Chapter 2
Materials and Methods

2.1 Array Comparative Genome Hybridization (Array CGH)

2.1.1 Patient and Control DNA Samples

The schizophrenia patient cohort consists of 100 Scottish patients, who were recruited through the inpatient and outpatient services of hospitals in South East Scotland. All patients met lifetime DSM-IV criteria for schizophrenia, based on consensus between two trained psychiatrists. All patients gave written consent for their DNA to be used in genetic studies. DNA samples were extracted from blood and were provided by Professor Douglas H Blackwood, the Royal Edinburgh Hospital.

A matched control cohort consists of 100 DNA samples of Scottish people with normal phenotype. The participants were recruited from surviving members of the Scottish Mental Survey of 1921. These participants resided in the Lothian area of Edinburgh, Scotland in 1932, when they were at the age of 11 years old, and their cognitive functions were assessed using the Scottish Mental survey. The participants were recruited between 1999 and 2001 at the age of 79 years, and their cognitive function assessed again. DNA samples were extracted from the blood of the patients at the mean age of 79 years, and were provided by Professor Ian J. Deary, University of Edinburgh.

Each array experiment was performed with a test DNA sample (schizophrenic patient or Lothian Birth control sample) hybridized against the standard reference DNA (*NA10851*) extracted from a normal HapMap lymphoblastoid cell line (*GM10851*; 46,XY) obtained from Coriell Institute for Medical Research.

2.1.2 DNA Labelling

The construction and experimental use of whole genome human tiling path BAC array at the Sanger Institute has been described previously (Fiegler et al. 2006; Fiegler et al. 2007). For each CGH experiment, the test DNA sample was labelled in Cy3-dCTP and the reference (*NA10851*) DNA in Cy5-dCTP using BioPrime labelling kit (BioPrime DNA Labelling Kit, Invitrogen Ltd). To avoid dye bias in labelling, a dye-reversal hybridisation (test DNA labelled in Cy5- dCTP and reference DNA in Cy3-dCTP) was performed for each test DNA. The labelling reaction consists of 150ng of DNA and 60 µL of 2.5x random primer solution, made up with nuclease-free water to 130.5 µL. After denaturing the DNA for 10 min at 100°C, 15 µL of 10x dNTP (deoxynucleoside triphosphate) mix (1 mM dCTP, 2 mM dATP, 2 mM dGTP, and 2 mM dTTP in TE buffer), 1.5 µL of 1 mM Cy5-dCTP or Cy3-dCTP (Amersham, GE Healthcare), and 3 µL of Klenow fragment were added on ice to a final reaction volume of 150 µL. The sample mixture was then incubated at 37°C overnight, and the random priming reaction terminated by adding 15 µL of stop buffer supplied in the kit.

Following DNA labelling, unincorporated nucleotides were removed using Microcon YM-30 Filter Devices (Millipore). 150 µL of the labelled test sample, combined with 150 µL labelled reference sample, was applied to a Microcon column placed in a 1.5 mL microfuge tub and spun at 12,000 rcf (relative centrifugal force) for 5 min. The flow-through containing unincorporated dyes was discarded. Labelled DNA was retained in the filter and was washed with 300 µL HPLC water and spun as before, with flow-through discarded. 100 µL of HPLC water was added to the sample and the filter column was placed upside-down to spin in a fresh tube at 2000 rpm (revolutions per minute) for 2 min to collect DNA sample. 3 µL of each sample was analysed on 2.5% agarose gel

electrophoresis and 1.5 µL of each sample was analysed in a NanoDrop™ ND-1000 or ND-8000 UV-VIS Spectrophotometer to check for successful labelling and amplification. The amplified sample should run as a smear, with the bulk fragments below 500 bp (Figure 2.1).

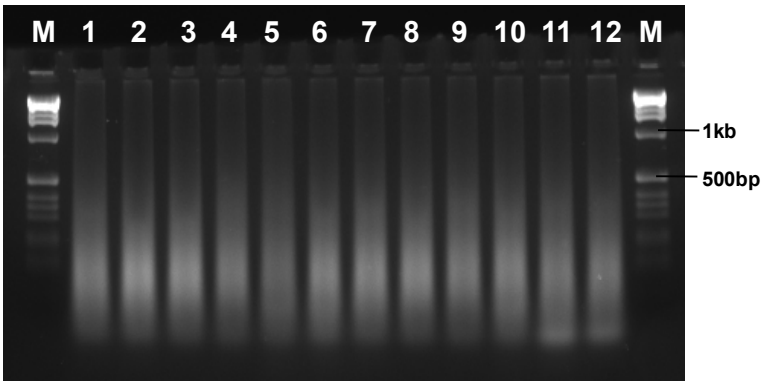

Figure 2.1 Amplified DNA samples after BioPrime Labelling Procedure. 3 µL of each labelled, amplified DNA sample was run on a 2.5% agarose gel stained with ethidium bromide. Most the DNA fragments are below 500bp.

