# 2. MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Chemicals

| Name | Company | Storage |
|---|---|---|
| Acrylamid-Bis | ROTH | 4°C |
| Agar-Agar | ROTH | RT |
| Agarose | GIBCO | RT |
| Albumin | AppliChem | 4°C |
| Ammonimum Acetate | ROTH | RT |
| APS | ROTH | 4°C |
| Ampicilllin | ROTH | 4°C |
| ATP | AppliChem | -20°C |
| 5-azacytidine | SIGMA | -80°C |
| Boric acid | ROTH | RT |
| Bromphenol blue | AppliChem | RT |
| Calcium Chloride | SIGMA | RT |
| Chloroform | MERCK | RT |
| Collagen | SIGMA | 4°C |
| Complete, mini protease inhibitor cocktail tablets | Roche | 4°C |
| DAPI | AppliChem | -20°C |
| dNTP set | Invitek | -20°C |
| Doxycycline | SIGMA | -20°C |
| DTT | AppliChem | -20°C |
| EDTA·$Na_2$ | ROTH | RT |
| EDTA solution | BioWHITTAKER | 4°C |
| Ethanol | ROTH | RT |
| Ficoll 400 | SERVA | RT |
| Formaldehyde | ROTH | RT |
| G418 | GIBCO | 4°C |

| | | |
|---|---|---|
| Glycerin | ROTH | RT |
| Glycogen | AppliChem | -20°C |
| HEPES | ROTH | RT |
| HEPES solution | BIOCHROM AG | 4°C |
| Hydrochloric Acid | SIGMA | RT |
| Hydroquinone | SIGMA | RT |
| Hygromycin | CALBIOCHEM | 4°C |
| IPTG | ROTH | -20°C |
| Potassium Chloride | ROTH | RT |
| L-Glutamine | SIGMA | -20°C |
| Lysozyme | ROTH | -20°C |
| Magnesium Chloride | ROTH | RT |
| Methanol | MERCK | RT |
| Methylene blue | ROTH | RT |
| Mineral oil | SIGMA | RT |
| Mycoplasma Removal Agent | ICN | 4°C |
| Sodium Acetate | MERCK | RT |
| Sodium Azide | ROTH | RT |
| Sodium Bicarbonate | BioWHITTER | RT |
| Sodium Carbonate | AppliChem | RT |
| Sodium Chloride | AppliChem | RT |
| Sodium Citrate | AppliChem | RT |
| Sodium Hydroxide | ROTH | RT |
| Sodium Hydrogensulfite | SIGMA | RT |
| Sodium Hydrogenphosphate | AppliChem | RT |
| PBS buffer (10X), powder | AppliChem | RT |
| Phenol | ROTH | RT |
| Phenylmethysulfonylfluoride (PMSF) | SIGMA | RT |
| Polyvinylpyrrolidon | AppliChem | RT |
| 2-propanol | ROTH | RT |
| Proteinase | SIGMA | -20°C |
| Salmon sperm DNA | AppliChem | -20°C |
| SDS | ROTH | RT |
| TEMED | ROTH | 4°C |
| Tris base | ROTH | RT |

| | | |
|---|---|---|
| Triton X-100 | SIGMA | RT |
| Trypan blue | GIBCO | RT |
| Trypsin solution 2.5% | GIBCO | 4°C |
| Trypron-Pepton | DIFCO | RT |
| Tween 20 | SIGMA | RT |
| X-gal | ROTH | -20°C |
| Xylencyanol FF | AppliChem | RT |
| Yeast extract | DIFCO | RT |

### 2.1.2 Solutions and media

Aqueous solutions or media were prepared using autoclaved dd$H_2O$ if not purchased. For sterilization, if necessary, solutions and media were autoclaved or passed through a 0.45µm filter (Schleicher & Schuell GmbH).

| Name | Composition |
|---|---|
| Ethidium Bromide | 100 mg/ml solution |
| LB (Luria Bertani) medium | 10 g/L tryptone<br>5 g/L yeast extract<br>10 g/L NaCl |
| STET | 50 mM Tris·HCl, pH 8.0<br>8% Sucrose<br>5% Triton X 100<br>50 mM EDTA |
| Digestion buffer | 100 mM NaCl<br>10 mM Tris·HCl, pH 8.0<br>25 mM EDTA, pH 8.0<br>0.5 % SDS<br>100 µg/ml proteinase K |

