# CHAPTER 2

# Materials and Methods

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## Materials

### 2.1 Composition of solutions

Denaturation solution

- 0.5 M NaOH
- 1.5 M NaCl

dNTP mix for $1^0$ DOP-PCR:

- 2.5 mM each dNTP

dNTP mix for $2^0$ DOP-PCR:

- 2.5 mM each dATP, dCTP, dGTP
- 1.25 mM dTTP

dNTP mix for nick translation:

- 0.5 mM each dATP, dCTP, dGTP

Flow sorter sheath fluid:

- 100 mM NaCl
- 10 mM Tris-base
- 1 mM $Na_2EDTA$
- 0.5 mM sodium azide

HSRE:

- 500 mM Tris-HCl pH 7.5
- 100 mM $MgCl_2$
- 1.5 M NaCl

Hybridisation buffer (FISH):

- 50% (v/v) deionised formamide
- 10% (w/v) dextran sulphate
- 2× SSC

Hybridisation buffer (library filter screening):

- 0.2% (w/v) ficoll
- 0.2% BSA
- 0.2% polyvinylpyrollidone
- 6× SSC
- 50 mM Tris-base
- 10% (w/v) dextran sulphate
- 1% (w/v) N-lauroyl sarcosine

Filtered through one layer of Whatman No. 4 filter paper

Hypotonic swelling solution:

- 75 mM KCl
- 0.2 mM spermine
- 0.5 mM spermidine

Lambda diluent:

- 10 mM Tris-HCl pH 7.5
- 10 mM $MgSO_4$

10× ligase buffer:

- 400 mM Tris-HCl pH 7.6

- 100 mM $MgCl_2$
- 10 mM DTT

6× loading buffer:

- 0.25% (w/v) bromophenol blue
- 0.25% (w/v) xylene cyanol
- 30% (v/v) glycerol

Made up in $T_{0.1}E$

LSRE:

- 50 mM Tris-HCl pH 7.5
- 10 mM $MgCl_2$

Lysis buffer:

- 50 mM glucose
- 10 mM $Na_2EDTA$
- 25 mM Tris-HCl pH 8.0

1× NDS:

- 500 mM $Na_2EDTA$
- 10 mM Tris-base pH 9.5
- 1% (w/v) N-Lauroylsarcosine

10× NEB buffer (modified):

- 66 mM Tris-HCl pH 8.8
- 16.6 mM $(NH_4)_2SO_4$
- 67 mM $MgCl_2$

Made up in $T_{0.1}E$

Neutralisation solution

- 0.5 M Tris-HCl pH7.4
- 1.5 M NaCl

10× nick translation buffer:

- 0.5 M Tris-HCl pH 7.5
- 0.1 M $MgSO_4$
- 1 mM DTT
- 500 µg/ml BSA

PBS:

- 2.6 mM $KH_2PO_4$
- 26 mM $Na_2HPO_4$
- 145 mM NaCl pH 7.2
- 2.7 mM KCl

Polyamine buffer:

- 80 mM KCl
- 20 mM NaCl
- 2 mM $Na_2EDTA$
- 0.5 mM EGTA
- 15 mM Tris
- 0.2 mM spermine
- 0.5 mM spermidine

- 3 mM DTT
- 0.25% (v/v) Triton X-100

Adjusted to pH 7.2 with HCl

Restriction enzyme diluent:

- 20 mM Tris-HCl pH 7.5
- 100 mM KCl
- 50 µg/ml gelatine
- 0.1% (v/v) 2-mercaptoethanol
- 50% (v/v) glycerol

5× SM:

- 500 mM NaCl
- 50 mM $MgSO_4$
- 250 mM Tris-HCl pH 7.5
- 0.05% (w/v) gelatine
- 50% (v/v) glycerol

20× SSC:

- 3 M NaCl
- 0.3 M tri-sodium citrate pH 7.0

50× TAE:

- 2 M Tris-acetate
- 0.05 M $Na_2EDTA$

10× TAK buffer:

- 300 mM Tris-HCl pH 7.9
- 600 mM potassium acetate
- 90 mM magnesium acetate
- 3 mg/ml BSA
- 5 mM DTT
- 800 µM SAM

Make up the solution omitting the DTT and SAM and store frozen in 1 ml aliquots. Add DTT and SAM just before use.

10× TAPS2 buffer:

- 250 mM TAPS pH 9.3
- 166 mM$(NH_4)_2SO_4$
- 25 mM $MgCl_2$
- 0.165% (w/v) BSA
- 0.1 mM 2-mercaptoethanol

10× TBE:

- 0.89 M Tris-borate
- 2 mM $Na_2EDTA$ pH 8.3

TE:

- 10 mM Tris-HCl pH 8.0
- 1 mM $Na_2EDTA$

$T_{0.1}E$:

- 10 mM Tris-HCl pH 8.0
- 0.1 mM $Na_2EDTA$

4× TNFM

- 4× SSC
- 0.05% (v/v) Tween 20
- 5% (w/v) non-fat milk powder

Filtered through several layers of Whatman No.4 filter paper

**2.2 Reagents and stains**

Note: All stains were made up in sterile distilled water, unless stated otherwise.

Restriction enzymes:

- *Mbo*I 5 U/µl (NEB)
- Dam methylase
- Calf Intestinal Alkaline Phosphatase
- *Sca*I 10 U/µl (NEB)
- *BamH*I

Haptens for $2^0$ DOP-PCR:

- Biotin-16-dUTP (Boehringer)
- Digoxygenin-11-dUTP (Boehringer)
- FluoroRed-dUTP (Amersham)
- FluoroGreen-dUTP (Amersham)
- FluorX-dCTP (Biological Detection Systems)

- Cy3-dUTP (Amersham)
- Oregon Green-dUTP (Molecular Probes Europe)

All at 1 nmol/µl

Chromomycin A3:

- 2 mg/ml in ethanol

DAPI:

- 80 ng/ml in 2× SSC

Hoechst 33258:

- 1 mg/ml

Turck's stain:

- 1% (v/v) acetic acid
- 0.1 mg/ml gentian violet

**2.3 Media**

LB broth:

- 1% (w/v) Bacto tryptone
- 0.5% (w/v) Bacto yeast extract
- 1% (w/v) NaCl

2× TY broth:

- 1.6% (w/v) Bacto tryptone
- 1% (w/v) Bacto yeast extract
- 0.5% (w/v) NaCl

**2.4 Cells and cell lines**

Note: The lymphoblastoid cell lines used had previously been immortalised by EBV transformation. The transformation process induces the EBV to randomly integrate into the host genome.

An anonymous normal human male lymphoblastoid cell line ("HRC575") was obtained from ECACC, Wiltshire, U.K.

A female Siamang gibbon, (*Hylobates syndactylus* , "HSY") lymphoblastoid cell line was kindly donated by Dr. Johannes Wienberg, Cambridge University Department of Pathology, Cambridge, U.K.

Canine blood samples for chromosome isolation and metaphase spread preparations were kindly donated by Dr. Matthew Breen, Animal Health Trust, Newmarket, U.K.

**2.5 Bacterial clones**

All BAC, PAC, cosmid and fosmid clones used for the high resolution FISH analysis of Siamang and dog metaphase chromosomes (were identified from the human chromosome 22 data base ("22ace", available online at http://webace.sanger.ac.uk/cgi-bin/webace?db=acedb22&frame=&.cgifields=db&Browse+DB=Browse+DB) and the Molecular Cytogenetics data base ("FISHLog") at The Sanger Centre. DNA from each clone was either isolated and subsequently labelled for FISH by nick translation by myself, or was obtained from frozen stocks previously isolated and labelled by members of the Molecular Cytogenetics team. Clones identified from FISHLog had been previously FISH-mapped to specific regions of human chromosome 22.

## Methods

### 2.6 Tissue culture

Note: All cell types were cultured under 5% $CO_2$ at 37˚C.

