

### **3 Methods**

#### **3.1 Isolation of DNA**

##### **3.1.1 Isolation of human genomic DNA from peripheral blood**

The blood sample was mixed with an equal volume of cold PBS and blood cells were collected by centrifugation at 1500 rpm for 10 min at 4°C. The blood cell pellet was first resuspended in 10-15 ml cold nuclei extraction buffer. The cell suspension was mixed with an additional 20-30 ml nuclei extraction buffer and placed on ice for 30 min. Cells were harvested by centrifugation at 2500 rpm for 10 min at 4°C and resuspended in 500µl DNA extraction buffer. An equal volume of DNA extraction buffer containing SDS and proteinase K was added slowly and the solution was incubated at 50°C overnight. The DNA solution was stepwise extracted with an equal volume of phenol, phenol/chloroform (1:1), and chloroform. The aqueous phase was separated from organic phase by centrifugation at 2500 rpm for 10 min. DNA was precipitated from the water phase with ethanol (EtOH) and washed with 70% ethanol. DNA was dissolved in 500 µl TE containing RNase. The DNA solution was incubated at 37°C for 30 min and stored at 4°C.

##### **3.1.2 Isolation of plasmid or cosmid DNA**

Bacteria were harvested from 1.5 ml of overnight culture by centrifugation at 9000 rpm for 1 min and resuspended in 100 µl Lysis buffer I. After addition of 200 µl Lysis buffer II, the cell suspension was incubated on ice for 5 min and mixed with 150 µl Lysis buffer III. Cell lysates were separated by centrifugation at 13000 rpm for 10 min. The aqueous phase was extracted with 500 µl phenol/chloroform (1:1) and centrifuged at 13000 rpm for 5 min. DNA in the aqueous phase was precipitated with 1 ml EtOH at -20 °C for 30 min and then collected by centrifugation at 13000 rpm for 10 min. The DNA pellet was washed with 200 µl 70% EtOH and air dried for 10 min. DNA was dissolved with 100 µl TE buffer with RNase and stored at -20°C.

##### **3.1.3 Isolation PAC or BAC DNA**

Bacteria containing PAC or BAC DNA were cultured in media containing kanamycin or chloramphenicol, respectively. Bacteria were grown as 1.5 ml culture for up to 16 hr at 37°C with vigorous shaking. Bacteria were harvested by centrifugation at 3000 rpm for 10 min. The pellet was resuspended in 0.3 ml P1 solution, mixed with 0.3 ml P2 solution, and placed at room temperature for 5 min. Cell lysates were mixed with 0.3 ml P3, incubated on ice for 5 min, and cleared by centrifugation. The DNA from the aqueous phase was precipitated by addition of 0.8 ml ice-cold isopropanol and collected by centrifugation at 13000rpm for 15 min at 4°C. The DNA pellet was washed with 0.5 ml of 70% EtOH and air dried at room temperature. When the color of DNA pellet turned from white to translucent, DNA was resuspended in 40 µl TE and stored at -20°C.

##### **3.1.4 Isolation of YAC DNA**

Yeast were cultured in 30 ml (-)Ura (-)Trp Yeast medium at 30°C for 2-3 days. Yeast cells were harvested by centrifugation at 3000 rpm for 10 min and the pellet was washed with 20 ml TE. The yeast cell pellet was resuspended in 500 µl of DNA extraction solution and

incubated at 50°C for 5 hr. The solution was stepwise extracted with 500 µl phenol, 500 µl phenol/chloroform (1:1) and 500 µl chloroform. The aqueous phase was separated from the organic phase by centrifugation at 13000 rpm for 15 min. DNA was precipitated from the aqueous phase with 1 ml EtOH containing 50 µl 3M NaOAc at -20°C for 30 min and collected by centrifugation. The DNA pellet was dissolved in 500 µl TE.

### **3.1.5 DNA extraction from agarose gel slices**

Macherey-Nagel Nucleospin Extract kit was used to extract DNA from agarose gel slices and isolate PCR products from PCR reaction mixtures.

The desired DNA fragment was excised from an agarose gel and mixed with 300 µl NT1 for each 100 mg agarose gel slices. The agarose gel slices in NT1 buffer were dissolved at 50°C for 10 min and then applied to a Nucleospin Extract column. The DNA in solution was adsorbed to the column during centrifugation. The column was washed with 600 µl NT3 buffer and 200 µl NT3 buffer. The DNA was eluted with 40 µl NE elution buffer by centrifugation.

### **3.1.6 Purification of Polymerase Chain Reaction (PCR) Products**

PCR product (20 µl) was mixed with 80 µl NT2 buffer and applied to a Nucleospin Extract column. The spin column was centrifuged, the flowthrough discarded and the column was washed with 600 µl NT3 buffer then twice with 200 µl NT3 buffer. The DNA was eluted with 40 µl NE elution buffer by centrifugation.

## **3.2 DNA Quantitation**

Absorption (A) of the sample was measured at several different wavelengths to assess purity and concentration of nucleic acids.  $A_{260}$  measurement is quantitative for pure nucleic acid preparations in microgram quantities. The ratio of absorption at 260 and 280 nm was used as an indicator of nucleic acid purity. The spectrophotometer was set to zero at 260 nm by using TE buffer as a reference. The reference was removed and the DNA solution was pipetted into the cuvette and read at 260 nm. The same procedure was performed at 280 nm.

To determine the concentration (C) of DNA, the  $A_{260}$  reading was used in combination with the following equation: Double-stranded DNA:  $C (\mu\text{g/ml}) = A_{260} / 0.020$

The value of  $A_{260} / A_{280} > 1.8$  indicated highly purified preparation of DNA. Proteins that absorbed at 280 nm reduced this ratio.

## **3.3 DNA gel electrophoresis**

DNA molecules carry an overall negative electric charge due to phosphate groups. Consequently, when DNA molecules are placed in an electric field they migrate towards the positive pole. Different sizes of DNA fragments can be separated in a gel which is usually made of agarose or polyacrylamide. The gel matrix is composed of a complex network of pores through which DNA molecules must pass to reach the positive pole. The larger the DNA molecule, the slower it migrates through the gel. Thus, different sizes of DNA can be separated through gel electrophoresis. Usually, agarose gels were used for separating DNA fragments which were longer than 500 bp and polyacrylamide gels are used for those fragments shorter than 500 bp.

### 3.3.1 Agarose gel electrophoresis

For 500 to 20 000 bp DNA fragments, a range of agarose gel concentrations from 0.5 to 2% (w/v) was usually used. The higher the agarose gel concentration, the better separation obtained for smaller DNA fragments. The suitable gel concentrations for separating different sizes of DNA fragments are listed in *Current Protocol in Molecular Biology* (Ausbel *et al.*<sup>46</sup>) and shown below.

Agarose gel (%)	Separation of DNA fragments(kb)
0.5	1~30
0.7	0.8 ~12
1.0	0.5 ~10
1.2	0.4~7
1.5	0.2~3

Agarose gels were prepared as follows:

Agarose was mixed with TAE buffer and boiled for 3 min. The melted gel solution was cooled down and poured into a gel chamber. The comb was set into the gel before the gel solution became solid. Electrophoresis was carried out in TAE buffer. The DNA sample was mixed with loading dye before loading to the gel. The dye ran as size indicator during electrophoresis. Usually, the voltage used for agarose gel electrophoresis was not more than 100 V. The gel was stained in an ethidium bromide (EtBr) solution (~2.4 µg/ml) for 15 min. The EtBr molecules intercalate with the DNA helix structure, and the EtBr-DNA complex is fluorescent under UV( $\lambda=254$  or 300 nm) irradiation allowing DNA fragments on the gel to be visualized.

