METHODS OF THE SCHMID-LAB

Bacteria

E. coli Overnight Culture

Solutions:

- LB (Luria bretani) broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl, in 1 L AD, pH 7.0. Sterilization by autoclaving
- Antibiotic stock solutions 1000x: ampicillin 100 mg/mL, kanamycin 25 mg/mL

For preparing a saturated E. coli over night culture, 5 mL LB broth containing antibiotics are inoculated with a single colony of the desired bacteria strain in a 50 mL tube. Incubation is carried out at 37°C with constant shaking at 200 rpm over night (o/n). After 12 hours the culture is in the state of stationary phase, no more increase of biomass is observed.

Preparation of Electro-competent E. coli

Solutions:

- LB broth
- AD sterile, 4°C
- 10 % glycerol, sterile filtered, 4°C
- liquid N_2

10 mL LB broth are inoculated with E. coli HB101 and an o/n culture are prepared. On the next day the o/n culture is diluted in 1 L LB broth and incubation is continued for around 3 hours. Optical density (OD) at 600 nm is measured after 1 hour respectively until OD 0.4 - 0.6 is reached. By that means the bacteria are in the state of exponential phase. The bacteria broth is then cooled down on ice for 15 – 30 minutes in order to stop the bacteria from dividing. Harvesting of bacteria is carried out by centrifugation at 2500 x g, 4°C for 15 minutes (centrifuge: Sorvall RC-5B), the supernatant is removed and collected for autoclaving. The bacteria pellet is resuspended in 1 L sterile AD (4°C) and again centrifuged at 2500 x g for 15 minutes at 4°C. The supernatant is discarded and the pellet is resuspended in 500 mL sterile AD (4°C) and again centrifuged at 2500 x g for 15 minutes at 4°C. The supernatant is discarded and the pellet is resupended in 20 mL 10 % sterile glycerol (4°C) and again centrifuged at 2500 x g for 15 minutes at 4°C. The supernatant is discarded and the pellet is resuspended in 2 - 5 mL 10 % sterile glycerol (4°C). 50 µL aliquots of this bacteria solution are prepared and quickly frozen in liquid N_2. Aliquots are stored at -80°C and can be used for 1 year.

Electroporation Transformation of E. coli

Solutions:

- SOC medium for recovery: LB broth containing glucose 20 mM, $MgSO_4$ 10 mM, $MgCl_2$ 10 mM

50 µL electro-competent bacteria aliquot is thawed on ice and transferred into prechilled electroporation cuvettes, 0.5 - 1 µg plasmid DNA (or 5 - 10 µL of ligation mix) is added (0.1 cm). The surface of the cuvette was dried prior to electroporation. Electroporation parameters (power supply : voltage: 1.25 – 1.9 kV, capacity: 25 µF, resistance: 200 Ω. A successful electroporation is achieved with a time constant between 4 – 5 ms. After electroporation the bacteria are resuspended in 1 mL of SOC medium and transferred in a 3059 Falcon tube. The bacteria are then incubated at 37°C at 200 rpm for 1 hour. After recovery the transformed bacteria are briefly centrifuged, resuspended in 100 µL SOC medium and the suspension is plated on LB agar plates containing appropriate antibiotics. The plates are incubated over night at 37°C.

Preparation of Heat-Shock Competent E. coli

Solutions:

- LB broth
- TSS Buffer: 1 % Bacto-Trypton, 0.5 % yeast extract, 0.5 % NaCl, 5 % DMSO (dimethylsulfoxide), $MgCl_2$ 50 mM, pH 6.5, 10% PEG (MW 3000, 3350), fill up with AD, sterile filtered, stored at 4°C.
- Glycerol 87 %
- Liquid N_2