*2.6.1 Lymphoblastoid cell culture*

Lymphoblastoid cells were cultured in suspension in 50 ml of RPMI-1640 (Sigma) supplemented with 16% (v/v) FBS (GibcoBRL), 100 U/ml Penicillin (Sigma), 100 µg/ml Streptomycin (Sigma) and 2 mM L-glutamine (Sigma) in 75 $cm^2$ tissue culture flasks. Once confluent, sub-culturing was carried out as follows:

1. Add 25 ml of fresh media to each flask and gently break up any cell clumps.

2. Distribute the media between three new 75 $cm^2$ flasks and add a further 25 ml of fresh media to each flask, effecting a 1/3 dilution of the confluent starting culture.

*2.6.2 Fibroblast cell culture*

Fibroblast cells were cultured in 50 ml of DMEM (Sigma) supplemented with 8% (v/v) FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 mM L-glutamine in 162 $cm^2$ tissue culture flasks. Once confluent, the adherent fibroblasts were sub-cultured as follows:

1. Discard the tissue culture medium and rinse the cell layer with PBS.

2. Discard the PBS and incubate the cell layer with 2.5 ml of Trypsin/EDTA solution (Sigma) for 2 to 5 minutes at room temperature. Tap the flasks to facilitate cell release.

3. Resuspend the cells in 150 ml of fresh media and distribute between three new 162 $cm^2$ flasks, effecting a 1/3 dilution of the confluent starting culture.

*2.6.3 Cell cryopreservation*

For frozen storage, cells were pelleted at 200 × *g*, and resuspended at approximately $1x10^7$ cells/ml in 10% (v/v) DMSO in FBS. Polypropylene tubes containing the cell suspension were cooled overnight to –70˚C, then transferred to the gas phase of a liquid nitrogen vessel (approximately –180˚C) for permanent storage. To recover from liquid nitrogen storage, cells were thawed rapidly at 37˚C then washed and resuspended in 10 ml of fresh media.

**2.7 Flow karyotype analysis and chromosome sorting**

*2.7.1 Lymphoblastoid cell chromosome isolation and staining for flow cytometric analysis*

Chromosomes were isolated from lymphoblastoid cells and stained for flow cytometric analysis by the following method:

1. To a 40% confluent lymphoblastoid culture add Colcemid Karyomax (GibcoBRL) to 0.1 µg/ml. Gently mix the culture and incubate for 6 hours at 37˚C.

2. Transfer the culture to a 50 ml centrifuge tube and pellet the cells at 200 × *g* for 10 minutes. Discard the supernatant and invert the tube on a tissue to drain.

3. Gently resuspend the cell pellet in 10 ml of hypotonic swelling solution and incubate at room temperature for 15 minutes.

4. Assess the proportion of cells arrested in metaphase by staining 10 µl of the cell suspension with 10 µl of Turck's stain and viewing in a haemocytometer with a phase-contrast microscope[a].

5. Transfer the swollen cell suspension to a 25 ml centrifuge tube and pellet the cells at 400 × *g* for 8 minutes. Discard the supernatant and drain the tubes as before.

6. Resuspend the cell pellet in 1.5 ml of ice-cold polyamine buffer and incubate on ice for 10 minutes.

7. Release the chromosomes into the solution by vortexing for 15 seconds at a speed which causes the suspension to swirl up around the wall of the tube.

8. Remove 10 µl of the suspension onto a microscope slide and stain it with 1 µl of 1 mg/ml propidium iodide. Check that the chromosomes are free in solution using a fluorescence microscope.

9. Centrifuge the chromosome suspension at 100 × *g* for 1 minute to pellet any chromosome clumps and cellular debris.

10. Transfer 1.4 ml of the supernatant to a tube suitable for use on the flow sorter. Add 70 µl of Chromomycin A3 stain and mix immediately.

11. Add 3.5 µl of 1 M $MgSO_4$ and 7 µl of Hoechst 33258 stain to the chromosome suspension. Mix well and incubate the preparation for at least 1 hour on ice.

12. Add 175 µl of 0.1 M sodium citrate and 175 µl of 0.25 M sodium sulphite solution 15 minutes prior to flow analysis and sorting. Mix the solution well and incubate on ice.

[a]Metaphase chromosomes, stained purple, are visible filling the cell.

*2.7.2 Generation of a bivariate flow karyotype and chromosome sorting*

Bivariate flow karyotypes were generated and chromosomes sorted by the following method:

1. Set up a modified commercial flow sorter (Coulter ELITE-ESP, Coulter Electronics, see Figure), equipped with two argon-ion lasers, for chromosome analysis and sorting following the manufacturer's instructions.

2. Operate the lasers at a power of 300 mW and align them on the sample stream such that the chromosomes pass in the sheath fluid first through the UV (351-364 nm) beam (exciting the Hoechst 33258 stain), then through the 457.9 nm beam (exciting the Chromomycin A3 stain).

3. Use fluorescent microspheres (Coulter Electronics) to align the optics in preparation for chromosome analysis, such that the cv of signals at the Hoechst and Chromomycin detectors is 1.6 or less.

4. Adjust the sample pressure such that the stained chromosomes flow in single file through the focus point of each laser at a rate of approximately 1,000 per second.

5. Set up the flow sorter software to display the fluorescent signals emitted from each chromosome event as a co-ordinate on a bivariate plot of Hoechst 33258 versus Chromomycin A3 fluorescence. The chromosome types can then be resolved on the plot by their size and AT to GC base pair ratios.

6. Use the software to define the chromosome type to be sorted and switch on the high-voltage deflection plates.

7. Utilising the electrostatic deflection of charged droplets containing the chromosome type of interest, sort 500 to 1000 copies directly into a 0.5 ml PCR tube containing 33 µl of sterile distilled water.

8. With the lid secure flick the bottom of the tube to suspend any chromosomes which may have adhered to the side of the tube. Spin the tube briefly in a microfuge to bring down the solution.

9. Store the flow-sorted chromosomes on ice if they are to be used for DOP-PCR the same day. Alternatively, they will remain stable for over 3 years stored at –20˚C.

**2.8 Generation of chromosome-specific paints by DOP-PCR**

Chromosome-specific paints were generated from flow-sorted chromosomes by the following methods:

*2.8.1 Primary DOP-PCR amplification of flow-sorted chromosomes*

Flow-sorted chromosomal DNA was randomly amplified by primary DOP-PCR by the following method:

1. For a 50 µl reaction in a 0.5 ml PCR tube, mix the following:

- X µl sterile distilled water (to a final volume of 50 µl)
- 500 to 1000 flow-sorted chromosomes
- 5.0 µl 10× TAPS2 buffer
- 4.0 µl $1^0$ DOP-PCR dNTP mix
- 5.0 µl 20 µM DOP-PCR primer (see Appendix)

2. Mix the tube contents and spin briefly in a microfuge to bring down the solution.

3. Place the tube on a PCR block and incubate at 94˚C for 8 minutes.

4. Add 0.5 µl (2.5 U) of *Taq* polymerase and continue the program with nine cycles of:

    - 94˚C for 1 minute
    - 30˚C for 1.5 minutes
    - Transition at 0.23˚C per second to 72˚C
    - 72˚C for 3 minutes

    followed by 30 cycles of:

    - 94˚C for 1 minute
    - 62˚C for 1 minute
    - 72˚C for 1 minute

    followed by:

    - 72˚C for 7 minutes

5. Mix 5 µl of the PCR products with 1 µl of 6× loading buffer and analyse by electrophoresis on a 1% w/v agarose gel.

*2.8.2 Secondary DOP-PCR amplification*

The products of a primary DOP-PCR amplification were further amplified and haptenised by the following method:

1. For a 50µl reaction in a 0.5 ml PCR tube, mix the following:

    - X µl sterile distilled water (to a final volume of 50 µl)
    - 5.0 µl 10× TAPS2 buffer
    - 3.5 µl $2^0$ DOP-PCR dNTP mix
    - 5.0 µl $2^0$ DOP-PCR hapten (see reagents and stains, this chapter)
    - 5.0 µl 20 µM DOP-PCR primer (see Appendix)
    - 2 µl (approximately 150 ng) $1^0$ DOP-PCR product

2. Mix the tube contents and spin briefly in a microfuge to bring down the solution.

3. Place the tube on a PCR block and incubate at 94˚C for 8 minutes.

4. Add 0.5 µl (2.5 U) of *Taq* polymerase and continue the program with 25 cycles of:

    - 94˚C for 1 minute
    - 62˚C for 1 minute
    - 72˚C for 1 minute

    followed by:

    - 72˚C for 7 minutes

5. Mix 3 µl of the PCR products with 1 µl of 6× loading buffer and analyse by electrophoresis on a 1% agarose gel.

**2.9 DNA Preparation**

*2.9.1 Preparation of high molecular weight gibbon genomic DNA from lymphoblastoid cells*

High molecular weight gibbon genomic DNA was prepared by the following method:

Safety Note: Carry out all manipulations involving phenol, chloroform and diethyl ether in a fume cabinet.