2.1.3 Sample Precipitation and Preparation

For each hybridisation, the combined Cy3 and Cy5 labelled DNA was precipitated with 135 µL of human Cot-1 DNA (1µg/µL, Roche Diagnostics) and 35 µL 3M NaAc (pH 5.2) in ice-cold 100% ethanol (EtOH) at -20oC overnight. The use of excess Cot-1 DNA suppresses repeat sequences that are present in both the test and reference DNA. In separate tubes, 70 µL herring sperm DNA (10 mg/mL, Sigma Aldrich) was simultaneously precipitated using 7 µL 3M NaAc and 100% EtOH as pre-hybridisation. Before hybridisation, samples were spun down and DNA pellets were washed in 500 µL 80% EtOH, then dried and resuspended in 120 µL hybridisation buffer (50 % formamide, 5-7.5 % dextran sulphate, 0.1 % Tween 20, 2x SSC, 10 mM Tris pH 7.4, 10mM Cysteamine). All samples were then denatured for 10 min at 72°C.

2.1.4 Array Hybridisation

Hybridizations were carried out on a Tecan HS Pro Hybridization Station (Tecan Group Ltd.). One microarray slide was fitted into each 63 x 20-mm hybridisation chamber. Slides were subjected to a short wash (0.5 min, PBS/Tween 20/2 mM cysteamine). Prior to sample hybridisation, 100 µL of the herring sperm mixture (containing herring sperm DNA) was injected to each hybridisation chamber for a 45-min pre-hybridisation at 37°C with medium agitation frequency. Subsequently, 100 µL of the hybridisation mixture (containing fluorescent samples) were injected and the samples were hybridised for 21 h at 37°C with medium agitation frequency. Slides were then subjected to a series of stringent washes with PBS/Tween 20/2 mM cysteamine (wash time 0.5 min, soak time 0.5 min, 15 cycles at 37°C), 0.1x SSC (wash time 1.0 min, soak time 2.0 min, 5 cycles at 54°C), PBS/Tween 20/2 mM cysteamine (wash time 0.5 min, soak time 0.5 min, 10 cycles at 23°C), and HPLC water (wash time 0.5, soak time 0.0, 1 cycle at 23°C) before drying for 2.5 min using nitrogen gas. Slides were removed for scanning and the Tecan stations were cleaned with HPLC water using the final system drying program. All hybridisation chambers and plugs were cleaned thoroughly using a cotton bud soaked in HPLC water, followed by a repeat cleaning step with 80% EtOH then left air dried.

2.1.5 Image Acquisition and Data Analysis

Arrays were scanned using an Agilent laser scanner (Agilent Technologies) at a 5 µm resolution setting. Initial data analysis was performed using the BlueFuse software (BlueGnome Ltd). Array spots were defined by the automatic grid feature of the software, manually adjusted where necessary. After correction of local background value, fluorescence intensities of all spots were extracted with a user-defined protocol. Spots with low signal intensities ("amplitude" < 100 in both channels) or inconsistent

fluorescence patterns ("confidence" < 0.5 or "quality" = 0) were excluded. Each spot was then normalised among the array blocks (sub-arrays).

Subsequent analysis was performed with a custom perl script Dyeswapper2.1 (Dimitrios Kalaitzopoulos and Richard Redon, Sanger Institute). The script merges the dye reversal experiments, and determines a central distribution for the log2 signal ratio of all clones, which for a normal individual (with majority of clones in the diploid state) should be a value close to zero. Outlier clones that fall outside a threshold of this central distribution were subsequently identified as copy number variations.

A variability measure, termed SDe, is also determined as an estimation of the standard deviation of the experiment. It is calculated based on the 68.2th percentile (equivalent to one standard deviation from the mean) of the absolute values for all combined log2 ratios for each chromosome (chromosome SDe) and for the whole genome (Global SDe). Dye-reversal experiments for each test DNA were fused and the signal intensity ratios of each clone in the two hybridizations were averaged into a combined ratio (cR). Clone replicates differ >8 SDe were excluded from further analysis. Dye-swap experiments were accepted for subsequent CNV calling only if the following criteria were fulfilled: (1) Global SDe < 0.06; (2) Global clone exclusion rate < 10%; (3) Clone exclusion rate per individual chromosome < 20%.

Copy number changes for each DNA profile were identified using the CNVFinder algorithm (Fiegler et al. 2006) which was based on SDe thresholds. A score (Si) was assigned to the combined ratios of each individual clones in an experiment for CNV calling.

If cR ≥6x SDe or cR ≤ – 6x SDe, then Si = 1 or Si = – 1, respectively.

If cR ≥4x SDe or cR ≤ – 4x SDe, then Si = 0.5 or Si = – 0.5, respectively.

If cR ≥3x SDe or cR ≤ – 3x SDe, then Si = 0.25 or Si = – 0.25, respectively.

If cR ≥1x SDe or cR ≤ – 1x SDe, then Si = 0.1 or Si = – 0.1, respectively.

A stringent 6 x / 4 x SDe ratio threshold was applied: a locus is considered deleted or duplicated where the hybridisation ratio of the corresponding clone exceeds the value of (+/-) 6 x SDe, or if two or more consecutive clones exceed the value of (+/-) 4 x SDe. The 3 x SDe and 1 x SDe thresholds were used to extend CNV regions and to merge clones into a CNV event, for neighbouring clones in the same CNV region with reduced signal response.

The normalized log2ratio for each clone was then plotted against its position along the genome, and the list of CNVs for each dye-swapped experiment was generated.

