3 Material and Methods

3.1 Equipment

Centrifuges	5810R, *Eppendorf AG*, Hamburg 5415D, *Eppendorf AG*, Hamburg J-6B, *Beckman Coulter GmbH*, Krefeld
Ultracentrifuge	L8-M, *Beckman Coulter GmbH*, Krefeld
Water baths	ss40-D, *Grant*, Cambridge/UK SW20, *Julabo*, Houston/USA
Freezer, -80°C	Forma, *ThermoQuest Analytische Systeme GmbH*, Egelsbach
Geiger Müller detector	LB 1210 B, *Berthold Technologies GmbH*, Wildbad, Germany
UV Cross-Linker	2400, *Stratagene*, *La Jolla*, CA/USA
Sonifier W250	Branson, *Dietzenbach*, Germany
Hybridization oven	Oncor, *Appligene*, UK
Thermostat	plus, *Eppendorf AG*, Hamburg
Filmprocessor	curix 60, *Agfa-Gevaert*, *NV*, Mortsel, Belgium
Autoclave	5075 EL, *Systec GmbH*, Wettenberg
Gel electrophoresis Equipment	*Amersham Pharmacia Biotech Europe GmbH*, Freiburg
Q-Fill	*Genetix*, Christchurch, Dorset/UK
Luminescent Image Analyser	LAS-1000, *Fuji*, Japan
Incubator	*Heraeus Instruments GmbH*, Wiesloch
Shaker	Rocky, *Fröbel Labortechnik*, Wasserburg
Plate sealer	*Genetix*, Christchurch, Dorset/UK
Scanners	Progress 3012, *Kontron-Elektronik AG*, Urdorf/Schweiz 1640XL Expression, *Epson*, Japan

Thermocyclers	PTC100 PTC200 PTC225 *MJResearch*, Inc.; Watertown/USA
Vortex	Vortex Genie 2, *Bender und Hobein AG*, Zürich/Switzerland
Pipettes, adjustable	Abimed, *Gilson*, Lübeck
Heating block	*Eppendorf AG*, Hamburg
Pipettes, adjustable, multichannel	*Coring Costar*, Acton, MA/USA
Paper cuter	560, *DAHLE*, Germany
Packing machine for microtitre plate blocks	*Lady Pack* (shrink wrap), *Pactur* Bologna/Italy
Tippy Pack (binding machine)	Spot, *Manfred Pütz GmbH*, Freiburg
Tube sealer	*Beckman Coulter GmbH*, Krefeld
Gel documentation system for ethidiumbromide-stained agarose gel	*Herolab GmbH*, Wiesloch

3.2 Kits

Anti-digoxigenin- AP, Fab fragments	*Boehringer Mannheim GmbH*
Nuclean (NC001) kit	*MoBiTech GmbH*, Göttingen
PCR DIG labeling mix kit	*Boehringer Mannheim GmbH*
Plasmid Maxi Kit	*Qiagen*, Hilden
QIAquick 96 PCR Purification Kit	*Qiagen*, Hilden
Random Primed DNA Labeling Kit	*Roche Mannheim GmbH*

3.3 Enzymes

DNA Polymerase I (E.coli), Large (Klenow) Fragment	*New England BioLabs GmbH*, Schwalbach/Taunus
DNA Taq-Polymerase	MPI for molecular genetic Berlin
*Eco*RV	*New England BioLabs GmbH*, Schwalbach/Taunus

Shrimp alkaline phosphatase	*Boehringer Mannheim GmbH*
PNK T4 Polynucleutide Kinase	*New England BioLabs GmbH,* Schwalbach/Taunus
Pronase from Streptomyces griseus	*Boehringer Mannheim GmbH*
*Rsa*I	*New England BioLabs GmbH,* Schwalbach/Taunus
T4 DNA Ligase	*New England BioLabs GmbH,* Schwalbach/Taunus

3.4 Chemicals and Substances

?-^{32}P-dATP, ? -^{33}P-dATP	*Amersham Pharmacia Biotech Europe GmbH,* Freiburg
? -^{32}P-dCTP	*Amersham Pharmacia Biotech Europe GmbH,* Freiburg
?-Mercapto-ethanol	*Fluka,* Buchs/Switzerland.
2 x YT broth agar	Bio 101, s*ystems, Q.BIOgene,* Germany
2 x YT broth	Bio 101, s*ystems, Q.BIOgene,* Germany
Acetic acid	*Merck*, Darmstadt
Adenosintriphosphate	*Boehringer Mannheim*, Manheim
Agarose	*Gibco Life Technologies*, Karlruhe
Ampicillin	*Sigma Chemistry*, Deisenhofen
Attophos	*Promega GmbH*, Mannheim
Bacto Agar	*Difco Laboratories*, Detroit/USA
Bacto Peptone	*Difco Laboratories*, Detroit/USA
Bacto Tryptone	*Difco Laboratories*, Detroit/USA
Bovine serumalbumin	*Sigma Chemistry*, Deisenhofen
Bromophenol blue	*Sigma Chemistry*, Deisenhofen
Calciumchloride	*Merck*, Darmstadt
Chloroform	*Merck*, Darmstadt
Choloramphenicol	*Sigma Chemistry*, Deisenhofen
Cresolred	*Merck*, Darmstadt

dATP, dCTP, dGTP dTTP sodium salt	*Amersham Pharmacia Biotech Europe GmbH,* Freiburg
EDTA (Titriplex? III)	*Merck*, Darmstadt
Ethanol	*Merck*, Darmstadt
Ethidium Bromide, 1% solution	*Fluka,* Taufkirchen
Formaldehyde	*Merck*, Darmstadt
Glucose	*Merck*, Darmstadt
Glycerol	*Merck*, Darmstadt
Hydrogen peroxide H_2O_2 30% (W/W)	*Sigma Chemistry*, Deisenhofen
Isoamylalcohol	*Merck*, Darmstadt
Isopropanol	*Merck*, Darmstadt
Kanamycin	*Sigma Chemistry*, Deisenhofen
LB medium	Bio 101, s*ystems, Q.BIOgene,* Germany
Lithiumchloride	*Sigma Chemistry*, Deisenhofen
Magnesium sulphate	*Merck*, Darmstadt
Magnesiumchloride	*Merck*, Darmstadt
Phenol	Roti-Phenol, *Carl Roth GmbH & Co. KG*, Karlsruhe
Potassiumhydroxide	*Merck*, Darmstadt
Sarcosyl	*Sigma Chemistry*, Deisenhofen
Sephadex G50	*Amersham Pharmacia Biotech*, Uppsala/Sweden
Sodiumacetate	*Merck*, Darmstadt
Sodiumchloride	*Merck*, Darmstadt
Sodiumdodecylsulphate	*Serva*, Heidelberg
Tris-HCl	*Merck* Darmstadt
Tween 20	*Merck*, Darmstadt

3.5 **Other materials**

3MM Chromatography paper	*Whatman GmbH,* Göttingen
Agar plates Bio Assay Dish	*Nunc GmbH & Co. KG*, Wiesbaden
E.coli (DH5? mer)	*Gico BRL*, Gaithersburg/USA

Quick-Seal centrifuge tube (13 x 51 mm)	*Beckman Coulter GmbH,* Krefeld
Mesh Sheets, Large (23x23 cm)	HB-OV-LM20, *Hybaid GmbH,* Heidelberg Germany
Saran Wrap, 300 mm x 30m SW2 Genetic	*Genetic Research Instrumentation Ltd*
Saran Wrap, 450 mm x 300m SW2 Genetic	*Genetic Research Instrumentation Ltd*
Nylon Transfer Membrane, 222 x 222 mm Hybond-N+	*Amersham Pharmacia Biotech Europe GmbH,* Freiburg
GB 005 Gel Blotting paper 25 Blatt/Sheet	*Schleicher & Schuell*, Germany
Micro Spin^{TM} G-50 Columns	*Amersham Pharmacia Biotech Europe GmbH,* Freiburg
Miracloth	*Fisher Scientific,* USA
Scientific Imaging Film XAR5 (35 x 43 cm)	*Kodak*, Germany
Schlauchfolie PE Starckwanding	*VWR International GmbH,* Germany
DNA Molecular Weight Marker III (Lambda DNA EcoRI and HindIII)	*Boehringer Mannheim*, Manheim
?-DNA/HindIII marker	*MBI Fermentas*, Germany
Midrange I Marker	*New England BioLabs GmbH,* Schwalbach/Taunus
PCR plates, Thermo-Fast^{R} 384	*Abgene*, Surrey/UK
Microtitre plates, 384-well	*Genetix, Christchurch*, Dorset/UK
PCR plates, Thermo-Fast^{R} 96	*Abgene*, Surrey/UK
Cell culture 96-well plates, U-form	*Greiner Labortechnik GmbH,* Frickenhausen
Polypropylene tubes 15 ml and 50 ml, sterile	*Greiner Labortechnik GmbH,* Frickenhausen
Replicator, 96-pin	*Genetix, Christchurch*, Dorset/UK
Replicator, 384-pin	*Genetix, Christchurch*, Dorset/UK