1. Culture a flask of gibbon lymphoblastoid cells until almost confluent, so that there are approximately $1\times10^6$ cells/ml in 50 ml of medium[a].

2. Transfer the culture to a polypropylene tube and pellet the cells by centrifugation at 400 × *g* for 10 minutes, discard the supernatant and drain the tube briefly by inverting it on a tissue.

3. Wash the cell pellet in 20 ml of sterile PBS and fully resuspend the cells to $1\times10^7$ cells/ml in 1× NDS + 100 µg/ml Proteinase K (GibcoBRL). Incubate the mixture o/n in a 50˚C water bath.

4. Add 4 volumes of 0.2 µm filtered $T_{0.1}E$. Invert the tube gently to mix and allow the solution to equilibrate to room temperature.

5. Add an equal volume of phenol (equilibrated with Tris) and mix gently, to form an emulsion, by slowly inverting the tube end-over-end for 10 minutes.

6. Separate the aqueous phase from the organic phase by centrifugation at 6000 × *g* for 10 minutes. Collect the aqueous phase into a fresh tube.

7. Carry out two further phenol extractions, until the phase interface is clear of white protein debris.

8. Add an equal volume of 1:1 phenol:chloroform and mix gently for 10 minutes. After centrifugation at 3000 × *g* for 15 minutes, collect the aqueous phase into a fresh tube.

9. Add an equal volume of chloroform and mix gently for 10 minutes. After centrifugation at 3000 × *g* for 15 minutes, collect the aqueous phase into a fresh tube.

10. Add an equal volume of diethyl ether and mix gently for 10 minutes. After centrifugation at 1000 × *g* for 10 minutes, discard the ether.

11. To precipitate the DNA, add 0.2 volumes of 10 mM ammonium acetate and mix gently. Add 2 volumes of 100% ethanol and invert the tube slowly end-over-end for 2-3 minutes.

12. Hook and swirl the precipitated high molecular weight DNA around a sterile inoculating loop and wash it by swirling in 40 ml of 70% ethanol for 2-3 minutes.

13. Transfer the DNA to a fresh 1.5 ml microfuge tube and air-dry for 1 hour at room temperature.

14. Dissolve the DNA in 500 µl TE by standing the tube o/n at 4˚C[b].

[a]For high molecular weight DNA extractions, it is possible to yield approximately 4 pg of DNA per cell.

[b]A high molecular weight genomic DNA solution should be stored at 4˚C.

*2.9.2 Preparation of DNA from BAC, PAC, fosmid and cosmid clones*

DNA from BAC, PAC, fosmid and cosmid clones was prepared by the following alkaline lysis “mini-prep” method:

Safety Note: Carry out all manipulations involving phenol in a fume cabinet.

1. Inoculate 10 ml of 2× TY broth in a 25 ml centrifuge tube with 1 µl of the appropriate clone glycerol stock, or a single colony. For cosmids and PACs include 30 µg/ml of kanamycin in the broth. For BACs in the vector pBACc3.6 and fosmids include 25 µg/ml of chloramphenicol, and for BACs in the vector pBeloBAC11 or pBAC108L include 12.5 µg/ml of chloramphenicol.

2. Allow the cultures to grow by incubating o/n at 37˚C with shaking at 210 rpm.

3. Make at least one back-up glycerol stock for each culture by mixing 200 µl of sterile 100% glycerol with 800 µl of culture[a].

4. Pellet the overnight culture by centrifuging at 3400 × *g* for 10 minutes at room temperature, discard the supernatant and drain the tube briefly by inverting on a tissue.

5. Gently resuspend the cell pellet in 200 µl of lysis buffer, transfer the suspension to a 1.5 ml microfuge tube and incubate at room temperature for 10 minutes.

6. Add 400 µl of fresh 0.2 M NaOH/1% (w/v) SDS (for 1 ml, mix 50 µl of 4 M NaOH with 100 µl of 10% (w/v) SDS and 850 µl of sterile distilled water). Invert the tube several times to mix and incubate on ice for 5 minutes.

7. Add 300 µl of 3 M sodium acetate (pH 5.2), mix the solution and incubate on ice for 10-30 minutes until the host cell debris forms a precipitate.

8. Pellet the debris in a microfuge at 12,000 × *g* for 5 minutes. Transfer the supernatant to a fresh tube. Microfuge the supernatant for a further 5 minutes, and transfer the clear supernatant again to a fresh tube.

9. Add 600 µl of isopropanol (propan-2-ol) and either place at -70˚C for 10 minutes, or -20˚C for between 30 minutes and o/n.

10. Pellet the precipitate in a microfuge at 12,000 × *g* for 5 minutes, discard the supernatant and briefly drain the tube on a tissue.

11. On ice, resuspend the pellet in 200 µl of 0.3 M sodium acetate (pH 7.0).

12. Add an equal volume of 1:1 phenol:chloroform mixture and vortex for 30 seconds to form an emulsion. Separate the aqueous from the organic phases by microfuging at 12,000 × *g* for 3 minutes and transfer 150 µl of the aqueous phase to a fresh tube.

13. Back-extract the phenol:chloroform by adding 50 µl of 0.3 M sodium acetate (pH 7.0), vortexing to form an emulsion and microfuging at 12,000 × *g* for 2 minutes. Transfer 50 µl of the aqueous phase to the other 150 µl.

14. Add an equal volume of isopropanol invert the tube to mix and place at -70˚C for 10 minutes.

15. After pelleting the DNA in a microfuge at 12,000 × *g* for 10 minutes, discard the supernatant and wash the DNA pellet with 500 µl of ice-cold 70% ethanol.

16. Immediately microfuge at 12,000 × *g* for 5 minutes. Discard the supernatant and air-dry the pellet for 25 minutes in a 37˚C oven.

17. On ice, resuspend the DNA in 50 µl of $T_{0.1}E$ with 1 µl of RNAseA (stock 10 mg/ml). Incubate the preparation at 55˚C for 15 minutes then store permanently at –20˚C.

[a]Store glycerol stocks permanently at -70˚C.

**2.10 Procedures for fluorescence *in situ* hybridisation (FISH)**

2.10.1 Fixed metaphase preparations from lymphoblastoid cell lines

Fixed metaphase preparations were prepared from lymphoblastoid cell lines by the following method:

1. To a 50% confluent culture, add BrdU to 10 µg/ml, mix well and incubate the cells for a total of 3 hours at 37˚C.

2. 1.25 hours prior to harvest add ethidium bromide to 10 µg/ml and mix well.

3. 45 minutes prior to harvest add Colcemid Karyomax (GibcoBRL) to 0.05 µg/ml and mix well.

4. At the time of harvest, transfer the contents of the flask to a 25 ml centrifuge tube and pellet the cells by centrifugation at 200 × *g* for 10 minutes.