| | |
|---|---|
| Ammonium acetate | 10 M |
| 2X HBS (HEPES Buffered Saline) | 8 g/L NaCl<br>0.21 g/L $Na_2HPO_4$<br>12 g/L HEPES<br>pH 7.05 |
| NaAc | 3 M, pH 5.2 |
| Proteinase K dissolving solution | 50 ml Glycerol<br>1 ml 1 M Tris·HCl, pH 7.5<br>0.29 g $CaCl_2$<br>adding $H_2O$ to 10 ml |
| 10X PBS<br>(Phosphate Buffered Saline) | 80 g NaCl<br>2 g KCl<br>11.50 g $Na_2HPO_4 \cdot 7H_2O$<br>2 g $KH_2PO_4$<br>adding $H_2O$ to 1 liter |
| 20X SSC | 175 g NaCl<br>88 g $Na_3Citrate \cdot 2H_2O$<br>adding $H_2O$ to 1 liter, pH 7.0 |
| 10X TBE | 108 g Tris base<br>55 g Boric acid<br>40 ml 0.50 M EDTA, pH 8.0<br>adding $H_2O$ to 1 liter |
| 50X TAE | 242 g Tris base<br>57.1 ml glacial acetic acid<br>37.2 g $Na_2EDTA \cdot 2H_2O$<br>adding H2O to 1 liter |

| | |
|---|---|
| TE | 10 mM Tris·HCl, pH 8.0<br>1 mM EDTA, pH 8.0 |
| Depurination solution | 0.25 M HCl |
| Denaturation solution | 1.50 M NaCl<br>0.50 M NaOH |
| Neutralizaiton solution | 1.50 M NaCl<br>0.50 M Tris·HCl pH 7.2<br>0.001 M EDTA |
| Church buffer | 1.00 % BSA<br>1.00 mM EDTA<br>7.00 % SDS<br>0.50 M $NaHPO_4$ |
| Blot washing solution I | 2X SSC, 0.10 % SDS |
| Blot washing solution II | 1X SSC, 0.10 % SDS |
| Blot washing solution III | 0.1X SSC, 0.10 % SDS |
| X-gal staining solution | 5 mM $K_3Fe[CN]_6$<br>5 mM $K_4Fe[CN]_6$<br>2 mM MgCl2<br>1 mg/ml X-gal<br>in 1X PBS |
| EDTA solution (cell culture grade) | BioWHITTAKER |
| Complete medium | 1X MEM (SIGMA)<br>1.5 mg/ml glucose<br>2 mM L-Glutamine<br>10% heat inactivated FCS |

| | |
|---|---|
| | 50 µg/ml penicillin<br>50 µg/ml streptomycin |
| Complete medium (high glucose) | 1X DMEM (SIGMA)<br>4.5 mg/ml glucose<br>2 mM L-Glutamine<br>10% heat inactivated FCS<br>50 µg/ml penicillin<br>50 µg/ml streptomycin |
| Optimum medium | Glumtamax-1, GIBCO |
| Freezing medium | 50 % complete medium<br>50 % conditioned medium<br>10 % DMSO |
| Trypsin solution | 2.5 %, GIBCO |
| Trypan Blue Stain | 0.4 %, GIBCO |

### 2.1.3 Enzymes

All restriction enzymes for cloning and Southern blotting were purchased from New England Biolabs, Amersham or MBI Fermentas GmbH. Additional enzymes used are listed in Table 1 below.

**Table 1. Additional enzymes**

| Enzyme | Concentration | Company |
|---|---|---|
| Taq DNA polymerase | 5 U/µl | Invitek or Eppendorf |
| T4 DNA ligase | 1 U/µl | Fermentas |
| Klenow fragment | 1 U/µl | Invitrogen |
| Shrimp Alkaline Phosphatase (SAP) | 1 U/µl | Amersham |
| Proteinase K | 10 mg/ml | SIGMA |
| DNase-free RNase | 1 mg/ml | ROTH |
| Pfu DNA polymerase | 5 U/µl | Applied Biosystems |
| Mung bean nuclease | 40 U/µl | Fermentas |

### 2.1.4 Kits

All kits, listed in Table 2, were used for purification as instructed by the manufacturer.

**Table 2. Kits**

| Kit Name | Company |
|---|---|
| QIAgen Plasmid Midi and Maxi Kits | Qiagen |
| Quich Spin Columns for DNA purification | Roche |
| RadPrime DNA Labeling System | Invitrogen |
| Deca DNA labeling Kit | Fermentas |
| Roti®-Fect Transfection reagent | ROTH |
| Calcium Phosphate Transfection Kit | Invitrogen |
| Anti-Acetyl Histone H4, ChIP grade | UPSTATE |
| Expand Long Template PCR System | Roche |
| Quick Ligation Kit | NEBiolabs |

### 2.1.5 Vectors

All vectors used for expression study as well as those used for intermediate cloning were listed in Table 3 below.

