2 Materials and methods

2.1 Cell culture

Lymphoblastoid cell lines were established from blood samples for patients t(2;7)(q37.3;p15.1), t(3;11)(q21;q12) and t(7;13)(q31.1;q21.3) by the European Cell and Culture Collection (ECCAC), Porton Down and the Cell Bank at Wessex Regional Genetics Laboratory (WRGL) for patient t(2;6)(q21.1;q25.1).

For patients AMD, AS and KK showing high viral load, lymphoblastoid cell lines were established from peripheral blood mononuclear cells (PBMCs) at the Department of Virology, Royal Free and University College Medical School of UCL, London.

All lymphoblastoid cell lines were cultured at 37°C in RPMI 1640 media (Invitrogen) supplemented with 16% Foetal Calf Serum (FCS; Invitrogen), 2mM L-glutamine/ 100U/ml penicillin/ 100µg/ml streptomycin (Sigma).

2.2 Isolation of Patient DNA

DNA was isolated from the patient derived cell lines by various methods for different protocols. Whole genomic DNA was used for array CGH and LR PCR. Flow sorted derivative chromosomes were used directly for the generation of fosmid libraries and for array painting onto WGTP arrays and custom-made oligonucleotide microarrays (NimbleGen Systems Inc.). After amplification, flow sorted derivative chromosomes were also used for STS PCR mapping and array painting onto fosmid microarrays.

2.2.1 Genomic DNA extraction

Whole genomic DNA was extracted using the Qiagen Blood and Cell Culture DNA Midi kit according to the manufacturer's protocol. Briefly, $2x10^7$ cells were centrifuged at 1500g for 10min. The pellet was washed twice with Phosphate

Buffered Saline (PBS; 1.37M NaCl/ 27mM KCl/ 100mM Phosphate buffer) and resuspended in 2ml ice-cold (4°C) PBS. 2ml ice-cold buffer C1 (Cell Lysis Buffer; 1.28M sucrose/ 40mM Tris-Cl pH7.5/ 20mM $MgCl_2$/ 4% Triton X-100) and 6ml ice-cold water were added to the cells, inverted and incubated on ice for 10min. The cells were centrifuged at 1300g for 15min and resuspended in 1ml ice-cold buffer C1 and 3ml ice-cold water before being recentrifuged at 1300g for 15min. 5ml buffer G2 (General Lysis Buffer; 800mM Guanidine HCl/ 30mM Tris-Cl pH8/ 30mM EDTA pH8/ 5% Tween-20/ 0.5% Triton X-100) was added and the nuclei resuspended by vortexing. 95µl 20mg/ml Qiagen protease was added prior to incubation at 50°C for 1hr. 100/G Genomic tips were prepared by allowing 4ml Buffer QBT (Equilibration Buffer; 750mM NaCl/ 50mM 3-[N-Morpholino] propanesulphonic acid (MOPS) pH7/ 15% isopropanol/ 0.15% Triton X-100) to flow through prior to addition of the nuclei. The tip with sample was then washed twice with 7.5ml Buffer QC (Wash Buffer; 1M NaCl/ 50mM MOPS pH7/ 15% isopropanol). Genomic DNA was eluted with 5ml Buffer QF (Elution Buffer; 1.25M NaCl/ 50mM Tris-Cl pH8.5/ 15% isopropanol). The DNA was precipitated by the addition of 3.5ml isopropanol and inversion of the collection tube. DNA was spooled using a sterile loop, transferred to a tube containing 50µl water and allowed to resuspend overnight at 4°C. The DNA concentration was measured using a TD-360 fluorometer (Turner Designs) and a 0.6% agarose gel with ethidium bromide was run to assess yield and quality.

2.2.2 Flow sorting of derivative chromosomes (Bee Ling Ng – Team 70)

Chromosomes from cell lines were prepared in polyamine buffer (pH7.5; 15mM Tris/ 2mM EDTA/ 0.5mM Ethylene glycol-bis N,N,N'N'-tetra acetic acid (EGTA; Sigma)/ 80mM KCl/ 3mM dithiothreitol/ 0.25% Triton X-100 (Sigma)/ 0.2mM spermine (Sigma)/ 0.5mM spermidine (Sigma)/ 20mM NaCl) and stained with 5ug/ml of Hoechst (Sigma) and 40ug/ml of Chromomycin A3 (Sigma). The stained chromosome sample was analysed and flow sorted using a MoFlo high speed cell sorter (Dako). 500 chromosomes for DOP PCR were sorted in 33µl

UV treated water and 250,000 chromosomes for array painting and fosmid library procedures were sorted in 250µl sheath buffer (10mM Tris-HCl (pH8.0)/ 1mM EDTA/ 100mM NaCl/ 0.5mM sodium azide).

"Bulk sorted" chromosomes (tubes of 250,000) were subjected to an overnight incubation at 42°C with 15µl 0.25M EDTA/ 10% sodium lauroyl sarcosine (Sigma) and 2µl 20mg/ml Proteinase K (Sigma). Chromosomes were then incubated at room temperature for 40min with 2µl 4mg/ml phenylmethanesulfonyl fluoride (PMSF; Sigma) in 96% ethanol to inactivate the proteinase K. Finally, chromosomes were precipitated with 10µl 5M NaCl, 2µl pellet paint (Novagen) and 770µl 100% ethanol at -20°C overnight.

2.3 Amplification of template DNA

2.3.1.1 REPLI-g

Flow sorted derivative chromosomes were amplified by Repli-G, a commercially available kit (QIAGEN) for whole genome amplification by Multiple Displacement Amplification (MDA). Replication is obtained using a DNA Polymerase capable of synthesising 100Kb of sequence at 30°C.

Tubes of 250,000 flow sorted derivative chromosomes were precipitated and resuspended in 20µl T0.1E (10mM Tris, 0.1mM EDTA) with 1µl of this used for the REPLI-g reaction as per the manufacturer's instructions. Briefly, 1µl of flow sorted chromosomes were added to 1.5µl TE (10mM Tris/ 1mM EDTA) and 2.5µl Buffer D1 (1 part Solution A (0.4M KOH/ 12.5mM EDTA)/ 7 parts nuclease free water) and incubated at room temperature for 3min. The reaction was stopped by the addition of 5µl Buffer N1 (1 part Solution B/ 9 parts nuclease free water). 27µl nuclease-free water, 12.5µl 4x REPLI-g buffer and 0.5µl REPLI-g DNA polymerase were added and the samples incubated at 30°C for 8hr and inactivated at 65°C for 3min.

2.3.1.2 DOP PCR

Flow sorted derivative chromosomes were amplified by degenerate oligonucleotide primer (DOP) PCR. The primers contained 6 bases of degenerate sequence and this coupled with initial low annealing temperatures allowed amplification at multiple sites along the template sequence.

Tubes of 500 flow sorted derivative chromosomes were amplified in 50µl reactions by DOP PCR. 5µl 10xTAPS2 buffer (250mM N-Tris (hydroxymethyl) methyl-3-amino-proane sulphonic acid pH9.3 (Sigma)/ 500mM KCl/ 20mM $MgCl_2$/ 10mM dithiothreitol/ 0.7% ß-mercaptoethanol/ 0.165% Bovine Serum Albumin), 4µl 2.5mM each dNTPs, 0.5µl 5U/µl Amplitaq (PerkinElmer), 2.5µl 1.25% W1 detergent (Sigma) and 5µl 20µM DOP primer were added to 500 chromosomes in 33µl water. For a negative control, 33µl of UV treated water was used. The sequence of the primers used is detailed in Table 2.1.

Primer name	Primer sequence
6MW	CCGACTCGAGNNNNNNATGTGG
DOP1	CCGACTCGAGNNNNNNCTAGAA
DOP2	CCGACTCGAGNNNNNNTAGGAG
DOP3	CCGACTCGAGNNNNNNTTCTAG

Table 2.1 DOP primers used for the amplification of chromosomes and clones by DOP PCR.

Samples were heated at 94°C for 9min followed by 10 cycles of 94°C for 1min, 30°C for 1min 30sec ramping at 0.23°C per second, 72°C for 3min followed by 30 cycles of 94°C for 1min, 62°C for 1min, 72°C for 1min 30sec followed by 72°C for 9min.