5. Discard the supernatant and resuspend the cells in 10 ml of 75 mM KCl pre-warmed to 37˚C. Transfer the cell suspension to a 15 ml polystyrene tube and incubate at 37˚C for 15-20 minutes.

6. Add 1 ml of fresh 3:1 methanol:acetic acid fix, invert the tube to mix then immediately pellet the cells by centrifugation at 400 × *g* for 10 minutes.

7. After carefully discarding the supernatant, resuspend the cell pellet in 2-3 ml of 3:1 fix. Centrifuge at 400 × *g* for 5 minutes and discard the supernatant.
8. Repeat the fixation and centrifugation steps a further 3 times. After the last centrifugation step, remove as much of the supernatant as possible.
9. Resuspend the fixed cell pellet in a small volume (1 to 2 ml) of 3:1 fix so that the solution remains cloudy.
10. Assess the fixed metaphase preparation by dropping a small aliquot onto a microscope slide from a pipette tip. Metaphase spreads can be detected under phase contrast using a light microscope.

For long-term storage, resuspend the fixed cells in approximately 15 ml of 3:1 fix. Seal the lid of the tube with parafilm and store the preparation at -20˚C.

*2.10.2 Fixed metaphase preparations from fibroblast cell lines*

Fixed metaphase preparations were prepared from fibroblast cell lines by the following method:

1. To a 50-60% confluent culture, add BrdU to 10 μg/ml, mix well and incubate the cells for a total of 4 hours at 37˚C.

2. 2.5 hours prior to harvest add Colcemid Karyomax to 0.05 μg/ml and mix well.

3. At the time of harvest, collect the culture media and rinse the adherent cell surface with 10 ml of sterile PBS. Collect the PBS and pool it with the media.

4. Incubate the adherent cells with Trypsin/EDTA solution (Sigma) and monitor cell release from the flask surface by viewing under a light microscope until 50% of the adherent cells have released.

5. Resuspend the released cells in the media and PBS collected previously, and transfer the suspension to a 50 ml polypropylene tube.

6. Pellet the cells by centrifugation at 200 × *g* for 10 minutes.

7. Continue the preparation from step 5 of the method for fixing lymphoblastoid cells *(2.10.1)* above.

*2.10.3 Metaphase spread slide preparation*

Fixed metaphase spreads were prepared on glass microscope slides by the following procedure:

1. Bring the tube containing the fixed metaphase suspension to room temperature.
2. After discarding approximately 12 ml of fix, resuspend the cell suspension in the 3 ml of fix remaining by flicking the tube.
3. Drop a single drop of the fixed preparation from a glass pipette onto a clean glass microscope slide, immediately followed with 1 drop of fresh 3:1 fix.
4. Allow the slide to air dry and examine it for metaphase spreads under phase contrast using a light microscope.
5. Mark the area of spread cells on the slide with a diamond pen.
6. Incubate the slides in 3:1 fix at room temperature for 30-60 minutes.
7. After air-drying, dehydrate the slides through a fresh 70%, 70%, 90%, 90% and 100% ethanol series for 1 minute each.
8. After air-drying, incubate the slides in acetone at room temperature for 10 minutes. Air-dry the slides.
9. If the slides are to be used the same day, incubate them at 65˚C in an oven for at least 1 hour.
10. If the slides are to be used the next day, incubate them at 42˚C o/n.
11. For long-term storage seal the slides in a box at room temperature.

*2.10.4 Probe labelling by nick translation*

Single-copy BAC, PAC, cosmid and fosmid DNA probes for use in FISH were labelled by nick translation by the following method:

1. For a 25 µl reaction to label 1 µg of DNA, add the following to a 1.5 ml microfuge tube on ice:
    - X µl sterile distilled water (to a final volume of 25 µl)
    - 2.5 µl 10× nick translation buffer
    - 1.9 µl nick translation dNTP mix
    - 0.7 µl Biotin-16-dUTP (or other hapten, see reagents and stains, 2.2, above)
    - 0.5 µl DNase I (1 µg/ml working solution)
    - 0.5 µl (5 U) DNA polymerase I
    - Y µl DNA (1µg)
2. Mix the tube contents and spin briefly in a microfuge to bring down the solution.
3. Incubate at 14˚C for 40 minutes to 1 hour.
4. To stop the reaction, add 2.5 µl of 0.5 M EDTA, mix well and transfer the tube to ice.
5. Mix 3 µl of the sample with 1 µl of 6× loading buffer and analyse by electrophoresis on a 1% agarose gel.
6. Meanwhile, precipitate the cut and labelled DNA by the addition of 2.5 µl of 3 M sodium acetate (pH 7.0) followed by 1 ml of 100% ice-cold ethanol. Mix the solution well and incubate at -70˚C for 30 minutes or -20˚C o/n.
7. Pellet the DNA by spinning in a microfuge at 12,000 × *g* for 10 minutes. Wash the pellet with 1 ml of 70% ice-cold ethanol and microfuge at 12,000 × *g* for 10 minutes.

8. Discard the supernatant and air-dry the pellet by incubating at 37˚C for 25 minutes with the tube lid off.

9. Resuspend the DNA on ice in 10 µl of $T_{0.1}E$ so that the concentration of DNA will be approximately 100 ng/µl.

*2.10.5 Hybridisation of human and gibbon single-copy probes and chromosome-specific paints onto human, pygmy chimpanzee, gibbon and lemur metaphases*

Human and gibbon DNA probes and chromosome-specific paints were hybridised onto human, pygmy chimpanzee, gibbon and lemur metaphases by the following method:

Safety Note: Carry out all manipulations involving formamide in a fume cabinet.

1. Add the following to a 1.5 ml microfuge tube on ice:
    - 0.5 µl labelled DNA (30-50 ng)
    - 1 µl human $C_0t$-1 DNA (1 µg)
    - 14 µl hybridisation buffer
2. Mix the tube contents, and spin briefly in a microfuge to bring down the solution.
3. Denature the probe mix by incubating at 65˚C for 10 minutes.
4. Pre-anneal the probe by incubating at 37˚C for 20 minutes to 1 hour. Meanwhile, denature the metaphase-spread slides in 70% formamide at 65˚C for 2 minutes.
5. Quench the denatured slides in 70% ice-cold ethanol for 2 minutes then dehydrate through a 70%, 70%, 90%, 90% and 100% ethanol series, allowing 1 minute in each.
6. Air-dry the slides
7. Pipette the probe mix onto the slide and cover it with a cover slip. Seal the edges of the cover slip with rubber cement.
8. Incubate the slides o/n at 37˚C.

*2.10.6 Hybridisation of human single-copy probes and chromosome-specific paints onto canine metaphases*

Human DNA probes and chromosome-specific paints were hybridised onto canine metaphases by the following method:

1. Add the following to a 1.5 ml microfuge tube on ice:
    - 1.5 µl labelled DNA probe (90-150 ng)
    - 1.0 µl sonicated dog genomic DNA (10 µg)
    - 14 µl hybridisation buffer
2. Follow the protocol above *(2.10.5)* from steps 2. to 7.
3. Incubate the slides over two nights at 37˚C.

*2.10.7 Hybridisation of canine chromosome-specific paints onto human metaphases*

Canine chromosome-specific paints were hybridised onto human metaphases by the following method:

1. Add the following to a 1.5 ml microfuge tube on ice:
    - 1.5 µl labelled DNA probe (90-150 ng)
    - 1.0 µl human $C_0t$-1 DNA (1 µg)
    - 0.9 µl sonicated salmon sperm DNA (9 µg)
2. Ethanol-precipitate the tube contents and resuspend the DNA pellet in 14 µl of hybridisation buffer.
3. Follow protocol *(2.10.5)* from steps 2. to 7.
4. Incubate the slides over two nights at 37˚C.