Adhesive PCR Film	*Abgene*, Surrey/UK
Airpore^{TM} Tape Sheets	*Qiagen, Hilden*
Bio-stat diagnostic system (Plate sealers)	*Bio-stat Ltd*, Stockport/UK
50 ml Reagent Reservoir Polystyrene 5	*Costar, Fisher Scientific*, USA
Size standard, DNA marker ? X174 DNA/BsuRI	*MBI Fermentas*, Germany

3.6 Oligonucleotides

3.6.1 BAC end-fragment primers

Forward-HindIII (FH)	5'-AAAACGACGGCCAGTGCCAAG-3'
Nested pKS-lac	5'- GACGGTATCGATAAGCTTGA–3'
Nested SP6	5'-TGACACTATAGAAGGATCCGCGG-3'
Nested T3-pKS-Sp	5'-AAAGGGAACAAAAGCTGGGTACC-3'
Nested T7-Bac-Sp	5'-TCACTATAGGGAGAGGATCCG-3'
Reverse-HindIII (RH)	5'-AGAGTCGACCTGCAGGCATGCAAG-3'
SP6-long	5'-ATTTAGGTGACACTATAGAA-3'
T3-long	5'-AATTAACCCTCACTAAAGGG-3'

3.6.2 cDNA primers

pM18F	5'-GCTGCGGAATTCCTCGAGCAC-3'
pM18R	5'-CGCGACCTGCAGCTCGAGCAC-3'
pUC118-F	5'-ACAGCTATGACCATGATTACG-3'
pUC118-R	5'-CCAAGCTTGCATGCCTGCAGG-3'
SK-Mitani-F	5'-TGGATCCCCCGGGCTGCAG-3'
SK-Mitani-R	5'-TACCGGGCCCCCCCTCGAG-3'

3.6.3 35-mer oligonucleotides from medaka expressed sequences

Lists of 35-mer oligonucleotides used for the hybridization approach are available at http://goblet.molgen.mpg.de/cgi-bin/hb/hb2004.cgi.

3.7 Software

Blastn (Altschul *et al.*, 1990)

Cap3 (Huang and Madan, 1999)

CrossMatch UWGC, Washington

Visual Grid GPC biotech, Munich

Probeorder (Mott *et al.*, 1993)

Pooled Probe Editor D. Groth, Max-Planck Institute for Molecular Genetics, Berlin

3.8 Buffers and reagents

3.8.1 DNA preparation solutions

Solution I

Tris-HCl	25 mM
EDTA (pH 8.0)	10 mM,
Glucose	50 mM

The volume was adjusted with sterile water.

Solution II

NaOH	0.2 M
SDS	1%

The volume was adjusted with sterile water

Solution III

Potassium acetate (pH 5.2)	3 M

The volume was adjusted with sterile water

Phenol:Chloroform:Isoamylalcohol (PCI) (25:24:1)

A mixture consisting of equilibrated phenol and chloroform:isoamylalcohol (24:1)

PCI is used to remove protein from preparation of nucleic acids. The chloroform denatures proteins and facilitates the separation of the aqueous and organic phases, and the isoamylalcohl reduces foaming during the extraction.

3.8.2 Hybridization solutions

Labelling solutions (LS, contains hexamers)

Hepes buffer (pH 6.6)	50 mM
Hexamers[1]	14 µl
TM buffer[2]	50 µl

[1]Hexanucleotides, Pharmacia $p(dN)_6$: 1:8 dilution of stock which is 45 OD units/ml

[2]TM buffer: 250 mM Tris-HCl (pH 8.0); 25 mM $MgCl_2$; 50 mM ß-mercaptoethanol.

Church buffer

Na_2HPO_4 (0.5 M pH 7.2)	500 ml
EDTA (0.5 M)	2 ml
10% SDS	500 ml

Wash solutions

Wash I was used both for filters that had been hybridized with end-fragment probes or oligonucleotide probes.

Wash I

2 x SSC

0.1% SDS

Wash II

The combination of wash II was different for hybridizing with end−fragment probes and oligonucleotide probes:

Wash II for oligonucleotide probes

0.5 x SSC

0.1% SDS

Wash II for end-fragment probes

0.1 x SSC

0.1% SDS

Strip solution

10% SDS

EDTA 0.5 M

NaP_i

Na_2HPO_4 (pH 6.8) 1M

NaH_2PO_4 (pH 6.8) 1M

Developing solutions

Developer

The solution A (AGFA G153) was added to 1250 ml distilled water, mixed and the solution B (AGFA G153) was added to it.

Fixer

The fixer solution (AGFA G354) was mixed with distilled water to 2.5 liter.

3.8.3 Spotting solutions

Denaturing solution

NaOH 0.5 M

NaCl 1.5M

Neutralisation solution (pH 7.4)

Tris-HCl 1M

Tris-base 1M

NaCl 1.5M

Pronase solution

Tris-HCl 50 mM

NaCl 100mM

Sarcosyl 1%

Pronase stock (50 mg/ml)

0.5 g Pronase was dissolved in 10 ml distilled water.

3.8.4 Other solutions

5 x TEN9

Tris-Base (pH 9.0)	250 mM
EDTA (pH 8.0	100 mM
NaCl	200 mM

The volume was adjusted with sterile water.

10 x HMFM (Hogness Modified Freezing Medium)

(For freezing of bacteria)

Solution A

$MgSO_4.7\ H_2O$	0.9 g
Na_3-citrate.$2H_2O$	4.5 g
$(NH_4)_2\ SO_4$	9 g
Glycerol (100%)	440 g

The volume was adjusted to 800 ml with sterile water and sterilized by autoclaving.

Solution B

KH_2PO_4	18 g
K_2HPO_4	47 g

The volume was adjusted to 200 ml with sterile water and sterilized by autoclaving.

Before using, 640 ml solution A was added to 160 ml solution B.

1 x TE (pH 7.6)

Tris.Cl (pH 7.6)	10 mM
EDTA (pH 8.0)	1mM

0.5 M EDTA (pH 8.0)

(Maniatis; 1989)

186.1 g of disodium ethylenediaminetetraacetate.2 H_2O was added to 800 ml of distilled water. The pH was adjusted to 8.0 with 10M NaOH and the volume was adjusted to 1000 ml with distilled water. The solution was dispensed into aliquots and sterilized by autoclaving.

20 x SSC

(Maniatis; 1989)

175.3 g of NaCl and 88.2 g of sodiumcitrate were dissolved in 800 ml of H_2O. The pH was adjusted to 7.0 with 10M NaOH. The volume was adjusted to 1 lit with H_2O and the solution was dispensed into aliquots and sterilized by autoclaving.

Attophos (for 50 ml)

Diethanolamine	12.6g
NaN_3	2.5 ml
$MgCl_2$ (1M)	11.5 µl
Attophos powder	145 mg

The distilled water was added to 50 ml.

PBS (Phosphate-buffered saline)

8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 were dissolved in 800 ml H_2O. The pH was adjusted to 7.4 with HCl. H_2O was added to 1 liter. The solution was dispensed into aliquots and sterilized by autoclaving.

Chloramphenicol solution

33 mg/ml of chloramphenicol was dissolved in 100% ethanol and kept at –20°C.

Ampicillin

50 mg/ml of ampicillin was dissolved in 100% ethanol and kept at –20°C.

Proteinase K stock

10 mg proteinase K was dissolved in sterile water and was kept as aliquot at –20°C.