2.2 Agilent Oligo Custom-Designed Array CGH

2.2.1 Custom Array Designs

Custom arrays were designed with Agilent's eArray microarray design tool. Multiplexed array formats of 4 x 44K were chosen. Probe lengths were optimized to 48-60 bp.

Two custom designs were made (with Richard Redon, Sanger Institute): the first targets 1.5 Mb at chromosome 7p12, with 34036 probes tiling across the region of the gene ATP binding cassette sub-family A (*ABCA13*) where a deletion CNV was identified. The second design targets 1.5Mb at chromosome 9q34, with 34761 probes tiling across a CNV downstream of the gene olfactomedin1 (*OLFM1*). Both designs include 2118 Agilent control probes plus around 9000 probes spaced evenly across all 24 chromosomes.

2.2.2 DNA Labelling

DNA labelling protocol was similar to that for WGTP tiling path array but in half volume reactions and with slight modification to the DNA clean-up steps. Briefly, 150 ng of DNA was added to 30 µL of 2.5x random primer solution (BioPrime Labeling Kit, Invitrogen), and made up with nuclease-free water to 65.25 µL. After denaturing the DNA for 10 min at 100°C, 7.5 µL of 10x dNTP mix (1 mM dCTP, 2 mM dATP, 2 mM dGTP, and 2 mM dTTP in TE buffer), 0.75 µL of 1 mM Cy5-dCTP or Cy3-dCTP (Amersham, GE Healthcare), and 1.5 µL of Klenow fragment (BioPrime Labeling Kit, Invitrogen) were added on ice to a final reaction volume of 75 µL. The reaction was incubated at 37^{o}C overnight over activated charcoal and stopped by 7.5 µL of stop buffer (BioPrimer Labeling Kit, Invitrogen).

Following DNA labelling, unincorporated nucleotides were removed using Microcon YM-30 Filter Devices (Millipore). 405 µL of 1x TE (Promega) was added to each labelled sample, loaded to a Microcon filter placed in a 1.5 mL microfuge tube. Samples were spun at 8,000 rcf for 10 min, followed by a wash with 480 µL 1x TE and spun at 8,000 rcf for 13 min. The filter was inverted and placed into a fresh 1.5 mL microfuge tube and spun for 1 minute. The volume of each eluate was measured and recorded. If the sample volume exceeded 21 µL, the sample would be returned to the filter to be spun again in the inverted position until all samples were < 21 µL. An appropriate volume of 1xTE was then added to bring each sample to 21 µL. 1.5 µL of each sample was analysed in a NanoDrop^{TM} ND-1000 or ND-8000 UV-VIS Spectrophotometer to determine yield and specific activity of the labelling and amplification reaction. Labelled DNA was either combined for the next step of hybridization preparation, or stored overnight at -20^{o}C in the dark for hybridisation on the next day.

2.2.3 Preparation of Labelled Genomic DNA

The appropriate Cy5 and Cy3 samples were combined for a mixture of 39 µL in a new 2 mL microfuge tube. For hybridisation preparation, the following reagents were added: 5 µL Cot-1 DNA (1.0 mg/mL, Roche Diagnostics), 11 µL Agilent 10x Blocking Agent and 55 µL Agilent 2x Hybridization Buffer, bringing the mixture to a final hybridization volume of 110 µL. The samples were mixed by pipetting. Samples were incubated in a 95^{o}C heat block for 3 min, followed by a 30-min incubation in a 37^{o}C heat block.

2.2.4 Array Hybridisation

A clean 4x44K gasket slide was loaded onto the Agilent SureHyb chamber base with the gasket label facing up. 100 µL of the hybridization mixture was slowly dispensed onto the gasket well in a “drag and dispense” manner. All four gasket wells were loaded. The

microarray slide was then placed onto the gasket slide with the “active” side (with the Agilent labelled barcode) facing down. To finish the chamber assembly set-up, the cover was placed onto the slides with the clamp slide across the assembly, followed by hand-tightening of the clamp. The assembled chamber was rotated to wet the slides and to assess the mobility of the bubbles. Stationary bubbles were removed by tapping the assembly on a hard surface. For hybridisation, the assembled slide chamber was left for 24 hours on a rotator rack in a 65 °C oven at a rotating speed of 20 rpm.

2.2.5 Slide Washing

After hybridisation, the chamber was disassembled by loosening the clamp, sliding the clamp off the assembly and removing the chamber cover. The array-gasket sandwich was removed from the chamber base and was submerged into a slide-staining dish (dish #1) which was filled with Oligo array CGH Wash Buffer 1. The sandwich was prised open using a pair of forceps such that the gasket slide would slip away from the microarray slide. The microarray slide was slotted into a slide rack placed in a second stain-dish (dish #2) filled with Oligo array CGH Wash Buffer 1. This step was repeated for up to 4 additional slides from the hybridisation.

The microarray slide(s) were incubated in dish #2 for 5 minutes at room temperature with magnetic stirring. The slide(s) were then transferred to the third slide-staining dish (dish #3), which was filled with Oligo array CGH Wash Buffer 2 pre-warmed to 37^{o}C on a hot plate with magnetic stirrer. The slides were incubated for 1 minute. The slides were then slowly removed from the slide rack. This removal step would take 5-10 seconds and the slides would be dried during the process.