*2.10.8 Direct detection for fluorescently-labelled probes*

After hybridisation, fluorescently-labelled probes were detected by the following method:

1. Pre-warm 3 coplin jars of 2× SSC and 2 coplin jars of 50% formamide/1× SSC to 42˚C. Remove the dried rubber cement sealing the cover slips and rinse the cover slips off in the first jar of 2× SSC.

2. Stringency wash the slides by incubating for 2× 5 minutes in 50% formamide/2× SSC at 42˚C.

3. Wash the slides by incubating for 2× 5 minutes in 2× SSC at 42˚C.

4. Rinse the slides in 4× SSC, 0.05% (v/v) Tween 20 for 5 minutes at room temperature.

5. Incubate the slides for 3 minutes in 50 ml DAPI stain in the dark.

6. Briefly rinse the slides in 2× SSC and quickly dehydrate through a fresh series of 70%, 70%, 90%, 90% and 100% ethanol. Air-dry the slides in the dark.

7. Apply 13 µl of citifluor AF1 antifade slide mount to each slide and overlay with a 22 x 32 mm cover slip.

8. Seal the cover slips with nail varnish and store in the dark.

*2.10.9 Three-layer immunochemical detection for biotinylated probes*

After hybridisation, biotinylated probes were detected imunochemically by the following three-layer method:

1. Pre-warm 3 coplin jars of 2× SSC and 2 coplin jars of 50% formamide/1× SSC to 42˚C. Remove the dried rubber cement sealing the cover slips and rinse the cover slips off in the first jar of 2× SSC.

2. Stringency wash the slides by incubating for 2× 5 minutes in 50% formamide/1× SSC at 42˚C.

3. Wash the slides by incubating for 2× 5 minutes in 2× SSC at 42˚C.

4. Incubate the slides in 4× TNFM at 37˚C for 10 minutes.

5. Meanwhile, dilute the detection reagents as follows (make 100 µl per slide plus at least 50 µl excess for each layer) in 1.5 ml microfuge tubes:

    - Avidin-FITC DCS or Avidin-Cy3 diluted 1/500 in 4× TNFM
    - Biotinylated anti-avidin diluted 1/250 in 4× TNFM

6. Incubate the detection solutions for 10 minutes at room temperature in the dark.

7. Pellet any debris by microfuging at 12,000 *g* for 10 minutes and use the supernatants.

8. Incubate the slides with 100 µl diluted Avidin-FITC or Avidin-Cy3 under a parafilm cover slip at 37˚C in a dark humidified box for 20 to 60 minutes.

9. Wash the slides for 3× 5 minutes in 4× TNFM at 42˚C.

10. Incubate the slides with 100 µl of diluted biotinylated anti-avidin for 20 to 60 minutes in a dark humidified box at room temperature.

11. Wash the slides for 3× 5 minutes in 4× TNFM at 42˚C.

12. Incubate the slides with 100 µl of diluted Avidin-FITC or Avidin-Cy3 for 20 to 60 minutes in a dark humidified box at room temperature.

13. Wash the slides for 2× 5 minutes in 4× TNFM at 42˚C.

14. Wash the slides for 2× 5 minutes in 4× SSC, 0.05% (v/v) Tween 20 at room temperature.

15. Follow method *(2.10.8)* from step 5.

## 2.11 Procedures for the construction of a genomic cosmid library

### *2.11.1 Partial restriction enzyme digestion and phosphatasing of high molecular weight genomic DNA*

High molecular weight gibbon genomic DNA was partially digested with *Mbo*I and phosphatased with CIAP in preparation for cloning, by the following method:

Note: Care was taken to minimise shearing of the high molecular weight gibbon DNA by carrying out all manipulations slowly and with cut-off pipette tips.

1. Using a cut-off pipette tip transfer 30 µg of high molecular weight gibbon DNA (dissolved in $T_{0.1}E$) to a 1.5 ml microfuge tube. Add 0.2 µm filtered 10 mM Tris-HCl pH 8.0 to a total volume of 450 µl. Resuspend the DNA by stirring gently with the pipette tip.

2. Add 50 µl of 10× TAK buffer without SAM or DTT added and mix by stirring gently with the pipette tip.

3. Allow the DNA to fully equilibrate by incubating o/n at 4˚C.

4. Add 0.25 µl of 0.2 µm filtered 1 M DTT (to give a 0.5 mM final concentration) and mix by stirring gently with the pipette tip. Incubate for 30 to 40 minutes at 4˚C.

5. On ice, aliquot the equilibrated high molecular weight DNA solution into ten 1.5 ml microfuge tubes, so that tube 1 has 60 µl and tubes 2 to 10 have 30 µl (1.8 µg DNA) each.

6. Make a 1/50 dilution of *Mbo*I to 0.1 U/µl in 1× TAK buffer without SAM or DTT added.

7. Serially dilute the *Mbo*I in tubes 1 to 9 as follows:

   I. To tube 1, add 0.1 U (1 µl of 0.1 U/µl) of *Mbo*I and stir gently with the pipette tip twenty times.

II. Using a cut-off tip transfer 30 µl of the solution from tube 1 to tube 2 and stir gently with the pipette tip twenty times.

III. Repeat the procedure in step ii. up to tube 9, then discard 30 µl of solution from tube 9 so that the quantity of *Mbo*I per tube will be as follows:

- Tube 1 0.05 U
- Tube 2 0.025 U
- Tube 3 0.0125 U
- Tube 4 0.00625 U
- Tube 5 0.003125 U
- Tube 6 0.0015625 U
- Tube 7 0.00078125 U
- Tube 8 0.000390625 U
- Tube 9 0.0001953125 U
- Tube 10 no-enzyme control

8. Incubate the tubes for 1 h at 37˚C in an oven.

9. Inactivate the restriction enzyme by incubating for 15 minutes at 70˚C on a hot block. After cooling to room temperature transfer the tubes to ice for 2 to 3 h.

10. Dilute CIAP from 10 U/µl to 0.6 U/µl in 1× TAK buffer with no SAM or DTT added.

11. Add 0.6 U (1 µl) of CIAP to each reaction tube and incubate for 30 minutes at 37˚C in a water bath.

12. At room temperature, using a cut-off pipette tip, remove 10 µl from each digested and phosphorylated sample, mix with 2 µl of 6× loading buffer and analyse by electrophoresis on a 0.3% agarose/1× TAE gel. Include 100 ng of *Hind*III-digested

Bacteriophage λ DNA and 25 ng of undigested Bacteriophage λ DNA as DNA size markers[a]. Run the gel at 1.5 V/cm o/n at room temperature.

13. Meanwhile, to inactivate the CIAP, add 2.2 µl of 150 mM NTA to the remaining 20 µl of each sample and incubate for 20 minutes at 68˚C on a hot block.

14. Immerse the tubes in ice to cool rapidly and spin the tubes briefly in a microfuge to bring down the solution.

15. Add 1.2 µl of 5 M NaCl to each sample and gently mix well.

16. Add 60 µl of 100% ethanol to each sample and gently mix well.

17. Allow the DNA to precipitate by incubating the tubes o/n at -20˚C[b].

[a]Denature the size markers at 65˚C for 2 minutes before use.

[b]Digested DNA in 100% ethanol may be stored for several months at -20˚C.

*2.11.2 Preparation and testing of Lawrist16 vector arms*

Lawrist 16 vector arms were prepared and tested by the following methods:

Safety Note: Carry out all manipulations involving phenol, chloroform and ether in a fume cabinet.

Method A: Preparation of Lawrist 16 vector arms

1. In a 1.5 ml microfuge tube combine the following:

    - 10 µl 10× NEB ScaI buffer
    - X µl DNA (20 µg)
    - Y µl sterile distilled water (to a total volume of 100 µl)
    - 9 µl (90 U) ScaI

2. Mix the tube contents, and spin briefly in a microfuge to bring down the solution.

3. Incubate for 2 h at 37˚C in an oven.

4. Remove a 1 µl aliquot and mix it with 2 µl of 6× loading buffer. Analyse the digestion products by electrophoresis on a 0.7% agarose/1× TBE gel. Include 200 ng of undigested Lawrist 16 as a control, and *Hind*III-digested Bacteriophage λ DNA as a size marker.