10 mL LB broth are inoculated with E. coli HB101 or another appropriate strain such as DH5α and an o/n culture is prepared. On the next day the o/n culture is diluted in 1 L LB broth and incubation is continued for around 3 hours. Optical density (OD) at 600 nm is measured after 1 hour respectively until OD 0.4 – 0.6 is reached indicating that the bacteria are in the exponential phase of growth. The bacteria broth is then cooled down on ice for 15 – 30 minutes in order to stop the bacteria from dividing. Harvesting of bacteria is carried out by centrifugation at 2500 x g, 4°C for 15 minutes (centrifuge: Sorvall RC-5B). The supernatant is removed and collected for autoclaving. The pellet is resuspended in a total volume of ~ 50 mL (36 mL TSS buffer + 12 mL of glycerol, 1/20 volume of diluted culture). Aliquots of 200 µL are prepared, quickly frozen in liquid N_2 and stored at -80°C.

Heat Shock Transformation of E. coli

Solutions:

- SOC medium: LB broth containing 0.36% glucose, $MgSO_4$ 10 mM, $MgCl_2$ 10 mM

An aliquot of 50 µL heat shock competent E. coli (DH5α, stored at –80°C) is quickly thawed in the palm and left for 10 minutes on ice. The aliquot is then transferred into a pre-chilled Falcon 2059 tube. About 1 µg of DNA (1 - 2.5 µL) is pipetted into the competent bacteria and the tube is gently swirled. After 20 minutes of incubation on ice the tube is placed in a prewarmed 42°C water bath for 90 seconds without moving the tube. In order to cool down the sample, the tube is immediately placed on ice for 1-2 minutes. Thereafter 800 µL of SOC medium are added to the transformation mix and the bacteria are incubated for 1 hour at 37°C and 200 rpm to allow recovery from the heat shock and start expression of the selection gene. Plating: For simple retransformations 100 µL are plated on appropriate LB (Luria Bertani) agar plates (prewarmed to 37°C) supplemented with the appropriate antibiotics (kanamycin 25 µg/mL or ampicillin 100 µg/mL). For clonings after a ligation the whole bacterial suspension is used by pelleting the bacteria of the transformation mix by briefly and gently spinning down, discarding the supernatant and resupension of the pellet in 100 µL SOC medium (or the reminder of the supernatant). This concentrated suspension containing all the bacteria is then plated on LB agar plates containing appropriate antibiotics. The plates are incubated over night at 37°C.

Glycerol Stock of E. coli

Solutions:

- Glycerol 87 %

For a -80°C E. coli glycerol stock, 400 µL of an E. coli o/n culture are mixed thoroughly with 100 µL 87 % glycerol in a 1.5 ml reaction tube and stored at -80°C. For inoculation of fresh LB broth, a yellow 200 µL tip is plunged into the frozen (!) stock and then pipetted up and down in the LB broth in order to thaw and resuspend the bacteria.

Yeast

LiAc Yeast Transformation

Solutions:

- Synthetic drop out solution 10 x in AD: L-isoleucine 300 mg/L, L-valine 1.5 g/L, L-adenine hemisulfate salt 200 mg/L, L-arginine HCl 200 mg/L, L-histidin HCl monohydrate 200 mg/L, L-leucine 1 g/L, L-lysine HCl 300 mg/L, L-methionine 200 mg/L, L-phenylalanine 500 mg/L, L-threonine 2 g/L, L-tryptophan 200, L-tyrosine 300 mg/L, L-uracil 200 mg/L
- SD -Trp medium (synthetic dropout medium): synthetic minimal medium lacking tryptophan: yeast nitrogen base without amino acids 6.7 g/L, 2 % dextrose (glucose) (sterile dextrose solution is added after autoclaving to avoid maillard reactions), pH adjusted to 5.8, for plates : agar 1.5 g/L
- YPD (yeast peptone dextrose) broth, yeast complete medium: yeast extract 10 g/L, peptone 20 g/L, 2 % dextrose (glucose), pH adjusted to 5.8
- Aqua dest. sterile
- LiAc 100 mM sterile
- LiAc 1 M sterile
- Bacterial RNA, used as carrier
- Poly-ethyleneglycol PEG 50 % (w/v) sterile filtered

