dried methanol: 1 part glacial acetic acid) at room temperature for 30 minutes. Slides were then dehydrated in a series of ethanols (70%, 70%, 90%, 90%, 100%) and air dried. The slides were fixed for 10 minutes in acetone and baked at 60^{o}C for two hours.

The nick translated probe was prepared for hybridisation to the slide. 0.5µl of probe was added to 1µl of Cot 1 DNA (Roche) and 11.5µl of Hybridisation buffer. This probe mix was denatured at 65^{o}C for 10 minutes before being pre-annealed at 37^{o}C for 30 minutes. Meanwhile the metaphase spread slides were denatured in 70% formamide, 0.6xSSC at 65^{o}C for 2 minutes. The denaturation was quenched in 70% ice cold ethanol and dehydrated through an ethanol series (70%, 70%, 90%, 90%, 100%) before air drying. The probe mix was applied to the metaphase spread and sealed under a 22x22mm coverslip with rubber cement. The slides were incubated overnight at 37^{o}C in a humid atmosphere.

After the overnight hybridisation the rubber cement was removed from the slide and the coverslips soaked off in 2xSSC. Slides were washed in 2xSSC, then 2 washes in

50% formamide, 1xSSC, followed by a wash in 2xSSC. All washes were performed at 42^{o}C for five minutes each. Slides were then mounted on a Cadenza immunostainer (Shannon).

2.7.5: Detection of labelled probes.

Three-layer detection was performed on the Cadenza. Antibodies for detection were diluted in blocking buffer (1% w/v blocking reagent (Boehringer), 0.05% Tween 20 (BDH), 1µl/ml Sodium azide, 4xSSC) and the Cadenza was used to incubate the antibody on the slide. Blocking buffer was applied to the slide before three-layer detection took place. The first layer was 4µg/ml avidin conjugated to Texas Red (Molecular Probes). The second layer was 4µg/ml biotinylated anti-avidin (Vector Labs) and/or 1:500 dilution of mouse anti-digoxin (Sigma). The third layer was 4µg/ml Avidin-Texas Red and/or 10µg/ml goat anti-mouse FITC (Sigma). In between incubation with the antibodies the slides were washed with 4xSSC, 0.05% Tween 20. After staining was completed the slides were removed from the Cadenza, washed in 2xSSC and stained with 0.08µg/ml 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI - Roche) in 2xSSC. The slides were then rinsed in 2xSSC, dehydrated in an ethanol series (70%, 70%, 90%, 90%, 100%) and air dried. 20µl aliquots of anti-fade solution (Citifluor – Citifluor Ltd) were applied to each cell spot and a 22mm x 50mm coverslip was laid over the slide and fixed in place with clear nail polish.

2.7.6: Acquisition of FISH images.

Slides were studied on a Zeiss Axioskop epifluoroescence microscope. Metaphase spreads were located at x200 magnification using a DAPI filter (exitation λ362nm emission λ465nm). Probes on the metaphases were detected using either a FITC filter (excitation λ495nm emission λ520nm) or a Texas Red filter (excitation λ596nm emission λ620nm) and captured at x1000 magnification using the Cytovision capture suite.

2.8: Real-Time PCR analysis of S and G phase DNA.

Selected Early and Late replicating Clones were chosen to verify the array results using real-time (quantitative) PCR.

2.8.1: Primer design

Four clones were chosen from the array for assessment by real time PCR. The clones chosen were a single late replicating clone (cN69F4), two mid replicating clones (cE140F8 & cB13C9) and an early replicating clone (bK57G9). Short amplicons (approximately 150bp) were designed as they are optimal for the real time PCR reaction. A pair of PCR primers were placed approximately every 10Kb along the selected clone using the Taqman primer design program, part of the Primer Express Software (ABI). Primer sequences had a Tm between 58-60^{o}C, a GC content between 20-80% (no more than 3/5 GC's in the 5' end of the primer), and a short amplicon (approx 150bp).

Primer pairs were checked using the Primer Test program in the Primer Express software to ensure they did not have excessive secondary structure or primer-dimer formation. Primer sequences are shown in Appendix 3.

2.8.2: Real Time PCR on S and G1 phase DNA.

Real time PCR was carried out using the same S and G phase DNA sorted in 2.3.2.3 and used for replication timing analysis. The DNA was first diluted to a concentration of approximately 5ng/ml to ensure quantisation by real-time PCR fell within the scope of the real-time PCR machine.

For each reaction, 12.5µl of 2x Sybr Green Reaction mix (including Taq polymerase and dNTP mix - ABI), 1µl of template DNA, 50mM forward primer and 50mM reverse primer was used. The reaction mix was made up to 25µl with sterile water. Each reaction was carried out on each plate in triplicate. A control PCR on each 96 well plate comprising standard male DNA (ABI) and a control primer was also included to allow comparison between different PCR runs.

The reaction had an initial incubation at 50^{o}C for 2 minutes followed by holding at 95^{o}C for 10 minutes. A two-step PCR was then carried out with 40 cycles of a denaturation step at 95^{o}C for 15 sec, and a hybridisation and elongation step at 60^{o}C for 60 sec. During each cycle of the PCR the Sybr-Green intensity of each well was measured. The amount of DNA present in the original sample is proportional to the cycle of PCR at which Sybr-Green intensity first appears. After the PCR was completed a standard disassociation curve for Sybr-Green was performed. This ensured the PCR had been successful, only a single product had been produced, and that primer dimers had not been produced. Under these conditions the Sybr-Green intensity measured during the PCR cycles is due to the amplification of the target and is quantitative.

The Sybr-Green intensities collected during the PCR reaction were analysed using the Sequence Detection System Software (ABI). Background threshold levels were set at the number of cycles before any Sybr-Green fluorescence was detected. The detection level was set at the point where the increase in Sybr-Green level became exponential. The cycle number at which the detection level is set is a measure of the DNA concentration in the original sample. This was compared to an internal standard control curve (0.625ng, 1.25ng, 2.5ng, 5ng and 10ng of DNA) to determine the starting quantity of DNA present. The ratio of amount of S:G1 phase DNA for each set of primers designed and the average ratio of S:G1 phase DNA for each clone was then calculated.