5. From the remainder of the *Sca*I digestion mix remove a 5 µl aliquot, (*Sca*I control) and store at –20˚C for use in Method B (see below).

6. Meanwhile, to the remainder of the *Sca*I digestion add 1× LSRE buffer to a volume of 370 µl.

7. Add 35 µl (35 U) of CIAP, pipette up and down a few times to mix and incubate for 45 minutes at 37˚C in a water bath.

8. To inactivate the CIAP add 45 µl of 150 mM NTA and incubate for 25 minutes at 68˚C in a hot-block.

9. Extract the DNA with an equal volume of 1:1 phenol/chloroform, then with chloroform alone, each time re-extracting the organic phase with an equal volume of TE. Finally, extract the aqueous phase with ether until no whiteness appears at the phase interface.

10. Place the tube at 68˚C for a few minutes with the cap open to evaporate the ether, then precipitate the DNA o/n at –20˚C with 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volumes of absolute ethanol.

11. Pellet the DNA in a microfuge at 12,000 *g* for 15 minutes at room temperature and discard the supernatant. Wash the DNA pellet with 1 ml of 70% ethanol, then microfuge for 2 minutes, discard the supernatant and air-dry the pellet.

12. Dissolve the DNA in 177.5 µl of TE. Remove a 5 µl aliquot (*Sca*1/CIAP control) and store at –20˚C for use in Method B.

13. To the remaining *Sca*I- and CIAP-treated DNA, add 20 µl of 10× *Bam*HI buffer and 7.5 µl (150 U) of *Bam*HI. Pipette up and down a few times to mix.

14. Incubate for 90 minutes at 37˚C in a water bath, then cool the reaction to room temperature.

15. Remove a 2.5 µl aliquot and mix with 2 µl of 6× loading buffer and analyse the digestion products by electrophoresis on a 0.7% agarose/1× TBE gel. Include *Hind*III-digested Bacteriophage λ DNA as a size marker.

16. To the remainder of the *Sca*I/CIAP/*Bam*HI reaction mix, carry out organic extractions and precipitate the DNA as described in steps 9, 10 and 11.

17. Dissolve the vector arms in 50 µl of TE and remove a 5 µl aliquot (*Sca*I/CIAP/*Bam*HI control) for use in Method B. Store the remainder of the prepared arms at –70˚C in 10 µl aliquots (approximately 200 ng/µl) in 0.5 ml microfuge tubes.

Method B: Testing of Lawrist 16 vector arms

1. To the 5 µl sample from step A5 (*Sca*I control) add 45 µl of TE.

2. Carry out organic extractions and precipitate the DNA as described in steps A9, A10 and A11.

3. Dissolve the *Sca*I control DNA in 5 µl of TE.

4. To this sample and those from steps A12 (*Sca*1/CIAP control) and A17 (*Sca*I/CIAP/*Bam*HI control) add 11 µl of TE.

5. Add 2 µl of 10× Nizetic ligase buffer and 0.2 µl of 100 mM ATP to each sample. Dilute the stock T4 DNA ligase to 50 U/µl in 1× Nizetic ligase buffer.

6. Divide each sample from step 5 into two 9 µl aliquots.

7. To one add 1 µl (50 U) of T4 DNA ligase. To the other, add 1 µl of 1× Nizetic ligase buffer.

8. Incubate o/n at 14˚C.

9. Analyse the quality of the vector arms by electrophoresis on a 0.7% agarose/1× TAE gel. Include *Hind*III digested λ DNA as a size marker and undigested vector DNA as a gel control.

*2.11.3 Ligation and packaging of partially -digested DNA*

Partially digested gibbon genomic DNA (from Method *2.11.1)* was ligated to Lawrist 16 vector arms (as prepared in Method *2.11.2*) and packaged into infective λ Bacteriophage particles by the following methods:

Method A: Ligation of vector arms to partially digested DNA

1. Pellet the partially digested DNA (stored in 100% ethanol at –20˚C) from Method *2.11.1* in a microfuge at 12,000 *g* for 15 minutes at room temperature. Align the tube hinge in the microfuge rotor so that it is possible to predict where the DNA pellet will be.

2. Carefully remove the supernatant with a P200 pipette tip. The DNA pellet will be visible as “speckles” at the bottom and up the side of the tube.

3. Wash the DNA pellet with 1 ml of 70% ethanol and microfuge at 12,000 *g* for 7 minutes.

4. Carefully remove the supernatant with a P1000, then a P200 pipette tip. Allow the DNA pellet to air dry with the tube lid off for 10 minutes at room temperature.

5. Resuspend the DNA in 10 µl of TE by working beads of TE over the surface of the DNA with a pipette tip. Incubate the sample on ice for 4 to 5 h, working the TE over the DNA pellet surface every hour.

6. Set up Test and Control ligation reactions as follows:

   Test reaction:

   - X µl vector arms (80-1520 ng)
   - Y µl genomic DNA (50-800 ng)
   - Z µl TE (to a final volume of 16 µl)
   - 1.6 µl 10× ligase buffer
   - 1.4 µl T4 DNA ligase (400 U/µl)
   - 1.0 µl 6 mM ATP

   Control reaction:

   - X µl vector arms (400 ng)
   - Y µl TE (to a final volume of 8 µl)
   - 0.8 µl 10× ligase buffer
   - 0.7 µl T4 DNA ligase
   - 0.5 µl 6 mM ATP

7. Flick the tubes to mix the reactants and spin briefly in a microfuge to bring down the solution. Incubate o/n at 14˚C on a PCR block.

8. Either store the ligation products at -70˚C or (if packaging the same day) keep them on ice.

Method B: Packaging ligated DNA

1. Package ligated DNA from Method A for 2 h using the Stratagene Gigapack Gold II, IIXL or Gold III packaging extract systems, according to the manufacturer's instructions.

2. Stop the reaction by adding 500 µl of lambda diluent, followed by 132 µl of 5× SM. Mix the samples gently by inversion.

3. Assess the library titre of each packaging reaction (see *2.11.4* below).

4. Freeze small (30 µl) aliquots of the packaged bacteriophage on dry ice, and store them at –70˚C.

*2.11.4 DH5αMCR E.coli plating cell preparation*

DH5αMCR cells were prepared by the following method:

1. Allow an antibiotic-free LB agar plate to equilibrate to room temperature.

2. Collect a DH5αMCR glycerol stock from -70˚C onto dry ice.

3. In a safety cabinet, gently scratch the surface of the (still frozen) glycerol stock with the tip of a sterile cocktail stick and streak the cells across the plate surface.

4. Take a fresh cocktail stick and cross hatch across the first streak. Repeat this process with a fresh cocktail stick each time, cross-hatching across the previous streaks, until the bottom of the plate is reached.

5. Allow the cell suspension to dry on the surface of the plate for a few minutes with the lid off.

6. Replace the lid and incubate the plates inverted at 37˚C o/n.

7. In a safety cabinet, use a sterile loop to transfer a small single separate colony to 40 ml of LB media in a sterile 250 ml conical flask.

8. Incubate the 250 ml flask with the lid loosened, shaking, o/n at 37˚C. Include a centrifuge tube containing 40 ml of LB media alone to act as a contamination control.

9. Transfer the cell suspension to a 50 ml centrifuge tube. Pellet the cells by centrifugation at 4,500 *g* for 15 minutes at room temperature.

10. Discard the supernatant into disinfectant and gently resuspend the cell pellet in 20 ml of autoclaved 10 mM $MgSO_4$.