10 mL of SD -Trp medium are inoculated with the appropriate yeast strain and incubated at 30°C while shaking at 200 rpm o/n. On the next day OD at 600 nm is measured and the yeast culture is diluted with YPD to OD_{600} 0.1. A total volume of 50 mL diluted yeast culture is used for further incubation. Every hour OD_{600} is measured until OD_{600} 0.4 is reached (3 - 5 hours). Then the cell number is calculated with a Thoma chamber. $2x10^7$ cells/mL are sufficient for 10 transformations. The yeast is then harvested by centrifugation at 3000 rpm for 5 minutes, the supernatant is carefully removed and collected for autoclaving. The pellet is resuspended in 25 mL sterile AD and again centrifuged at 3000 rpm for 5 minutes. After removing of the supernatant the pellet is resuspended in 1 mL LiAc 100 mM. Excess of LiAc is removed by spinning the tubes for 15 seconds at full speed in a tabletop centrifuge and carefully removing the supernatant. The yeast pellet is brought to a final volume of 500 µL with LiAc 100 mM. Aliquots of 50 µL are prepared. One 50 µL aliquot of this yeast suspension is used for one transformation. 50 µL aliquots are again briefly centrifuged to pellet the cells, the supernatant is removed and on top of the yeast pellet, layers of the following transformation solutions are pipetted in following order: 240 µL 50 % PEG, 36 µL LiAc 1 M, 3.3 µL of bacterial RNA (31 µg/µL), 70.7 µL sterile AD, 1 µg plasmid DNA (1µg/µL). The tube is then thoroughly mixed by vortexing for 1 minute until the yeast pellet is completely dissolved and placed for 30 minutes in a 30°C water bath. The tube is then transferred to a 42°C water bath for 25 minutes in order to perform the heatshock. The transformation mix is then briefly centrifuged for 15 seconds at 4 000 x g (7 000 rpm) in a table top centrifuge, the supernatant is discarded and the pellet is resuspended in 1 mL sterile AD. 50 µL of this transformed yeast suspension are plated on SD – Leu, - Trp, - Ade plates and incubated at 30°C for some days.

Mammalian Cell Culture

Cell culture of 293, HeLa, MEF or stable transfected 293 Cells

Solutions:

- Dulbecco's modified Eagle's medium complete (DMEM complete) containing 10 % FCS (fetal calf serum), penicillin (100 u/mL), streptomycin (100 µg/mL), glutamine 2 mM (stock solutions of penicillin, streptomycin and glutamine: 100x)
- DMEM G418 medium: DMEM complete containing G418 500 µg/mL
- G418 stock solution: 500 mg/mL G418 in Hepes 100 mM
- PBS deficient: NaCl 8 g, Na_2HPO_4 1.44 g, KH_2PO_4 0.24 g, with AD to 1 L, pH adjustment with HCl to pH 7.4 (provided by Novartis facility)
- Trypsin solution 0.25 % in Hepes 10 mM

293 cells (immortalized human embryonic kidney cells, adherent), HeLa (human cervix carcinoma cells, adherent) and MEF (mouse embryonic fibroblasts, adherent) are cultured in DMEM complete medium, in addition to this DMEM complete medium, the medium for stable 293 cell lines with a genomic integration of a neomycin-resistance plasmid containing the gene of interest, contains 500 µg/mL G418 as permanent selection agent. Cells are cultivated in 75 cm^2 tissue culture flasks at 37°C, in a humidified (90 %) and CO_2 (5 %) containing atmosphere. All cell types are trypsinized and passaged every second or third day in proportion of 1:4 or 1:6, respectively. For passaging the cell culture medium is removed, about 13 mL of PBS def. (room temperature) are used to wash the cells. PBS def. is removed as well and about 3 mL trypsin solution (37°C) are pipetted into the flask and spread evenly all over the bottom. Excess of trypsin is removed and the flask is placed in the incubator for around 3 minutes. With an inverse microscope (Nikon TMS F 4x or 10x objective) it is checked if the cells are already detached and round in shape. Trypsinized cells are gently resuspended by several times up/down pipetting in 8 mL (to 12 ml – according to the desired passaging ratio) complete DMEM and 2 mL are transferred into a new tissue culture flask containing 13 mL of DMEM complete or DMEM complete including G418. Tissue culture flasks prepared for MEFs have to be gelatinized prior to passaging as follows: 15 mL of 1 % gelatin solution (autoclaved and sterile filtered, 37°C) are pipetted in a 75 cm^2 tissue culture flask and incubated at 37°C for 30 minutes, the gelatin solution is discarded afterwards. After this procedure, passaging is performed as usual.