**Table 3. Vectors**

| Vector | Size (bp) | Resistance | Source |
|---|---|---|---|
| pEGFP-C1 | 4731 | Kanamycin/Neomycin | Clontech |
| PCMVß | 7164 | Ampicillin | Clontech |
| pCpGvitro-hygro-LacZ | 8751 | Hygromycin | InvivoGen |
| pEFG3 | 3704 | Ampicillin | Strathdee. CA |
| pUB/Bsd | 4245 | Ampicillin | Invitrogen |
| pHygEGFP | 5792 | Ampicillin | BD Biosciences |
| pTRE-d2EGFP | 3988 | Ampicillin | Clontech |
| pEYFP | 3355 | Ampicillin | Clontech |
| pECFP | 3355 | Ampicillin | Clontech |
| pIREShyg2 | 5788 | Ampicillin | BD Biosciences |

### 2.1.6 DNA markers

SmartLadder (Eurogentec)

1 Kb DNA Ladder (Fermentas)

### 2.1.7 Bacterial material

DH5a, XL1-blue and GM48 E.coli strains were used for all the DNA recombination techniques.

### 2.1.8 Mammalian cell lines

HeLa (human cervical carcinoma cell,)

Flp-In 293 (genetically engineered human embryonic kidney cell, (Invitrogen Co., the Netherlands))

### 2.1.9 Antibiotics

All antibiotics used in the transformation or stable clone selection experiments are listed in Table 4 below.

**Table 4. Antibiotics**

| Name | Stocking concentration |
|---|---|
| Ampicillin | 100 mg/ml |
| Hygromycin | 100 mg/ml |
| Blasticidin | 3 mg/ml |
| Kanamycin | 50 mg/ml |
| G418 | 50 mg/ml |
| Zeocin | 100 mg/ml |

## 2.2 Methods

### 2.2.1 DNA isolation

Genomic DNA was extracted from HeLa or HEK 293 cell lines according to “Current protocols in molecular biology”. Essentially, cells were harvested and suspended in 1 ml digestion buffer/ 10E8 cells for overnight digestion at 50°C by proteinase K, followed by phenol/chloroform/isoamyl alcohol extraction. The aqueous layer containing DNA was precipitated by ½ vol of 7.5 M ammonium acetate and 2 vol of 100% ethanol. DNA was dissolved in TE buffer and stored at

-20°C. Approximately 750 µg genomic DNA was obtained from cells harvested from one confluent 10 cm Petri dish.

Plasmid DNAs were minipreped by STET buffer. Plasmid DNAs used for transfection were isolated using QIAprep plasmid Maxiprep kits according to the supplied protocols.

### 2.2.2 Recombinant DNA techniques

All the restriction enzyme reactions were performed according to the provided protocols on the enzyme sheets. If necessary, the digested DNA fragment ends were dephospharated with SAP or blunted with mung bean nuclease before preceding the ligation reaction. Ligation reactions were carried out following the Quick Ligation Kit protocols.

pFRT-CMV-EGFP$^{WT}$ was constructed by cloning EGFP gene fragment (from pEGFP-C1, Clontech. In this study this EGFP was called EGFP$^{WT}$.) into the expression vector pcDNA/FRT between NheI and KpnI sites. In order to obtain pFRT-EF-EGFP$^{WT}$, a synthesized polylinker was ligated to pcDNA5/FRT cut with MluI and XhoI. The original multiple cloning sites were destroyed. The new polylinker sequence is designed as,

5'-CGCGT-XhoI-NotI-HindIII-NheI-EcoRV-KpnI-BamHI-G- 3'

3' -A-XhoI-NotI-HindIII-NheI-EcoRV-KpnI-BamHI-CAGCT- 5'.

The EF promoter was obtained by digesting the pEFG 3 (donated by Strathsee CA) plasmid with SalI and HindIII, and then inserted at the new polylinker site. The EGFP gene fragment from pEGFP-C1 was inserted between NheI and KpnI sites. pFRT-EF-EGFP$^{CpG-}$ was constructed by substituting the NheI-EGFP-BamHI fragment with NheI-EGFP$^{CpG-}$-BamHI fragment cut out from pEGFP$^{CpG-}$ plasmid (a newly synthesized EGFP gene by removing all CpG dinucleotides in the coding sequence based on codon usage, constructed and contributed by Hampf M and Gossen M, MDC, Germany). pFRT-CMV-EGFP$^{CpG-}$ was obtained by substituting the EF promoter on plasmid pFRT-EF-EGFP$^{CpG-}$ with CMV promoter fragment from the original expression vector pcDNA5/FRT cut with MluI and HindIII.