11. Store the cells at 4˚C for up to four weeks.

*2.11.5 Plating libraries on E. coli and assessing library titres*

Library titres were assessed by the following method:

1. In a safety cabinet set up seven transduction reactions in 1.5 ml microfuge tubes as follows:

   I. 1 µl library packaged extract + 99 µl lambda diluent

   II. 50 µl packaged self-ligation + 50 µl lambda diluent

   III. 10 µl 5× SM + 90 µl lambda diluent

   IV. 100 µl lambda diluent

   V. 100 µl DH5αMCR plating cells

   VI. 30 µl lambda diluent alone

   VII. 20 µl packaged extract alone

2. Add 100 µl of fresh (less than four weeks old) DH5αMCR plating cells to reactions i. to iv. and mix the tube contents gently. Incubate all the tubes at 37˚C in a hot block for 20 minutes.

3. Dilute the infected cells in reaction tubes i. and ii. with 1 ml of LB media each and invert several times to mix.

4. Incubate the reactions for 45 minutes at 37°C in a hot block.

5. Pellet the cells by microfuging tubes i. and ii. for 2 minutes at 6000 rpm.

6. Remove the supernatant with a pipette tip leaving approximately 30 µl in the tube.

7. Gently resuspend each cell pellet in the residual media by pipetting up and down.

8. Using a sealed, bent glass pasteur pipette, spread each sample on a separate 8x20 cm plate containing 1% LB agar + 30 µg/ml kanamycin.

9. Allow 10 minutes for the plate surface to air-dry with the lid off.

10. Replace the lids and incubate the plates upside down for approximately 16 h, at 37°C.

11. Count the number of colonies on each plate. (The test plates iii. to vii. should have no colony growth.)

12. Assess the library titre by multiplying the number of colonies on plate i. by the total volume (µl) of the library (see *2.11.4*, Method B), to give the number of potential colonies of that library. Although some colonies from the packaging reaction of self-ligated vector arms are to be expected, the number of colonies should only be a fraction of that obtained by the genomic DNA packaging.

*2.11.6 Plating libraries onto filters and their replication for screening by hybridisation*

Method A:

Master filters, each supporting approximately 20,000 colonies from a library, were set up by the following method:

Note: Carry out all manipulations up to step 14 in a safety cabinet.

1. Estimate the volume of packaged library extract required to generate 20,000 colonies (see *2.11.5* above).

2. Calculate the number of transduction reactions required, allowing 10 µl of packaged extract per reaction (e.g. if 60 µl of packaged extract is required for 20,000 colonies, then 6 transduction reactions are required, each containing 10 µl of packaged extract and 90 µl of lambda diluent.)

3. For each transduction reaction aliquot 90 µl of lambda diluent into a 1.5 ml microfuge tube.

4. Add 10 µl of packaged library extract and rinse the pipette tip by pipetting up and down gently 3 or 4 times.

5. Add 100 µl of DH5αMCR cells and mix gently by pipetting up and down twenty times.

6. Set up control reactions containing lambda diluent alone, DH5αMCR cells alone and LB broth alone.

7. Incubate all the tubes at 37˚C in a hot block for 20 minutes.

8. Add 1ml of LB broth to each tube and invert them several times to mix.

9. Incubate for 45 minutes in a hot block at 37°C.

10. Pellet the cells by microfuging for 2 minutes at 6000 r.p.m.

11. Discard the supernatant from all but one of the tubes where approximately 200 µl should be retained.

12. Pool and resuspend all the pellets in the 200 µl of supernatant.

13. Using a sealed, bent pasteur pipette, spread the cells (or control reaction) onto a Hybond N+ (Amersham) nylon transfer membrane (8x20 cm) supported on a 1% LB agar plate including 30 µg/ml of kanamycin.

14. Allow the membrane surface to air dry for 10 minutes.

15. Incubate the plates upside down for approximately 16 h, at 30°C and then for approximately 7 h at 37°C, until the colonies have grown to 0.1-0.5 mm in diameter.

16. Transfer the plates to 4˚C to delay colony growth. Store the plates at 4˚C until further use.

Method B:

Master filters were replicated for hybridisation by the following method:

Note: Carry out all filter manipulations in a safety cabinet.

1. Bring the plate and filter to be replicated to room temperature (the colonies are difficult to replicate if too cold).

2. Using clean forceps, carefully peel the filter off the plate and lay it colony-side up on a clean plate of glass.

3. Label the replica filter appropriately and carefully place it writing-side down on top of the master filter. Allow a slight over-hang of the replica filter over the master filter to ease their separation in step 6.

4. To assist in cell transfer, apply firm pressure to the filters (e.g. by placing a 2.5 kg metal thermal cycler insert on top) for 2 to 4 minutes.

5. Using a 24-guage syringe needle, punch holes at the corners of both filters in distinct patterns to allow alignment of the master and replica when picking colonies at a later date.

6. Transfer the replica filter to a fresh 1% LB agar plate including 30 µg/ml of kanamycin.

7. Incubate the replica plate upside down for 3 to 4 h at 37°C, until the colonies have grown to an appropriate size.

8. Transfer the master filter to a fresh 1% LB agar plate containing 30 µg/ml kanamycin and 25% (v/v) glycerol. Incubate the plate upside down for 2 h at 37°C.

9. Wrap the plate tightly in Parafilm, and store it upside down at -70°C. This plate will be used for picking positive colonies.

10. Process the replica filter as described in Method *2.11.7* before use for hybridisation screening.

*2.11.7 Library filter processing*

Library filters were processed by the following method:

1. Place two sheets of 3MM paper on two biohazard trays. Saturate one tray with 10% SDS and the second tray with denaturation solution.

2. Ensure that there are no air bubbles under the saturated paper, and drain away the excess solution.

3. Place the filters on the 3MM paper with 10% SDS, colony-side up for 4 minutes.

4. Transfer the filters onto the tray with denaturation solution for 10 minutes.

5. Air-dry the filters on a clean piece of 3MM for 10 minutes.

6. Neutralise the filters by washing in 1 l of neutralisation solution for 5 minutes on an orbital shaker.

7. Repeat step 6.

8. Wash the filters in 1 l of 50 mM Tris-HCl pH 7.4/ 0.15 M NaCl (a 1/10 dilution of neutralisation solution) for 5 minutes, shaking on an orbital shaker.

9. Rinse the filters in 1 l of 2× SSC/ 0.1% SDS for 5 minutes, shaking on an orbital shaker.

10. Rinse the filters in 1 l of 2× SSC for 5 minutes, shaking on an orbital shaker.

11. Rinse the filters twice in 1 l of 50 mM Tris-HCl pH 7.4 for 5 minutes each, shaking on an orbital shaker.

12. Air-dry the filters, colony-side up on clean 3MM paper.

13. Cross-link the DNA to the filters by exposing the filters colony-side down on Saran-wrap to UV light on a transilluminator for 2 minutes.

14. Store the filters at room temperature in a sealed plastic bag.

*2.11.8 Filter Screening*

Method A: PCR-labelling of gibbon STSs

1. Using the appropriate primer pairs, perform a PCR using gibbon genomic DNA as a template. Electrophorese the products on a 2.5% agarose gel and stain with ethidium bromide to visualise the bands.

2. Over a UV transilluminator wearing a face shield and gloves, excise each product and transfer to 100 µl of $T_{0.1}E$ in a 1.5 ml microfuge tube.

3. Store o/n at 4˚C.

4. Make a PCR master mixture of an appropriate volume by combining multiples of the following single reaction volumes:

    - 6.83 µl $T_{0.1}E$
    - 1.5 µl 10× Perkin Elmer PCR buffer (Perkin Elmer)
    - 0.12 µl (0.6 U) AmpliTaq polymerase
    - 0.3 µl mixture of 3 unlabelled dNTPs (5 mM each)

5. Aliquot 8.75 µl of this mixture into PCR tubes and add 5 µl of $T_{0.1}E$ from the agarose slice from step 2.

6. Add 0.75 µl of primer mixture (100 ng/µl each). Add one drop of mineral oil.

7. With the protection of appropriate safety shielding, pipette 0.5 µl of $\alpha$-$^{32}$P dCTP (3,000 Ci/mmol) under the mineral oil and mix slightly with the pipette tip.