10 x PCR buffer

Tris-Base	35 mM
Tris-HCl	15 mM
Tween 20	0.1%
KCl	50 mM
Cresol red	15 µM

3M sodium acetate pH 5.6

(Maniatis; 1989)

408.1 g of sodium acetate .3 H_2O was dissolved in 800 ml of H_2O. The pH was adjusted to 5.2 with glacial acetic acid. The volume was adjusted to 1 lit with H_2O and dispensed into aliquots and sterilized by autoclaving.

1 M Tris

(Maniatis; 1989)

121.1 g of Tris base was dissolved in 800 ml of H_2O. The pH was adjusted as desired. The volume was adjusted to 1 lit with H_2O and dispensed into aliquots and sterilized by autoclaving.

50 x Tris-acetate (TAE)

(Maniatis; 1989)

Tris base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

The volume was adjusted to 1 lit with distilled H_2O.

RNase stock

10 mg RNase was dissolved in 1 ml H_2O and was incubated at 95°C for 15 min (DNase will be destroyed)

3.8.5 Media

LB Medium (Luria-Bertani Medium)

(Maniatis; 1989)

Per liter:

To 950 ml of deionized H_2O, was added:

bacto-tryptone	10g
bacto-yeast extract	5 g
NaCl	10 g

It was shaken until the solutes had dissolved. The pH was adjusted to 7.0 with 5 N NaOH. The volume of the solution was adjusted to 1 liter with deionized H_2O and sterilized by autoclaving.

2 x YT Medium

(Maniatis; 1989)

Per liter:

To 900 ml of deionized H_2O was added:

bacto-tryptone	16g
bacto-yeast extract	10 g
NaCl	5 g

It was shaken until the solutes had dissolved. The pH was adjusted to 7.0 with 5M NaOH. The volume of the solution was adjusted to 1 liter with deionized H_2O and sterilized by autoclaving.

3.9 Alkaline lysis maxi preparation of plasmid DNA

(Modification after Birnboim and Doly, 1979)

Plasmid purification protocols are based on an alkaline lysis procedure under appropriate low-salt and pH conditions. Exposure of bacterial suspensions to the strongly anionic detergent at high pH opens the cell wall, denatures chromosomal DNA and proteins, and releases plasmid DNA into the supernatant.

3.9.1 Preparation of culture

One bacterial colony from solid culture was transferred into 3 ml liquid medium (2 x YT) containing appropriated antibiotics (for BAC clones 33 mg/ml chloramphenicol and for pBluescript II KS clones 50 mg/ml ampicillin). The culture was incubated at 37°C with vigorous shaking overnight (preculture). 500 ml of 2 x YT broth containing antibiotic was inoculated with 1 ml of preculture. Incubation was followed as described for preculture.

3.9.2 DNA preparation

To collect bacterial cells the overnight culture was centrifuged at 4,000 rpm in a Beckman J-6B centrifuge. The supernatant was removed. The bottle was placed upside down on paper towels to drain the rest of medium. The pellet was resuspended in 10 ml of solution I containing 100 µg RNase (10 µl of RNase stock). The bacterial cells were lysed by addition of 20 ml solution II. The sample was mixed by gently inverting the bottle several times. The bottle was then stored at RT for 10 min. The lysate was neutralized by addition of 15 ml of ice-cold solution III, mixed and the bottle was then kept on ice for 10 min. During this step the chromosomal DNA was precipitated (the precipitate consists of chromosomal DNA, RNA, potassium salts, SDS, proteins and membrane complexes). The precipitate was collected by subsequent centrifugation of the sample at 4000 rpm. The supernatant (containing plasmid DNA) was filtered through Miracloth and collected in a new 50-ml falcon tube. Furthermore 1 vol. of isopropanol was added to precipitate bacterial plasmid and after mixing the vial was incubated at RT for 15-30 min. The sample was again centrifuged at 4,000 rpm at RT for 20 min (salt may precipitate if centrifugation is processed at 4°C). The supernatant was decanted and the DNA pellet was washed with 70% ethanol. The pellet was then resuspended in 10 ml of TE (pH 8.0). To purify

the plasmid DNA, 5 ml of PCI (Phenol/ Chloroform/ Isoamylalcohol) were added to the sample. It was mixed and centrifuged at 4,000 rpm for 10 min. The supernatant was transferred into a new tube. To precipitate RNA, 1 vol. of ice-cold 5 M LiCl were added to the sample and the tube was stored on ice for 10 min. After centrifugation at 4000 rpm, the supernatant was transferred into a new tube. The DNA was precipitated by addition of 2 vol. of 100% ethanol. The sample was incubated on ice for 20 min. It was followed by centrifugation at 4,000 rpm. The pellet was washed with 500 µl 70% ethanol, air-dried and dissolved in 400 µl TE.

3.10 BAC-DNA minipreparation

3.10.1 Culture preparation

A 96-deep-well plate containing 1.5 ml medium (2 x YT plus 33 µg/ml chloramphenicol) in each well was inoculated from BAC library plates, using a 96-pin replicating tool. The plate was covered with a gas permeable Airpore tape sheet and the cultures were grown for 18-22 hours at 37°C with agitation at 300 rpm in an incubator shaker. Agitation and gas permeable sheet provide the aeration during the growth of cultures.

Bacterial cells from 96-well plate were pelleted by centrifugation at 4,000 rpm in an eppendorf centrifuge 5810R for 15 min at 4°C. The supernatants were decanted from the pellets, and the 96-well block was left inverted on paper towel for 5 min to drain excess culture media.

3.10.2 DNA isolation

DNA preparation was performed using a alkaline lysis procedure similar as above:
25 µl of solution I and 1 µl of RNAase stock (10 µg/µl) were added to each well of the 96-well plate and the bacterial pellets were resuspended by vigorous vortexing. 75 µl of solution II was added to lyse the bacterial cells. The plate was then vortexed carefully. Furthermore 62.5 µl of solution III were added to each well and after gentle vortexing the plate was incubated on ice for 15 min. The cell debris was pelleted by centrifugation at 4,000 rpm at a temperature of 4°C for 30 min. The clear supernatant was transferred into a fresh 96-well cell culture plate with round bottom. At room temperature, 100 µl of isopropanol were added to the samples, to precipitate the BAC DNAs. To achieve the best result of precipitation the plate may be incubated overnight at –20°C. To collect the DNAs, samples were centrifuged at 4,000 rpm for 45 min on the next day.

The supernatants were decanted and pellets were washed twice with 70% ethanol. The pellets were dried at 37°C and resuspended in 50 µl 1 x TE (pH 8.0).

3.11 Purification of closed circular DNA by equilibrium centrifugation in CsCl-ethidium bromide gradients

3.11.1 Preparation of culture and plasmid DNA isolation

The preculture was prepared as described in (see 3.9.1). A 2-liter Erlenmeyer flask filled with 800 ml of 2 x YT medium plus 50 mg/ml ampicillin was inoculated with 3 ml of preculture. The culture was incubated overnight at 37°C with vigorous shaking.

To collect bacterial cells, the culture was centrifuged in a 1-liter bottle in a Beckman J-6B centrifuge at 3,800 rpm for 30 min at 4°C. The supernatant was carefully decanted and the bottle was placed upside down on paper towels to remove rest of medium for 5 min. The pellet was completely resuspended in 10 ml of solution I by vigorous vortexing until no cell clumps remained. 30 ml of solution II was then added. The sample was mixed gently by inverting 4-6 times and incubated on ice for 5 min. This mixture was not vortexed in this step to avoid shearing of genomic DNA. The lysate appeared viscous. 22.5 ml of solution III were added, mixed immediately by inverting 4-6 times and the bottle was incubated on ice for 30-60 min. The sample was moved to a 250-ml GSA bottle and centrifuged at 11,000 rpm for 60 min. The supernatant containing DNA was collected in a new bottle.

3.11.2 DNA precipitation

The double-stranded DNA was precipitated by adding 45 ml of isopropanol followed by incubation for 5 min at room temperature. The DNA was pelleted by centrifugation at 9,000 rpm for 30 min at 4°C (the isopropanol pellet has glassy appearance and may be difficult to see). The supernatant was removed and the pellet was dried by placing the bottle in an inverted position on a paper towel to allow all of the fluid to drain away. The pellet was resuspended in 5 ml of 2 M ammonium acetate (CH_3COONH_4) and then incubated on ice for 5 min to remove RNA and proteins. After centrifugation at 7,000 rpm for 15 min the supernatant was transferred into a new 50-ml falcon tube.