Freezing of Mammalian Cell Lines

Solutions:

- Freeze medium: DMEM, 10 % (v/v) DMSO (dimethylsulfoxide), 20 % (v/v) FBS (fetal bovine serum), sterile filtered

293, HeLa, MEF, stable transfected 293 cell lines are passaged as described above, seeded in 75 cm^2 cell culture flasks and grown until 100 % confluency is reached. The cells are trypsinized as described above, resuspended in DMEM complete medium (4°) and centrifuged at 1000 rpm (400

g), 4°C for 5 minutes in a cell centrifuge with a swing-out rotor. The supernatant is removed and the cell pellet of one 75 cm^2 cell culture flask is resuspended in 2 mL freeze medium (4°C). 1 mL of resuspended cells are pipetted in pre-chilled cryotubes, placed in an isopropanol-box ("Mr Freeze", 4°C) and then transferred to -80°C in order to freeze the cells in a controlled and slow procedure. After 24 hours the cell aliquots are transferred and stored in the liquid nitrogen tank at -196°C.

Thawing of Mammalian Cell Lines

A cell aliquot of 293, HeLa, MEF or stable transfected 293 cell lines stored in liquid nitrogen is quickly thawed in a 37°C water bath with constant moving to prevent local overheated spots warmer than 4°C. The thawed cell suspension is resuspended and diluted in about 20 mL DMEM complete medium and the cells are centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant is removed by suction, the cell pellet is resuspended in DMEM complete medium or supplemented with G418 and the cells are seeded in 75 cm^2 cell culture flasks.

Transfections

$CaCl_2$ Transfection

Solutions:

- $CaCl_2$ 2 M in Aqua dest:
- HeBS (Hepes buffered saline): Hepes 50 mM, NaCl 280 mM, Na_2HPO_4 1.5 mM, pH adjustment to exactly pH 7.05 with HCl, sterile filtered (0.2 µm pore size), stored at -20°C

Preparation: 293 or HeLa cells are passaged and seeded at a density of 500.000 cells/6 well (40 % confluence). After one day (80 % confluence) transfection is carried out. Transformation mix: 2 µg of DNA (1 µg/µL) are diluted in 60 µL of aqua dest. in a polystyrene tube and 9 µL of 2 M $CaCl_2$ are added and mixed, 71 µL of HeBS are added dropwise by unscrewing a P200 Gilson Pipette while constantly vortexing at half speed. After incubation of 5 minutes at room temperature the solution is pipetted dropwise as evenly as possible on the surface of the cell culture dish. Once the DNA/calcium-precipitates get in contact with the cells the tissue culture dish should not be moved a lot. Normally the proteins of interest are expressed sufficiently after 24 hours of transfection (when CMV promoter containing plasmids are used).

Polyethylene-imine Transfection Method (for 293 and HeLa cells)

1. Reed SE, Staley EM, Mayginnes JP, Pintel DJ, Tullis GE: **Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors.** *J Virol Methods* 2006, **138:**85-98.

