# 3. Materials

## *3.1. Chemicals*

If it is not mentioned otherwise deionized water (Millipore) was used as solvent.

| | |
|---|---|
| Acetazolamide | Sigma-Aldrich, St. Louis, USA |
| Acetic acid 96% | Merck, Darmstadt, BRD |
| Acryl-Bisacrylamide (30% or 40%) | 30 or 38g acrylamide, 0.8g or 2 g N'N'-methylenbisacrylamide per 100ml (Carl Roth, Karlsruhe, BRD) |
| Agar, bacteriological grade | ICN Biochemicals, USA |
| Agarose | Gibco BRL, UK |
| Ammonium acetate | Merck |
| Ammonium persulfate (APS) | Bio-Rad Laboratories, BRD |
| Bromphenol Blue | Sigma |
| Bovine serum albumin (BSA),10 mg/ml | New England Biolabs, USA |
| Chloroform | Merck |
| Coomassie-Brilliant Blue G-250 | Merck |
| Diethylpyrocarbonate (DEPC) | Sigma |
| Dimethylsulphoxide (DMSO) | Serva, Feinbiochemical, Heidelberg, BRD |
| Dithiothreitol (DTT), 0.5M and 0.1M | Gibco BRL |
| DNA from salmon testes | Sigma |
| Ethylenediamine-tetraaceticacid (EDTA) | Roth |
| Ethanol 99% | Merck |
| Ethidium bromide | Merck |
| Formaldehyde 37% | Merck |
| Formamide 100% | Merck |
| D-(+)-Glucose | Sigma |
| Glycerol | Sigma |
| Hydrochloric acid 37% | Roth |
| Isoamyl alcohol 98% | Roth |
| Isopropanol, saturated with NaCl | Roth |
| Lennox L Broth Base (LB Broth Base) | Gibco BRL |
| Lithium chloride | Serva Electrophoresis, Heidelberg, BRD |
| Magnesium chloride | Merck |
| 2-Mercaptoethanol (2-ME) | Gibco BRL |
| Methanol 99% | Roth |
| Phenol/Tris-saturated | Roth |
| Phenol/water-saturated | Sigma |
| Polyoxyethylensorbitanmonolaurat (Tween 20) | Sigma |
| Polyvinylpyrrolidone | Sigma |
| Potassium chloride | Roth |
| Potassium acetate | Roth |
| Sephadex (G-50) | Pharmacia, Fine Chemicals, Sweden |
| Silicon solution | Serva |
| Sodium acetate | Roth |
| Sodium azide | Sigma |

| | |
|---|---|
| Sodium chloride | Roth |
| Sodium hydroxide | Roth |
| Sodium lauryl sulfate (SDS) | Sigma |
| Sodiumhydrogen phosphate | Roth |
| Sulfonamide insolubilized, cross-linked at 4% beaded agarose | Sigma |
| TEMED (Tetra-methyl-ethylenediamine) | Gibco BRL |
| Tris (Tris (hydroxymethyl) aminomethan) | ICN |
| Triton®X-100 | Sigma |
| TRIzol®Reagent | Gibco BRL |
| Urea | Serva |
| Xylene Cyanole FF | Sigma |

## ***3.2. Solutions and Buffers***

Standard laboratory chemicals were purchase from Sigma, Roth or Merck.

| | |
|---|---|
| Calf Intestine Phosphatase (CIP) buffer (10x) | Boehringer Mannheim |
| Carnoy's fluid | ethanol - chloroform - acetic acid (6:3:1) |
| Complete Proteinase Inhibitor Cocktail® | Boehringer |
| Coomassie Blue staining solution | 0,25g of Coomassie-Brilliant Blue G-250; 90ml methanol : $H_2O$ (1:1 v/v); 10 ml acetic acid |
| Denhardt's solution (100x) | 2% (w/v) BSA; 2% ficoll; 2% polyvinylpyrrolidone |
| Denaturing solution | 0,5N NaOH; 1,5M NaCl |
| Destaining Coomassie Blue solution | 90ml methanol : $H_2O$ (1:1 v/v);10ml acetic acid |
| DNase buffer (10x) | 100mM Tris-HCl, pH 8,3; 500mM KCl; 15mM $MgCl_2$; in DEPC $H_2O$ |
| ES cell lysis buffer | 100mM Tris-HCl, pH 8,5; 5mM EDTA, pH 7,5; 200mM NaCl; 0,2% SDS; 100µg/ml fresh proteinase K |
| First strand buffer (5x) | Gibco BRL |
| Forward buffer | Promega, Mannheim, BRD |
| Guanidium thiocyanate solution | 4M guanidium thiocyanate; 25mM sodium citrate; 0,5% SDS; 100mM 2-ME |
| Gel loading buffer (GLB) | 0,4% bromphenol blue; 0,4% xylene cyanole FF; 50% glycerol |
| $H_2O$ treated by DEPC | 1ml DEPC in 1l $H_2O$ disperse by shaking, 1hr incubation; autoclave |
| Hybridization solution | 6x SSC; 0,5% SDS; 100µg/ml DNA from salmon testes |
| Klenow buffer (10x) | Boehringer |
| MOPS buffer | 0,4M MOPS; 50mM sodium acetate; 10mM EDTA in DEPC $H_2O$; pH 7,0 |
| Neutralizing solution | 1,5M NaCl; 0,5M Tris, pH 7,4 |
| Plasmid isolation solutions | |
| Sol-I | 50mM glucose; 10mM EDTA; 25mM Tris-HCl, pH 8,0 |