2.4 Construction of microarrays

2.4.1 Genomic clone microarrays (fosmid and plasmid microarrays)

2.4.1.1 Clone selection

For fosmid microarray production, fosmid clones were selected at full redundancy from Build35 of the UCSC genome browser (http://genome.ucsc.edu/) to cover the spanning BAC clone and approximately 100Kb of sequence both proximal and distal to this region. In addition, BAC clones were selected at 10Mb spacing from the ”Golden Path”. Fosmid and BAC clones were picked from libraries held within the Sanger Institute.

Plasmid clones were selected to provide maximum coverage of the HHV-6 genome and were kindly supplied by the Centre of Disease Control, Atlanta.

2.4.1.2 Picking

Clones were picked into LB media with appropriate antibiotic (12.5µg/ml chloramphenicol for fosmids, 20µg/ml chloramphenicol for BACs or 100µg/ml ampicillin for plasmids) and incubated at 37°C overnight. All clones were tested for T4 bacteriophage λ and *Pseudomonas aeruginosa* contamination before being sent for prepping.

2.4.1.3 DNA prepping (Carol Carder – Team 63)

Clones were grown overnight in 2ml 96-well boxes (Beckman) at 37°C with shaking in 2xTY media with the appropriate antibiotic (12.5µg/ml chloramphenicol for fosmids, 20µg/ml chloramphenicol for BACs or 100µg/ml ampicillin for plasmids). 250µl of the overnight culture was transferred into a 96 well plate (Costar), centrifuged for 2min at 800g at 20°C and the pellets resuspended in 25µl Solution 1 (50mM Glucose/ 10mM EDTA/ 5mM Tris pH8.0). 25µl Solution 2 (0.2mM NaOH/ 1% SDS) was added and the plates incubated at room temperature for 5min followed by the addition of 25µl 3M KOAc pH5.5 and a

further 5min incubation at room temperature. The contents of each well were transferred to a 96 well filter plate (Millipore), centrifuged for 2min at 800g at 20°C into 100µl isopropanol and incubated at room temperature for 30min. The plates were spun at 1300g for 20min at 20°C and the pellets air dried for 15min. The pellets were washed twice with 70% ethanol and air dried before being resuspended in 5µl T0.1E (10mM Tris, 0.1mM EDTA) with 10µg/ml RNase (Sigma).

2.4.1.4 DOP PCR amplification of plasmid, fosmid and BAC clones

All clones were amplified using 3 separate DOP primers (DOP 1, DOP 2 and DOP 3 in Table 2.1). 50µl DOP PCR reactions were performed in 96 well plates (Costar). 28µl water, 5µl 10xTAPS2 buffer (250mM N-Tris (hydroxymethyl) methyl-3-amino-proane sulphonic acid pH9.3 (Sigma)/ 166mM $(NH4)_2SO_4$/ 25mM $MgCl_2$/ 0.7% ß-mercaptoethanol/ 0.165% Bovine Serum Albumin), 5µl 20µM DOP primer, 4µl 2.5mM each dNTPs, 2.5µl 1% W1 detergent (Sigma) and 0.5µl 5U/µl Amplitaq (PerkinElmer) were added to 5µl of clone DNA (section 2.4.1.3) and subjected to temperature cycling on an MJ-Thermocycler; 94°C for 3min followed by 10 cycles of 94°C for 1min 30sec, 30°C for 2min 30sec ramping at 0.1°C per sec to 72°C, 72°C for 3min followed by 30 cycles of 94°C for 1min, 62°C for 1min 30sec, 72°C for 2min and finally 72°C for 8min. 5µl of each reaction was analysed using 2.5% agarose gel electrophoresis with ethidium bromide staining (Figure 2.1).

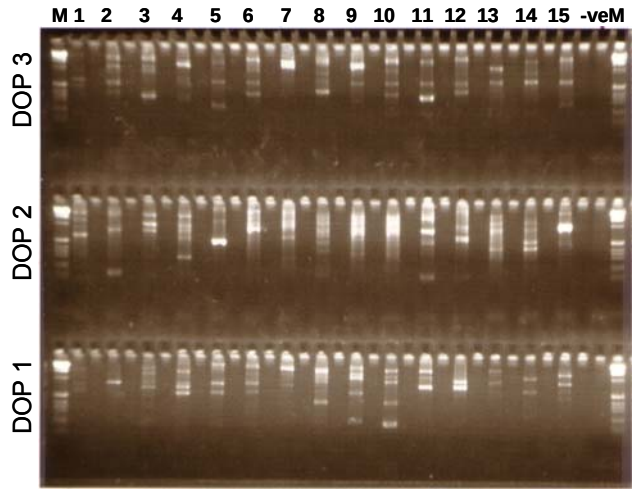

Figure 2.1 PCR amplification of 15 plasmid clones using primers DOP 1, DOP 2 and DOP 3 run on 2.5% agarose gel run with 1Kb marker. Unlabelled wells were left unloaded.

2.4.1.5 Amino-linking of DOP PCR products

Prior to amino-linking PCR, all 3 DOP PCR reactions for each clone were combined. 3µl of this combined product was used as template in an amino-linking PCR to attach an amino group to the 5' end of products to allow for covalent bonding of the product to the glass microarray slides.

90µl amino-linking PCR reactions were performed using 3µl of DOP template, 63.6µl water, 9µl 10x Amino-linking buffer (500mM KCl/ 25mM MgCl2/ 50mM Tris pH8.5), 9µl 2.5mM each dNTPs, 4.5µl 200ng/µl Amino-primer (GGAAACAGCCCGACTCGAG with an amino group attached to the 5' end) and 0.5µl 5U/µl Amplitaq (PerkinElmer). Reactions were performed on an MJ-Thermocycler; 95°C for 10min followed by 35 cycles of 95°C for 1min, 60°C for 1min 30sec, 72°C for 7min followed by 72°C for 10min. 2µl of each reaction was analysed using 2.5% agarose gel electrophoresis with ethidium bromide staining (Figure 2.2).

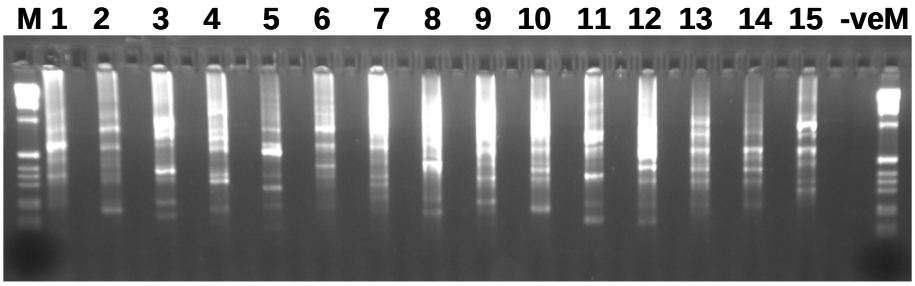

Figure 2.2 Amino-linking PCR for 15 plasmid clones run on 2.5% agarose gel with 1Kb marker. Unlabelled wells were left unloaded.

2.4.1.6 Spotting onto slides (Cordelia Langford & Team 77)

45µl PCR products (section 2.4.1.5) were added to 15µl 4x spotting buffer (1M sodium phosphate buffer pH8.5/ 0.001% N-lauroyl-sarcosine/ 0.4% azide) in multiscreen filter plates (Millipore) and filtered by centrifugation at 550g for 10min into 96 well plates (Falcon). Using a Qiagen RapidPlate liquid handling robot, 15µl of each filtered sample was transferred into a Genetix X6004 384-well plate. DNA products were arrayed onto Amine Binding slides (Motorola) at 20-25°C at 40-50% humidity using a MicroGridII array gridding robot (Genomic Solutions) equipped with tungsten spotting pins. The microarray slides were incubated for 24-72hr at room temperature in 70-80% humidity, incubated for 5min in 1% ammonium hydroxide at room temperature followed by a further incubation for 5min in 0.1% sodium dodecyl sulphate at room temperature before being rinsed in Double Distilled Water (DDW) at room temperature followed by immersion in 95°C DDW for 2min. Slides were then transferred to ice-cold DDW and then briefly washed in room temperature DDW before drying by centrifugation at 150g for 5min at room temperature. Microarray slides were stored in a dessicator at room temperature until use.