### 3.3.2 Pulsed field gel electrophoresis (PFGE)

Pulsed field electrophoresis is a technique for resolving large DNA molecules up to chromosomes which are not able to be separated by a standard agarose gel electrophoresis. By changing the electric field between spatially distinct pairs of electrodes, megabase sized DNAs are able to reorient and migrate at different speeds through the pores in an agarose gel. According to the instruction manual of Bio-Rad CHEF-DR III system, there are several parameters influencing PFGE.

- Agarose concentration: Gel concentration affects the size range of DNA molecules to be separated, and the sharpness or tightness of the bands. A 1.0% (w/v) agarose gel is useful for separation of DNA molecules up to 3 Mb in size. Gel concentrations between 0.5 and 0.9% are useful for extremely high molecular weight DNA of greater than 3 Mb. For improving band tightness, 1.2- 1.5% agarose concentrations are used. However, running time increases proportionately.
- Buffer concentration and temperature: In PFGE, the mobility of the DNA is sensitive to changes in buffer temperature. It is recommended that the buffer is chilled to 14°C to maintain band sharpness and to dissipate heat generated during prolonged electrophoresis. Tris-Borate-EDTA (TBE) buffer at a concentration of 0.5x is commonly used.
- Switch times: In PFGE, DNA molecules change direction or reorient themselves in the gel matrix according to the switches of electric field. Larger molecules take longer to reorient and therefore have less time to move during each pulse, so they migrate slower than smaller molecules. As DNA size increases, an increased switch time is needed to resolve DNA of different sizes.

- d. Voltage: DNA migration increases with increasing voltage. In general, lower voltage should be applied with increasing sizes. At high field strengths (6 V/cm), some very large DNAs (>3 Mb) can not be resolved on the gel and the voltage needs to be reduced. Some large DNA molecules do not enter the gels at high electric field strengths. Therefore, in selecting the field strength, a compromise between running time and resolution has to be made.
- e. Field angle: Movement of DNA molecules up to 1 Mb is independent of the angle between 90° and 120°. Decreasing the included angle will decrease the resolution of smaller DNAs by causing them to pile up on each other. The field angle should be decreased to less than 120° when separating DNA fragments greater than 2 Mb.
- f. Electrophoresis running time: The running time is determined by the migration rates of the DNA molecules. As the migration rate of the DNA decreases, the running time needs to be increased to adequately resolve the DNA fragments.

### **3.3.2.1 Sample Preparation**

Large DNA fragments, such as YACs, are so fragile that they are sheared by mechanical forces during preparation. To avoid breakage of large DNA molecules, intact cells embedded in agarose were lysed and deproteinized in agarose blocks.

#### **3.3.2.1.1 YAC Plug Preparation**

The method of yeast culture in medium was the same as described previously<sup>45</sup>. The cells were harvested by centrifugation. The pellet was washed with 20 ml TE followed by 20 ml SCE. The cells were collected by centrifugation at 3000 rpm for 10 min and resuspended in 800 µl Yeast lysis buffer (8 mg lysozyme /ml SCE). The cell solution was mixed with 800 µl 1.5% LMP agarose in SCE at 50°C. The mixtures were aliquoted to each block on ice. The agarose blocks were transferred into 10 ml SCE containing 10 mM DTT and incubated at 37°C for 60 min. The blocks were transferred into 10 ml proteinase K solution and incubated at 50°C for 48 hr, following two washes of 15 ml TE for 15 min. The blocks were incubated in 10 ml TE containing 0.04 mg/ml PMSF at 50°C for 30 min and washed two times with TE as described previously. The blocks were stored in 20 ml 0.5 M EDTA pH 8.0 at 4°C.

#### **3.3.2.1.2 Liquid Samples**

PAC and BAC DNA were digested with *Not* I before carrying out PFGE, the DNA solution can be directly loaded into the wells of the gel. In addition, using a thin well comb (0.75 mm) improved the resolution and sharpness of the bands.

### **3.3.2.2 Parameters for PFGE**

Equipment: Bio-Rad CHEF-DR III system

Running buffer: 0.5 x TBE

Gel: 1% Agarose-Pulse Field in 0.5 x TBE buffer

	YAC	PAC/BAC
DNA	1/3 YAC plug	15 µl (~100ng)
Switch time	50→100 sec	1→20 sec
voltage	6 V	5V
Angle	120°	120°
Time	24hr	22hr

The PFGE was performed in 0.5 x TBE buffer at 14°C. The gel was stained for 30 min in 500 ml 0.5 x TBE containing 100 µl EtBr (10 mg/ml) and destained for 10min in 0.5 x TBE. DNAs were visualized by UV irradiation. YAC sizes were determined by using the chromosomes of *Saccharomyces cerevisiae* (strain YPH49) as size markers which range from 260 kb to 2000 kb. BAC and PAC sizes were determined by using 50 kb size ladders (New England BioLabs) which range from 48.5 kb -1000 kb.

### 3.3.3 Denaturing polyacrylamide gel electrophoresis

Denaturing polyacrylamide gels can distinguish molecules differing in length by just a single nucleotide. In this study, denaturing polyacrylamide gels are used to separate the sequencing products and the PCR products which were amplified by different polymorphic markers.

Denaturing gels were prepared as follows:

After addition of 40 µl TEMED and 150 µl 10% APS, 60 ml of acrylamide gel solution was poured. The composition of acrylamide gel solution is as follows.

Components	6% Polyacrylamide gel	8% Polyacrylamide gel
10 M Urea	375 ml	350 ml
10 X TBE	50 ml	50 ml
40% Acrylamide/bis-acrylamide	75 ml	100 ml
total volume: 500 ml		

Electrophoresis:

The gel was prerun at 60 W for a minimum of 30 min. Samples were denatured with the loading buffer at 94 °C for 5 min before loading on the denaturing gel. Running time depended on the size of the desired DNA fragment using the loading dye as a size indicator. The relationship between gel concentration and dye mobility was adapted from *Current Protocol in Molecular Biology* and is shown below.

Polyacrylamide gel (%)	Migration of bromphenol blue (bases)	Migration of xylene cyanol (bases)
6	26	105
8	19	75

After electrophoresis, the gel was dried on Whatman 3MM paper for 45 min at 75 °C on a gel dryer and subjected to autoradiography. Autoradiography required a few hours to several days depending on the intensity of the signal.

### 3.3.4 Single-stranded conformation polymorphism gel electrophoresis

Single-stranded conformation polymorphism (SSCP) analysis was used to detect mutations based on single-nucleotide changes in DNA sequences that alter the mobility of single-

stranded DNA in nondenaturing gels. The DNA fragments containing mutations were amplified using polymerase chain reaction (PCR) and rendered single-stranded by heating in a denaturing buffer. The denatured strands were separated on polyacrylamide gels under conditions that resolve two molecules by conformational changes caused by differences in as little as one base.

SSCP gels were prepared as follows:

For 0.5 x MDE gel	15 ml	2 x MDE gel solution
	3.6 ml	10 x TBE buffer
	41.4 ml	dH <sub>2</sub> O

The gel solution was mixed with 500 µl APS and 50 µl TEMED and allowed to polymerize. The electrophoresis was carried out in 0.6 x TBE .

Electrophoresis:

The desired DNA fragments were amplified by a standard PCR procedure incorporating [ $\alpha$ -P<sup>32</sup>] dCTP in the amplified fragments. PCR stop buffer was added to 20 µl of PCR product and samples were denatured at 90°C for 3 min before loading on a 0.5 x MDE gel. Electrophoresis was carried out at 400 V at 4°C overnight. The gel was dried and subjected to autoradiography.

### 3.4 Enzymatic manipulation of DNA

#### 3.4.1 Digestion of DNA with restriction endonucleases

Restriction endonucleases are isolated from bacteria and can recognize short DNA sequences and cleave double-stranded DNA at specific sites within or adjacent to recognition sequences. The amount of enzyme, DNA, buffer and ionic concentration, and the temperature and duration of the reaction were adjusted according to different applications.