Linear PEI (0.323 g/L in A. dest = 7.5 mM): stirred on low heat for 1 h until all particles are in solution, pH set to 7.0

PROTOCOL for PEI transfection (Kalsoom)

Mix the contents of soln A and B separately
Add sol B to sol A, mix and vortex briefly.
Let it at room tepmrature for 15-20 minutes
Wash the cells with SFM once and then add SFM:
Drop wise add the transfection mix to the wells
Put in the incubator at37C.
Change the SFM with full media after 3-4 hours
Incubate for 24-48 hour at 37C:

Well size	Soln A		Soln B		Total Volume for one well
	DNA/RNA	Heps buffer	PEI	Heps buffer	
1-12well	0.75ug	46.2ul	3.1	46.2ul	92ul
1-6well	1.5ug	114ul	7.6	114ul	223 ul

Solution: PEI 25000 sigma density 1.03g/ml
Stock soln. of PEI 40mM: 10g PEI in 10 ml distilled water Mix 4-5 hours,
Working soln. 87ul of this in 100ml water. Mix 4-5 hours maintains pH 7.0 with HCl. Filter steril 2times and store at 4C.
2X HEPS pH 7.05: (500ml): 8gNaCl, 0.2g Na2HPO4.7H2O, 6.5g Heps or 5.96g Reeacid. Filter sterile and store at -20C.

Lipofectamine Transfection of Mammalian Cell lines

Solutions:

- Sterile DMEM medium without any additives (DMEM ∅)
- DMEM complete
- Lipofectamine Plus reagent
- Lipofectamine reagent

Preparation: 293 or HeLa cells are passaged and seeded at a density of 500,000 cells/6 well (40 % confluence). After one day (80 % confluence) transfection is carried out. For transformation of one 6-well 293 (HeLa) cells, 100 µL DMEM ∅ medium is mixed with 1 µg (1.5 µg) plasmid DNA and 4 µL (5 µL) lipofectamine Plus reagent and incubated for 15 minutes at room temperature. 100 µL of DMEM ∅ are mixed with 3 µL (3 µL) lipofectamine reagent added to the incubated solution and mixed gently. The lipofectamine/DNA solution is further incubated at room temperature for 15 min. Thereafter, the transformation mix is diluted with 800 µL DMEM ∅. The culture medium of 293 or HeLa cells is removed by suction, the cells are carefully washed once with at least 1.5 mL DMEM ∅ medium, the wash DMEM is removed and the transformation mix is carefully added to the cells. The 293 (or HeLa) cells are incubated with the transformation mix in the incubator for 3 hours (7 hours). After the transformation period the transformation medium is removed and replaced by DMEM complete medium. 1 day after transfection, cells are investigated.
For Lipofectamine2000 transfection follow the guidelines of the manufacturer (transfection is possible in presence of serum)

Electroporation of mammalian cells

Electroporation is the cost effective method for introducing foreign DNA into the primary and other hard to transfect cells. In this protocol HUVEC transfection is shown as an example. Electroporation was carried out when the cells were at passage 5.

1. One day before electroporation, the cells were trypsinized and seeded in 100cm cell culture flasks.
2. The next day the cells were ready for electroporation, the cells were washed with sterile PBS, harvested by trypsinization and centrifuged at 4°C.
3. The cells were resuspended in electroporation buffer, counted and 5.4x106 cells/400ul was used for each transfection. Every step was carried out at ice.
4. 20 µg of the plasmid DNA was used for each electroporation. The DNA was mixed with cells and transferred to 4mm electroporation cuvette and placed on ice.
5. The cells were than electroporated at 960 µF and 200volts and put immediately on ice.
6. The electroporated cells were mixed with 6ml of media and seeded in 1% gelatin coated dishes. The cells from one electroporation are enough to put in three wells of a 6-well plate.
7. After 4 hr the media was changed. The cells were analysed after 24-48 hr of transfection.