2.2.6 Image Acquisition and Data Analysis

Slides were scanned at 5 μm resolution using an Agilent Scanner with Agilent Scanner Control software v7.0. The image was analyzed using Agilent's Feature Extraction (FE) software V9.5. A protocol which incorporated a custom data filtering and normalization method (Dr Richard Redon & Tomas Fitzgerald, Sanger Institute) was applied for data analysis.

2.3 Polymerase chain reaction (PCR)

All oligonucleotides for use as PCR primers were designed using the web-based program Primer3 (Rozen and Skaletsky 2000) with default parameters unless otherwise stated. Oligonucleotide sequences are listed in appendix 1.

2.3.1 PCR genotyping of the 3p26 Deletion near *CHL1*

General PCR amplification was performed in a 25 µL reaction containing approximately 50-200 ng genomic template DNA, 1.25 U *AmpliTaq* DNA polymerase (Applied Biosystem), 125 µM each dNTP, 5 µM each primer and 1x amplification buffer II with $MgCl_2$ (Applied Biosystem). The cycling protocol is shown in table 2.1.

Table 2.1 Cycling protocol for general PCR amplification A generic PCR cycling protocol consists of an initial denaturation of 94°C for 60 sec, followed by 30 cycles of 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing), and 72°C for 60sec/kb of expected PCR products (extension).

Temperature (°C)	Time (s)	Cycle number
94	60	1
94	30	30
55	30	
72	60/kb	
72	120	1

PCR products were resolved by agarose gel electrophoresis to confirm the presence of the product at expected size.

A deletion at 3p26 5' upstream of the gene Close Homolog of L1 (*CHL1*) was PCR genotyped. Amplification was performed in 25 µL reaction containing approximately 50 ng genomic template DNA and PCR reagent mix as mentioned above. To distinguish the deletion allele from the wild-type allele, PCR primers tiling across the CNV region were designed to validate the deletion polymorphism and to sequence the CNV breakpoints (Figure 2.2). A set of one forward primer (CHL1-fwd) and two reverse primers (CHL1-fwd-5 + wt-rev-2 & del-rev-1) were chosen as the genotyping primers, which generated a ~400bp band corresponding to the wild type allele, and a ~700 bp band corresponding to the deletion allele. Each DNA sample was amplified using the same forward primer and both of the reverse primers. PCR products for each sample were pooled and run on a large 2.5% agarose electrophoresis gel at 200V for 80 min. Figure 2.2 shows the gel electrophoresis analysis for this PCR assay.

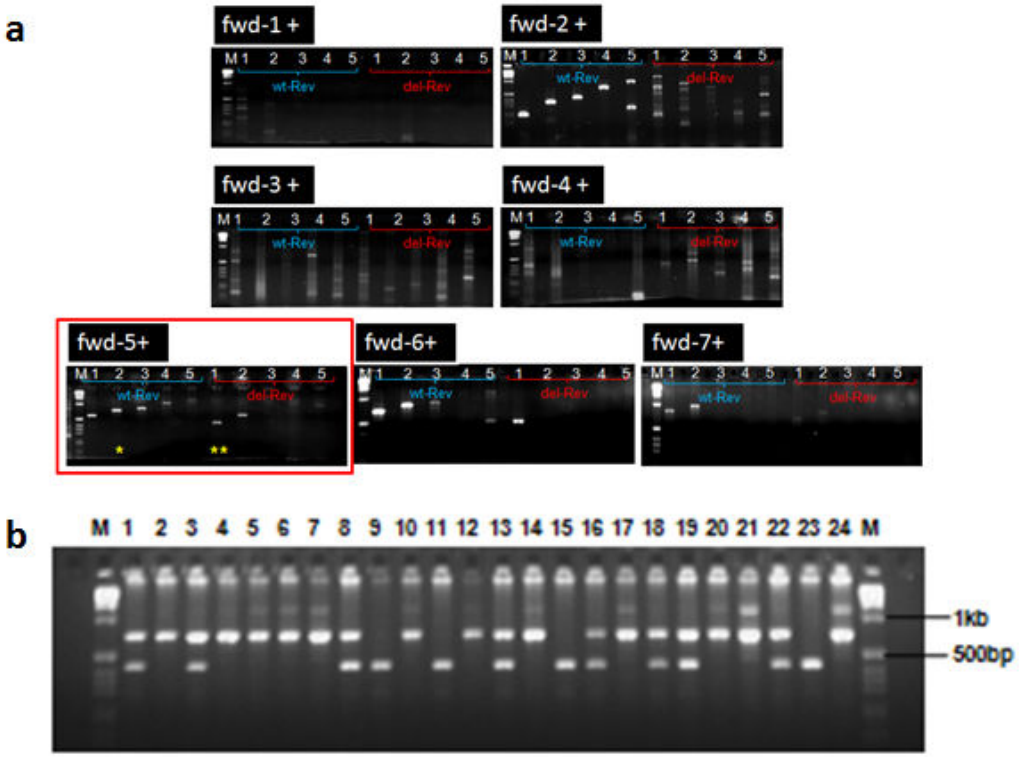

Figure 2.2 PCR genotyping of a deletion at 3p26 5' upstream of Close Homolog of L1 (*CHL1*). a) PCR results from a series of forward primers used in combination of two series of reverse primers, one for the wild-type allele (wt-Rev) and another for the deletion allele (del-Rev). **b)** The selected set of genotyping primer: Under gel electrophoresis analysis, the ~700bp band indicates the presence of the wild-type allele whereas the ~400bp indicates the presence of the deletion allele. A single band at ~700bp or at ~400bp represents homozygous wild-type and homozygous deletion DNA sample respectively, whereas a sample heterozygous for the deletion will show both bands.