##### 3.4.1.1 Single digestion

Single digestion refers to digesting a single DNA sample with a single restriction endonuclease. A total of 20 µl reaction including 0.1~ 4 µg DNA, 1 to 5 U restriction endonuclease /µg DNA and specific buffer concentration was convenient for analysis by gel electrophoresis. The reaction was incubated at 37°C for 1.5-2 hr and stopped at 70°C for 10 min. The volume of restriction endonucleases was not more than 1/10 the reaction volume because glycerol in enzyme buffer can interfere with the reaction.

##### 3.4.1.2 Double digestion

Double digestion refers to digesting a single DNA sample with two different restriction enzymes. Many restriction endonucleases are active in a wide variety of buffers, so it is usually possible to choose a standard buffer in which two enzymes can retain their activities. Alternatively, DNA can be first digested with the restriction enzyme in low salt buffer followed by the adjusting the reaction mixtures to a high salt condition and digested with the other enzyme.

### 3.4.1.3. Restriction endonuclease digestion of YAC plugs

To cleave the desired DNA fragments from YAC plugs, it was necessary to treat the YAC plugs before restriction enzyme digestion. One YAC DNA plug was cut into three slices and the slices were washed twice with TE for 30 min. The slice was mixed with 60  $\mu$ l dH<sub>2</sub>O and 10  $\mu$ l 10 x restriction enzyme buffer and dissolved at 70°C for 20 min. The sample was cooled down to 37°C and 15-30 U of restriction enzyme were added. The reaction was incubated at 37°C for 1.5-2 hr.

### 3.4.2 Dephosphorylation of DNA with calf intestine phosphatase (CIP)

Calf intestine phosphatase catalyzes the hydrolysis of 5'-phosphate residues from DNA, and therefore can be used to prevent self-ligation of cloning vector termini. The CIP reaction conditions for dephosphorylation of DNA were as follows:

10 x CIP buffer	5 $\mu$ l
dH <sub>2</sub> O	14.5 $\mu$ l
Vector-DNA(0.5-1.0 pmol / $\mu$ l)	30 $\mu$ l
CIP (20 U/ $\mu$ l)	0.5 $\mu$ l
total volume: 50 $\mu$ l	

The reaction was incubated at 37°C for 1 hr and stopped at 75 °C for 10 min. The dephosphorylated DNA was precipitated with ethanol as described previously.

### 3.4.3 Preparation of blunt ends by repairing of 5' Overhangs with Klenow Fragment

If the 5' termini of vector and insert DNAs were incompatible, both could be modified with Klenow fragment to generate blunt ends for ligation. The reaction conditions are as follows:

DNA (1-2 $\mu$ g)	15 $\mu$ l
10 x reaction buffer	2.5 $\mu$ l
dH <sub>2</sub> O	2.5 $\mu$ l
dNTP mix (125 $\mu$ M)	4 $\mu$ l
Klenow-polymerase (1-5U)	1 $\mu$ l
total volume: 25 $\mu$ l	

The reaction was incubated at room temperature for 20 min and stopped at 75°C for 10 min. The DNA was precipitated with ethanol.

### 3.4.4 Ligation of DNA with T4 DNA ligase

T4 ligase, originally isolated from phage T4, catalyzes the formation of phosphodiester bonds between 5' phosphate and 3' hydroxyl termini in duplex DNAs. Using ATP as a cofactor, T4 DNA ligase catalyzes the repair of single-stranded nicks in duplex DNA and join duplex DNA restriction fragments with either blunt or sticky ends. The standard reaction conditions are as follows:

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Vector DNA (100 ng/μl)	1 μl
Insert DNA (100 ng/μl)	4 μl
10 X ligation buffer	1 μl
dH <sub>2</sub> O	3 μl
T4 DNA ligase	1 μl

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total volume: 10μl

Ligation was performed at 16°C for 16 hr and stopped by incubation at 75°C for 10 min. The ligation product was ready for transformation. The pBluescript II KS (+) was used as a vector in this study to ligate CAG positive fragments subcloned from a cosmid library.

Another method is used when insert DNA is embedded in low melting point (LMP) agarose gel. The procedure is as follows:

An agarose slice containing the desired DNA fragment was excised from a 0.7% LMP gel. The excised agarose volume should not be more than 20 μl. The agarose slice was dissolved in 60 μl dH<sub>2</sub>O at 70°C for 10 min until the agarose slice was dissolved. The agarose concentration was less than 0.2% in the solution. The sample was incubated at 32°C and added with 1 μl vector DNA (100 ng), 8 μl 10 x ligation buffer, 2 μl 10 mM ATP, 1 μl T4 DNA ligase. Ligation was performed at 32°C overnight and stopped by incubation at 75°C for 10 min. The ligation product was ready for transformation.

### **3.5 Transformation of *E.coli* cells with plasmids**

#### **3.5.1 Preparation of Competent Cells**

A single colony of *E. coli* (JM83 or XL1-Blue) from a freshly streaked LB agar plate was transferred into 50 ml LB medium followed by incubation at 37°C overnight with vigorous shaking. A 100 μl aliquot of the bacterial culture was transferred to 100 ml dYT medium and incubated at 37°C with shaking until the bacterial concentration reached  $5 \times 10^7$  cells/ml ( $O.D \approx 0.5$ ). Bacteria were collected by centrifugation at 2500 rpm at 4°C for 10 min. The cell pellet was resuspended in 15 ml TBF I and incubated on ice for 60 min. Bacteria were collected by centrifugation at 2500 rpm at 4°C for 10 min and resuspended in 2 ml TBF II. Competent cells were aliquoted and stored at -70°C.

#### **3.5.2 Transformation**

Competent cells were thawed on ice for 5 min. Plasmid DNA was mixed with the competent cells, the mixture was incubated on ice for 15 min followed by heat shock at 45°C for 90 sec in a water bath. A 200 μl aliquot of dYT was added to the cells followed by incubation at 37°C for 45 min. Cells were spread on the LB-antibiotics agar plates and incubated at 37°C overnight.

### **3.6 Southern Blotting**

Southern blotting is the transfer of DNA fragments from an agarose gel to a membrane via upward capillary transfer of a high-salt buffer and subsequent hybridization with a complementary probe. The transfer results in immobilization of the DNA fragments so that the membrane carries a semipermanent copy of the banding pattern of the gel. After



immobilization, the DNA can be used for hybridization analysis which facilitate identification of bands with a sequence identical to a labeled probe.

DNA fragments were separated by agarose gel electrophoresis, denatured in denaturing solution for 1 hr, and soaked for 45 min in a neutralization solution. The blotting apparatus was set up in a chamber containing 20 x SSC and Whatman 3 MM paper saturated with 20 x SSC. The gel was turned upside down on the saturated Whatman 3 MM paper. The blotting membrane (Hybond N/N+) was laid exactly on the gel. Two sheets of Whatman 3 MM paper were placed on the blotting membrane. Additional paper towels were placed on the Whatman 3 MM paper. A 500 g weight was placed on top of this pile of papers and transfer of the DNA to the membrane was allowed to proceed overnight. The blotting membrane was labeled at the well edges and baked in a vacuum oven at 80°C for 2 hr.

### **3.7 Hybridization**

The principle of hybridization is that a single-stranded DNA or RNA molecule (the so-called probe) can bind to a single-stranded DNA or RNA with a complementary sequence. The stability of the hybrid depends on the extent of the base pairing and base composition. In practice, a labeled probe is used to hybridize the target DNA that has been immobilized on a membrane. Hybridization analysis is usually performed with a radiolabeled DNA probe (100-1000 bp) and are sensitive enough to detect single-copy genes in complex genomes.