2.4.2 PCR product arrays

2.4.2.1 PCR primer design (James Morris – Team 117)

18-22bp oligonucleotide primers with 40-60% GC content were designed to repeat-masked sequence to amplify 800-1200bp products tiling the region of interest. Forward primers were synthesised with an amino group attached to their 5' end.

2.4.2.2 PCR amplification of products for microarray production

Primer pairs were used to amplify products from genomic DNA (Promega) for patient t(2;7)(q37.3;p15.1) or viral DNA (kindly supplied by Hoe Nam Leong and Duncan Clarke at UCL, London) for the HHV-6 microarray. All primer pairs were also tested on water as a negative control.

60µl PCR reactions were conducted in 96 well plates (Costar) with 15ng template DNA in 5µl (or 5µl water for negative control), 1.5µl 200ng/µl forward primer, 1.5µl 200ng/µl reverse primer, 6µl 10xPCR Buffer (500mM KCl/ 50mM Tris pH8.5/ 25mM $MgCl_2$), 3µl 10mM each dNTPs, 0.375µl 5U/µl Amplitaq (PerkinElmer) and 42.625µl water. Samples were subjected to temperature cycling of 95°C for 5min followed by 35 cycles of 95°C for 1min, 65°C decreasing by 0.3°C per cycle for 1min 30sec, 72°C for 1min 30sec followed by 72°C for 5min. PCR products were spotted onto glass slides as described in section 2.4.1.6.

2.5 Hybridisation and analysis of microarrays

2.5.1 Hybridisation and analysis of in-house arrays (PCR, fosmid, WGTP)

All microarrays were hybridised on an HS4800 Hybridisation Station (Tecan) using small chambers (51 x 20mm) (fosmid microarrays and PCR product microarrays) or large (63.5 x 21mm) chambers (Whole Genome Tile Path

(WGTP) microarrays). All details given remain the same regardless of which microarray was used unless otherwise stated.

2.5.1.1 Labelling

Template DNA (150ng genomic DNA for CGH, DNA from 250,000 flow sorted derivative chromosomes or 1µl REPLI-g amplified DNA for array painting) was labelled using reagents from the BioPrime Labelling Kit (Invitrogen) with modifications as stated to allow for labelling of the DNA with fluorescent dyes. DNA was mixed with 60µl Random Primers Solution and water to make up to 130.5µl, heated to 100°C for 15min and then cooled on ice prior to the addition of 15µl 10xdNTP mix (1mM dCTP/ 2mM dATP/ 2mM dGTP/ 2mM dTTP), 1.5µl 1mM Cy3 or Cy5 labelled dCTP (NEN Life Science) and 3µl Klenow fragment. Samples were incubated at 37°C overnight and the reactions terminated by the addition of 15µl Stop Buffer. For CGH onto the WGTP microarray, 300µl labelling reactions were set up per sample and per reference in duplicate with the dyes reversed so that dye swap experiments could be performed.

Labelled samples were cleaned up using Microcon YM-30 columns (Millipore); 150µl labelled sample and 150µl water (300µl combined labelling reactions for WGTP array) were applied to the column and spun at 12,000g for 5min, then washed with 300µl water and spun as before. 100µl water was added to the sample and the inverted column spun in a fresh tube at 400g for 2min to collect the sample. 3µl of each sample was analysed using 2.5% agarose gel electrophoresis with ethidium bromide staining to check for successful amplification.

2.5.1.2 Precipitation of samples

For each microarray a sample tube and prehybridisation tube was required; for the sample tube 80µl Cy3 labelled DNA, 80µl Cy5 labelled DNA and 135µl Human Cot1 DNA (Invitrogen) were precipitated with 35µl 3M Sodium Acetate

pH5.2 and 1ml 100% ethanol. For prehybridisation tubes, 100µl 10mg/ml Herring Sperm DNA (Sigma) was precipitated with 10µl 3M Sodium Acetate pH5.2 and 500µl 100% ethanol. Tubes were incubated at -20°C for a minimum of 1hr, centrifuged at 18,000g for 30min at 4°C and washed with 80% ethanol. Pellets were resuspended in 120µl preheated hybridisation buffer (50% deionised formamide (Fluka)/ 2xSSC/ 5% dextran sulphate (Amersham)/ 10mM Tris pH7.4/ 0.1% Tween 20 (BDH)/ 0.2M Cysteamine (Sigma)) for sample tubes and 165µl for prehybridisation tubes (for WGTP microarrays, both tubes were resuspended in 165µl).

2.5.1.3 Hybridisation and washing

All sample and prehybridisation tubes were denatured at 70°C for 10min. 100µl from the prehybridisation tubes (140µl for the WGTP microarray) was immediately injected onto the microarray slide within Tecan chambers whilst the sample tubes were incubated at 37°C for 45min prior to injection of 100µl (140µl for the WGTP microarray). Samples were hybridised to the microarray at 37°C for 21hr (64hr for PCR product microarrays) with gentle agitation before being washed 15 times in 1xPBS/ 0.05% Tween20/ 2mM Cysteamine at 37°C for 1min, 5 times in 0.1xSSC at 54°C (52°C for PCR product microarrays) for 3min, 10 times in 1xPBS/ 0.05% Tween20/ 2mM Cysteamine at 25°C for 1min, before being rinsed in water at 25°C for 30sec prior to drying with nitrogen. Slides were scanned immediately after drying.

2.5.1.4 Scanning and image quantification

Fosmid and PCR product microarrays were scanned at a 10µm resolution and WGTP microarrays were scanned at a 5µm resolution on a G2565 scanner (Agilent Technologies). Fosmid and PCR product microarray images were quantified using GenePix Pro version 6.0 software (Axon Instruments) and the data analysed using a custom-made Excel spreadsheet. WGTP microarrays were quantified using BlueFuse for microarrays (BlueGnome).

2.5.2 Hybridisation and analysis of NimbleGen oligonucleotide microarrays (carried out by NimbleGen Systems)

Labelling, hybridisation and scanning were performed by NimbleGen Systems Inc. Briefly; 1µg genomic DNA (section 2.2.1) or Repli-G amplified flow sorted chromosomes (section 2.3.1.1) was denatured at 98°C in the presence of 1 O.D. of 50-Cy3- or 50-Cy5-labeled random nonamer (TriLink Biotechnologies) in 62.5mM Tris-HCl pH7.5/ 6.25mM $MgCl_2$/ 0.0875% ß-mercaptoethanol. The denatured sample was chilled on ice, then incubated with 100U (exo-) Klenow fragment (NEB) and 6mM each dNTPs for 2hr at 37°C. Reactions were terminated by addition of 0.5M EDTA (pH8.0) and the products precipitated with isopropanol and resuspended in water. The Cy3 and Cy5 labelled samples were combined (15µg each) and dried by vacuum centrifugation. The sample was rehydrated in 40µl of NimbleGen Hybridisation Buffer, denatured at 95°C for 5min, then cooled to 42°C. Hybridisations were carried out for 18hr at 42°C. The arrays were washed using a NimbleGen Wash Buffer System and immediately dried down by centrifugation. Arrays were scanned at 5µm resolution using the GenePix4000B scanner (Axon Instruments). Data were extracted from scanned images using NimbleScan 2.0 extraction software (NimbleGen Systems, Inc.)

2.6 Fluorescence in situ hybridisation

2.6.1 Preparation of slides for FISH hybridisation

2.6.1.1 Metaphase spreads

20ml of lymphoblastoid cell line culture was grown to confluency and subcultured. Approximately 24hr after subculturing, 100µl 3mg/ml Bromodeoxyuridine (BrdU;Sigma) in 2% ethanol was added and the cells incubated for 3hr at 37°C. 20µl 10mg/ml ethidium bromide and 40µl 10µg/ml colcemid (Invitrogen) was added prior to a further incubation at 37°C for 2hr. The cells were collected by centrifugation at 300g for 5min and the cells resuspended in 10ml 75mM KCl prewarmed to 37°C before incubation at 37°C for 12min. 3ml ice-cold fixative (3:1

methanol (BDH): glacial acetic acid (VWR International)) was added and the cells collected by centrifugation as before, then resuspended in 10ml fixative. The cells were collected and resuspended in fixative a further 2 times to remove cytoplasm and finally resuspended in approximately 5ml fixative. Metaphase suspensions were stored at -20°C in tubes sealed with parafilm for up to 3 years.