The DNA was collected by adding 0.7 vol. isopropanol and centrifugation at 4,000 rpm for 15 min. The pellet was dried at room temperature. Afterwards the pellet was resuspended in 4 ml of 1 x TE.

3.11.3 DNA purification

Plasmids, which were purified by CsCl-ethidium bromide gradients, were very pure for molecular experiments. Using this method separated the plasmid (closed circular DNA) and chromosomal (linear DNA) DNA. In this method crude DNA and ethidium bromide were mixed with CsCl. The mixture was centrifuged at high speed. The centrifugal force was sufficient to generate and maintain a density gradient of cesium atoms. During the formation of the gradient, DNA of different buoyant densities migrates to positions in the tube at which the density of the surrounding CsCl solution equals that of the DNA itself. Separation depends on differences of amounts of ethidium bromide that can be bound to linear and closed circular DNA molecules. Binding of ethdium bromide causes a decrease in the buoyant density of both linear and closed circular DNAs. However, because linear DNAs bind more ethidium bromide, they have a lower buoyant density than closed circular DNAs in CsCl gradients containing saturating amounts of ethidium bromide. Closed circular DNAs come at a lower position than linear DNAs in CsCl gradients.

4.4 g CsCl was added to the 4 ml of dissolved DNA prepared above and the suspension was transferred into a Beckman Quick-Seal centrifuge tube (13 x 51 mm).

400 µg ethidium bromide were added into the tube. The tube was sealed with a Beckman tube sealer very tightly. The tube was centrifuged at 48,000 rpm in a Beckman ultracentrifuge L8-M for more than 8 hours at 25°C.

After centrifugation two bands of DNA, located in the center of the gradient, were visible in ordinary light. The upper band, which usually contains less material, consists of linear bacterial (chromosomal) DNA and nicked circular plasmid DNA. The lower band consists of closed circular plasmid DNA. The deep red pellet on the bottom of the tube consists of ethidium-bromide/RNA complexes.

A hypodermic needle was inserted into the top of the tube to allow air to enter. The band of circular plasmid DNA (the lower band) was collected with a syringe.

3.11.4 Removal of ethidium bromide by washing the DNA with isoamylalcohol

The ethidium bromide was removed from the DNA using water-saturated isoamylalcohol. First a solution of 1:1 isoamylalcohol and water was prepared, mixed and left until the two phases separated. The upper phase, which was isoamylalcohol, was collected. An equal volume of isoamylalcohol was added to the DNA sample. The two phases were mixed and centrifuged at 14,000 rpm for 2 min. The lower phase was collected and transferred into a new tube. The extraction was repeated 4-5 times until the pink color had disappeared. Then 3 volumes of 1 x TE were added to it and mixed.

3.11.5 DNA precipitation and washing

1 volume isopropanol was added to the tube and it was centrifuged at 4500 rpm for 20 min. The pellet was washed with 1 volume 70% ethanol and dried at room temperature.
The DNA was redissolved in 100 µl of 1 x TE and stored at –20°C.

3.12 Preparation of genomic fish DNA

20 medaka fishes, which were frozen at –80°C, were disrupted into powder by using a mortar. The sample was kept on dry ice during the grind. The powder was transferred into a 50 ml falcon tube. 40 ml of 1 x TEN9 buffer were added to the sample and it was mixed thoroughly at RT. Subsequently, 200 µl RNase stock (10 µg/µl) were added to the tube. The tube was incubated on a rocker platform for 10 min at RT. Then 1 ml proteinase K (10 mg/ml) and 1 ml of 20% SDS were added to the tube and it was incubated on a rocker platform at 55-65°C for 36 h. To remove the proteins from the sample, phenol extraction was performed, using 1 vol. PCI. After addition of PCI to the sample, the tube was left on the rocker at RT until phases were sufficiently mixed. The sample was centrifuged at 4,000 rpm in an eppendorf centrifuge 5810R for 15 min at RT. The supernatant was transferred into a new tube and PCI extraction was repeated. In the next step the aqueous phase was extracted again using a chloroform-isoamylalcohol mixture. To precipitate the DNA 1/10 vol. of 3M NaAc (pH 6.0) and 1 vol. isopropanol were added to the tube and it was shaken on a rocker. The precipitated DNA should be observed as tiny filaments. The DNA was collected by centrifugation at 4,000 rpm and it washed twice with 70% ethanol. After air-drying the DNA at RT, it was dissolved in 1 x TE on a rocker at 37°C overnight.

The DNA was physically sheared to small size by sonification. Two different dilutions (1:10 and 1:50) of DNA were prepared and checked on a 0.8% agarose gel. Sonification was considered optimal, if the bulk of DNA had a size of less than 2000 bp.

3.12.1 Determination of DNA concentration

3 µl DNA was added to 750 µl H_2O in a quartz cuvette.
The OD was measured at 260 nm. The concentration could be calculated as follows:

$$DNA\,in\,?g/?l\,? \frac{OD_{260}\,?\,50?\,Dilution}{1000}$$

3.13 Digestion

3.13.1 Digestion of BAC DNA for amplification of end fragments

Digests were carried out in a 96-well format. Twenty ng BAC-DNA were digested in a volume of 20 µl containing 1 x NEB I buffer and 1 unit *Rsa*I restriction enzyme. *Rsa*I was chosen because its recognition site (5'...GT$^?$ AC...3') is short, such that it occurs quite often in the DNA sequence and because it produces blunt ends. A reaction mixture for 96 digests was prepared in a reagent reservoir. The digest mixture consisted of buffer, restriction enzyme and H_2O (without DNA), which then was transferred into Thermo-Fast 96-well plate, using 12-channel multipipette. Subsequently, the DNA was added to the reaction mixture in each well. The plate was covered with Bio-stat foil and then incubated at 37°C overnight in a MJResearch PTC 100 thermocycler. To inactive the restriction enzyme, the plate was incubated at 65°C in the same apparatus for 15 min.

3.13.2 Digestion of pBluescript II KS (-) vector for ligation

The pBluescript II KS (-) plasmid DNA was isolated and purified, using protocol 3.11. The purified plasmid was digested with *Eco*RV (5'...GAT$^?$ ATC...3'), which produces blunt ends. 50 ng purified plasmid DNA were digested in a volume of 20 µl containing 1 x NEB III buffer and 40 units *Eco*RV restriction enzyme. Digested pBluescript II KS (-) was used for ligation of BAC inserts.

3.14 Dephosphorylation

Dephosphorylation was carried out using SAP (Shrimp Alkaline Phosphatase). SAP removes the phosphor group of the 5' end of DNA, which prevents the fragments from self-ligation.
SAP is active in all restriction enzyme buffers. 1 unit of enzyme could be added directly to the restriction digest and incubated at 37°C for 1 hour. To inactivate SAP, the sample was heated to 65°C for 15 min.

3.15 Ligation of BAC fragments into a plasmid vector

Digested DNA was ligated into pBluescript vector cut by *Eco*RV. The ligation mixture consisted of 5 ng digested BAC DNA, 20-50 ng linearized pBluescript vector, 1x T4 DNA ligase buffer and 200 units of T4 DNA Ligase in a volume of 20 µl. Ligations may be prepared in a 96 well format. The plate was incubated at room temperature overnight.

3.16 Amplification

To amplify a segment of DNA located between two regions of known sequence the polymerase chain reaction (PCR) was used.

3.16.1 Amplification of BAC end-fragments

To amplify BAC end fragments, the ligation products (see 3.15) were used as template. For Cab clones (vertor: pBACe3.6), the primers Sp6-long plus T3-long and T7-Bac-Sp plus T3-pKS-Sp were used to amplification. The reaction mixture consisted of 10 ng template DNA, 10 pmol of each primer, 125 µM of each dNTP, 3 units of *Taq* DNA Polymerase, 1 x PCR buffer (see 3.8.4) and distilled water to 30 µl. The PCR program was used:
Reactions were heat denatured for 3 min, and then processed by 35 cycles of 94°C (30 sec), annealing (30 sec), extension at 72°C (90 sec) and final incubation at 72°C for 10 min.
The annealing temperature for Sp6-long/T3-long was 52°C and for T7-Bac-Sp/ T3-pKS-Sp was 65°C.
As the Hd-rR and HNI library were cloned in the pBAC-Lac vector, to amplification, the BAC specific primer Reverse-HindIII (RH) or Forward-HindIII (FH) in combination with pBluescript specific primer, pKS-lac were utilized. The reaction mixture was prepared as described above. The annealing temperature for theses reactions was 58°C.