Basing on plasmid pEGFP-C1, I constructed 8 plasmids, namely pCMV-EGFP$^{WT}$, pCMV-EGFP$^{CpG-}$, pEF-EGFP$^{WT}$, pEF-EGFP$^{CpG-}$, pfCMV-EGFP$^{WT}$, pfCMV-EGFP$^{CpG-}$, pfEF-EGFP$^{WT}$ and pfEF-EGFP$^{CpG-}$. The latter 4 plasmids have two sequences free of CpG dinucleotide flanking on the transgene expression unit, otherwise identical to the former 4 plasmids, their counterparts. The pEGFP-C1 plasmid was cut with MluI and inserted an EcoRV linker with MluI overhang (5'-CGCG GATATC-3'), and pEGFPEcoRV was named to the new plasmid. pEGFPEcoRV was cut with AseI and NheI, where a linker (AseI overhang-ScaI-MluI-NheI overhang) was inserted to construct pEGFPScaMluEco plasmid. This new plasmid was digested with MluI and BamHI, at which site I inserted fragments MluI-CMV-EGFP$^{WT}$-BamHI, MluI-CMV-EGFP$^{CpG-}$-BamHI, MluI-EF-EGFP$^{WT}$-BamHI and MluI-EF-EGFP$^{CpG-}$-BamHI cut from pFRT-CMV-EGFP$^{WT}$, pFRT-CMV-EGFP$^{CpG-}$, pFRT-EF-EGFP$^{WT}$ and pFRT-EF-EGFP$^{CpG-}$ constructs, respectively. Those new constructs are pCMV-EGFP$^{WT}$, pCMV-EGFP$^{CpG-}$, pEF-EGFP$^{WT}$, pEF-EGFP$^{CpG-}$. All the final plasmid maps are listed in Appendix V.

### 2.2.3 Cell culture

All cell lines were cultured under standard conditions. HeLa cells were maintained in the complete MEM (minimum essential medium) containing 1.5 mg/ml glucose, 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin. Cultures at ~80% confluence were routinely split 1:10 in T25 flasks as follows. After removal of the growth medium, cells were washed once with 1X PBS. 0.5 ml trypsin was then added to the flasks and placed at 37$^0$C for about 3 minutes. After the cells were detached from the flasks, 5 ml pre-warmed culture medium was added, and the cells split into new flasks in a proper dilution ratio.

Flp-In 293 cells were maintained in the complete DMEM (Dulbecco modified Eagle's medium) containing 4.5 mg/ml glucose, 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 µg/ml penicillin and 50 µg/ml streptomycin. Maintenance was done in the same way as HeLa cells except passaged in a ratio of 1:5.

Plating cells for transfection was done essentially as described above with the few following exceptions. Cells were harvested by centrifugation at 1200 rpm for 3 minutes and counted in the Neubauer Haemocytometer. ~ 4X 10E5 cells were seeded in 6-well plates one day before transfection. Cells should be 50-80 % confluent on the day of transfection.

Cells were frozen in the proper freezing medium with 10 % DMSO at a concentration of 3X 10E6/ml. The cells were kept in a cryo-freezing box at -80°C for 48 hours before transferred to the liquid nitrogen (-180°C).

### 2.2.4 Transfection

#### 2.2.4.1 Calcium phosphate transfection

The calcium phosphate transfection method is based on the formation of a calcium phosphate-DNA precipitate. The calcium phosphate is thought to facilitate the binding of the DNA to the cell surface; subsequently, the DNA enters the cell by endocytosis.

The transfection was done in this study according to either the “Calcium phosphate transfection kit manual” or the method established in the lab. Both are essentially as described below. Cells were plated one day before transfection as described above and the medium was changed 4 hours before transfection. In one eppendorf tube, 25 µl of 500 mM $CaCl_2$ and 5 µg of plasmid DNA were added, and the final volume was adjusted to 50 µl with dd$H_2O$. The formed DNA-$CaCl_2$ complex was shot to 50 µl of 1X HBS prepared in another tube. Aeration was cared to form during the shooting in order to obtain the maximum contact between the DNA-$CaCl_2$ complex and the HBS buffer, therefore very fine DNA-$CaCl_2$ precipitate. The DNA precipitate was dropwise added to the cells after incubation for 25-30 mins at RT. The cells were further incubated overnight at 37°C in a $CO_2$ incubator before either checked for gene expression if for transient transfection, or plated onto 10 cm Petri dish for obtaining stable clones with proper antibiotic selection.

#### 2.2.4.2 LipoFection

LipoFection was performed with Roti®-Fect transfection. Roti®-Fect is a liposome formation of a polycationic lipid in combination with a neutral colipid. It can condense DNA to compact structures with Roti®-Fect reagent and ensure high efficient uptake of mammalian cells.