Two drops of metaphase suspension were dropped onto a glass slide and the slide incubated above a 50°C water bath to air dry. Slides were stored in a sealed box at room temperature for up to 7 days prior to use.

2.6.1.2 Extended chromatin fibres

Cells were collected from 2-3ml of an actively growing culture by centrifugation at 300g for 5min. The cells were washed twice in 3ml PBS, re-spun and finally resuspended in PBS to a concentration of 2-3x10^6 cells/ml.

Using a pipette, 10µl of cell suspension was spread over a 1cm area on the upper portion of a microscope slide and allowed to air dry. The slide was clamped to a Perspex block (made in-house) in a vertical position (Figure 2.3). To lyse the cells, 150µl lysis buffer (0.25M NaOH/ 27.4% ethanol) was allowed to pass along the slide by gravity flow until the meniscus stopped moving. A further 150µl 96% ethanol was then added and allowed to flow down the slide to remove the lysis buffer. The slide was gently separated from the Perspex block. This mechanical process allowed the DNA to be pulled into fibres and extend along the slide. The slides were air dried and stored in a box with desiccant for up to 6 months prior to use.

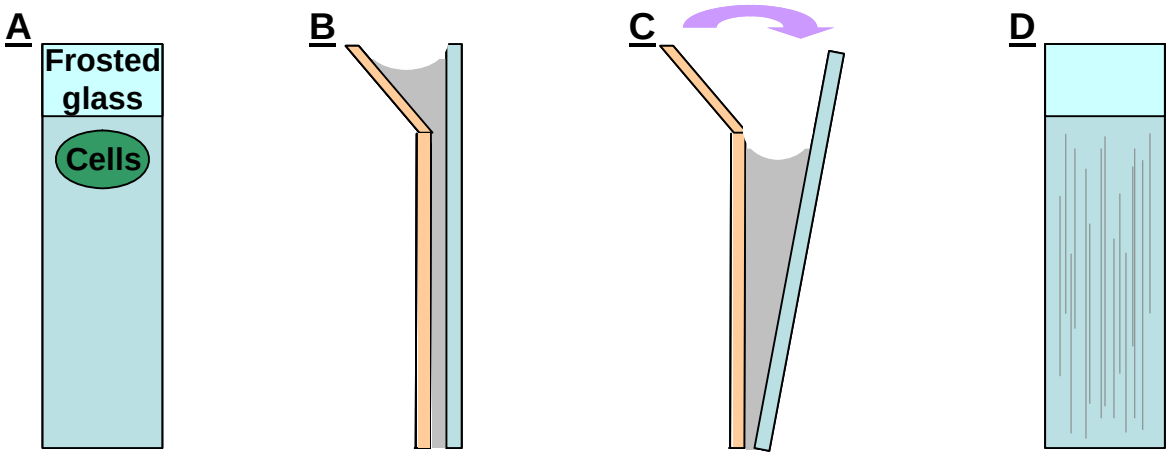

Figure 2.3 Schematic showing the production of extended chromatin fibres. A The cell spot was created on the upper portion of the microscope slide. B Cross section of the microscope slide (blue) clamped to the Perspex block (orange). The lysis buffer and ethanol (grey) pass along the microscope slide, extending the DNA from the cell spot into fibres. C Gentle separation of the slide from the holder allows the fibres to extend down the slide. D The fibres adhere along the length of the microscope slide.

2.6.2 Preparation of clone insert DNA

2.6.2.1 Preparation of fosmid clone insert DNA

Fosmid clone insert DNA was prepped from 40ml cultures using the Sigma PhasePrep BAC DNA Kit according to the manufacturer's instructions; cells were collected from an overnight 40ml culture by centrifugation at 4000g for 10min. 2ml Resuspension Solution was used to resuspend the cells prior to the addition of 2ml Lysis Solution. The tubes were inverted 5 times to allow mixing and incubated for 5min at room temperature. 2ml 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 transferred to a fresh tube and this centrifugation step repeated. 3.6ml isopropanol was added to the clear supernatant, the tubes inverted and centrifuged at 15,000g for 20min at 4°C. The pellet was washed with 2ml 70%

ethanol and the pellet air dried briefly. The pellet was resuspended in 650µl Elution Solution followed by the addition of 1µl of 1/10 RNase cocktail and incubated at 60°C for 10min. 50µl 3M Sodium Acetate Buffer Solution pH7 and 120µl Endotoxin Removal Solution were added and the tubes incubated on ice for 5min, 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. 700µl DNA Precipitation Solution and 2µl Pellet Paint (Novagen) were added and the DNA collected by centrifugation at 21,000g for 20min at 4°C. The pellets were washed with 500µl 70% ethanol and air dried prior to resuspension in 20µl water. DNA concentration and quality was assessed using an ND-1000 spectrophotometer (Nanodrop) and 1% agarose gel electrophoresis with ethidium bromide staining (Figure 2.4).

2.6.2.2 Preparation of plasmid clone insert DNA

5ml LB broth with 100µg/ml ampicillin was inoculated with bacterial glycerol stock and incubated at 37°C with shaking for 8hr. 1ml of this culture was used to inoculate 100ml of LB media with 100µg/ml ampicillin and incubated at 37°C with shaking overnight. Plasmid clone insert DNA was extracted from this culture using the Plasmid Maxi purification system (QIAGEN) according to the manufacturer's instructions. Briefly, bacterial cells were pelleted by centrifugation at 6000g for 15min at 4°C and resuspended in 10ml Buffer P1 (Resuspension Buffer; 50mM Tris-Cl pH8/ 10mM EDTA/ 100µg/ml RNase A). Following the addition of 10ml Buffer P2 (Lysis Buffer; 200mM NaOH/ 1% SDS) the cells were incubated at room temperature for 5min, then 10ml of ice-cold Buffer P3 (Neutralisation Buffer; 3M KOAc pH5.5) was added prior to incubation on ice for 20min. The samples were then centrifuged at 20,000g for 30min at 4°C and the supernatant removed and re-spun at 20,000g for 15min at 4°C. The supernatant was applied to a QIAGEN-tip 500 (already equilibrated with 10ml Buffer QBT (Equilibration Buffer; 750mM NaCl/ 50mM 3-[N-Morpholino] propanesulphonic

acid (MOPS) pH7/ 15% isopropanol/ 0.15% Triton X-100)) and washed with 2x30ml Buffer QC (Wash Buffer; 1M NaCl/ 50mM MOPS pH7/ 15% isopropanol). The DNA was eluted with 15ml Buffer QF (Elution Buffer; 1.25M NaCl/ 50mM Tris-Cl pH8.5/ 15% isopropanol), precipitated by the addition of 10.5ml isopropanol and collected by centrifugation at 15,000g for 30min at 4°C. The pellet was washed with 5ml 70% ethanol and centrifuged at 15,000g for 10min at 4°C. The pellet was air dried and dissolved in 500µl water. DNA concentration and quality was assessed using an ND-1000 spectrophotometer (Nanodrop) and 1% agarose gel electrophoresis with ethidium bromide staining (Figure 2.4).

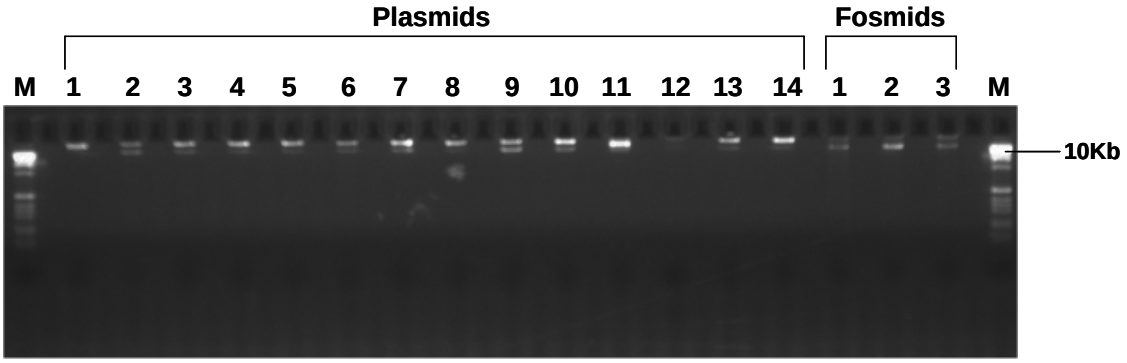

Figure 2.4 Isolated fosmid and plasmid insert DNA.