3.16.2 Amplification of cDNA clone inserts

cDNA libraries obtained from our collaborator Prof. Hiroshi Mitani from the university of Tokyo (Table 3-1). Fresh cultures of cDNA colons were prepared on 20 x20 cm LB agar plates containing 50 mg/ml ampicillin, using 96-pin replicating tools. The plates were incubated at 37°C overnight.

1) OLa libarary	
RNA source	Male and female adult whole body of HNI inbred strain (male and female)
Vector	pME18s (AB009864 1193, 1194 modified from CC to AT)
Cloning site	*Dra*III
Host strain	*E .coli* Top10
Produced by	S. Sugano, K. Kawakami, M. Suzuki (The Institute of Medical Science The University of Tokyo)
2) OLb libarary	
RNA source	Cultured medaka cell, OLHNI derived from caudal fin of adult male HNI inbred strain
Vector	pBluescriptSK(+)
Cloning site	*Eco*RI, *Sal*I (disappeared after cloning)
Host strain	*E .coli* XL1 Blue MRF'
Produced by	H. Mitani
3) OLc library	
RNA source	Cultured medaka cells, OLHNI derived from caudal fin of adult male HNI inbred strain (irradiated with 20J/m^2 UV ± PR)
Vector	pUC118
Cloning site	*Eco*RI, *Sal*I (disappeared after cloning)
Host strain	*E. coli* SURE
Produced by	H. Mitani
4) OLd library	
RNA source	Ovary of HNI inbred strain
Vector	pUC118
Cloning site	*Eco*RI, SalI (disappeared after cloning)
Host strain	*E. coli* XL1 Blue MRF'
Produced by	H. Mitani, K. Naruse, M. Kondo
5) OLe library	
RNA source	Liver of HNI inbred strain (male and female)
Vector	pUC118
Cloning site	*Eco*RI, SalI (disappeared after cloning)
Host strain	*E. coli* XL1 Blue MRF'
Produced by	H. Mitani, K. Naruse, M. Kondo

6) OLf library	
RNA source	Cultured medaka cells, OLHNI derived from caudal fin of adult male HNI inbred strain (with gamma-irradiation)
Vector	pBluescriptSK(+)
Cloning site	*Eco*RI, SalI (disappeared after cloning)
Host strain	*E. coli* SURE
Produced by	H. Mitani

Table 3-1: Information on the cDNA libraries available at http://mbase.bioweb.ne.jp/~dclust/lib_info.htm

To amplify the inserts in pME18s vector (OLa clones), the primers pM18F and pM18R were used. The inserts in pBluescript SK(+) vector (OLb and OLf clones) were amplified using SK-Mitani-F and SK-Mitani-R primers and to amplify the inserts in pUC118 vector (OLc, OLd and OLe clones) the primers pUC118-F and pUC118-R were used.

The reactions mixture consisted of 10 pmol of each primer, 125 µM of each dNTP, 3 units Taq DNA Polymerase, 1 x PCR buffer and distilled water to a final volume of 30 µl, prepared in 96-well plates. Reactions were inoculated with bacterial colonies (bacterial clones were directly used as template cDNA), using a 96-pin replicating tool. Amplification was carried out in a thermocycler using the following conditions: Initial denaturation step (94°C, 3 min) followed by 35 cycles (94°C, 30 s; 65°C, 30s for clones in pME18s and pBluescript SK (+) vectors and 60°C, 30 s for clones in pUC118 vector;72°C, 90 s) and a final incubation step at 72°C for 10 min.

3.17 Purification of PCR-products

3.17.1 QIAquick kit-Method

This protocol is for the purification of single- or double stranded PCR products in the size of 100 bp – 10 kb from salts, primers, nucleotides and polymerase using vacuum. The kit was assembled as described in the handbook.

3 volumes of PM buffer (binding buffer) were added to 1 volume of PCR reaction and mixed. The samples were applied to the wells of the QIAquick plate and drawn through the silica-gel membrane using a vacuum system. The DNA was adsorbed to the silica-gel membrane and washed twice with 900 µl of PE buffer, removing the salts. Applying the maximum vacuum for 10 min dried the membranes. The waste tray was replaced by a 96-well microtiterplate. To elute

the DNA, 80 µl of EB (elution buffer) buffer (10 mM Tris-Cl, pH 8.5) or H_2O was added to the center of each well of the QIAquick 96 plate and after 1 min the plate was vacuumed for 5 min.

3.17.2 Silica Matrix DNA Purification kit (NC001 nuclean I kit)

This method is based on the high affinity of nucleic acids to the large surface of the silica particles in the presence of a chaotropic salt. Chaotropic salts increase the solubility of nonpolar substances in water. They denature proteins because they have the ability to disrupt hydrophobic interactions, but they do not denature DNA or RNA.

5 volumes of NuMelt buffer (NaI solution, protected from oxidation) were added to DNA solution and mixed. The vial of NuBeads (SiO_2 particles in suspension) was thoroughly mixed and for each µg of DNA 1 µl of NuBeads was added to the reaction tube. After mixing, the tube was incubated on ice for 5 min. The NuBeads particles were briefly centrifuged for 10 sec. The supernatant was decanted and the pellet was washed twice with 600 µl ice cold Nuwash buffer (washing solution). The pellet was briefly dried in a 55°C heating block for 5 min and then resuspended in 20 µl TE buffer. The tube was centrifuged at 14,000 rpm in an Eppendorf centrifuge 5415 D for 1 min and the supernatant containing DNA was carefully transferred into a new tube.

3.18 Bacterial transformation by heat shock

The tube of competent cells (E.coli DH5? mer) was thawed on wet ice. 1-50 ng DNA was added into the tube. The cells were incubated on ice for 30 min. Heat shock was carried out at 42°C in a heating block for 1 min. It was followed by incubation of cells on ice for 2 min. Then 1 ml of 2 x YT broth medium (without antibiotic) was added to the cells. The transformation mixture was incubated at 37°C while shaking vigorously for 1 hour. Afterwards the cells were plated on appropriate selection agar. To provide growth of transformed bacterial cells, the plate was incubated at 37°C overnight.

3.19 Agarose gel electrophoresis

Mixtures of differently sized DNA fragments, which were created by sonification, digestion, PCR and other methods, may be separated by gel electrophoresis based on their different rates of migration in an electrical field, depending on their corresponding fragment sizes.

1% agarose gel was prepared using 1 x TAE buffer. The same buffer was used as the running buffer. All agarose gel contained 1 µg/ml ethidium bromide to visualize DNA bands. Before loading of samples, 1 µl 6 x DNA loading buffer was added to 5 µl of the DNA sample. To estimate the DNA fragment length, 0.5 µg of a marker, containing fragments of known size, would be loaded in two different wells of the gel. The DNA bands were photographed under UV light.

3.20 Library replication

The 384-well microtiter plates were labeled with the necessary information (name of the library, number of copy, replication date). The plates were filled with LB broth medium containing appropriate antibiotic and anti-freezing (1 x HMFM) buffer, using the Q-Fill machine.
Master library plates (kept at –80°C) were thawed at room temperature whereby a sterile whatman paper was placed between the plate and its lid to adsorb condensed water. To produce the copy, the new plates were inoculated with the bacteria from original plates using 384-pin replication tools. The bacterial cultures were incubated at 37°C for 16-18 h. After incubation, the growth of bacterial cultures was checked by visual inspection. The plates were packed in 3 blocks of 8 plates each using packing machines. The plates can be stored at –80°C indefinitely.

3.21 Colony filter spotting and processing

Spotting refers as to the automated transfer of bacterial clones from microtiterplates (MTP) onto positively charged nylon membranes (222 x 222 mm Hybond-N+). Towards this end, small aliquots of the bacterial culture are transferred from wells of an MTP onto the filter by 384-pin tools (gadgets). The spotting was done on the Amazon robot at the Max-Planck-Institute for molecular genetics in Berlin.