| | |
|---|---|
| Sol-II | 0,2N NaOH; 1% SDS; |
| Sol-III | 3M potassium acetate; 2M acetic acid |
| Prehybridization solution | 6x SSC; 0,5% SDS; 100µg/ml DNA from salmon testes; 5x Denhardt's solution |
| Protein electrophoresis buffer | 25mM Tris-base; 250mM glycine (pH8,3); 0,1% SDS |
| Restriction enzyme buffers (10x) | Gibco BRL, Boehringer, New England BioLabs |
| RNA-Sample buffer | 66% formamide; 7,5 formaldehyde; 1,5x MOPS buffer |
| RNA-Transfer buffer | 57,7mM $Na_2HPO_4$; 43,3mM $NaH_2PO_4$ |
| RNA- Gel loading buffer | 66% formamide; 7,5% formaldehyde; 1,5x MOPS buffer; 0,4% bromphenol blue |
| STET buffer | 8% (w/v) sacharose; 0,1% (v/v) Triton X-100; 50mM EDTA, pH 8,0; 50mM Tris-HCl, pH 8,0 |
| 4x SDS/sample buffer | 0,2M Tris-HCl, pH 6,8; 8% SDS; 40% (w/v) glycerol; 0,004% bromophenol blue; 0,1M DTT |
| 20x SSC | 3M NaCl; 0,3M sodium citrate pH 7 |
| T4 DNA ligase buffer(10x) | Eurogentec; Belgium |
| T4 DNA polymerase buffer (10x) | Gibco BRL |
| TAE buffer | 40mM Tris-acetate, pH 8,0; 1mM EDTA |
| Taq-polymerase buffer (10x) | Gibco BRL |
| TBE buffer | 90mM Tris-borate, pH 8,0; 1mM EDTA |
| TE buffer | 10mM Tris-HCl, pH 7,5; 1mM EDTA |
| TENS lysis buffer | 50mM Tris-HCl, pH 8,0; 0,1mM EDTA, pH 8,0; 100mM NaCl; 1% SDS; 400-100µg/ml fresh proteinse K |
| Tissue fixation solution | 4% formaldehyde; 1,5% acetic acid; in PBS |
| Transfer buffer (5x) | 200mM glycine; 250mM Tris-base; 0,185% SDS; pH 8,3; fresh 20% methanol |

## ***3.3. Enzymes***

| | |
|---|---|
| Calf Intestine Phosphatase (CIP) | Boehringer |
| Klenow polymerase | Boehringer |
| Lysozyme | Sigma |
| Proteinase K | PAN Biotech; BRD |
| RNase A | Boehringer |
| RNase T1 | Boehringer |
| Sp6 RNA polymerase | Boehringer |
| T7 RNA polymerase | Pharmacia |
| T4 DNA polymerase | New England BioLabs |
| T4 DNA ligase | Eurogentec, Belgium |
| Taq-polymerase | Gibco BRL |
| Restriction enzymes | Gibco BRL, Boehringer and New England BioLabs |
| Recombinant RNasin | Promega |

| | |
|---|---|
| SuperScrip®II reverse transcriptase | Gibco BRL |
| Terminal deoxynucleotidyl transferase | Gibco BRL |

### ***3.4. Nucleotides and Radioactive Nucleotides***

| | |
|---|---|
| dNTPs (0,1M) | Pharmacia |
| NTPs (0,1M) | Pharmacia |
| γ - 32P*- dATP (3.000 Ci/mmol) | Amersham |
| α - 32P*- GTP (800 Ci/mmol) | Amersham |
| α - 32P*- dCTP (3.000 Ci/mmol) | Amersham |

### ***3.5. Antibody***

*Not labeled antibodies*

| | |
|---|---|
| anti-PG-MN (rabbit IgG) | Ortova Gut *et al.* |
| anti-uvomorulin (rat IgG; DECMA-1 hybridoma) | Sigma |