#### **3.7.1 DNA probe preparation**

##### **3.7.1.1 Random primers labeling**

Appligene-Oncor Nonaprimer labeling kit was used for random prime labeling and purification. A 1 µl aliquot of human genomic DNA (~300 ng) was mixed with 6 µl dH<sub>2</sub>O and denatured at 95-100°C for 10 min. The denatured DNA was chilled on ice for 5 min and mixed with 4 µl Nonaprimer mix, 3 µl dATP/dTTP/dGTP (1:1:1), 5 µl [ $\alpha$ -P<sup>32</sup>]dCTP and 1 µl Klenow enzyme. The reaction mixture was incubated at 37°C for 30 min. The [ $\alpha$ -P<sup>32</sup>]dCTP labeled DNA fragments were mixed with 60 µl adsorb solution and 2 µl DNAprep resin. The resin was centrifuged and washed twice with 100 µl wash solution. The labeled DNA fragments were eluted thrice with 100 µl elution solution.

##### **3.7.1.2 5'-end labeling of oligonucleotides**

The reaction mixture composed of 1 µl (15 pmol/µl) oligonucleotide, 1 µl 10x reaction buffer, 4 µl [ $\gamma$ -P<sup>32</sup>]ATP, 2 µl dH<sub>2</sub>O and 2 µl (1:10 diluted) T4 polynucleotide kinase incubated at 37°C for 1 hr. The reaction was stopped at 65°C for 10 min. The oligonucleotide was precipitated by a solution containing 30 µl EtOH, 1 µl Glycogen and 1.1 µl 3 M NaOAc at -20°C for 30 min. The oligonucleotide was collected by centrifugation, washed with 70% EtOH and dissolved in 100 µl TE.

#### **3.7.2 Prehybridization and hybridization**

The membrane was prehybridized in prehybridization solution ( $\geq 1$  ml /10 cm<sup>2</sup> membrane) at 42°C for at least 4 hr. The DNA probe was denatured at 94 °C for 10 min and added to hybridization solution. The membrane was transferred to the hybridization solution and

incubated at 42 °C overnight. The membrane was stepwise washed by increasing washing stringency. The procedures were as follows:

<u>Washing buffers</u>	<u>Temperature</u>	<u>Time</u>
4 X SSC	room temperature	3 x 15 min
2 X SSC/0.5% SDS	45°C	20 min
1 X SSC/0.5% SDS	45°C	20 min
0.5 X SSC/0.2% SDS	60°C	30 min
0.2 X SSC/0.5% SDS	60°C	30 min
0.1 X SSC/0.1% SDS	60°C	30 min

The radioactive signals were controlled with Geiger counter to avoid an over stringent wash. The membrane was packed in plastic wrap and subjected to autoradiography.

### 3.8 Polymerase chain reaction (PCR)

PCR is a rapid method for *in vitro* amplification of a specific fragments of DNA. The basis of PCR is that primers hybridize to DNA templates and then DNA polymerases synthesize the DNA segments between two primers from 5'→ 3'. These products accumulate with each subsequent cycle of denaturation, annealing to primers, and amplification. If *n* cycles of PCR are carried out,  $2^{n-2}$  fold amplification of product can be synthesized. PCR was performed in 1x PCR buffer containing 0.25 M of each dNTP, 0.5 µM of forward/reverse primers, 2.5 U Taq polymerase and 30 ng genomic DNA in a reaction volume of 20 µl. The standard PCR conditions were 30 cycles at: 94 °C for 30 sec, 45-65°C for 60 sec, and 72°C for 15 sec. The reaction was preceded by 5 min denaturation at 94 °C and followed by a final extension at 72°C for 5 min. To incorporate [ $\alpha^{32}$ P]-dCTP into the PCR product during strands synthesis, a modified dNTP mix was used. The modified dNTP mix contains less dCTP (0.1 mM) and the concentration of dATP, dGTP and dTTP is unchanged. In this case, 0.1 µl [ $\alpha^{32}$ P]-dCTP is used in a 20 µl PCR reaction and the other components of PCR are the same as described above.

### 3.9 Reverse transcriptional polymerase chain reaction (RT-PCR)

#### 3.9.1 Total RNA isolation from human blood

QIAamp RNA Mini kit was used for isolation of total RNA. Human blood (2 ml) was mixed with 10ml buffer EL and incubated for 15 min on ice. Blood cells were collected by centrifugation at 400 x g for 10 min at 4°C. The cells pellet was resuspended into 4 ml buffer EL and repelleted. Pelleted leukocytes were resuspended into 600 µl buffer RLT containing  $\beta$ -mercaptoethanol. Lysates were transferred into a QIA shredder spin column and homogenized by centrifugation for 2 min at maximum speed. The homogenized lysates were mixed with 600 µl 70% ethanol and transferred into a new QIAamp spin column. The nucleic acids were adsorbed to the column by centrifugation for 15 sec at  $\geq 8000$  x g. The QIAamp spin column was washed with 700 µl buffer RW1 followed by 500 µl buffer RPE containing ethanol. RNA was eluted twice with 30-50 µl RNase-free water and stored at -80°C.

### 3.9.2 First strand cDNA synthesis and RT-PCR

An 11 µl aliquot of total RNA (1-5 µg) was mixed with 1 µl Oligo (dT)<sub>15</sub> (500µg/ml) and heated at 70°C for 10 min. To the mixtures of RNA and Oligo (dT) 4 µl 5x first strand buffer, 2 µl 0.1 M DTT and 1 µl 10 mM dNTP mix were added. This mixture was first incubated at 42°C for 2 min before the addition of 1 µl (200 U) SUPERScript II. The cDNA synthesis reaction was incubated at 42°C for 50 min and inactivated at 70°C for 15 min. The cDNA was ready for PCR amplification. The standard PCR procedure was performed by using 1-2 µl cDNA as template. The primers for amplifying *SPAST* cDNA are listed.

Table 3. Primers used for *SPAST* cDNA amplification and sequencing

Primer	Sequence (5'→3')	T <sub>m</sub> (°C)	Product size (bp)
SPA_Db	TAGCAGTGGCTGCCGCCGT	62	655
SPA_Dm	AAGCGGTCCTTGGCCATAAC		
SPA_Dc	GGCGGCAGTGAGAGCTGTG	60	543
SPA_Dn	CTAGCTCTTTCACACTGTTC		
SPA_Ad	AACAGGCCTTCGAGTACATC	60	746
SPA_Am	CTGTGAACAACCTCAGGCCTC		
SPA_Ba	CTACAACCTGCTACTCGTAAG	58	763
SPA_Bm	CAGTGCTGCATCTTTTGCC		
SPA_Ca	TGGAGATGACAGAGTACTTG	56	766
SPA_Cm	CTGGAATACTTTCATCTGC		

The primers are adapted from the database of Genoscope, Centre National de Séquenage, France.( <http://www.genoscope.cns.fr/>)

### 3.10 DNA sequencing

Chain termination (or the Sanger method) is the most commonly used technique for sequencing DNA. All reagents needed for *in vitro* DNA synthesis are combined in this reaction, including a DNA polymerase and a 2', 3'-dideoxynucleotide (ddNTP). ddNTPs can be incorporated by DNA polymerases into growing DNA chains through 5' phosphate groups. However, the 3'-OH group necessary for phosphodiester bond formation and chain elongation is lacking so the chains terminate at the exact point at which the ddNTP is incorporated. Four sets of reactions are performed on each template, differing only by ddNTPs species. The dNTP:ddNTP ratio is selected so that the resulting labeled strands formed a nested set of molecules up to several thousand bases long, each terminating at a specific base. These are able to be separated according to size by denaturing acrylamide gel electrophoresis.

#### 3.10.1 Sequencing plasmid DNA with thermo sequenase fluorescent labeled primer cycle sequencing kit

In this study, a thermo sequenase fluorescent labeled primer cycle sequencing kit was used to sequence the subcloned DNA fragments derived from the cosmid library.