3.21.1 Preparation of filters and robot

To prevent contamination during the spotting process, the ethanol bath for cleaning and the gadget were treated with 3% H_2O_2 for 15 min. The plexiglas plates that are used as support for membranes were cleaned with 70% ethanol.
Positively charged Hybond N+ nylon membranes were labeled with identification codes, the name of the library and date.

For production of high-density colony filters, the membranes were fixed on gel blotting paper (GB 005), which provides wetness, optimal pH and optimal osmotic condition to the bacteria. The filters and GB papers were moistened with the 2 x YT medium containing 33 mg/ml chloramphenicol. To assemble this system, the GBs were placed on the plexiglas plates. Air bubbles, which were between the plates and GBs, were removed by rolling the GBs with a glass pipette in all directions. The nylon membranes were laid onto the GBs avoiding air bubbles to be trapped underneath.

4	40	52	4	10	2	38	50	2	8
28	46	34	58	34	26	44	32	56	32
52	64	0	22	70	50	62	0	20	68
22	16	40	64	10	20	14	38	62	8
46	28	58	70	16	44	26	56	68	14
1	37	49	1	7	6	42	54	6	12
25	43	31	55	31	30	48	36	60	36
49	61	0	19	67	54	66	0	24	72
19	13	37	61	7	24	18	42	66	12
43	25	55	67	13	48	30	60	72	18
5	41	53	5	11	3	39	51	3	9
29	47	35	59	35	27	45	33	57	33
53	65	0	23	71	51	63	0	21	69
23	17	41	65	11	21	15	39	63	9
47	29	59	71	17	45	27	57	69	15

4	58	76	4	28	70	2	56	74	2	26	68
10	70	94	0	82	88	8	68	92	0	80	86
46	40	0	16	34	52	44	38	0	14	32	50
34	64	0	46	28	22	32	62	0	44	26	20
10	22	76	64	88	40	8	20	74	62	86	38
16	82	52	94	0	58	14	80	50	92	0	56
1	55	73	1	25	67	6	60	78	6	30	72
7	67	91	0	79	85	12	72	96	0	84	90
43	37	0	13	31	49	48	42	0	18	36	54
31	61	0	43	25	19	36	66	0	48	30	24
7	19	73	61	85	37	12	24	78	66	90	42
13	79	49	91	0	55	18	84	54	96	0	60
5	59	77	5	29	71	3	57	75	3	27	69
11	71	95	0	83	89	9	69	93	0	81	87
47	41	0	17	35	53	45	39	0	15	33	51
35	65	0	47	29	23	33	63	0	45	27	21
11	23	77	65	89	41	9	21	75	63	87	39
17	83	53	95	0	59	15	81	51	93	0	57

Figure 3-1: Spotting pattern for 5x5 pattern (above) and 6x6 pattern (below). 0 is the position of guide dots.

Afterwards the plexiglas plates were placed in fixed positions into the robot with the labeled side of filters facing to the left. The UV light was turned on for 20 min to sterilize filters, gadget and inside area of the robot. The microtiterplates (clone library) were placed into the stacker. Before starting the spotting process, the spotting pattern was chosen (Figure 3-1). The spotting routine included a cleaning cycle. Once spotting of a plate had been finished, the roboter arm holding the gadget moved to the tray holding 70% EtOH. Thereafter spotting was continued with the next library plate. After finishing the spotting process with all clones of the library, the filters were spotted with black ink (guide dots). The position of guide dots in both spotting pattern is indicated "0" (Figure 3-1).

Then, the nylon filters were transferred onto Nunc agar plates containing 2 x YT agar with 33 mg/ml chloramphenicol. Air bubbles between filter and agar surface were avoided and the plates were incubated at 37°C for 20 hours. Usually 14 filters were spotted in each run. After each run, the robot surface was cleaned with 70% ethanol and the gadget was washed with 3% H_2O_2.

3.21.2 Filter processing

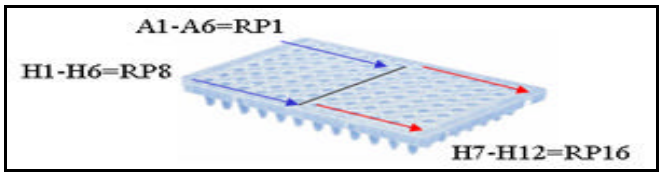
The first step in the filter processing, to produce the high-density colony DNA filters, is a denaturing step. Removal of air bubbles during the filter processing is necessary. To lyse the bacterial colonies and to denature double stranded DNA, filters were placed onto a GB paper moistened with denaturing solution for 4 min. This step was repeated. Afterwards, filter and GB paper together were transferred onto a glass plate, which was placed above hot water steam in a 95°C water bath for 4 min. Then to neutralization the filter, it was moved from the water bath and placed on a new GB paper moistened with neutralisation buffer for 4 min. Subsequently the proteins were digested by pronase. 100 ml of pronase solution, which was kept at 37°C, was poured into a Nunc plate. 0.5 ml of fresh pronase stock (50 mg/ml) was added to it. The filter was submerged in this solution so that the surface of filter was totally covered with this solution. The plate was incubated at 37°C for 30-45 min. Thereafter, the filter was rinsed in a plastic box containing 250 ml washing solution for 10 min. 2-4 filters could be washed at the same time in each plastic box. After the washing step, the filter was dried first for a couple of hours on a clean GB002 paper and then at 37°C for 2-3 days. Finally, the dried filter was irradiated in the UV Cross-Linker for 1 min (125 mjoules).

3.22 Selection of 35-mer oligonucleotide probes

To select the 35-mer oligonucleotide probes, which were used for hybridization against medaka BAC filters, a non-redundant set of medaka cDNA obtained by sequence clustering (see 3.25.1) was aligned against the *Takifugu rubripes* genomic sequence v3.0 assembly from August 2002 (see 2.5.3). cDNA matches on fugu scaffolds were identified by blastn (Altschul *et al.*, 1990) and exon alignments were refined using CrossMatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm). The results were produced in html format. Oligonucleotide probes were chosen from matches 100 kb apart. In additional, oligonucleotides were designed from scaffold matches corresponding to genetically mapped medaka genes listed in MBase (http://mbase.bioweb.ne.jp/~dclust/medaka_top.html). 35-mers were ordered from Metabion (Martinsried, Germany) and shipped lyophilized in 96-well plates.

3.23 Pooling scheme

To reduce the number of hybridization, the hybridization probes were pooled either according to a 2D (dimensional) or in a 3D scheme.



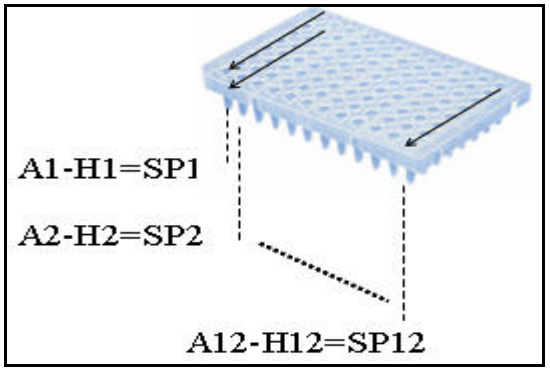


Figure 3-2: Pooling scheme. Above: row pool (RP), below: column pool (SP).

3.23.1 2D Scheme

Oligonucleotieds in 96-well plates dissolved to a concentration of 10 pmol/µl were pooled according to a 2D scheme into 12 column (SP) and 16 half-row pools (RP) (Figure 3-2). Each column pool consisted of 8 probes and each row pool consisted of 6 probes. That means to hybridize all probes in one 96-well microtiterplate, 28 hybridizations were needed, 12 hybridizations with column pool probe and 16 with row pool probes.

3.23.2 3D scheme

Oligonucleotides from six 96-well microtiterplate were pooled according to a 3D scheme, into block column pools (BSP), block row pools (BRP) and block plate pools (BPP)(Figure 3-3). Each BSP consisted of 48 probes, each BRP consisted of 36 and each BPP of 48 probes. In this way to hybridize all 576 probes contained in the six 96-well plates, 40 hybridizations were needed (12 BSP +16 BRP +12 BPP).