2.3.2 Long Range Polymerase Chain Reaction (LR-PCR)

Oligonucleotides for use as long-range PCR primers were designed using Primer3 (Rozen and Skaletsky 2000) with the following parameters: primer melting temperature (Tm) ranges from 58°C to 63°C (optimal 61°C) and primer length ranges from 28 to 32 bp (optimal 30bp).

Long range PCR was performed using Elongase® Enzyme Mix (Invitrogen). PCR amplification was performed in 50µl reaction containing approximately 100 ng of genomic template DNA in a mixture containing 200 µM dNTP, 10 µM each of forward and reverse primer, 1 µl of enzyme mix and the two supplied 5x Buffers combined to give a final Mg2+ concentration of 1.8 mM. The cycling protocol is shown in table 2.2:

Table 2.2 Cycling protocol for long-range PCR amplification Long-range PCR cycling protocol consists of an initial denaturation of 94°C for 30 sec, followed by 35 cycles ^ of 94°C for 30 sec (denaturation), 61°C for 30 sec (annealing), and 68°C for 10 min (extension).

Temperature (°C)	Time	Cycle number
94	30 s	1
94	30 s	
61	30 s	35
68	10 min	
72	2 min	1

PCR products were resolved by 0.8-1% agarose gel electrophoresis to confirm the presence of the product at expected size (Figure 2.3). Selected PCR products were sent for sequencing.

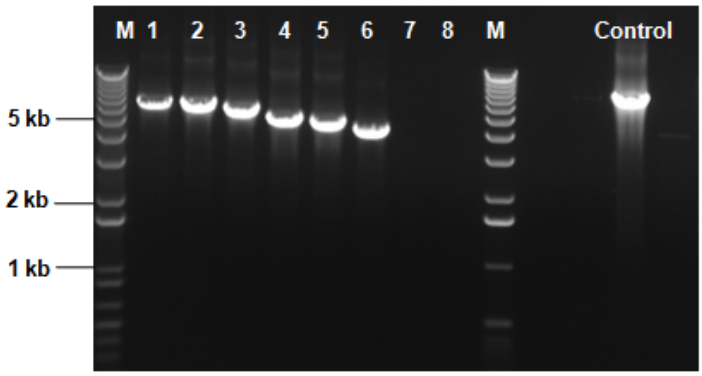

Figure 2.3 Long Range PCR gel electrophoresis analysis of a tandem duplication at Chr1p36. Lane 1-8: Long range PCR results from combinations of the same forward primer with different reverse primers tiling across the breakpoint.

2.3.3 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

2.3.3.1 SYBR Green Method

Oligonucleotides for use as PCR primers in SYBR Green qPCR assays were designed using Primer3 (Rozen and Skaletsky 2000) with parameters as follows: amplicons were designed to be 80 to 150 bp; primer melting temperature (Tm) ranged from 57°C to 63°C (optimal 60°C); primer length from 18 to 27 bp (optimal 20bp); and primer GC content from 20 to 80%. For internal control, a primer set for the gene Beta-2-Microglobulin (β2M) was used as control primer.

SYBR qPCR amplifications were performed in 25 µL reactions containing 10ng of genomic DNA, 12.5 µL of 2x qPCR MasterMix Plus SYBR® green I (Eurogentec) reaction buffer (with HotGoldStar *Taq* polymerase) and 300 nM of forward and reverse primers. For each target region or primer, reactions were performed in triplicate with the test DNA sample(s), and separate triplicate reactions were performed using a reference genomic DNA *NA10851* or *NA15510* (Coriell Institute for Medical Research) as a calibrator. Test primer(s) and control primer for each DNA sample were run on the same plate.

2.3.3.2 Taqman Method with MGB Probes

Oligonucleotides for use as PCR primers and probes in Taqman qPCR assays were designed using the software PrimerExpress® v2.0 (Applied Biosystem) with default settings for MGB probe and primer design. The best score MGB probes and primers with probes ranging from 15-22 nucleotides (nt) were selected.

Taqman qPCR amplifications were performed as duplex reactions with assays for the test and control in the same well. The reaction mixture for a 25 µL reaction was as follows:

Table 2.3 Reagent mixture for each Taqman quantitative PCR assay.

Reagent	Final Concentration/Amount
Control primer (Sigma Aldrich)	900nM
Test primer (Sigma Aldrich)	300nM
5'VIC labeled MGB control probe (Applied Biosystem)	100nM
5'FAM labeled MGB test probe (Applied Biosystem)	100nM
Taqman Universal qPCR Master Mix (Applied Biosystem)	1x
Additional AmipliTaq Polymerase (Applied Biosystem)	2.4 unit
Additional dNTP	50 µM
DNA	~10ng

2.3.3.3 Thermal Cycler and Reaction Condition

Reactions for SYBR or Taqman assays were run in Optical 96-well Reaction Plates (Applied Biosystem) using an ABI Prism 7900 Thermal Cycler with the standard thermal cycling mode as shown in Table 2.4. At the end of the PCR reactions, samples were

subjected to a dissociation curve analysis to confirm specificity of amplification. Figure 2.4 shows examples of a) a dissociation curve showing multiple dissociation peaks (which are discarded for further experiments), and b) a dissociation curve giving a discrete dissociation peak (which are kept for further experiments and analysis),

Table 2.4 Cycling protocol for Quantitative PCR amplification. Quantitative PCR cycling protocol consists of an initial heat activation step (for Hotstart Polymerase in the mastermix) of 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec (denaturation) and 60°C for 60 sec (annealing and extension).