2.6.3 Fluorescence labelling of probes

2.6.3.1 Whole genome amplification labelling of clone DNA

10ng of prepped DNA (section 2.6.2) was amplified using the GenomePlex Complete Whole Genome Amplification Kit (Sigma) according to the manufacturer's instructions. Briefly; 10ng (1ng/µl) DNA was fragmented with 1µl 10xFragmentation Buffer at 95°C for 4min and immediately cooled on ice. The fragmented DNA was then used to create a library following the addition of 2µl 1xLibrary Preparation Buffer and 1µl Library Stabilisation Solution. The samples were heated at 95°C for 2min and cooled on ice prior to the addition of 1µl Library Preparation Enzyme. Samples were incubated at 16°C for 20min, 24°C

for 20min, 37°C for 20min and 75°C for 5min. For amplification of the library, 47.5µl nuclease-free water, 7.5µl 10xAmplification Mastermix and 5µl WGA DNA Polymerase were added and the samples subjected to temperature cycling of 95°C for 3min followed by 17 cycles of 94°C for 15sec and 65°C for 5min. Amplification was verified by running 6µl of the amplified library by gel electrophoresis on a 1.5% agarose gel with ethidium bromide staining.

1µl of the amplified DNA was labelled in a 25µl reaction with 2.5µl 10x Amplification Mix (special order from Sigma; A5604 without dNTPs), 1.5µl 1mM Biotin-16-dUTP (Roche), 1µl 1mM dTTP, 1.75µl WGA DNA polymerase (Sigma) and 17.25µl water. The samples were subjected to temperature cycling of 95°C for 3min followed by 17 cycles of 94°C for 15sec and 65°C for 5min. The fragment sizes of the labelled product were assessed by gel electrophoresis running 1µl on a 1% agarose gel with ethidium bromide (Figure 2.5).

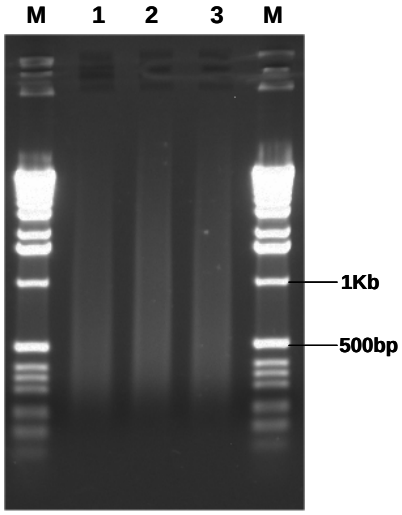

Figure 2.5 Amplified, labelled DNA for FISH.

To reduce the fragment sizes prior to hybridisation to the metaphase slides, 4µl 1µg/µl DNAse1 (Sigma) was added to the remaining 24µl of labelled DNA and incubated at 15°C for 1hr 30min. The fragment sizes were reassessed by gel electrophoresis as before. Optimal fragment sizes for FISH were considered to be 50-500bp in length (Figure 2.6).

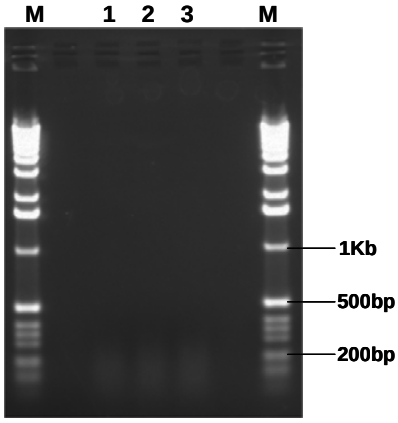

Figure 2.6 Amplified, labelled DNA after nick translation.

2.6.3.2 Nick translation of clone DNA

1µg of prepped plasmid or fosmid DNA (section 2.6.2) in 16µl water was added to 4µl biotin or dig nick translation mix (Roche) and incubated at 15°C for 1hr 30min. The reaction was stopped by the addition of 1µl 0.5M EDTA (pH8.0) and heating at 65°C for 10min. Fragment sizes were analysed by gel electrophoresis running 3µl of the reaction on a 1% agarose gel with ethidium bromide.

2.6.3.3 DOP PCR labelling of flow sorted chromosomes

2µl of a primary DOP reaction (section 2.3.1.2) was labelled in a 25µl secondary DOP reaction with 12µl DDW, 2.5µl 10xTAPS2 Buffer (250mM N-Tris (hydroxymethyl) methyl-3-amino-proane sulphonic acid pH9.3 (Sigma)/ 500mM KCl/ 20mM $MgCl_2$/ 10mM dithiothreitol/ 0.7% ß-mercaptoethanol/ 0.165% Bovine Serum Albumin), 2µl 20µM DOP primer (the same primer as used in the primary reaction), 2µl $^1/_2$T dNTPs (2.5mM each dATP, dCTP, dGTP and 1.25mM dTTP),

2.5µl 1mM Spectrum Green (Vysis), 1.25µl 1.25% W1 detergent (Sigma) and 0.25µl 5U/µl Amplitaq (PerkinElmer). Due to the higher annealing temperature used during a secondary DOP reaction the amplification was more specific than in a primary reaction. Samples were subjected to cycling conditions of 94°C for 4min followed by 35 cycles of 94°C for 1min, 62°C for 1min, 72°C for 1min 30sec followed by 72°C for 8min 30sec. Amplification was assessed by gel electrophoresis running 5µl on a 2.5% agarose gel with ethidium bromide.

2.6.4 Pre-treatment of metaphase spreads and DNA fibre slides

All slides were passed through an ethanol series (1min each in 70%, 70%, 90%, 90%, 100%) and allowed to air dry. Slides were pre-treated with 0.003% pepsin (Sigma)/ 0.01M HCl in 2xSSC at 37°C for 5min, followed by 3 washes in 2xSSC at room temperature for 5min, and a further ethanol series as before.

2.6.5 Hybridisation of FISH slides

For both metaphase spread and DNA fibre slides, 0.5µl of probe (section 2.6.3) was mixed with 1µl Cot1 DNA (Invitrogen) and 13.5µl hybridisation buffer (50% deionised formamide (Fluka)/ 2xSSC/ 10% dextran sulphate (Amersham)/ 10mM Tris pH7.4/ 0.1% Tween20 (BDH)). Samples were denatured at 65°C for 10min, prior to pre-annealing at 37°C for 50min before addition to the slides. Slides were denatured in 70% formamide/ 0.6xSSC at 65°C for 1min 30sec prior to quenching for 1min in ice-cold 70% ethanol and passing though an ethanol series as before. Hybridisations were performed under a coverslip sealed with rubber cement (Marubawerke GmbH & Co.) at 37°C overnight with humidity.

2.6.6 Detection of FISH slides

Indirectly labelled probes (sections 2.6.3.1 and 2.6.3.2) were detected using the schema detailed in Table 2.2. Slides were soaked in 2xSSC for 15min to remove the coverslip, washed in 2xSSC for 5min at 44°C, twice in 50% formamide/1xSSC for 5min at 44°C followed by a final wash in 2xSSC for 5min at

44°C. Slides were washed in 4xSSC/ 0.05% Tween20 (BDH) prior to antibody detection. Each slide was detected using 200µl of the relevant antibody (Table 2.2) in blocking buffer (1% blocking agent (Roche) in 4xSSC/ 0.05% Tween20/ 1µg/ml sodium azide) for 25min at 37°C and rinsed 3 times in 4xSSC/0.05% Tween for 4min at room temperature between layers.