3.24 Hybridization

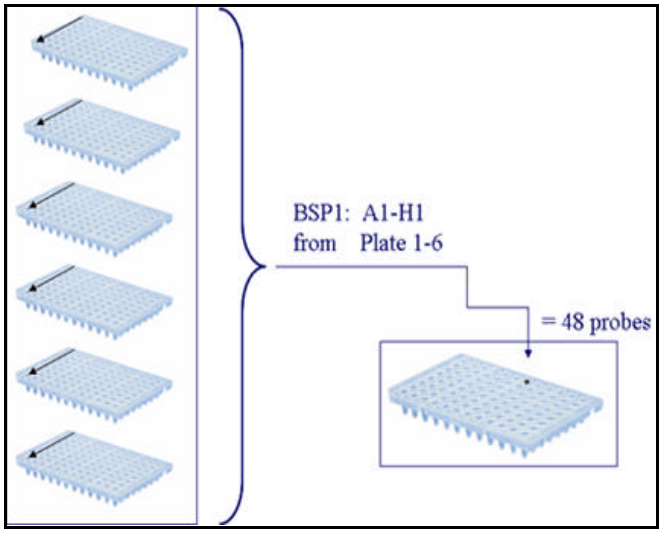
For hybridization both non-radioactive labeling with DIG-dUTP and radioactive labeling with ^{32}P or ^{33}P isotope were employed.

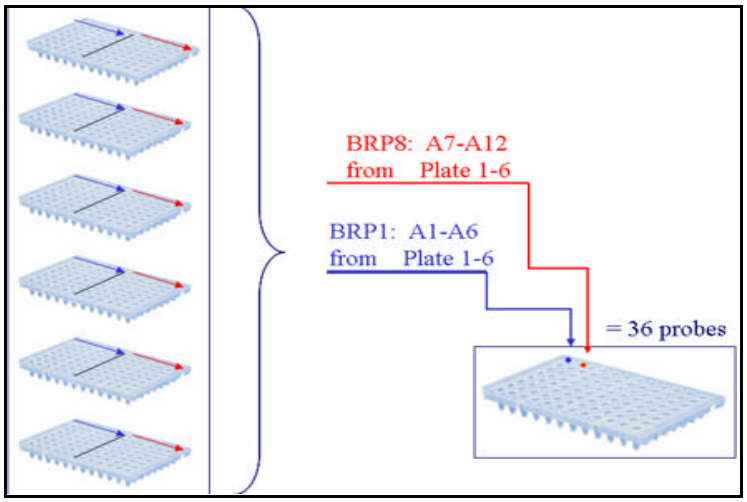
3.24.1 Non-radioactive hybridization with digoxigenin (DIG-dUTP) labeled probes

3.24.1.1 Labeling and sample preparation

DNA probes were labeled with DIG-dUTP by PCR, using the PCR DIG labeling mix kit from Boehringer. The detection of DIG labeled DNA occurred by Anti-Digoxigenin-AP Fab fragments using the appropriate kit. The PCR reaction consisted of 20 ng template DNA, 1 x PCR buffer, 10 pmol of each forward and reverse primer, 3 units *Taq* DNA, 1 x Boehringer DIG labeling mix (instead of dNTPs) and distilled water to 30 µl. The PCR reactions were cycled as follows: Reactions were heat denatured for 3 min at 94°C, then they were processed by 35 cycles of 94°C (30 sec), annealing at 65°C (30 sec), extension at 72°C (90 sec) and final incubation at 72°C for 10 min. For hybridization single stranded DNA was required. To produce single

stranded DNA, amplified DNA was denatured in a thermocycler at 94°C for 5 min and then immediately added into 10 ml of hybridization buffer (Church buffer).





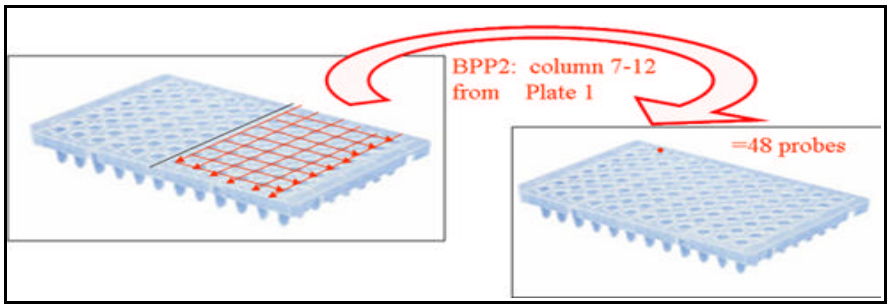


Figure 3-3: 3D pooling scheme of six 96-well plates. Above: Block Column Pool (BSP). Middle: Block Row Pool (BRP) and below Block Plate Pool (BPP).

3.24.1.2 Prehybridization

To prevent non-specific hybridization and for subsequent reduction of background, prehybridization is required. To prehybridize the filter, it was put in a plastic box containing 500 ml Church buffer preheated to 58°C. The box was incubated at 65°C on a rocking platform for more than half an hour.

3.24.1.3 Hybridization

The Church buffer from the prehybridization step was decanted. The filter was placed in a plastic bag. The hybridization mixture (labeled DNA in 10 ml Church buffer) was added to the filter. Air bubbles were removed and the filter was covered equally with the buffer. The plastic bag was sealed and incubated in a 65°C oven on a rocking platform over night.

3.24.1.4 Washing

On the next day, the filter was transferred into a plastic box. To remove probe that had bound non-specifically to the DNA on the filter, the filter was washed consecutively with wash I and wash II solutions as follows: 600 ml of wash I was added to the box. It was incubated on a rocking platform at 65°C for 45 min. Afterwards, the wash I solution was removed and the wash process was repeated with wash II solution.

3.24.1.5 Blocking

The wash II solution was discarded and the filter was incubated in blocking buffer, a mixture of 500 ml 1x PBS and 5% BSA (20 ml of blocking buffer was kept for the next step), at RT without shaking for 45 min.

3.24.1.6 Antibody binding, removal of unbound antibody, and antibody detection

The blocking buffer was decanted. The Anti-DIG Fab fragment was diluted 1:5,000 by adding 4 µl of it into 20 ml of 1x PBS + 5% BSA. The filter was placed into a new plastic bag and the antibody mixture was added to it. The air bubbles were removed and the plastic bag was sealed, followed by incubation at RT for 1 hour. To avoid the influence of air bubbles, which prevent the contact between DNA and antibody, the bag that holds the filter was squeezed during the incubation few times using a paper towel. Afterwards, the antibody solution was discarded. To remove unbounded antibodies, the filter was washed twice with 500 ml 1 x PBS in a plastic box

on a rocker at RT for 30 min each. Thereafter, the filter was incubated twice in 200 ml of 100 mM Tris pH 9.0-9.5 and 1 mM $MgCl_2$, each time for 10 min. 5 ml of 1 x Attophos solution (see 3.8.4) were added to the filter in a plastic bag and subsequently incubated in darkness overnight. The complex of DIG-antibody was detected by Attophos (alkaline phosphates substrate). The formation of the fluorchrome occurs when an alkaline phosphatase-labeled antibody liberates the phosphate group from the non-fluorescent Attophos molecule. Attophos substrate (2´-[2-benzothiazoyl]-6´-hydroxybenzothiazole phosphate [BBTP]) is cleaved by alkaline phosphatase to produce inorganic phosphate (P_i) and the alcohol, 2´-[2-benzothiazoyl]-6´-hydroxybenzothiazole (BBT). After Attophos reaction the filter was washed with 1 x TE. Then it was wrapped in saran foil and scanned with a Luminescent Image Analyser. The signals were visible in UV light.

3.24.2 Radioactive hybridization

For hybridization of radioactive probes the kind of DNA (oligonucleotide, DNA fragment or cDNA fragment) determines, which method should be used for labeling.

3.24.2.1 Labeling by hexamer priming (Feinberg and Vogelstein, 1983)

This technique is based on the insertion of radioactive nucleotides during the synthesis of DNA probes from the provided DNA template. After denaturing the double stranded provided DNA by heating at 94°C, the complementary strand was synthesized from the 3'OH termini of annealed random hexanucleotides primers using Klenow enzyme.