The transfection was performed according to the supplied instructions. Essentially, 800 ng of plasmid DNA was added to 60 µl of optimum medium (free of serum and antibiotics) in a first eppendorf tube; 6 ml of Roti®-Fect transfection reagent was added to 60 µl of optimum medium in a second tube. The above two solutions were combined and mixed gently by carefully pipetting several times. I normally incubate this mixture for 35 mins at RT to allow the DNA-lipid complex to form. During the incubation time, 0.8 ml of fresh complete medium was changed to the cells on 6-well plates. The cells were incubated in a 37°C $CO_2$ incubator for 5 hours after the addition of the DNA-lipid complex. 1.2 ml of complete medium was supplemented to the cells and incubated further for 15-20 hours in 37°C $CO_2$ incubator before checked for transient expression or plated for stable clone selection.

### 2.2.5 Southern Blotting

Genomic DNAs were digested with appropriate restriction enzymes, separated in 1% agarose gels with a SmartLadder molecular weight marker for reference, and transferred to Hybond-N+ membranes (Amersham) on vacuum blotting apparatus (BIO-RAD). The membranes were rinsed twice in 5X SSC, and the DNA was crosslinked to the membranes by UV. The membrane was ready for hybridization.

DNA probes were radioactively labeled with DNA labeling kit according to the provided protocols. Normally 25 ng of appropriate DNA fragment was used as template, and denatured at 95°C for 5 min before the reaction buffer, dATP, dGTP, dTTP and $\alpha$-$^{32}$P dCTP were added to the reaction tube. The $^{32}$PdCTP labeled probes were then synthesized at 37°C for 10 min with the presence of 1 µl of Klenow Fragment.

The labeled probes were purified with Quick Spin Columns Sephadex G-50 by centrifuging at 1100 g for 4 min. Eluted DNA probes were denatured at 95°C for 10 min and chilled on ice immediately before ready for Southern hybridization.

The membrane blots were prehybridized in the Church buffer for 1 hr at 65°C in the hyb-oven before the labeled probes were added. The blots were then further incubated with the labeled probes overnight at 65°C in the hyb-oven.

I normally performed three times washing at 65°C, once in each washing solution I, II, and III, respectively, if necessary, repeated once in washing solution III. Autoradiographs were exposed at -80°C from O/N to one week, as necessary for clarity of the observed signals.

### 2.2.6 Subcloning by limited dilution

Limited dilution is one way to obtain clones of single cell origin. Cells were harvested and counted using Trypan blue on a Neubauer Haemocytometry, then were diluted to a concentration of 1 cell/200 µl complete medium. The cell suspension was seeded to each well of 96-well flat bottom microtiter plates. The plates were checked under microscope to record the single cell seeding events one day after the plating. After further incubation of the plates in a 37°C $CO_2$ incubator for 10 days without medium change, subclones could be visible as round colonies and be picked up for further analysis.

### 2.2.7 Flow cytometry

#### 2.2.7.1 Fluorescence-activated cell scanning (FACS)

Flow cytometry, or fluorescence-activated cell scanning or sorting (FACS analysis), is the measurement (meter) of characteristics of single cells (cyto) suspended in a flowing saline stream when they flow past a series of detectors. The fundamental concept is the cells flow one at a time through a region of

integration where multiple biophysical properties of each cell can be measured at rates of over 1000 cells per second. These biophysical properties are then correlated with biological and biochemical properties of interest. In order to make the measurement easy, the cells, if not auto fluorescent, are usually stained with fluorescent dyes which bind specifically to cellular constituents. The dyes are excited by the laser beam and emit light at longer wavelengths. The emitted light is picked up by detectors, and these analogue signals are converted to digital so that they may be stored, for later display and analysis.

Cells subjected for FACS analysis in this study were either expressing fluorescent protein EGFP or stained with propidiumiodide (PI) as described. Cells grown on 6-well plates were harvested by centrifuging at 1200 g for 3 min as described, and then suspended in 400 µl of PBS with 0.1% EDTA in Falcon tube 2054. The cells were scanned on the FACSCalibur station (Becton Dickinson Inc.) using proper laser settings according to the suggested manufacture protocols. In order to keep the resolution as accurate as possible, the acquisition flow rate was controlled around 200 events per second by choosing proper scanning speed on the FACScalibur or diluting the cells with PBS. Dead cells and cell debris were excluded by gating the cells with a FSC threshold of 200. Doublet discrimination (DMM) to distinguish between clumped and mitotic cells was set for FL2. One negative cell line was included as autofluorescence control in each FACS analysis in order to adjust the instrumental setting properly for the positive fluorescent samples. The instrumental settings were kept consistent for all batches of samples during the time course. 10,000 cells were set as the defined scanning events for every sample. Data acquisition and control of the flow cytometer was performed with the CellQuest program (BD Biosciences, Heidelberg, Germany) on an Apple G4 computer (Apple Computers, Cupertino, Ca) according to the manufacturer's suggestions.