	Biotin-16-dUTP		Digoxigenin-11-dUTP
1st layer	Avidin-Cy3 GE Healthcare 1µg/µl	Avidin-FITC Vector Laboratories 8µg/µl	Mouse-Anti-Digoxigenin Sigma; D8156 1/500 dilution of supplied stock
2nd layer	n/a	Anti-Avidin-FITC Vector Laboratories; SP2040 1/125 dilution of supplied stock	Goat-Anti-Mouse-Texas Red Invitrogen 8µg/µl

Table 2.2 Detection systems used for biotin and digoxigenin labelled probes.

Slides hybridised with directly labelled probes (section 2.6.3.3) were soaked in 2xSSC for 15min to remove the coverslip, then washed in 2xSSC for 5min at 42°C, twice in 50% formamide/0.5xSSC for 5min at 42°C followed by a final wash in 2xSSC for 5min at 42°C.

All slides were rinsed in 2xSSC at room temperature for 5min before staining for 3min in 0.2µg/ml DAPI (Sigma) in 2xSSC. Slides were then mounted with Citifluor mounting fluid (Citifluor, Ltd) and sealed with nail varnish.

2.6.7 Microscope analysis of FISH slides

Slides were visualised using an Axioskop microscope (Zeiss) with a CoolSNAP HQ camera (Photometrics) and narrow band pass filters (Chroma). Images were acquired at x100 magnification using SmartCapture X imaging software (Digital Scientific).

2.7 PCR

2.7.1 STS PCR

STS PCR was used to refine the translocation breakpoints to regions less than 5Kb prior to LR PCR amplification across the junction and also to create pools of PCR products for fosmid library screening. When mapping breakpoints, each STS primer pair was tested on whole genomic DNA from a normal individual as a positive control, water as a negative control, and both derivative chromosomes from the translocation and spanning BAC clone DNA as a control if available. When creating pools of products for library screening, each primer pair was individually used to amplify a product from genomic DNA (Promega) and tested on water as a negative control.

PCR primer pairs were designed to repeat-masked sequence using a perl script written in-house (Dimitris Kalaitzopoulos – Team 70). Oligonucleotides were designed to be 18-22bp in length with a melting temperature between 57°C and 63°C and GC content of 20-80%. Primer pairs were designed to amplify products of 90-120bp at the specified spacing along a target sequence.

15µl STS PCR reactions were performed in 96 well plates (Costar) on an MJ-Thermocycler. 5µl template DNA (50ng genomic DNA (Promega), 1/5 dilution of DOP amplified derivative chromosomes (section 2.3.1.2) or 5µl of colony DNA (created from a single colony resuspended in 50µl T0.1E) or water was amplified using 5.4µl 28% w/v Sucrose/cresol red, 2.5µl 10xNEB buffer (660mM Tris/ 167mM $(NH_4)_2SO_4$/ 67mM $MgCl_2$), 2.5µl 5mM each dNTPs, 0.495µl 0.5% Bovine Serum Albumin, 0.21µl 5% ß-mercaptoethanol and 0.18µl 5U/µl Amplitaq (PerkinElmer). 0.75µl of primer mix (100ng/µl of both forward and reverse primers) was added prior to temperature cycling of 94°C for 5min followed by 35 cycles of 94°C for 30sec, 58°C for 30sec, 72°C for 30sec followed by 72°C for 5min. 7.5µl of each reaction was run on a 2.5% agarose gel with ethidium

bromide. When creating PCR product pools for library screening, the whole 15µl reaction was run and the bands excised into 200µl T0.1E (10mM Tris, 0.1mM EDTA pH8).

2.7.2 Colony PCR

Colony PCR was used to verify that clones identified by library screening contained the translocation junction. The forward and reverse primers detailed in Table 2.3 were used to amplify products from clone DNA; Clones were picked and resuspended in 50µl T0.1E. 5µl of this DNA was used as template for the PCR reaction with 5µl (50ng) of patient DNA as a positive control and 5µl water as a negative control. Reactions were performed as detailed in Section 2.7.1 above.

Junction	Forward primer sequence	Reverse primer sequence
Derivative 7	GTAGTGATTCGGCCTTGCAT	TGGCCATATTTGGCTTTTTG
Derivative 13	TCCATTCATGTTGCTGCATT	GGAAGACAGGATGGATTCAAA

Table 2.3 Primers used to amplify products across the derivative chromosome 7 and 13 translocation junctions for patient t(7;13)(q31.1;q21.3).

2.7.3 LR PCR amplification of junction fragments

Oligonucleotide primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to be 28-32bp in length, have a GC content of 40-60% and a melting temperature of 58-63°C.

25µl LR PCR reactions were performed in 96 well plates (Costar) on an MJ-Thermocycler. To 5µl of template (100ng genomic DNA (section 2.2.1)), 5µl Q solution, 2.5µl Hotstar buffer, 3.5µl 2mM each dNTPs, 0.5µl HotstarTaq DNA Polymerase, 0.5µl diluted Proofstart (0.8µl Proofstart, 1µl 10xProofstart buffer, 8.2µl water) and 7.6µl water were added. 0.2µl 100µM forward primer and 0.2µl 100µM reverse primer were added prior to temperature cycling of 95°C for 15min followed by 40 cycles of 95°C for 20sec, 57°C for 1min, 68°C for 10min followed

by 68°C for 10min. 5µl of each reaction was analysed using 0.6% agarose gel electrophoresis with ethidium bromide staining.

2.8 Sequencing

2.8.1 Sequencing of LR PCR products (Nik Matthews – Team 56)

Amplified junction fragment DNA (section 2.7.3) was prepared for sequencing using the ExoSAP method (Amersham). Cleaned fragments were sequenced from both ends using the di-deoxy chain termination method, with V.3.1 Big Dye Terminator chemistry (Applied Biosystems). Resulting sequencing reactions were analysed on 3700 ABI sequencing machines (Applied Biosystems).

2.8.2 End sequencing of fosmid clones (Teams 42 and 56)

Fosmid DNA was extracted using Millipore filter plates on a vacuum manifold and sequenced using V.3.1 Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems) using a MJ thermocycler. Reactions were run on an ABI 3730 capillary sequencer (Applied Biosystems).

2.8.3 Full sequencing of fosmid clones (Teams 41, 53, 57, 116)

Random shotgun sequences of fosmid clones were generated from pUC19 plasmids with inserts of mainly 2–4 kb which were sequenced from both ends using the di-deoxy chain termination method. The resulting sequencing reactions were analysed on ABI 3730 sequencing machines (Applied Biosystems), and the data generated processed by a suite of in-house programs before assembly with the PHRED and PHRAP (http://www.phrap.org/) algorithms. The GAP4 program was used to assess and close sequence gaps.

2.9 Custom-made library production

2.9.1 In-house cosmid library

2.9.1.1 Extraction of high molecular weight DNA

$5x10^7$ cells were collected by centrifugation at 250g for 10min and resuspended in 5ml PBS. The cells were pelleted by centrifugation as before and resuspended in 1ml TE pH8 (10mM Tris/ 1mM EDTA). 10ml Lysis Buffer (10mM Tris pH8/ 0.1M EDTA/ 0.5% SDS/ 20µg/ml RNase (Sigma)) was added and the sample incubated at 37°C for 1hr. 55µl 20mg/ml Proteinase K (Sigma) was added and the sample incubated at 50°C for 3hr. The solution was cooled to room temperature and the DNA extracted with equal volumes of phenol/chloroform followed by chloroform. Each time, the two phases were separated by centrifugation at 5000g for 15min and the aqueous phase removed with a wide bore tip. DNA was precipitated with 0.2 volumes of 10M ammonium acetate and 2 volumes ethanol. DNA was spooled with a sterile loop, transferred to a tube containing 500µl 70% ethanol and spun at 5000g for 5min. The pellet was washed with 70% ethanol, air dried and resuspended in 10ml TE pH8.

2.9.1.2 Preparation of vector arms

Lawrist 16 cells were streaked onto LB agar containing 100µg/ml ampicillin and 20µg/ml kanamycin and incubated at 30°C until colonies were visible. A single colony was selected and the plasmid DNA collected (section 2.6.2.2). DNA was dissolved in 1ml TE (10mM Tris/ 1mM EDTA) and quantified using a TD-360 fluorometer (Turner Designs).