*Antibodies cojugated with horse-reddish peroxidase*

| | |
|---|---|
| anti-rabbit Ig | Santa Cruz |
| anti-rat Ig | Santa Cruz |

### ***3.6. Filters and Membranes***

| | |
|---|---|
| Nylon membrane | Pall; Biodyne B transfer membrane, UK |
| PVDF transfer membrane | Hybond-P; Amersham Pharmacia Biotech |
| Whatman paper | Schleicher and Schoell |

### ***3.7. Plasmids***

| | |
|---|---|
| pcDNA3 | Invitrogen |
| pMN/CA9-neo | self cloned |
| pB-B | self cloned |
| pBleuscript II KS | Stratagene |
| pBAC108L | Genome Systems |

### ***3.8. Kits***

| | |
|---|---|
| ECL® Western blotting detection reagent | Amersham |
| JetStar®- Plasmid Maxi kit | Genomed, Oeynhausen, BRD |
| JetSorb®- Gel Extraction kit | Genomed |
| Rediprime®II | Amersham |
| 3' RACE System for Rapid Amplification of cDNA Ends, version 2.0 | Gibco BRL |
| 5' RACE System for Rapid Amplification of cDNA Ends, version 2.0 | Gibco BRL |
| TA cloning®kit | Invitrogen, BV, Netherlands |

## *3.9. Bacterial Strains and Cells*

| | |
|---|---|
| Escherichia coli | strain DH5α, Stratagene |
| Embryonic stem (ES) cells | E14 (Hooper et al, 1997) |
| Embryonic fibroblasts | Isolated from heterozygote animals from ICSBP-/- and wild type 129/Bl6 mixed background mating |
| GR/3 | Generous gift from Dr. Dicson, ICRF, England |
| MM5 | Generous gift from Dr. Dicson |
| NMG | Generous gift from Dr. Dicson |
| TS/A | Generous gift from Dr. Bulfone-Paus, Universitaetsklinikum Benjamin Franklin, Germany |
| RENCA | Generous gift from Dr. Maurer, MPG, Germany |
| J558 | Generous gift from Dr. Bulfone-Paus |
| NIH 3T3 | Generous gift from Dr. Zavada, Academy of science of Czech republic |

## *3.10. Animals*

| | |
|---|---|
| Wild types | C57 BL/6J<br>129/Ola<br>BALB/c<br>C57 BL/6J x 129/Ola mixed background (Harlan Winkelmann or selfmated) |
| Blastocysts donors | C57BL/6J |
| Foster mothers | Ola:MF1; Harlan |
| MN/CA IX deficient mice | C57 BL/6J x 129/Ola mixed background, selfmated |
| Vasectomized males | ICR (CD-1); Harlan |

## *3.11. Laboratory Equipment*

| | |
|---|---|
| Blotting device, semi-dry, for protein gels | Hoefer TE 70, Amersham |
| Cell electroporator | Gene Pulser®, Bio-Rad |
| Centrifuges | Eppendorf 5417C; Beckmann Avanti(J-25); Hettich Rotina 48R |
| Gel electrophoresis | Hoefer SE 200, Amersham |
| Homogenizer | Bachofer |
| Hybridization incubator | Sauer |
| Incubator shaker | New Brunswick Scientific |
| Laminar flow unit | MSC II, Holten LaminAir |
| PCR-machine | Eppendorf; Perkin Elmer, USA |
| Spectrophotometer | Photometer Ultraspec®III; Pharmacia |
| Phosphoimager | Molecular Dynamics |
| Ultrasonic homogenizer | Branson Utrasonic, Danbury |
| Vortex | Vortex Genie 2-Mixer, Berder und Hobein AG, BRD |

### ***3.12. Primers***

All the primers were commercially synthesized (Metabion) and are written in 5'-3' sense