Temperature (^{o}C)	Time	Cycle number
95	10 min	1
95	15 s	40
60	60 s	

2.3.3.4 Standard Curve Generation

For each set of primers, a standard curve was prepared using 2-fold serial dilutions of a reference genomic DNA *NA10851* or *NA15510* from Coriell Institute for Medical Research. Dilutions were made for at least 5 concentrations from 50ng to 3.125ng to completely cover the range of expected DNA copy number changes. Each concentration was done in quadruplicate. Standard curve for the duplex reactions were also performed for the Taqman assays. The slope of the standard curves and qPCR efficiency for each primer was determined. Primer sets with standard curve slope values ranging from -3.0 to -3.9 were accepted. Figure 2.5 shows an example of a typical standard curve.

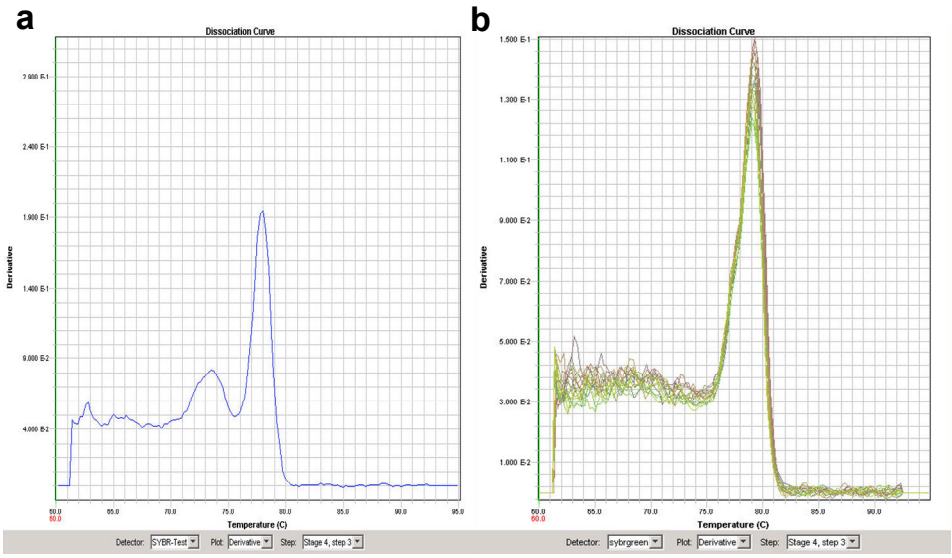

Figure 2.4 Dissociation curves analysis for different primer sets. (a) Example of a primer set giving multiple dissociation peaks. **(b)** Example of a dissociation curve giving a discrete dissociation peak.

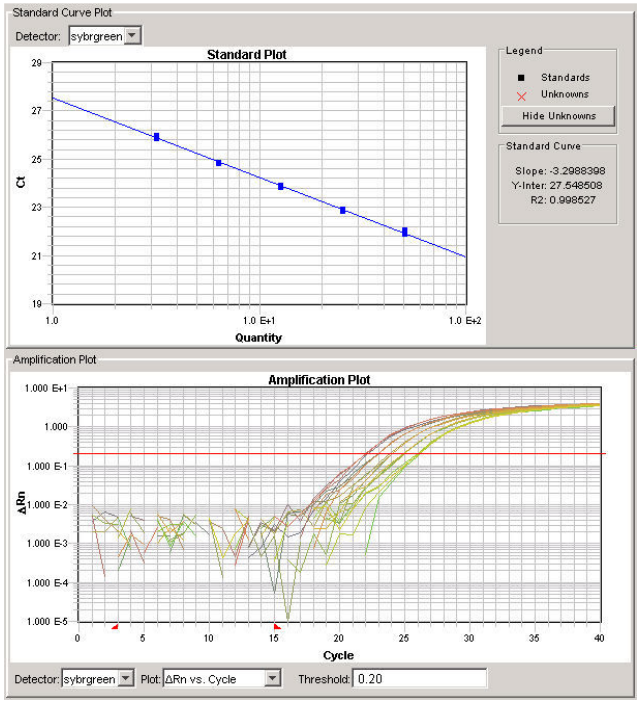

Figure 2.5 A typical standard curve of the control primer. (a) A standard plot of Ct against DNA quantity (in $\log_{10}$ scale) in five 2x dilution from 50 ng to 3.125 ng. **(b)** The amplification plot of the 5 DNA dilutions.