8. Place the tubes into a thermal cycler and perform PCR using the following cycling profile:

    - 94˚C for 5 minutes

    followed by 20 cycles of:

    - 93˚C for 30 seconds

- 55˚C for 30 seconds
- 72˚C for 30 seconds

followed by:

- 72˚C for 5 minutes

Method B:

1. Tightly roll each processed filter for analysis by hybridisation and insert them in pairs into 15 ml polypropylene tubes. Add 15 ml of prehybridisation buffer to the tubes, replace the lids and incubate for at least 1 h at 65˚C with shaking. (If a large number of filters are to be probed, these may be stacked colony-side up and probed in a small sandwich box with a tight lid.)

2. Prepare the following competition mixture by combining the following in a 1.5 ml microfuge tube:

   - 125 µl 20× SSC
   - 125 µl (1.25 mg) sonicated human placental DNA (stock 10 µg/µl)
   - X µl (50 µg) gibbon genomic DNA
   - Y µl $T_{0.1}E$ (to a final volume of 500 µl)
   - 15 µl radiolabelled probe (from Method A above)

   For multiple probes to be hybridised, pool together in the competition mix and modify the volume of $T_{0.1}E$.

3. Mix by inverting the tube and place in a boiling water bath for 5 minutes.

4. Snap chill on ice, then add to the filters in the prehybridisation solution.

5. Incubate o/n at 65˚C with shaking.

Method C: Post-hybridisation filter washes and autoradiography

1. Taking the appropriate precautions to prevent radioactive exposure, discard the hybridisation buffer and briefly rinse each filter in 2× SSC.

2. Wash the filters in 1 l of 2× SSC for 5 minutes at room temperature on an orbital shaker.

3. Wash the filters twice for 30 minutes in 1 l of 0.5× SSC/ 1% sarcosyl at 65˚C on an orbital shaker.

4. Wash the filters twice for 5 minutes in 1 l of 0.2× SSC at room temperature on an orbital shaker.

5. After blotting off any excess liquid, lay the filters face-down on a clean sheet of Saran-wrap. Place a second sheet of Saran-wrap on top of the filters and fold over the edges to prevent the filters from over-drying.

6. Expose the filters to () film in an autoradiograph cassette with a () screen for at least 4 h at –70˚C.

*2.11.9 Colony PCR*

Library colonies were analysed for the presence of STSs by colony PCR using the following method:

1. In a safety cabinet, touch a sterile toothpick onto a well-separated colony.

2. Suspend the cell sample by swizzling the toothpick 100 µl of $T_{0.1}E$ in a 96-well microtitre plate. Store the microtitre plate at 4˚C until use.

3. Make a colony PCR master mixture of an appropriate volume by combining multiples of the following single reaction volumes:

    - 6.175 µl $T_{0.1}E$
    - 1.5 µl 10× NEB PCR buffer
    - 0.75µl dNTP

- 0.495 µl BSA (5 mg/ml stock)
- 0.21 µl 2-mercaptoethanol (1/20 stock)
- 0.12 µl (0.6 U) AmpliTaq Polymerase

4. Aliquot 9.25 µl per reaction in a microtitre plate and add 5 µl of $T_{0.1}E$ from each colony sample.

5. Add 0.75 µl of primer mixture (100 ng/µl) and seal the wells with a rubber plate-sealer.

6. Place the plate on a thermal cycler and perform PCR using the following cycling profile:

    - 94˚C for 5 minutes

    followed by 20 cycles of:

    - 93˚C for 30 seconds
    - 60˚C for 30 seconds
    - 72˚C for 30 seconds

    followed by:

    - 72˚C for 5 minutes

7. Add 3µl of 6x loading buffer to each reaction well. Analyse 10 µl of the PCR products by electrophoresis on a 2.5% agarose gel.

**2.12 Procedures for the construction of a vectorette library from a gibbon cosmid clone**

*2.12.1 Restriction enzyme digestion of a gibbon cosmid clone*

In preparation for library construction, gibbon cosmid clones were restriction digested by the following method:

1. In a 1.5 ml microfuge tube, mix the following:

- X µl sterile distilled water (to a final volume of 18 µl)
- 2 µl 10× manufacturer's recommended restriction enzyme buffer
- 0.2 µl 100 mM spermidine
- Y µl (200 ng) DNA

2. Pipette the tube contents up and down to mix and briefly spin in a microfuge to bring the liquid down.

3. Add 1 µl of restriction enzyme and pipette up and down to rinse the tip.

4. Incubate for 1 h at 37˚C in an oven.

5. Add a further 1 µl of restriction enzyme, again pipetting up and down to rinse the tip.

6. Incubate for 2 h at 37˚C in an oven.

7. At room temperature, remove 10 µl of the digested sample and mix it with 3 µl of 6× loading buffer. Analyse the sample by electrophoresis on a 1% agarose gel. Include a control sample of 150 ng of uncut DNA on the gel.

8. To the remainder of the digested DNA, add 40 µl of sterile distilled water.

9. Add 5 µl of 3 M sodium acetate and mix well. Add 100 µl of ice-cold absolute ethanol and mix well. Incubate o/n at –20˚C to precipitate the DNA.

*2.12.2 Ligation of digested cosmid DNA to vectorette bubbles*

Digested gibbon cosmid DNA was ligated to annealed vectorette bubbles by the following method:

1. Pellet the DNA from *2.12.1* by microfuging at 12,000 *g* for 15 minutes at room temperature. Discard the supernatant and wash the pellet with 500 µl of ice-cold 70% ethanol.

2. Microfuge at 12,000 *g* for 10 minutes, discard the supernatant and air-dry the DNA by incubating at room temperature for 30 minutes with the tube lid open.

3. Resuspend the DNA in 100 µl of 1× vectorette ligation buffer on ice.

4. Add 10 µl of 1 pmol/µl annealed vectorette bubbles (appropriate for the restriction enzyme used) and pipette up and down to mix.

5. Add 1.1 µl of rATP, followed by 0.5 µl (2.5 U) of T4 DNA ligase (Boehringer Mannheim).

6. Pipette up and down gently to mix, then incubate for 1 h at 37˚C in a hot block.

7. Dilute the reaction by adding $T_{0.1}E$ to a final volume of 500 µl.

8. Store the vectorette library frozen at –20˚C.

*2.12.3 Rescue of unknown gibbon sequences by vectorette PCR*

Unknown gibbon sequences were rescued by vectorette PCR by the following method:

1. Make a vectorette PCR master mixture of an appropriate volume by combining multiples of the following single reaction volumes:

    - 9.425 µl $T_{0.1}E$
    - 1.5 µl 10× NEB PCR buffer
    - 0.75µl dNTP
    - 0.495 µl BSA (5 mg/ml stock)
    - 0.21 µl 2-mercaptoethanol (1/20 stock)
    - 0.12 µl (0.6 U) AmpliTaq Polymerase

2. Aliquot 13.25 µl per reaction in a microtitre plate and add 1 µl of vectorette library template.

3. Add 0.75 µl of each primer (100 ng/µl).

4. Place the plate on a thermal cycler and perform PCR using the following cycling profile:

    - 94˚C for 5 minutes

    followed by 35 cycles of:

    - 94˚C for 30 seconds
    - 55˚C/60˚C/65˚C for 30 seconds
    - 72˚C for 3 minutes

    followed by:

    - 72˚C for 5 minutes

5. Add 3 µl of 6× loading buffer to each microtitre well, and analyse 10 µl of the vectorette PCR products by electrophoresis on a 2.5% agarose gel.

6. With the agarose gel on a transilluminator, excise vectorette PCR product bands of appropriate size and specificity using sterile inoculating loops. Transfer the excised gel pieces to PCR tubes containing 50 µl of $T_{0.1}E$, and store at 4˚C.