To denature the template DNA, 10  $\mu$ l plasmid DNA ( $\sim 2.0\mu$ g) was mixed with 2  $\mu$ l 2 M NaOH and incubated at room temperature for 10 min. The denatured DNA was precipitated with EtOH and dissolved in 23  $\mu$ l sterile d H<sub>2</sub>O.

Two common primers, M13 and reverse primers, were used to sequence the insert DNA cloned into pBluescript II KS (+). The sequences of primers are as follows:

M13(-20) primer: 5'-GTAAAACGACGGCCAGT -3'

Reverse primer : 5'-GGAAACAGCTATGACCATG-3'

The sequencing mixture comprising 1  $\mu$ l DMSO and 1.5  $\mu$ l fluorescent labeled primer (1 pmol/ $\mu$ l) was mixed with denatured 23  $\mu$ l of plasmid DNA solution. Aliquots of 6  $\mu$ l of this mixtures were mixed with 2  $\mu$ l of reagents A, C, G, T, respectively. Cycle sequencing conditions were 30 cycles at 94°C for 15 sec, 60°C for 15 sec, and at 70°C for 15 sec. After cycle sequencing, 4  $\mu$ l stop buffer were added to each reaction and samples were denatured at 90°C for 5 min. The samples were loaded on a 4% denaturing acrylamide gel. Sequences were analyzed by MWG CL4200 autosequencer.

### 3.10.2 Sequencing PCR product with thermo sequenase radiolabeled terminator cycle sequencing kit

A thermal sequenase radiolabeled terminator cycle sequencing kit was used to sequence all 17 exons, 5' UTR, 3'-UTR, intron 6, intron 10, and intron 11 of the *SPAST* gene. Each fragment was amplified by PCR and purified after agarose gel electrophoresis with a Nucleospin extraction kit.

In the cycle sequencing reactions [ $\alpha$ -<sup>33</sup>P]ddNTPs were used as terminators. The sequencing reaction was performed according to the manufacture instructions. The cycle sequencing conditions were 30 cycles at 94°C for 30 sec, T<sub>m</sub> for 30 sec, and 72°C for 90 sec. T<sub>m</sub> was dependent on the sequencing primer used. The cycle sequencing products were loaded on a denaturing polyacrylamide gel and the sequences were read on an autoradiograph. The sequence and T<sub>m</sub> of primers for amplification and sequencing of the various portions of *SPAST* gene are listed.

Table 4. The primers for amplification and sequencing of *SPAST*.

Location	Primer	Sequence (5'→3')	T <sub>m</sub> (°C)	Product size (bp)
5' UTR	spg4utr5af	GCCTTTGCGGTCTGGGTTCTGTGC	64	661
	spg4utr5ar	GGCTTGGGGTCGCTCTGCTGGTTC		
	spg4utr5bf	GGTCGCCTGGCAGAAAAAGAT	62	748
	spg4utr5br	CAAACAGCGGGTAGGAGAAATAGT		
exon1	spgex1pza	GCCACCGACTGCAGGAGGAGAAGG	65	721
	spgex1pzb	CCGCAGAAAAGGGACGCAGGTGTT		
	spgex1pbf	GTCGGTCTGCGGGAGGCGGGTTAT	65	705
	spgex1pbr	TGTGGGGGAAGGCTGGTGTCTGAA		

exon2	spgex2pf spgex2pr	TTTTTATGTATTACCTCTCAA AAAAATAAATAAATAAATAAG	45	266
exon3	spgex3paf spgex3par	CTTCTTTTGGGTATACATTTTCT AGCAAGCGTCCATCTCAA	55	340
exon4	spgex4paf spgex4par	ATTTCTGTTATTTTCGTGACT ATGCAAGCTTTATTATTTTATG	54	335
exon5	spgex5pf spgex5pr	CCTATGAAGATCCTGGTAC TTTATAGCAAGTTGCCCTG	56	806
exon6	spgex6pbf spgex6pbr	TCTCTAGTGAATACAGTTTTACC ACAGGGCCCAGTTATTACAA	60	552
intron6	spgin6pf spgin6pr	TTAAGGGTACTCCGAAAAC GACCAGCTATATCATCAAAT	54	457
exon7	spgex7paf spgex7par	ACTGGGCCCTGTTTGTAT TATCCATTTTCCTATTCTAT	55	352
exon8	spgex8pf spgex8pr	CTGTTTGGGAAGATGCT GTAAATAATAGACTCAAGGACAAG	53	273
exon9	spgex9pf spgex9pr	TGGCCTCATAGCTTACATTTTATAG CCAGCCAGTTTACGGTATTTTATT	55	315
exon10	spgex10pf spgex10pr	GTAGTACTCTCCCCTTTCTCA TAATGTTTCCAATCGTATCTT	55	307
intron10	spgin10pf spgin10pr	GTAGTACTCTCCCCTTTCTC TGCTCCCCTTCTCTTCTTCAC	60	505
exon11	spgex11pf spgex11pr	ACTCACATAGCTTGGTCTT CATGAGTAAATATTGTCTGTAA	55	418
intron11	spgin11pf spgin11pr	AGACGCCTAAAACTGAAT TTGCCTAAAACCGTAACTA	52	482
exon12	spgex12pf spgex12pr	ATGGCCAAGGTAAAAATACAA CTGGAAGAAAATAGTGAAT	54	281

exon13	spgex13pf	CTTTTCCTGTCATTTGCTGTTT	60	311
	spgex13pr	CATTTTGTCTCTCTGGGGTAA		
exon14	spgex14paf	GGAGGCTGAGATGGGAGGATT	60	406
	spgex14par	AAGGCAAAGGAGGTAGAGGATGAG		
exon15	spgex15paf	AAAAAGCGGGAGGGGAAATA	60	328
	spgex15par	GAAGGCTGGGTGGGAGAATCA		
exon16	spgex16paf	TTCAACTGCAAAATGTATGTA	54	250
	spgex16par	ATGGTGAAATGCCCTCTC		
exon17	spgex17p1f	ATAACATTAAGAAACAGCAGCATC	60	845
	spgex17p1r	AAAAGGTAAAAAGAACACAACAAT		
	spgex17p2f	AATTGTTGTGTTCTTTTAC	50	745
	spgex17p2r	TGACATTTTACATAGCATC		
	spgex17pe	GGAATGCCAAACACTCTT	53	312
	spgex17pf	AAAAATACTGCAGGTCACAT		
3' UTR	spg4utr3af	ATAACATTAAGAAACAGCAGCATC	62	850
	spg4utr3ar	AAAAGGTAAAAAGAACACAACAAT		
	spg4utr3bf	AATTGTTGTGTTCTTTTAC	50	747
	spg4utr3br	TGACATTTTACATAGCATC		
	spg4utr3cf	GGAATGCCAAACACTCTT	53	313
	spg4utr3cr	AAAAATACTGCAGGTCACAT		

*Note:* The sequences are adapted from the database of Genoscope, Centre National de Séquençage, France ( <http://www.genoscope.cns.fr/>). The primers are designed according to the sequence of the exons including at least 30 nucleotides of the preceding and followed introns.

### 3.10.3 Sequencing DNA with a T7 sequenase kit

A T7 sequenase kit was used to prepare a basewise size ladder by using phage DNA M13 as a template. The preparation was as follows:

Master mix A was composed of 20 µl DNA (1~2µg), 4 µl primer (4 ng/µl) and 4 µl annealing buffer. Master mix B was composed of 3.6 µl dilute buffer, 6.5 µl label-mix, 1.6 µl <sup>35</sup>S dATP and 0.4 µl T7 polymerase. Master mix A was first incubated at 60°C for 10 min then at room temperature for 10 min. Master mix A and 12 µl Master mix B were mixed and placed at room temperature for 5 min. Aliquots of 9 µl of this mixture were added

to 5 µl ddATP, ddCTP, ddGTP, and ddTTP. This reaction was incubated at 37°C for 5 min and stopped with 10 µl stop buffer at 90°C for 3 min.