2.3.3.5 DNA Quantification and Data Analysis

Quantification of DNA was based on increased fluorescence in each reaction, expressed in terms of threshold cycle (Ct) values generated by the thermal cycler. Data was exported using the associated SDS 2.2 software in text format. The relative copy number fold change of each target genomic region was normalized to the calibrator DNA. This Test-versus-Calibrator fold change was calculated using the formula below. Standard deviation for each fold-change analysis was also calculated using the following formula (Pfaffl 2001):

$$FoldChangeRatio = \frac{\left(E_{test}\right)^{\Delta Ct(|control-affected|)}}{\left(E_{reference}\right)^{\Delta Ct(|control-affected|)}}$$

where E is a measure of the efficiency or the amplification of the primer. E is calculated by:

$$E = 10^{-1/slope}$$

2.4 Fluorescence In-Situ Hybridisation (FISH)

2.4.1 Growing Cell lines

Lymphoblastoid cell lines were obtained from the Coriell Institute of Medical Research. Cell lines were grown in RPMI 1640 medium (Invitrogen), supplemented with 20% fetal calf serum (Invitrogen) and 2mM L-glutamine/ 100 U/mL penicillin/ 100 µg/mL streptomycin (Sigma). Cultures were incubated at 37ºC and 5% CO2.

2.4.2 Preparation of Extended Chromatin Fibre Slides

Approximately 2-3 ml of healthy cell suspension of each cell line was centrifuged at 1200 rpm for 5 min. Cell pellets were washed twice with PBS and re-suspended in PBS to a final concentration of approximately 2-3 x 10^6 cells/mL. Using a pipette, 10-12 µL of the cell suspension was spread onto the upper part of a polylysine-coated slide (Sigma). Slides were air-dried and fitted into a Perspex Candenza block assembly (made in-house) one at a time, clamped in a nearly vertical position in a bent metal rack. By applying solution to the gap at the top of the Candenza assembly, the slide was washed with 150 µL lysis solution (5 parts 70mM NaOH, 2 parts absolute ethanol) followed by 150 µL of 96% ethanol. The slide was allowed to drain until the meniscus stopped falling (approximately 30s). The Candenza clamps were removed and the slide was carefully lifted away from the Candenza assembly with extended chromatin fibres attached to the slide surface.

Fibre slides were air-dried and fixed with acetic acid (3:1 96% ethanol/acetic acid fixative) for 5 minutes, then dehydrated in an ethanol series (70%, 90%, 100%). Finally, slides were pepsin treated at 37ºC for 5 minutes by incubating in the fixing solution (0.01M HCL with 0.01% pepsin (Sigma)), and were dehydrated again through an ethanol series. Air-dried slides were then stored in a sealed box at –20ºC.

2.4.3 Preparation of Fosmid Clone Insert DNA

Fosmid clones corresponding to DNA sequences within or near the copy number variation regions were ordered from the Sanger Institute. Details of the fosmid clones used in the study are listed in Appendix A. Each fosmid clone was inoculated in 10 mL LB media with 12.5 µg/mL chloramphenicol and was cultured at 37ºC overnight. On the next day, the 10 mL culture was pelleted at 2,000g for 10 minutes. Genomic DNA was isolated from each clone using Phase Prep BAC DNA Kit (Sigma Aldrich) with modification to manufacturer's protocol. 300 µL Resuspension Solution was used to resuspend the cells, followed by the addition 300 µL Lysis Solution. The tubes were then inverted 5 times to allow mixing and incubated for 5 min at room temperature. 300 µL chilled Neutralisation Solution was added and the tubes inverted 8 times before incubation on ice for 5min. The tubes were centrifuged at 15,000g for 20 min at 4°C and the clear supernatant was transferred to a fresh 1.5 mL microfuge tube. 540 µL isopropanol was added to the clear supernatant, and the tubes were centrifuged at 15,000g for 20 min at 4°C to precipitate the DNA. The pellet was washed with 2 mL 70% ethanol and air dried briefly.

The pellet was then resuspended in 540 µL Elution Solution, followed by the addition of 1 µL 1:10 RNase cocktail and a 10-min incubation at 60°C to digest residual RNA (ribonucleic acid). 40 µL Sodium Acetate Buffer Solution (3M, pH 7.0) was added to the tubes to adjust the salt concentration. To remove endotoxin and other impurities, 100 µL of Endotoxin Removal Solution was added followed by a 5-min incubation on ice. The tubes were then warmed at 37°C for 5min prior to centrifugation at 16,000g for 3min. The clear upper phase containing the DNA was transferred to a clean tube and the lower blue phase containing endotoxins and other impurities was discarded. This endotoxin

removal step was repeated once more. 540 µL DNA Precipitation Solution was then added with 1 µL pellet paint to precipitate the DNA again by centrifugation at 21,000g for 20 min at 4°C. The pellets were washed with 150 µL 70% EtOH, air dried and resuspended in 20 µL water. The DNA concentration and quality was assessed using a NanoDrop^{TM} ND-1000 or ND-8000 UV-VIS Spectrophotometer.