50 ng DNA in a volume of 11 µl was boiled to denature its double strand structure at 94°C for 1 min. The DNA was chilled immediately on ice to avoid reannealing. The following solutions were added in this order:

- 18 µl of LS (labeling solution containing hexamers)
- 3 µl of 5 mg/ml acetylated BSA
- 3 µl of 100 mM cold nucleotide mix (dATP, dTTP and dGTP)
- 1 µl Klenow enzyme (5 U/µl)

Finally, 3 µl (30 µCi) of ?-^{32}P-dCTP isotope were added to the mixture. The reaction was incubated at RT overnight.

3.24.2.2 Labeling with "Random Primed" kit (Roche)

This method follows the same principle as described above. Using this kit labeling of DNA amounts as small as 10 ng is possible.

40 ng DNA was added to distilled water and the final volume was adjusted to 11 µl. The DNA was denatured by heating at 96°C for 10 min and subsequently cooled on ice.
1 µl dATP (solution 2), 1 µl dGTP (solution 4) and 1 µl dTTP (solution 5) were added to the reaction tube. It was followed by adding 2 µl of hexanucleotide mixture (solution 6) and 1 µl Klenow enzyme (solution 7). In the hot lab, 2.5 µl (25 µCi) ?-^{32}P-dCTP were added to the reaction tube. The tube was incubated at 37°C for 30 min. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0).

3.24.2.3 Oligonucleotide labeling

The oligonucleotide was labeled by 5' phosphorylation using T4-polynucleotide kinase (PNK). PNK catalyzes the transfer of P$_i$ from the ?-position of ATP to the 5'-hydroxyl terminus of polynucleotides.

2.5 µl oligonucleotide (10 pmol/µl), 1 x PNK buffer and 7.5 µl H_2O were added into a new tube. It was followed by addition of 2.5 µl (25 µCi) ?-^{32}P-dATP or ? -^{33}P-dATP and 1µl (10 U) PNK enzyme to the tube in the hot lab. The mixture was incubated at 37°C for 1h. The labeled oligonucleotide was purified using a Micro Spin G-50 column. The probe was added into the middle of Micro Spin G-50 column. Then it was centrifuged at 3,000 rpm for 2 min using an Eppendorf centrifuge 5415 D. The labeled oligonucleotide was collected in a fresh eppendorf tube. The dye and not incorporated radioactive nucleotides remained in the Micro Spin column.

3.24.2.4 Prehybridization

To reduce nonspecific absorption of the radiolabeled probe the prehybridization was done. A colony filter was placed in a hybridization bottle. In the case of hybridization with more than one filter per bottle, a mesh sheet was put between two filters. The tube was incubated for more than one hour with 50 ml of preheated Church buffer in a mini hybridization oven with rocker. For hybridization with probes generated by random priming, the incubation was carried out at 65°C and for 35mer-oligonucleotide probes at 58°C.

3.24.2.5 Hybridization

The prehybridization buffer was decanted. The purified labeled probe was added to 15 ml of fresh preheated Church buffer. Then it was mixed and poured into the hybridization bottle containing the filter. The bottle was incubated at 65°C for DNA fragment probes and at 58°C for oligonucleotide probes in the hybridization oven overnight.

3.24.2.6 Washing

The hybridization solution was removed. The filter was transferred into a plastic box and washed first with wash solution I and then with wash II. Wash I solution is in common for oligonucleotide and DNA fragment probes, but different wash II was used (see 3.8.2). To wash the filters, which were hybridized with DNA fragments, 600 ml of wash I was added into a plastic box and it was incubated at 65°C in a water bath for 45 min. The solution was decanted and washing was repeated with wash II (specific for end-fragment probes) for an incubation time of 20 min at the same temperature.

For the filters, which were hybridized with 35mer-oligonucleotides, the incubation times were reduced to 25-30 min for the wash I and 10-15 min for the wash II. The time of washing became shorter once filters had been used for hybridization several times due to reduction of the quality of DNA bound to the filters.

3.24.2.7 Exposing and developing

The filter was monitored with a Geiger Müller detector. If the level of cpm (counts per minute) was above 50 counts, filters were washed again with solution II for about 7 min. Two filters were placed on a plastic pad in the size of an X-ray cassette. The filters were blotted dry with whatman paper and wrapped in saran foil. Air bubbles between filters and saran wrap were removed. The filters were packed in a cassette and exposed to X-ray films. The cassette was incubated at –80°C for 4-7 days for exposure (this time was prolonged for hybridization with ^{33}P labeled probes for up to 2 weeks). The localized radioactivity in the bound probe reduced the silver in the X-ray film. The exposed x-ray film was developed chemically with an AGFA machine.

3.24.2.8 Stripping

Each filter could be used up to 10 – 15 times for hybridization, but bound probes had to be removed from filters after each hybridization.

The hybridized filter was stored at 4°C for at least one half-life period depending on the isotope used. Up to six filters were placed into a plastic box containing stripping solution (see 3.8.2), which was placed in a shaking water bath at 65°C for 1 h. After stripping, the filters were checked with a Geiger Müller counter. The filters with more than 10 cpm were stored again at 4°C for one week and after this time stripped again. The filters with less than 10 cpm were kept in 1 x TE in a plastic box, ready for the next hybridization.

3.24.2.9 Competitive hybridization

Repetitive elements or vector fragments in hybridization samples cause a strong background during hybridization, but may be removed by the use of competitive DNA. Vector DNA with a small insert or without an insert and sonified genomic DNA were used as competitive DNA, which functions by binding to repetitive elements or vector fragments in the hybridization probe. For preparation of competitive DNA, 100 µg DNA (mix of sonified genomic DNA from fish and vector DNA) were mixed with 15 µl 1 M NaP_i and 5 µl 0.5 M EDTA and the volume was adjusted to 65 µl using distilled water. This mixture was added to the labeled BAC end-fragment or labeled cDNA fragment probe in the hot lab. The tube was incubated first at 95°C for 10 min (to denature the DNA) and then at 65°C for 3 h using a thermocycler.

3.25 Software tools for constructing a physical map

3.25.1 Clustering medaka ESTs for aligning on the fugu genome

To cluster the 103,144 EST sequences, which were downloaded from the NCBI website (http://www.ncbi.nlm.nih.gov/), a software developed in house was used (Poustka *et. al*, 2003). First EST sequences were checked for repeats and vector sequences (step one). Processed ESTs from step one were preclusterd into groups under the condition that they overlapped by at least 80 bp in an all against all comparison using Blastn.

The preclusters were used as input sequence sets for creating the final clusters and the calculation of a consensus sequence by Cap3 (Huang and Madan, 1999).

3.25.2 Image analysis

Autoradiograms obtained by hybridization were scanned using an Epson scanner. The image files were saved as tiff format. For image analysis, the tiff files were imported into the VisualGrid program (GPC Biotech, Munich, Germany, http://www.gpc-biotech.com/en/index.html). This software was used to overlay the macroarray images manually with a grid.

Two software packages were used to analyze images overlaid with a grid, first by VisualGrid, which performed manual and semi-automatic analyses of images. The positive signals were manually detected. VisualGrid calculated the location of the clones corresponding to these signals in the microtiterplates.

Second, by the automatical image analysis, which was performed by an in house developed program (Steinfath *et al.*, 2001). According to the grid, this software calculates the position of signals on the filter and identifies their coordinates in microtiterplates.

3.25.3 Pooled Probes Editor (PPE)

Pooled probes editor was developed by Dr. Detlef Groth in MPI for Molecular Genetics in Berlin in 2001. This software calculated the plate coordinates from pool coordinates.

The results of hybridization of column and row pools, obtained from image analysis software, were compared against each other by PPE and the signals, which were in common in SP and RP and (BSP, BRP and BPP) respectively, were identified. These signals belong to the probe at the intersection of the pooled row and column in the microtiterplate (Figure 3-2).

3.25.4 Wprobeorder

Wprobeorder was used to construct contigs of overlapping clones based on distance measure and simulated annealing algorithms (Mott *et al.*, 1993; Grigoriev *et al.*, 1998). The data of hybridizations of probes were used as input for this software.

This program gives a detailed output in HTML format, including statistics on probe and clone hits, and a description of each individual contig and its connections to other contigs. It also produces a schematic view of the whole physical map as an HTML file with links to individual contigs.