#### 2.2.7.2 Fluorescence-activated cell sorting (FACSorting)

Fluorescence-activated cell sorting is designed to selectively deposit cells from particular populations into tubes or other collection vessels. These sorted cells essentially unharmed by the process and can then be used for further culturing or experimental analysis. In order to sort the cells, the FACSorter

electronics interprets the signals collected for each cell as it is interrogated by the laser beam, and compares the signals with the sorting criterion settings. If the cell meets the criteria, an electrical charge is applied to the liquid cell stream which is being accurately broken into droplets containing the cell. This charge is applied to the stream at the precise moment the cell of interest is about to break off the stream, and removed once the charged droplet has broken from the stream. As the droplets fall, they pass between two metal plates strongly positively or negatively charged. These charged droplets are drawn towards the metal plate of the opposite polarity, and deposited in the collection vessel.

FACSorting in this study was set for collecting EGFP positive or negative cell populations. Cells grown in T75 flasks were harvested and counted. The cells were diluted with PBS at a concentration of 2X 10E6 cells per ml. The cell suspension was pipetted into 5 ml Falcon round bottom tubes (Falcon tube 35-2235) through cell-strainer cap to break up any clumped cells. The cells were sorted on FACSVantage (Becton Dickinson Inc.) and the subpopulations were collected into 15 ml Falcon tubes with 3 ml complete medium.

### 2.2.8 X-gal staining of cell monolayers

LacZ gene product, ß-galactosidase, can be detected by histochemical staining. ß-galactoside hydrolyzes X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) to an insoluble dense blue compound 5-bromo-4-chloro-indigo (HORWITZ et al., 1964; Davies and Jacob, 1968). Thus, cells expressing ß-galactosidase are stained in blue and can be easily distinguished from those without lacZ expression.

Staining of cell lines used in this study was performed in 6-well plates. Briefly, cells were first washed three times with 1X PBS, and then fixed with 3% formaldehyde for 3 min at RT. After fixation, cells were permeabilized with 50% methanol (pre-cooled at -20°C) for 5 min at -20°C. After washed with 1X PBS, cells were incubated in X-gal staining solution without X-gal for 5 min at RT. This is followed by incubating cells in X-gal staining solution with 1 mg/ml of X-gal at 37°C for appropriate time.

### 2.2.9 Flp-In system

#### 2.2.9.1 Constitution of the Flp-In system

Flp-In system allows integration and expression of the transgene in mammalian cells at a specific genomic location. This special gene expression system is commercially available from the Invitrogen Corporation (Invitrogen Co., the Netherlands).

This system includes:

- A host cell line, i.e. Flp-In 293 cell. The host cell line was generated by stable transfection of the pFRT/lacZeo plasmid. The FRT site is located downstream of the ATG initiation codon of the lacZeo fusion gene in this vector, as shown in Fig. 3. Zeocin resistant clones were screened for single FRT site integration with Southern blot analysis. The host cell line is thus those containing single FRT site and Zeocin resistant clones. The phenotype of the host cell line is lacZ positive and Zeocin resistant.

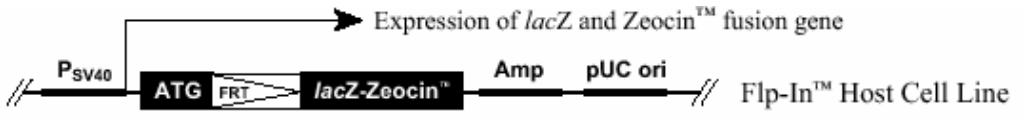


**Fig. 3. The location of the FRT site in the host cell line genome.**

- A Flp recombinase expression vector, pOG44.
- A FRT containing plasmid, pcDNA5/FRT, into which the transgene under study can be inserted. Based on it, four expression vectors were constructed as shown in section 2.2.2 and used in this study. They are pFRT-CMV-EGFP$^{WT}$, pFRT-CMV-EGFP$^{CpG-}$, pFRT-EF-EGFP$^{WT}$, and pFRT-EF-EGFP $^{CpG-}$ (Appendix V).

The major features of the Flp-In system are illustrated in Fig. 4.