### 3.11 Cosmid library construction

Cosmid vectors are valuable tools for isolation and physical mapping of large fragments of DNA ranging in size from 10 to 42 kb. SuperCos is a cosmid vector containing bacteriophage promoter sequences flanking a unique cloning site. It has been engineered to contain genes for amplification and expression of cosmid clones in eukaryotic cells. In addition, most genomic inserts can be excised as a single large restriction fragment using the *Not* I restriction site that flanks the SuperCos 1 polylinker.

#### 3.11.1 Preparation of host cells

XL1-Blue MR was used as the host cell and its genotype was previously described in Material section. A single colony of XL1-Blue MR was inoculated in 50 ml NZYC medium and incubated at 37°C overnight. The host cells from 10 ml culture were harvested by centrifugation at 2500 rpm for 15 min and resuspended into 25 ml 10 mM MgSO<sub>4</sub>. The host cells were stored for up to 2 days at 4°C.

#### 3.11.2 Preparation of a -80°C glycerol stock

A 10 ml aliquot of LB medium was inoculated with a single colony of host cells and incubated at 37°C for 6-8 hr. The cells culture was mixed with 4.5 ml solution of a sterile glycerol-LB (1:1) medium. The mixtures were aliquoted and stored at -80°C.

#### 3.11.3 Preparation of vector DNA

SuperCos 1 vector (~85 µg) was restricted with 5 µl *Xba* I (20 U/µl) in a total of 100 µl buffer H at 37°C for 2 hr. The linear SuperCos 1 vector is about 7.6 kb. The volume of the digestion reaction mixtures was adjusted to 200 µl with TE. The solution was extracted with 200 µl phenol/chloroform (1:1) and chloroform. The DNA was precipitated with 500 µl EtOH containing 25 µl 3M NaOAc pH5.5 at -20°C for 30 min and collected by centrifugation. The pellet was washed with 70% EtOH and air dried. The DNA was dissolved in 20 µl 1x dephosphorylation buffer. The *Xba* I cut vector DNA was dephosphorylated with 2 µl CIP (10 U/µl) at 37°C for 90 min and extracted with phenol/chloroform as described above. The DNA was dissolved with 74 µl 1X buffer B. The vector was restricted with 6 µl *Bam*H I (20 U/µl) at 37°C for 2 hr. The two DNA fragments are 1.1 and 6.5 kb. The DNA was extracted with phenol/chloroform and dissolved in 20 µl TE. The restricted vector was stored at -20°C.

#### 3.11.4 Partial digestion of YAC DNA

In order to clone DNA fragments into the *Bam*H I site of the SuperCos 1 cosmid vector, the YAC DNA was partially digested with *Sau* 3A. An optimal concentration of *Sau* 3A for obtaining appropriate partial digestion products was previously determined.

The YAC DNA agarose plug was washed twice with TE and dissolved in 120 µl 1x buffer A at 70°C. *Sau* 3A (0.03 U/µl) was added to the solution and incubated at 37°C for 15 min. The reaction was stopped at 70°C for 10 min. The partially digested DNA was dephosphorylated

with 5 µl CIP at 37°C for 60 min. The DNA was extracted with phenol/chloroform, precipitated with EtOH /0.3 M NaOAc pH 5.5, and dissolved in 12 µl TE.

### 3.11.5 Ligation

The reaction mix was composed of 12 µl partially digested YAC DNA [Sau 3A-CIP], 3 µl (1 µg/µl) SuperCos 1 DNA [*Xba* I-CIP/*Bam*H I], 2 µl 10x ligase buffer, and 2 µl 10 mM ATP and 1 µl T4 DNA ligase. The ligation mixture was incubated in a water bath at 32°C overnight.

### 3.11.6 Packaging

The packaging extracts (Gigapack III Gold Packaging Extract; Stratagene) were quickly thawed at room temperature until the contents of the tube began to thaw. The ligation product was added immediately to the packaging extract and stirred gently with a pipette tip. The packaging mixture was spun quickly for 5 sec and incubated at room temperature (~20°C) for 2 hr. To the mixture 470 µl SM buffer and 10 µl Chloroform were added. The cosmid library was ready for titring and could be stored at 4°C for 1 month.

### 3.11.7 Titration of the cosmid packaging reaction

The cosmid library was diluted 1:10 and 1:50 in SM buffer. The diluted cosmid library (25 µl) was mixed with the equal volume of host cells in 10 mM MgSO<sub>4</sub> and incubated at room temperature for 30 min. Host cells were regrown in 200 µl dYT at 37 °C for 60 min and collected by centrifugation at 9000 rpm for 1 min. The cell pellet was resuspended in 50 µl LB medium. The cell suspension was spread on LB-Amp agar plates and incubated at 37 °C overnight. The colony forming units per milliliter (cfu/ml) were estimated.

### 3.11.8 Screening the cosmid library

The titer of the cosmid library was determined by serial dilution as described above. The appropriate amount of host cell suspension for plating was calculated and the suspension was diluted and ~10<sup>4</sup> cfu was spread on the LB-Amp plates (10 x 15 cm in diameter petri dish). The plates were incubated at 37°C overnight. Hybond N/N+ Nylon membranes (15 cm in diameter) were used to perform colony lifts. Host cells were regrown on LB-Amp plates at 37 °C for 4-6 hr until the bacterial colonies began to reform and then stored at 4°C. Colony lift and hybridization on Nylon membranes were prepared as follows.

The membranes were placed colony-side up for 10 minutes on the surface of Whatman 3 MM paper prewetted with 0.5 M NaOH and transferred to a sheet of Whatman 3MM paper prewetted with 1 M Tris-HCl (pH 7.6) for 10 min. Membranes were transferred to a sheet of Whatman 3 MM paper prewetted with 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl for 15 min. Cells debris were removed by carefully brushing the membranes in the solution of 1 M Tris-HCl (pH 7.6) and 1.5 M NaCl. The membranes were air dried and baked at 80°C for 2 hr.

Prehybridization and hybridization were performed at 42°C. The membranes were washed with 4 x SSC, 0.5% SDS buffer followed by washes of increasing stringency until the background diminished. The membranes were packed with plastic wrap and subjected to autoradiography.



### 3.11.9 Purification of the positive clones

The autoradiograph and original host plate were aligned. The positive candidate colonies were transferred in array to a new LB-Amp plate and incubated at 37°C overnight. The selective colonies were rescreened by hybridization as described previously<sup>46</sup>. A 4 ml aliquot of LB-Amp medium was inoculated with each positive clone and incubated at 37°C overnight. The bacterial culture was used for isolating the cosmid DNA as described previously and the remaining 1 ml culture was used for preparation of glycerol stock.

### 3.12 YAC contig construction

#### 3.12.1 Yeast artificial chromosome (YAC)

A total of 26 YACs were chosen by searching databases, the Whitehead Institute for Genome Research (<http://www-genome.wi.mit.edu/>), Genethon (<http://www.genethon.fr/genethon-en.html>), and the previously reported YAC contig (De Jonghe *et al.*<sup>84</sup>) and obtained from CEPH (Centre d'Etude Polymorphisme Humain, Paris, France). The YAC clones were plated on (-)Ura, (-)Trp agar plates and then single colonies were transferred into selective media for YAC DNA isolation using standard procedures.

#### 3.12.2 Sequence tagged site (STS)

STS markers which had been assigned to 2p21-p24 (<http://www.ncbi.nlm.nih.gov/SCIENCE96>, <http://www.ncbi.nlm.nih.gov/genemap98>, <http://www.ncbi.nlm.nih.gov/genemap99>) were used to perform a series of PCR reactions with YAC DNAs. The sequences and annealing temperatures of primers are shown in Table 5.