20µg of the DNA was digested in a 100µl reaction with 10µl 10xHSRE buffer (500mM Tris-HCl pH7.5/ 100mM $MgCl_2$/ 1500mM NaCl) and 9µl 10U/µl *Sca1* (NEB) at 37°C for 2hr. 1µl (200ng) of the digestion was checked on a 0.6% agarose gel alongside 200ng of the undigested DNA. A 5µl aliquot was removed as a *Sca1* control. To the remainder of the digestion, 276µl 1xLSRE buffer

(50mM Tris-HCl pH7.5/ 100mM $MgCl_2$) and 35µl 1U/µl Calf Intestinal Alkaline Phosphatase (CIAP; Roche) were added and the sample incubated at 37°C for 45min. 45µl 150mM Nitriloacetic Acid (NTA; Sigma) was added and the tube incubated for a further 25min at 68°C. Samples were then extracted with 450µl phenol/chloroform, then chloroform, each time re-extracting the organic phase with 450µl TE. Finally the aqueous phase was extracted with ether and the tube incubated at 68°C to evaporate any remaining ether. The DNA was precipitated with 45µl 3M sodium acetate and 1125µl ethanol at -20°C overnight. DNA was collected by centrifugation at 15,300g, the pellet washed with 70% ethanol, air dried and resuspended in 177.5µl TE. A 5µl aliquot was removed as a *Sca1*/CIAP control. 20µl 10xHSRE buffer and 7.5µl 20U/µl *BamH1* (NEB) were added and the tube incubated for 90min at 37°. DNA was extracted with equal volumes of phenol/chloroform, chloroform, and ether as described above. DNA was precipitated with $^{1}/_{10}^{th}$ volume sodium acetate and 2.5 volumes ethanol and resuspended in 100µl TE. A 5µl aliquot was removed as *Sca1*/CIAP/*BamH1* control.

45µl TE was added to all three 5µl control aliquots and the DNA extracted with 50µl phenol/chloroform and precipitated with 5µl sodium acetate and 125µl ethanol at -20° overnight. DNA was collected by centrifugation at 15,300g for 15min, washed with 70% ethanol, air dried and resuspended in 5µl TE and analysed using 0.6% gel electrophoresis with ethidium bromide staining.

2.9.1.3 Partial digestion of DNA

DNA (section 2.2.2) was divided into 7x150ng aliquots and partially digested using dilutions of *Mbo1* (NEB) and dam methylase (NEB). 150ng DNA in 18µl was added to 2µl 10xTAK buffer (300mM Tris-HCl pH7.9/ 600mM KOAc/ 90mM MgAc2/ 5mM DTT/ 3mg/ml Bovine Serum Albumin/ 800µM S-adenosyl methionine (SAM; Sigma)). 6µl was removed from each tube and retained as an

undigested control. 1µl of the appropriate enzyme mix from Table 2.4 was added to the relevant tube.

Tube name	Enzyme proportions	Enzyme mix
	Mbo1 **:dam methylase**	
BASIC	1:720	4µl *Mbo1* (0.1U/µl)
		36µl dam methylase (8U/µl)
1.3	1:2,320	4µl BASIC
		8µl dam methylase (8U/µl)
1.2	1:3,120	4µl BASIC
		12µl dam methylase (8U/µl)
1.1	1:7,920	1µl BASIC
		9µl dam methylase (8U/µl)
1.0	1:15,920	2µl 1.2
		8µl dam methylase (8U/µl)
dam	-	1µl dam methylase (8U/µl)

Table 2.4 Enzyme mixtures for partial digestion of genomic DNA.

All samples (including the undigested controls) were incubated for 2hr 30min at 37°C. 1µl 0.02U/µl CIAP (Roche) was added to tubes 1.0-1.3 and BASIC+ and the incubation continued for a further 30min at 37°C.

7µl was removed from each of the sample tubes and run with the undigested controls on a 0.3% agarose gel followed by ethidium bromide staining to assess for the best level of digestion (the fragment sizes averaging 36Kb).

11µl T0.1E (10mM Tris, 0.1mM EDTA) was added to tubes 1.0-1.3, BASIC+ and BASIC-. 2.2µl 150mM NTA was added to 1.0-1.3 and BASIC+ and all tubes incubated at 68°C for 20min prior to the addition of 1.2µl 5M NaCl and 60µl ethanol. Samples were precipitated overnight at -20°C.

2.9.1.4 Recovery of digested DNA

DNA for the selected digestion and BASIC+ and BASIC- tubes was collected by centrifugation at 15,300g for 15min, washed with 70% ethanol, air dried and

dissolved in 4µl T0.1E for the selected best digestion tube or 8µl for BASIC tubes.

2.9.1.5 Ligation of vector arms to digested DNA

2µl (400ng) vector arms were ligated to 50ng genomic DNA in an 8µl reaction with 0.8µl 10xligation buffer (400mM Tris-HCl pH7.9/ 100mM $MgCl_2$/ 10mM DTT), 0.7µl 400U/µl T_4 DNA Ligase (NEB) and 0.5µl 6mM ATP at 14°C overnight. To assess the background of non-recombinant clones, a control ligation was conducted where the genomic DNA was replaced with 4µl T1.0E. To assess the phosphatasing, the BASIC+ and BASIC- samples were split in half. The first half was ligated without arms and the second without arms and ligase. Both BASIC samples were run on a 0.3% agarose gel as before.

2.9.1.6 Packaging of ligated DNA

4µl of the ligation reaction (section 2.9.1.5) was added to a vial of Gigapack Gold III packaging extract (Stratagene) and incubated at room temperature for 2hr. The reaction was stopped by the addition of 500µl λ diluent (10mM Tris-HCl pH7.5/ 10mM $MgSO_4$) and 132µl 5xSM (500mM NaCl/ 50mM $MgSO_4$/ 250mM Tris-HCl pH7.5/ 0.05% gelatine/ 50% glycerol).

2.9.1.7 Preparation of plating cells

40ml LB broth was inoculated with a single colony of DH5αMCR E.coli and incubated at 37°C with shaking overnight. Cells were pelleted at 3,200g for 15min and resuspended in 20ml 10mM $MgSO_4$.

2.9.1.8 Plating of library

Varying strengths of packaged phage and λ diluent (ranging from 1:5 to 1:100 dilutions) totalling 100µl were added to 100µl plating cells and incubated at room temperature for 20min. The infected cells were then diluted with 1ml LB broth and incubated at 37°C for 45min to allow expression of antibiotic resistance. The cells were then pelleted by centrifugation at 3,300g for 2min. The majority of the

supernatant was removed, leaving approximately 30µl remaining for resuspension of the cells which were then plated on LB agar with 30µg/ml kanamycin and incubated at 37°C overnight.

2.9.2 EpiFOS fosmid library

Flow sorted derivative chromosomes were used to generate custom-made fosmid libraries using the EpiFOS™ Fosmid Library Production Kit (Epicentre) according to the manufacturer's instructions.

2.9.2.1 Size modification of DNA

Flow sorted chromosomes (section 2.2.2) were resuspended in 200µl TE (10mM Tris-HCl (pH7.5)/ 1mM EDTA). 100ng of DNA was run on a 0.3% agarose gel using fosmid control DNA to assess size. To reduce its size, the DNA was sheared though a 21 gauge syringe needle (Microlance), and a further 100ng of DNA checked gel electrophoresis as before. The DNA was then concentrated to less than 52µl using an YM-30 microcon column (Millipore).

2.9.2.2 End-Repair of sheared DNA

In an 80µl reaction, the sheared DNA was mixed with 8µl End-Repair 10xBuffer, 8µl 2.5mM each dNTP mix, 8µl 10mM ATP and 4µl End-Repair Enzyme Mix and incubated at room temperature for 45min. Following the addition of blue loading buffer, the samples were incubated at 70°C for 10min to inactivate the enzyme.

2.9.2.3 Size selection of end-repaired DNA

End-repaired DNA was separated using a 0.3% low melting point agarose (Sigma) gel. A gel slice containing DNA of approximately 30-40Kb in size was excised from the gel and melted at 70°C for 15min. Following the addition of pre-warmed GELase 50x Buffer and GELase Enzyme Preparation (to a final concentration of 1x buffer and 1U enzyme per 0.1g excised agarose) the samples were incubated at 45°C for 3hr, followed by 70°C for 10min. 500µl aliquots were chilled on ice for 5min and then centrifuged at 9,300g for 20min.