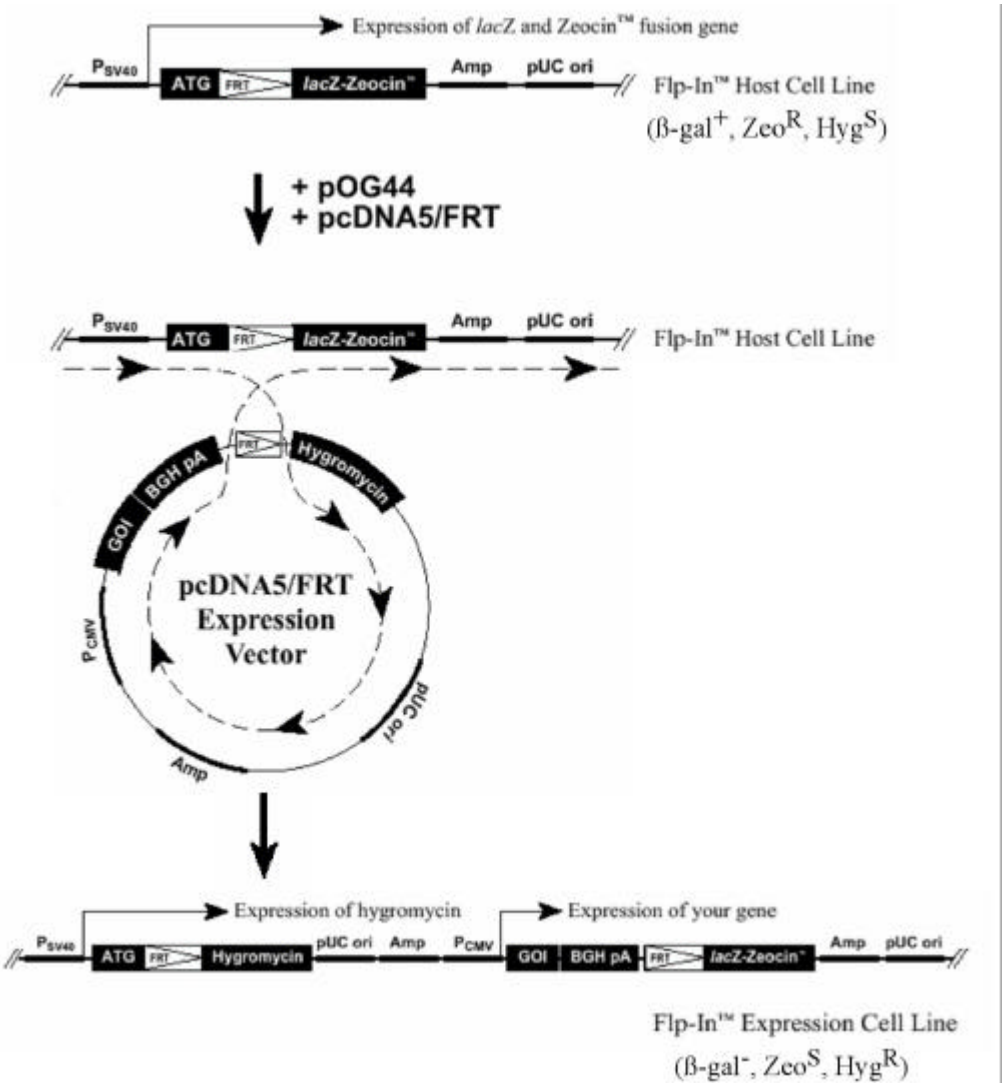


**Fig. 4. Scheme of Flp-In system.**

(adapted from the Flp-In system manual, Invitrogen Co.)

### 2.2.9.2 Construction of stable clones using the Flp-In system

In the Flp-In system, the integration of the expression construct or the transgene into the host cell genome occurs via Flp recombinase-mediated intermolecular DNA recombination. Flp recombinase is expressed by the pOG44 plasmid. The recombination occurs between the specific FRT sites on the interacting DNA molecules. The DNA strand exchange requires only the small 34 bp minimal FRT site (Fig. 5).

The FRT site, originally isolated from Saccharomyces Cerevisiae, serves as a binding site for Flp recombinase and has been well characterized (Senecoff et al., 1985; Jayaram, 1985; Gronostajski and Sadowski, 1985; Sauer, 1994). The minimal FRT site consists of a 34 bp sequence containing two 13 bp imperfect inverted repeats separated by an 8 bp spacer that includes an Xba I restriction site. An additional 13 bp repeat is found in most FRT sites, but is not required for cleavage (Andrews et al., 1985).

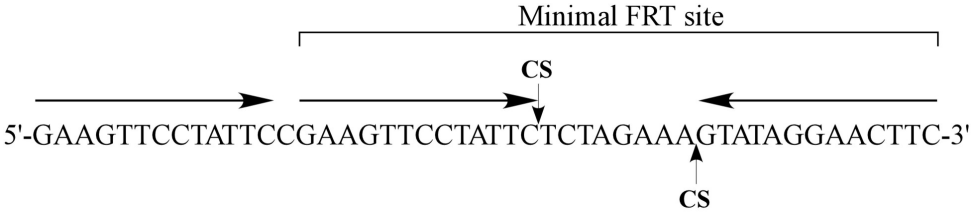


**Fig. 5. The feature of the FRT site.**

CS, cleavage site.