2.4.4 Amplification and Labeling of DNA probes

2.4.4.1 GenomePlex® Whole Genome Amplification

10ng of the isolated fosmid DNA (at concentration of 1 ng/µL) was WGA amplified using the GenomePlex® Complete Whole Genome Amplification Kit (Sigma) following manufacturer's instructions. Briefly, 10 ng (1 ng/µL) DNA was fragmented with 1 µL 10x Fragmentation Buffer at 95°C for 4 min and immediately cooled on ice. The fragmented DNA was then used to create a library following the addition of 2 µL 1x Library Preparation Buffer and 1 µL Library Stabilisation Solution. The samples were heated at 95°C for 2 min and cooled on ice prior to the addition of 1 µL Library Preparation Enzyme. Samples were incubated at 16°C for 20 min, 24°C for 20 min, 37°C for 20 min and 75°C for 5 min. For amplification of the library, 47.5 µL nuclease-free water, 7.5 µL 10x Amplification Mastermix and 5 µL WGA DNA Polymerase were added and the samples subjected to a PCR cycling of 95°C for 3min followed by 17 cycles of 94°C for 15 sec and 65°C for 5 min.

2.4.4.2 DNA Fluorescent Labelling

Amplified DNA from the clones was labelled with either Biotin-18-dUTP or Digoxigenin-11-dUTP (Roche Diagnostics) in a PCR reaction containing 1 µL of the amplified DNA from WGA, 60 µM of either Biotin or Digoxigenin labelled dNTP, 40 µM dTTP, mM MgCl2, and 2.5 U BioTaq polymerase (Bioline) in a 25 µL reaction volume. The thermal

conditions were 3 min at 94°C, followed by 17 cycles of 15s at 94 °C and 5min at 65 °C. The fragment sizes of the labelled product were assessed by gel electrophoresis running 1 µL on a 1% agarose gel with ethidium bromide.

2.4.4.3 Probe Fragmentation

To reduce the fragment sizes prior to hybridisation to the fibre slides, 4 µL 1 µg/µL DNAse I (Sigma) was added to the remaining 24 µL labelled DNA and incubated at 15°C for approximately 1hr 30 min. The fragment sizes of each sample were reassessed on 1% agarose gel electrophoresis stained with ethidium bromide, while the remaining samples were kept on ice. Optimal fragment sizes for FISH were considered to be 50-500 bp in length (Figure 2.6). DNase I digestion was resumed if necessary.

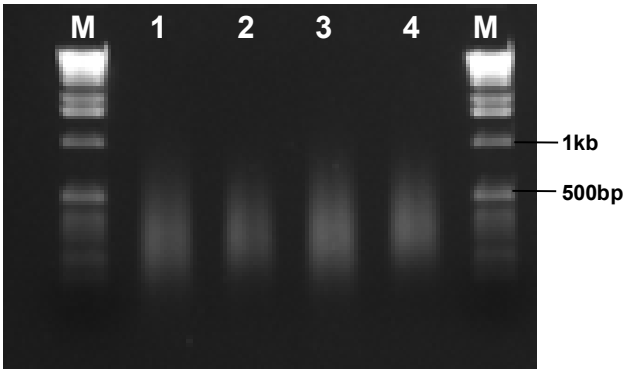

Figure 2.6 Labelled DNA probes for Fluorescent In Situ Hybridisation. Fosmid probes were labelled with either Biotin or Digoxygenin dUTP. Probes were digested by DNase I to reduce DNA fragment sizes to approximately 50-500 bp for optimal hybridisation results. Digested DNA fragment sizes were checked using a 1% agarose gel stained with ethidium bromide.

2.4.5 Immunofluorescence and Image Acquisition

FISH was carried out as follows: Before hybridization, slides were denatured in 70% formamide/ 2x SSC for 2 min at 70°C. Slides were then quenched in 70% ice-cold ethanol, dehydrated through an ethanol series and air-dried.

For each hybridization, approximately 100 ng of each biotin- or digoxigenin-labelled probe was combined with 1 µL Cot1 DNA (1 µg/µL, Roche Diagnostics) and 10 µL hybridization buffer (50% deionised formamide, 2x SSC, 10% dextran sulphate, 0.1% Tween 20, 10mM Tris pH7.4). Labelled probe mixtures were denatured at 65°C for 10 min, followed by a pre-annealing incubation at 37°C for 30 min to 3 hours. Denatured probe mixtures were added to the slides, covered with 22 x 36 mm plastic coverslips, sealed with rubber cement and incubated at 37°C overnight for hybridization. On the next day, rubber cement were removed and slides were incubated twice in 2xSSC (43°C, 5 min), once in 50% formamide for 5 min at 43°C, and once again in 2xSSC (43°C, 5 min). Slides were then transferred to 4xST (4 x SSC, 0.05% Tween) for 5 min at room temperature.

Hybridised probes were detected by two layers of antibodies. Biotin-labeled probes were detected by avidin-FITC (Vector Laboratory), followed by biotinated antiavidin D as secondary antibody (Vector Laboratory). Digoxigenin-labeled probes were visualized by monoclonal mouse anti-dig antibody (Sigma) followed by Texas Red-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) as secondary antibody. Slides were incubated sequentially in each antibody layer (200 µL under Nescofilm®) for 20 min at 37°C in the dark. In between the two layers of antibody detection, slides were washed in 4xST (5 min, room temp) three times.

After probe detection, each slide was mounted with DAPI (4′,6-diamidino-2-phenylindole) (Vector Labs, Orton Southgate, UK) under a 22 x 50 mm plastic coverslip. Edges of the coverslips were sealed with nail varnish. Slides were stored at 4°C.

Images were captured and processed with the SmartCapture software (Digital Scientific, Cambridge, UK).