The upper 90-95% of supernatant was transferred to a clean tube and precipitated with 1/10th volume of 3M Sodium Acetate pH7 and 2.5 volumes 100% ethanol at room temperature for 10min. The DNA was collected by centrifugation at 16,000g for 20min, washed twice with 70% ethanol, air dried and resuspended in 200µl TE buffer. The DNA was concentrated using an YM-30 microcon column (Millipore).

2.9.2.4 Ligation of size-selected DNA

10µl ligation reactions were performed using the size-selected DNA, 1µl 10xFast-Link Ligation Buffer, 1µl 10mM ATP, 1µl 0.5µg/µl pEpiFOS-5 Vector and 1µl Fast-Link DNA Ligase and incubation at room temperature for 2hr followed by 70°C for 10min.

2.9.2.5 Preparation of E.coli host strain

2 days prior to the packaging reactions EPI100^{TM}-T1^{R} cells were streaked onto LB agar plates and the plate incubated at 37°C overnight. The day preceding the packaging reaction, a single colony was picked from this plate and used to inoculate 50ml LB broth supplemented with 10mM $MgSO_4$ and incubated at 37°C overnight with gentle shaking. The day of the packaging reactions, 5ml from the overnight culture was used to inoculate 50ml LB broth supplemented with 10mM $MgSO_4$ and incubated at 37°C with gentle shaking to an OD_{600} of 0.8-1.0.

2.9.2.6 Packaging of ligated DNA

1 tube of MaxPlax Lambda Packaging Extract was thawed on ice and 25µl of the extract was transferred to the tube containing the 10µl ligation reaction; the other 25µl was returned to -70°C. After incubation of the sample tube at 30°C for 90min, the remaining 25µl of packaging extract was added, and the tube incubated for a further 90min at 30°C. After the second incubation, 940µl of Phage Dilution Buffer (10mM Tris-HCl pH8.3/ 100mM NaCl/ 10mM $MgCl_2$) and 25µl chloroform were added and the packaged phage stored at 4°C.

2.9.2.7 Plating of library

Before plating the whole library, a series of plating dilutions were performed to determine the optimum plating strength. In all cases, the packaged phage was diluted with Phage Dilution Buffer. 10µl of the diluted packaged phage was added to 100µl of the prepared EPI100^{TM}-T1^{R} cells (section 2.9.2.5) and incubated at 37°C for 20min prior to plating on LB agar plates with 12.5µg/ml chloramphenicol and incubation at 37°C overnight.

2.10 Generation of fosmid library filters

Polygrid filters were printed for all libraries created. Round filters were only created for the patient t(7;13)(q31.1;q21.3) library. For all libraries created, clones were picked from round agar plates into LB broth with 12.5µg/ml chloramphenicol in 384 well plates (Genetix) by a robot developed in-house and incubated at 37°C overnight with shaking.

2.10.1 Printing of polygrid filters from 384 well plates (Mark Maddison – Team 63)

Clones were gridded from 384 well plates onto Hybond-XL filters (Amersham) on top of LB agar plates with 12.5µg/ml chloramphenicol using a robot developed in-house with Black Magic Ink (Higgins) used to highlight the spot locations. Filters were incubated overnight at 37°C.

2.10.2 Replication of clones onto round filters from agar plates

Round agar plates with the plated library clones were used to generate lift filters for library screening. Hybond N+ filters (Amersham) were placed on top of the library plates and then moved to fresh agar plates with 12.5µg/ml chloramphenicol and incubated at 37°C overnight.

2.10.3 Preparation of clone DNA on filters for hybridisation

For both polygrid and round filter systems, the filters were placed colony side up on 3MM paper (Whatman) saturated with 10% SDS for 5min, then transferred to

paper saturated with denaturing solution (0.5M NaOH/ 1.5M NaCl) for 10min before being transferred to fresh paper and allowed to air dry for 15min. Filters were then subjected to a series of washes with gentle shaking; 10xNeutralising Solution (0.5M Tris-Cl pH7.7/ 1.5M NaCl) for 5min twice, 1xNeutralising Solution for 5min, 2xSSC/ 0.1% SDS for 5 min, 2xSSC for 5min and 100mM Tris-Cl pH7.4 for 5min before being allowed to air dry. DNA on the filters was crosslinked for 2min on a 90W UV-B transilluminator TL-312A (Spectroline) prior to use in hybridisations.

2.11 Radioactive screening of fosmid library filters

2.11.1 Generation of probe for library screening

2.11.1.1 Generation of probe by Inter-Alu PCR

100ng of spanning BAC DNA was amplified in a 25µl reaction using 2.5µl 10xNEB Buffer (670mM Tris-HCl pH8.8/ 166mM $(NH_4)_2SO_4$/ 67mM $MgCl_2$), 2.5µl 10xdNTP-C (5mM each dATP, dGTP, dTTP), 0.6µl (3000Ci/mmol) ^{32}P dCTP (Amersham), 0.825µl 5mg/ml Bovine Serum Albumin, 2.5µl 100ng/µl ALE1 primer (GCCTCCCAAAGTGCTGGGATTACAG), 2.5µl 80ng/µl ALE3 primer (CCAYTGCACTCCAGCCTGGG) 0.35µl 5% ß-mercaptoethanol and 0.3µl 5U/µl Amplitaq (PerkinElmer). Samples were subjected to temperature cycling of 94ºC for 5min followed by 30 cycles of 93ºC for 1min, 65ºC for 1min, 72ºC for 5min followed by 72ºC for 5min under mineral oil (Sigma).

125µl 20xSSC, 125µl Cot1 DNA (Invitrogen), 25µl radiolabelled probe and 225µl water were mixed together and incubated at 100ºC for 5min, prior to incubation on ice for 2min. The completed probe was then added to the pre-hybridised filters.

2.11.1.2 Generation of probe by STS PCR

A secondary round of PCR was performed to label products obtained in section 2.7.1. 1µl of the primary product was labelled in a 15µl reaction with 0.6µl 100ng/µl both forward and reverse primer mix, 1.5µl 10xNEB Buffer (670mM Tris-HCl pH8.8/ 166mM $(NH_4)_2SO_4$/ 67mM $MgCl_2$), 9.9µl T0.1E (10mM Tris, 0.1mM EDTA), 0.6µl dNTP-C (5mM each dATP, dGTP, dTTP), 0.5µl (3000Ci/mmol) ^{32}P dCTP (Amersham), 0.495µl 5mg/ml Bovine Serum Albumin, 0.21µl 5% ß-mercaptoethanol, 0.18µl 5U/µl Amplitaq (PerkinElmer). Samples were subjected to temperature cycling of 94ºC for 5 min followed by 25 cycles of 94ºC for 30sec, 55ºC for 30sec, 72ºC for 30sec followed by 72ºC for 5min under mineral oil (Sigma).

Samples were denatured at 94ºC for 5min prior to quenching on ice for 2min. The probes were then pooled on ice prior to addition to the pre-hybridised filters.

2.11.2 Hybridisation of radioactive DNA probe to fosmid library filters

Filters were pre-hybridised in filtered hybridisation buffer (4mg/ml Ficcoll (Sigma)/ 4mg/ml Bovine Serum Albumin (Sigma)/ 4mg/ml polyvinylpyrrolidone (Sigma)/ 6XSSC/ 50mM Tris pH7.4/ 200mg/ml Dextran Sulphate (Amersham)/ 1% sarkosyl) at 65°C at least 2hr prior to the addition of the radioactive probe. Following the addition of the probe, the filters were incubated at 65°C overnight with gentle shaking.

2.11.3 Washing and detection of fosmid library filters

Hybridised filters were rinsed twice in 2xSSC for 5min with gentle shaking, washed in 0.5xSSC/ 1% sarkosyl washing solution at 65°C twice for 30min with gentle shaking and finally rinsed for 5min in 0.2xSSC with gentle shaking prior to laying out and wrapping in clingfilm. Filters were exposed to X-ray film (Fuji) overnight and developed using a Kodak processor.