Electroporation Buffer:

20mM HEPES

135mM KCl

2mM MgCl2

0.5% Ficoll 400

pH 7.6 with NaOH

TIPS:

1. DNA eluted with water works better than TE buffer.
2. Proceed quickly after mixing DNA and cells. Keeping DNA and cells mixture longer results in low efficiency.
3. After electroporation , before seeding the cells place them on room temperature for 10 min.

Electroporation of Endothelial Cells

(Angiogenesis 2004; 7:235-41)

day one:

1. seed cells for electroporation:
   $5{,}4 * 10^6$ / T162

day two

→ ***prepare new culture dishes (+medium and fibronectin) before starting electroporation and place in incubator in the meantime***

→ ***prepare eppis with DNA, prepare + label electroporation cuvettes***

2. ECs should be 70-90% confluent
3. wash cells with $HBSS^{def}$ once, harvest cells by trypsinization
4. stop with RPMI 1640 (Gibco) + 10% FCS (4 °C)
5. centrifuge 5 min at 4 °C, 1300 rpm
6. resuspend ECs in 1 ml RPMI 1640 (Gibco) + 10% FCS
7. count cells (in the meantime: put cells and medium on ice)
8. dilute ECs to $2{,}5 – 5*10^6$ cells/ml in RPMI 1640 + 10% FCS (4 °C)
9. mix 20 µl plasmid DNA with 400 µl cells, incubate eppi on ice for 10 min
10. transfer mix to 4mm-cuvette (BioRad electroporation cuvette) and electroporate immediately at 200 V, 1200 µF (should result in 40-45 msec pulse length)

Note: alternatively, 960 µF are also acceptable (pulse length: 30-35 msec)

11. leave cuvette at RT for 10 min, then re-seed cells in prepared 10cm-culture dishes with 10 ml EGM2-MV and fibronectin
12. analyse cells after 24-48 h

Expected transfection efficiency: 50-70 %

Expected cell death: 20-50 %

Lentiviral Transduction

Day1:

4x106 293 cells were plated in 10cm2 tissue culture plate in 10ml of DMEM, 24 hours before transfection.

Day2:

At the time of transfection, the plate was taken out of incubator, 5ml of media was sucked out from the 10cm2 plate and chloroquin was added to the final concentration of 25uM. The plate was put back in incubator and DNA was mixed in a falcon tube in the following order:

i. 5ug of VSVG (envelop protein)
ii. 7.5ug of packaging protein
iii. 10ug of the Target vector

After mixing the DNA,125ul of 2M CaCl2 and 875ul of H2O were added by tapping gently.

1ml of 2xHBS was then added drop wise into the DNA while bubbling with a pipette. When finished the mixture was continued to bubble for 1 min. The plate was taken out of the incubator and transfection mixture was added drop wise on the plate. The plate was swirled gently and returned to the incubator. After 4 hours of transfection the media was replaced with the fresh DMEM and the plates were transferred to the virus room incubator.

Day4:

48 hours later the viral supernatant was harvested and filtered through 0.45um filter. To the filtrate Polybrene was added to the final concentration of 10ug/ml and the filtrate was added to the target cells for 24 hours.

Day5:

After 24 hours the filtrate was removed, cells were washed with PBS and the fresh medium with the antibiotic (puromycin) in the final concentration of 1ug/ml was added to the cells. The cells were grown in the resistant media until all the cells in the control (untransfected) flask were dead. The cells were then checked for transfection and proceeded for the desired assay.

Generation of Stable 293 Cells

293 cells are seeded at a density of 500,000 cells/6-well. The next day $CaCl_2$ transfection with linearized and EtOH/acetate precipitated plasmid DNA is carried out as described above. 1 day after transfection cells are trypsinized and reseeded in a 10 cm cell culture Petri dish (about 60 cm^2) and DMEM complete medium is exchanged with DMEM complete medium containing 900 µg/mL G418. About 2 weeks later, colonies that survived the G418 selection are checked by fluorescence microscopy for the expression of the transfected GFP-fusion protein and positive colonies are picked

under the microscope (4x magnification objective) under sterile laminar flow by scraping them off with the tip of a pipette, careful suction of the colony and placement into a well of a 24-well plate containing the selection medium. 1 d after the isolation, the adherent colony is separated into single cells by trypsinization and reseeding of the cell suspension in the same well. After reaching confluence, the cells are further expanded up to 75 cm^2 flasks and aliquots are frozen in liquid nitrogen.