Flp-In 293 cells were cotransfected with pOG44 and the EGFP expression vector at a ratio of 9:1 (w/w) by means of calcium phosphate precipitation. 5 µg of plasmid DNA in total was used to transfect the cells seeded in 6-well plate. 24 hours after transfection, cells were split into fresh medium with a confluence of less than 25%. 100 µg/ml of hygromycin was applied to the cells after the cells were attached to the bottom of the Petri dish. Selective medium was replenished every 3 days till the foci were formed on the dish. Foci were observed under fluorescent microscope and those EGFP positive foci were marked and picked up. The obtained EGFP stable clones gained the phenotype of hygromycin resistance and transgene expression, while lost the phenotypes of ß-galactosidase expression and Zeocin resistant. Single copy integration was further examined by Southern hybridization.

### 2.2.10 Stable clone construction by the “Sorting-Subcloning” approach

I introduced a brand new method to construct stable clones in cell lines. This method was named as “Sorting-Subcloning” in our lab. This method bypasses using antibiotics to select stable clones after transfection. Instead, after transfection with the EGFP expression vectors cells are subjected to two rounds

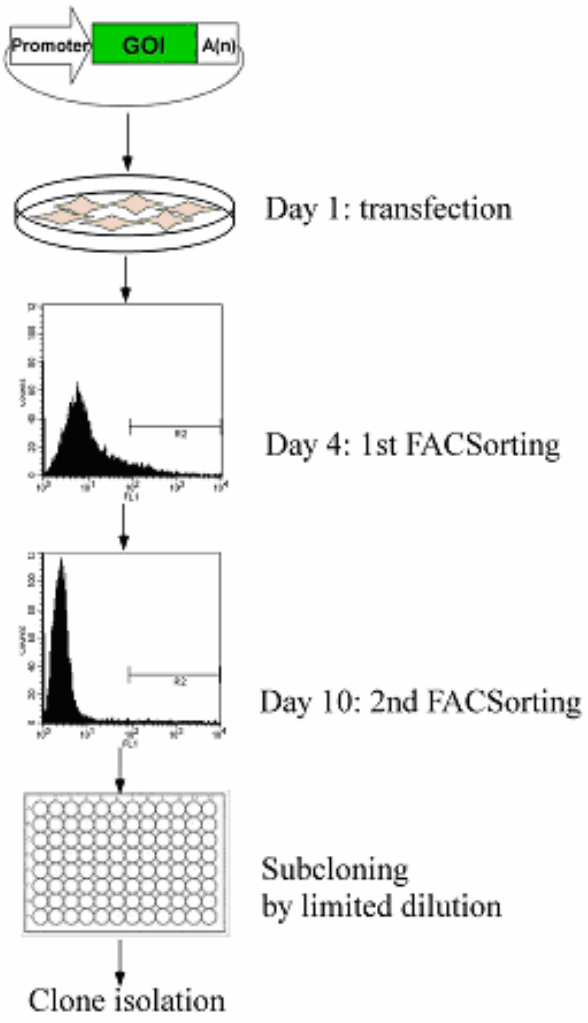


**Fig. 6. The flow chart of the “Sorting-Subcloning” approach.**

Cells are transfected with the transgene expression plasmid on day 1. Two rounds of FACSorting are done consecutively on day 4 and 10, respectively. EGFP positive cells are collected, denoted by the R2 sorting gate on the FACS profile. Subcloning is done through limited dilution when enough cells are available. Clones formed on the 96-well plate are examined by fluorescent microscopy and isolated according to appropriate criteria.

of FACSorting in order to enrich transfected cells. Clones of single cell origin are obtained by subcloning, which is practically done by limited dilution (Fig. 6). In more detail, the first FACSorting is normally done at the fourth day after the transfection. The second round of FACSorting is due a week after the first round of FACSorting. The second round of FACSorting should generate a cell population with more than 80% of cells expressing the transgene. This is optically reflected by the distinct positive peak on the FACS profile. Subcloning can be done as long as enough cells are available. Clones of single cell origin are isolated around two weeks after the limited dilution and expanded. DNAs used for transfection in this method were either linearized expression plasmids or only the essential transcription components of the transgenes. For the latter case, the DNA fragment was shortened as "**cass**" in this thesis, including the transgene promoter, the coding region and ploy(A) signal.