Table 5. Sequence tagged sites (STSs) for YAC, BAC and PAC mapping

Name	Primer	Sequence (5'→3')	T <sub>m</sub> (°C)	Product size (bp)
D2S352	D2S352f D2S352r	GCAAAGTCGTTCTCAGGTG CTACAGGGCTTCAGCATCC	55	205
D2S367	D2S367f D2S367r	TTCTTTGGTCTAAGGGTCAC AGCTTCTTGTTACAGGTGT	55	148
D2S400	D2S400f D2S400r	AATGTGACAAAGCCCAGTGTTAGC GATAATCTCCCTGAGTATGTGTGCC	55	190
D2S1794	D2S1794f D2S1794r	TTCTTGCCAGAATGAAGTAAGC TTGAGGACAAGAGCAGTATAGAGG	55	124
D2S1998	D2S1998f D2S1998r	TGAAACCCCATTAAGAAGAGATC GGGAGAAGCTTTTCAGTTAATATTCC	55	203

D2S2203	D2S2203f D2S2203r	AAGTGCTTAGAAGGGTTCCTGAC ATATGCTCTCTGGTGACTGTAGGTG	55	264
D2S2005	D2S2005f D2S2005r	ATGAACAGATTTCACTGCTACTCTC GCCTTTTTCTGAGTTTCTTCTCA	55	226
D2S2255	D2S2255f D2S2255r	CTCCAGGCTACTTTGAGGATTTT CCATTTGTTTGCAGATTCTTGTAT	55	214
D2S2283	D2S2283f D2S2283r	GATCATGGCCCCAATA GCCCCAGGTAACCACT	55	273
D2S2347	D2S2347f D2S2347r	TGCCTAAGCCCATGTC CTGCGTCACTTCACATAGA	55	272
D2S2351	D2S2351f D2S2351r	TGAACTTTGCAGTGAGAAA TTTACTGTGTATGTGTGTGTACTCT	55	241
D2S3008	D2S3008f D2S3008r	CCAGCAGGCAGAGTGAGAATC TGTTTAAAGCACAAGGGCAGT	63	224-292
A004H42	A004H42f A004H42r	AACAAAATTATTACAGGTGG GGTTTATAGTAGTCCATGCTATA	55	168
A008N33	A008N33f A008N33r	TAGCAGATACGGTCAAGACTAGT GTGGTTAAATAGTTGACGGATTA	56	105
Cda15g09	Cda15g09f Cda15g09r	GCTTGAAATTCAGGTCCTCTC GGGTTATTTGGGGAAGTGTTG	56	249
Cda0sd05	Cda0sd05f Cda0sd05r	GCAGTGGCACAATTATAAC AATAAAGTAAGCTCCCGTC	54	155
GAAT- P33068	GAAT-P33068f GAAT-P33068r	TCCTTCATCATGTGATTCCC TTACCAGTGGCCATTACTGC	55	152
N52847	N52847f N52847r	TAGCAGCTTGGCTAAGAACG AGGCCAGTGATTTTGTTC	56	110
SGC32192	SGC32192f SGC32192r	TTTTAAAAAGAGACATGGGTCTCA CTGTAGTGCCACTACTCTGAAAGC	55	136
SGC32499	SGC32499f SGC32499r	AAATGTCAGCCCTTCACAGC TACAGTATAGTCAGTGGCAGTTTCC	55	127
SGC33436	SGC33436f SGC33436r	TCTGCTTTTAAAGATTCTTCATAGC AGAGTGGAATAAGACTGGCTATC	55	150

SHGC12567	SHGC12567f SHGC12567r	AGAGAGTCCTCAGCAGAGTCTTC TATTCACCATTAGGCATAACAGTT	58	224-245
SHGC13568	SHGC13568f SHGC13568r	TGACCCCCAAGCTTTCTGT CCTACAGAAGCAGCAACCGT	57	139
stSG1707	stSG1707f stSG1707r	TGCACTGCAGTAGTAAGCACG CCCCTGTACTCAATGCCCT	59	196
stSG2515	stSG2515f stSG2515r	TGTTGGTGGCAAAGAAAACA AAGGAAAAGGGTGAAGGGAA	57	186
stSG3789	stSG3789f stSG3789r	GCAGGTGAAAGCACCTTTTC CCCAGGGAATTAGCTGATGA	55	131
stSG4150	stSG4150f stSG4150r	TGCACTGCAGTAGTAAGCACG GCCCCTGAACCAATCACTT	58	138
stSG47880	stSG47880f stSG47880r	CTTTGCCATGAATGAGAGCA TTGAAGAGGGGATCTTGGTG	55	128
stSG15464	stSG15464f stSG15464r	CAGCTTGATGAATTTTCACAAA TTTTGTATGGCCTTAAATCTGG	55	204
stSG22421	stSG22421f stSG22421r	ACGAGTCTCGACGTGCAAG TCACTGTTATGGTCCTGTCAGG	56	148
stSG29624	stSG29624f stSG29624r	CAAAGCTCTGCCCTGTAAGG ACTGTTGTGGCCTTAATGTCG	56	175
stSG30783	stSG30783f stSG30783r	TCTACCCTCCAGAAGCTTGTG TGCTCAACTGCACTGTGAAA	55	146
stSG31920	stSG31920f stSG31920r	TAAATCAATAAAGCCAGCCTCT AGCAGACATTGCACGGGT	55	122
stSG32067	stSG32067f stSG32067r	GGTCCGAGTTCAAATTCTGC CGAGCTCCGTCTCAAAAAAA	55	84
stSG44150	stSG44150f stSG44150r	TAAAATGTGCTTTCGCTGCA CGTTATTAAGCTATTGAAGGCC	55	81
stSG48207	stSG48207f stSG48207r	AGCACGGCAACAAAGAAATT CGTCTTGAAAGGTGAAAGGTG	56	128
stSG51853	stSG51853f stSG51853r	AAAAACCCACTTTATGCAACTG ACCATTACAGTTCGGTTTTGG	55	135

stSG52124	stSG52124f stSG52124r	TAAGCATGGAAGCTACATCAGC GCCTGGATTTTCCCCTTTAT	56	189
stSG62506	stSG62506f stSG62506r	ACCTAGTAGGGGCAAGTTTTT TTCTCTGGTGCTTAAATGATGC	55	140
stSG63103	stSG63103f stSG63103r	TAAGGGCACCAAAAACCAAC CCTCATCCCAGCCATGTC	55	189
WI9534	WI9534f WI9534r	TTTTATTAACAATTTGTTGGTGGC AAGGAAAAGGGTGAAGGGAA	55	199
WI9668	WI9668f WI9668r	AAACAGTTTAAATTCAAGCAATCTGG AGTGTTGGAAAAAAGATTAGGGC	55	281
WII1827	WII1827f WII1827r	TTTTTTGCTGTAAAACAAAACACA CATAAAAGTTGGGGAAAGAGACC	55	111
WII16788	WII16788f WII16788r	TGTCTGGAAATACTTTCCCAT CAGACTCATCAAGTTTCAGCAA	55	150
WII16901	WII16901f WII16901r	ACATATGTGCTGTCTACTATGAGCC AAATAGGTACTCCTTGGAATCTGTG	55	103

### 3.13 BAC and PAC contig construction

To construct a physical BAC and PAC map, human genomic libraries, RPCI-1, RPCI-6, RPCI-11 and human chromosome 2 specific library LL02NP04“AI” were screened either by hybridization or PCR. The sequences and annealing temperatures of the STSs used to screen several genomic libraries were shown in the section of YAC contig construction.