Reporter Gene Assays

Luciferase Assay

(Naila Malkani)

In the firefly luciferase activity assay D-luciferin is used as substrate for firefly luciferase. The oxidation of luciferin to oxyluciferin in the presence of ATP and magnesium is catalysed by luciferase, which results in bioluminescense.

Day1:
Seed the cells in 24 well plate, according to the experiment design.

Day2:
Transfect the cells with reporter plasmid, beta-gal and gene of interest with proper positive and negative controls.

Day3:
After 26-30 hrs of transfection lyse the cells. Take out the media, wash the cells with 1xPBS and put 50µl of lysis buffer (PI added). Transfer the plate to - 80°C.

When you want to proceed with the assay, thaw the plates and transfer the contents of each well into the eppendorf tubes. Centrifuge at 14000rpm at 4°C for 20 minutes. Use the supernatant for proceeding the assay.
In a 96well white plate add 20µl of the supernatant and 50µl of the assay buffer. Measure the luminescence at the reader (synergy) by injecting 50µl of the injection buffer.
For normalization, measure beta-gal activity. Transfer the contents of the above plate (almost 110µl from each well) to a normal transparent 96 well plate and 50µl of beta-galactosidase substrate (CPRG) was added to each well. The yellow CPRG solution turned orange to red depending on the beta-galactosidase activity which is measured at 595nm. The normalized activity is measured by dividing firefly luciferase values to respective beta-galactosidase values.

Lysis buffer:
0.1M KH2PO4 (pH 7.8 by NaOH)

Assay buffer: (1ml)

MgSO4 (1M) = 20ul

ATP (100mM) = 200ul

Glycyl gliaia buffer (25mM) = 780ul

Injection buffer: (3ml)

1M Luciferin = 750ul

Glycyl gliaia buffer 25mM = 2250ul

Recipes:

0.1M KH2PO4

136g KH2PO4 / 1 liter mQ H2O pH 7.8 by NaOH .

25mM Glycyl glycine

3.3g of glycyl glycine /1 liter mQ H2O pH 7.8

1mM luciferin

14mg D-Luciferin +3.5ml TrisHCl pH 7.5 make volume upto 50ml with mQ H2O.

CPRG

27.1mg of CPRG in 20ml PBS+0.5%BSA

TIPS:

After getting supernatant, use immediately for assay, avoid repeated freeze thaw.

Warm the luciferin to 37°C before using.

β-Galactosidase Assay with CPRG

(Johannes Schmid)

- Lyse cells
  (recommended cell lysis buffer: 0.25M Tris/HCl pH 8.0, 0.25% (v/v) NP40,
  2.5 mM EDTA)

- Pipet about 10 µl of extract into a 96-well plate (appropriate neg. control / blank: 10 µl of mock transfected cells, or non-transfected cells – as there is a slight endogenous β-Gal activity) – leave one well empty for blank (A-1)

- Add 100 µl substrate solution to the wells (also to the blank-well)

- Incubate until red color develops (min to hours – depending on β-Gal activity, if you have low activity you can also incubate at 37°C)
- Optional: Stop with 50 µl of Stop solution (only necessary if you want to time it exactly, e.g. by adding the substrate in a timed way and stopping the reaction in the same way)
- Measure with ELISA Reader at 570 nm (Filter #3)