| |
|---|
| Oligo-d(T)16<br>TTT TTT TTT TTT TTT T<br>Human *MN/CA9* cDNA primers used to amplify mouse *MN/CA9* cDNA<br>KAS2 sense<br>ATC CAC GTG GTT CAC CTC AG<br>KAS3 sense<br>TGA CTT CAG CCG CTA CTT CC<br>ICA antisense<br>CTT TGG TTC CCC TTC TGT GC |
| Mouse β-Actin control primers<br>β-Actin sense<br>TGG AAT CCT GTG GCA TCC ATG AAA C<br>β-Actin antisense<br>TAA AAC GCA GCT CAG TAA CAG TCC G |
| Primers for 5' RACE to detect 5' extremity of *MN/CA9* wild type mouse cDNA<br>1) NOCA antisense = A1<br>AGC TGT AGG AGG AAG GCG AT<br>2) RACEA antisense = A2<br>TGA CAG CAA AGA GAA GGC CA<br>3) PROTA antisense = A3<br>CAG GGA AGG AAG CCT CAA TC<br>Primers for 5' RACE to detect 5' extremity of *MN/CA9* mutated mouse cDNA<br>1) 900A antisense<br>GAT ACA TCC AAA CCT GGG ATC TCA A<br>2) 800A antisense<br>TCC TGC AGA AAG GCA GCC AAA ACT G<br>3) 700A antisense<br>CAG GGA AAC GGT GAC CAT TGA CTG T |
| Primers for 3' RACE<br>RACES sense = S1<br>CAG GAG AGC CCA GAA GAA AA<br>PROTS sense = S2<br>TGA AGG GTC TCT GAC CAC AC |
| Primers for RT-PCR detection of mutated *MN/CA9-/-* cDNA<br>MNG sense = A<br>GAC ACC CCA GTC AGC TGC ATG GCC T<br>MNA antisense = D<br>TGT GCT CAG GAG CCT CGG GAG TCG A<br>Neo sense = C<br>CGA AGG AGC AAA GCT GCT ATT GGC C<br>NeoV = B<br>CAT TCT CAG TAT TGT TTT GCC AAG TT<br>1403A = F<br>AAG GAG GCT GTA TAA CAG GCA GGA C<br>EXS1 |

| |
|---|
| AGT CAA GGT TCC CAC GGG GAT GAA |
| Primers for primer extension<br>EXT30 antisense<br>AGC TGA CTG GGG TGT CCC AGG ACA CAC TGT<br>EXT40 antisense<br>GAG GCC ATG CAG CTG ACT GGG GTG TCC CAG GAC ACA CTG T |
| Primers for restriction mapping<br>5' sense<br>CTC TTT ACT GTG ATT ATT AAC CAA CCC<br>3' sense<br>AAT TCA CAT GGA CTG AAG AGA TAA AAG G |
| Internal primers for genotyping of *MN/CA9* gene mutation<br>MNG sense<br>GAC ACC CCA GTC AGC TGC ATG GCC T<br>MNA antisense<br>TGT GCT CAG GAG CCT CGG GAG TCG A<br>Neo sense<br>CGA AGG AGC AAA GCT GCT ATT GGC C |

## ***3.13. Media and Supplements for Bacterial and Cell Culture***

| | |
|---|---|
| DMEM high glucose | Gibco BRL |
| RPMI 1640 | Gibco BRL |
| PBS | Gibco BRL |
| Fetal calf serum (FCS) | Biospa, 30min inactivated at 56°C |
| 1x trypsin/EDTA | Gibco BRL |
| Transfection medium | 10mM HEPES in PBS |
| Not essential amino acids | Gibco BRL |
| Glutamin | Gibco BRL |
| Leukemia inhibitory factor (LIF) | ESGRO (Gibco BRL) |
| Complete medium | 90% RPMI; 10% FCS |
| ES cell medium | 500ml DMEM<br>15% FCS; 0,1mM glutamin; 0,2 not essential amino acids; 0,1mM 2-ME; 1600 units LIF, 6ml nucleoside solution; 1x penicillin/streptomycin |
| Feeder medium | 500ml DMEM<br>10% FCS; 0,1mM glutamin; 0,2mM not essential amino acids; 0,1mM 2-ME;<br>1x penicillin/streptomycin |
| Nucleoside solution | 80mg adenosine; 85mg guanosine; 73mg cytidine; 73mg uridine; 24mg thymidine dissolved in 100ml $H_2O$ at 40°C, filtered to sterilize and stored at 4°C |
| Penicillin/streptomycin | Gibco BRL |
| G418 sodium salt | Gibco BRL |
| 2x freezing medium | 20% DMSO in FCS |
| Luria Broth (LB) medium | 20g LB Broth base; 5g NaCl, add 1l $H_2O$ |
| LB agar | 20g LB Broth base; 5g NaCl; 15g agar, add 1l $H_2O$ |

## *3.14. Material for Injection and Embryo-Transfer*

| | |
|---|---|
| Glass for injection capillaries | Borosilicat |
| Glass for holding capillaries | Borosilicat |
| Glass for transfer capillaries | Borosilicat |
| Injection chamber | Nunc Chamber slide |
| Injection medium | M2, Sigma |
| Injection microscope | DM/IRB Leica, BRD |
| Micromanipulator | Leica |
| Micro-drop-culture | M16 drops pre-incubated by 5% $CO_2$ and covered with paraffin oil |
| Paraffin oil | Embryo tested, Sigma |
| Wound clips and applicator | Clay Adams |

## *3.15. Hormones and Narcotics*

| | |
|---|---|
| Pregnant mares serum | Sigma |
| Human chorionic gonadotropin | Sigma |
| Pentobarbital | Sigma |