#### 3.13 1 High density filter libraries

The high density filters are ready for prehybridization. A standard hybridization was performed as described previously. Several STSs or ESTs that assigned to chromosome 2p21-24 from the GenBank 98 and 99 Map were chosen as probes for hybridization. The probes were first synthesized by PCR and then labeled with [ $\gamma$ - $^{32}$ P]ATP at 5'ends. The 5' labeling was described in the hybridization section. To reduce the removal of DNA from filters, the filters were reused for hybridization in subsequent steps without removing the hybridized probes. The new positive hybridizing signals appeared on the autoradiogram after the hybridization with a new probe. If the background was too high, filters were stripped by washing at room temperature in 0.2 M NaOH for 30 min, and soaked in 0.1 x SSC/0.1% SDS/0.2 M Tris-HCl pH 7.5 for 15 min.

The interpretation of positive signals was according to the instructions of German Resource Center and UK HGMP Resource center because different spotting strategies of libraries were used. The positive clones were confirmed by PCR and the sizes of the clones were determined by PFGE.

### 3.13.2 DNA pool libraries

Two DNA pool libraries, RPCI-6 and RPCI-11, were used for PCR screening. RPCI-6 was a PAC library consisted of 36 primary pools. RPCI-11 was a BAC library consisted of 144 primary pools. DNA primary pools were first screened and the secondary pools were determined by the primary pools screening results. The secondary pools were screened further to obtain the positive candidate clones. The DNA primary pool sample (5 µl) was diluted with 45 µl sterile water and 1 µl diluted DNA was used as template for a standard 20 µl PCR. PCR was performed as described before. The lyophilized DNA secondary pool sample needed to be resuspended in 5 µl sterile water and the subsequent procedures of PCR screening is the same as described above.

### 3.14 Genotyping and linkage analysis

DNA was extracted from peripheral blood as described previously and was amplified with different STRP markers by standard procedures of PCR. [ $\alpha^{32}\text{P}$ ]-dCTP was incorporated in the PCR products during amplification. Amplified DNA was electrophoresed on 6% polyacrylamide/7M urea gels, and alleles were scored on the basis of autoradiographs. The microsatellite polymorphic markers are listed in Table 6. Two-point LOD scores were calculated using the LINKAGE version 5.1 program package. The following assumptions were made: a gene frequency of  $10^{-5}$  for autosomal dominant SPG; equal allele frequencies for each marker; equal recombination rates in males and females and 90% as the penetrance rate.

Table 6. STRPs markers for linkage analysis at SPG 3,4, 6 loci.

Name	Primer	Sequence	Tm(°C)	Product size(bp)
<b>SPG3</b>				
<i>D14S63</i>	D14S63A	GGCCAGGTTTCAATCAGTTT	55	208
	D14S63B	GCCAGAGAGCCACACTGTAT		
<i>D14S66</i>	D14S66A	GGCAACAGACTTGACCAATC	55	185
	D14S66B	CGTTCAGTAAGCAGAGAGCA		
<i>D14S69</i>	D14S69A	AAAGCCCACTGCTAGTCAC	55	204
	D14S69B	TTCAGATGCCAATTAAGGGA		
<i>D14S75</i>	D14S75A	TGTCCCCAGGTGTTA	55	185
	D14S75B	CCAAGTGGCCTTGCC		
<i>D14S79</i>	D14S79A	AGGTTGATAGACCATGGAGACA	55	82
	D14S79B	TTTTATTGTTATGTGGCTTTCA		
<i>D14S266</i>	D14S266A	ACAAGCCCCATATATTCATG	55	135
	D14S266B	AATAGACTTCCAAATCTTCAGATA		
<i>D14S269</i>	D14S269A	CACATGGCATTACCAC	55	221
	D14S269B	GCAACATGCTTGACAGG		
<b>SPG4</b>				
<i>D2S146</i>	D2S146A	TCATCCTTACTCTAAGCAAAGATCC	55	189
	D2S146B	CACCACATTCAAATGCCTCC		
<i>D2S170</i>	D2S170A	TTGCTCAATAATGTCAGGTG	55	216
	D2S170B	CGCATGAGAGGCGTCT		
<i>D2S352</i>	D2S352A	GCAAAGTCGTTCTCAGGTG	55	205
	D2S352B	CTACAGGGCTTCAGCATCC		
<i>D2S367</i>	D2S367A	TTCTTTGGTCTAAGGGTCAC	55	148
	D2S367B	AGCTTCTTGTTACAGGTGT		
<i>D2S400</i>	D2S400A	AATGTGACAAAGCCCAGTGTTAGC	55	190
	D2S400B	GATAATCTCCCTGAGTATGTGTGCC		
<b>SPG6</b>				
<i>D15S122</i>	D15S122A	GATAATCATGCCCCCA	55	146
	D15S122B	CCCAGTATCTGGCACGTAG		
<i>D15S128</i>	D15S128A	GCTGTGTGTAAGTGTGTTTTATATC	55	206
	D15S128B	GCAAGCCAGTGGAGAG		
<i>D15S156</i>	D15S156A	CAGCCACCGCATTCTA	55	229
	D15S156B	CAACACCATTATTGAAGAGAC		
<i>D15S165</i>	D15S165A	GTTTACGCCTCATGGATTTA	55	208
	D15S165B	GGGCACACAGTCCCAA		

### 3.15 WWW. web sites

Organization/Database	Address
The National Center for Biotechnology Information	<a href="http://www.ncbi.nlm.nih.gov/">http://www.ncbi.nlm.nih.gov/</a>
Medline	<a href="http://www.ncbi.nlm.nih.gov/PubMed/">http://www.ncbi.nlm.nih.gov/PubMed/</a>
Online Mendelian Inheritance in Man (Human Genes and Genetic Disorders)	<a href="http://www.ncbi.nlm.nih.gov/Omim/">http://www.ncbi.nlm.nih.gov/Omim/</a>
Cooperative Human Linkage Center (Human Genome Map)	<a href="http://lpg.nci.nih.gov/CHLC/">http://lpg.nci.nih.gov/CHLC/</a>
Whitehead Institute (Physical Mapping)	<a href="http://www-genome.wi.mit.edu/">http://www-genome.wi.mit.edu/</a>
Online Linkage Analysis Software	<a href="http://linkage.rockefeller.edu/">http://linkage.rockefeller.edu/</a>
Stanford Human Genome Center (Radiation Hybrids Mapping)	<a href="http://www-shgc.stanford.edu/">http://www-shgc.stanford.edu/</a>
European Bioinformatics Institute (Sequence Database)	<a href="http://www.ebi.ac.uk/">http://www.ebi.ac.uk/</a>
Radiation Hybrids database	<a href="http://www.ebi.ac.uk/RHdb/">http://www.ebi.ac.uk/RHdb/</a>
Human Gene Mutation Database	<a href="http://www.archive.uwcm.ac.uk/">http://www.archive.uwcm.ac.uk/</a>
UK Human Genome Mapping Project Resource Cernter (HGMP)	<a href="http://www.hgmp.mrc.ac.uk/">http://www.hgmp.mrc.ac.uk/</a>
Sanger Institute (Human Genome Sequences)	<a href="http://www.sanger.ac.uk">http://www.sanger.ac.uk</a>
Genome database	<a href="http://www.gdb.org/">http://www.gdb.org/</a>
German Resource Center (German Human Genome Project Funded)	<a href="http://www.rzpd.de/">http://www.rzpd.de/</a>
CEPH (YAC Mapping)	<a href="http://www.cephb.fr/">http://www.cephb.fr/</a>
Genethon (SSLP Maps)	<a href="http://www.genethon.fr/">http://www.genethon.fr/</a>
Genoscope (Human Chromosome 2p21-p24 Sequences)	<a href="http://www.genoscope.cns.fr/">http://www.genoscope.cns.fr/</a>