Lysis Buffer:
0.25M Tris/HCl pH 7.4 (or better 8.0)
0.25% (v/v) NP40
2.5 mM EDTA

CPRG-substrate solution: 1 mg/ml (= 1.65 mM)
in PBS + 10 mM KCl, + 1 mM $MgCl_2$
alternative substrate buffer:
60 mM Na_2HPO_4 pH 8.0, 1 mM $MgCl_2$, 10 mM KCl, 50 mM Mercapto-ethanol

Stop solution: 0.5M Na_2CO_3

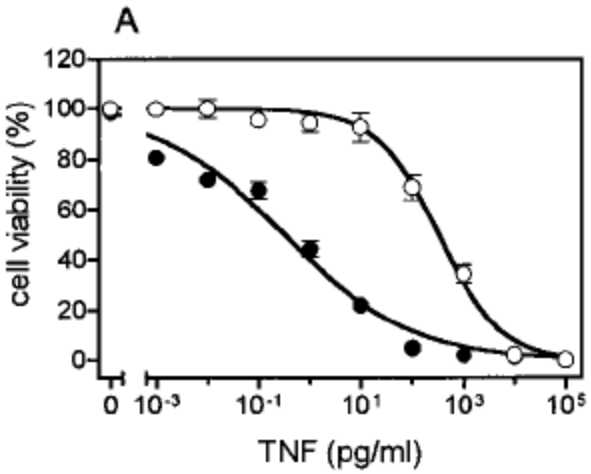
Cell Viability Assays

Crystal violet staining (can be used as proliferation or cytotoxicity assay)

HeLa cells and transfectants derived thereof were plated at a density of 1.5×10^4 cells/well in triplicates in 96-well microtiter plates in 100 µl of Click RPMI 1640 overnight at 37°C. On the next day, the reagents of interest were added in the presence of 2.5 µg/ml cycloheximide. The plates were incubated for additional 12- to 24-h culture, and cell viability was determined by crystal violet staining. Briefly, supernatants were discarded and the cells were washed once with PBS, followed by **crystal violet staining** (**20% methanol, 0.5% crystal violet) for 15 min**. The wells were washed with H2O and air dried. Residual dye was diluted with methanol for 15 min, and OD at 550 nm was measured with a R5000 ELISA plate reader (Dynatech, Guernsey, U.K.).

Ref.: ***The Journal of Immunology,* 1998, 161: 3136–3142.**

Example:



DNA Methods

General Cloning Methods

Crude Plasmid DNA Preparation by Alkaline Lysis

Solutions: Qiagen Plasmid Preparation Kit

- Resuspension buffer (P1): Tris/HCl 50 mM pH 8.0, EDTA 10 mM, RNase A 100 µg/mL , stored at 4°C
- Lysis buffer (P2): NaOH 200 mM, 1 % SDS
- Neutralization buffer (P3): KAc 3 M pH 5.5
- Isopropanol, room temperature
- Ethanol 70 %, 4°C

50 mL Falcon tubes filled with 5 mL of LB broth containing appropriate selective antibiotics (ampicillin: 100 µg/mL or kanamycin: 25 µg/mL) are inoculated with one colony of the desired transformed E. coli strain and incubated o/n at 37°C with vigorous shaking at 200 rpm. On the next day, a glycerol stock is made and then the cells are harvested with centrifugation at 6000 rpm? for 15 minutes at 4°C (centrifuge: Heraeus model Biofuge Primo R), the supernatant is removed and collected for autoclaving. Leftovers of LB broth can decrease the yield of plasmid DNA, therefore the Falcon tubes are placed upside down on a paper towel for some minutes in order to remove as much LB broth as possible. The E. coli pellet is resuspended in 250 µL of Resuspension buffer, transferred to 1.5 mL reaction tubes, then 250 µL of Lysis buffer are added and the lysis mix is carefully inverted 5 – 6 times until the solution is viscous. Shearing of genomic DNA by heavy shaking should be avoided. After 5 minutes incubation time at room temperature 250 µL of Neutralization buffer are added and again the mix is carefully inverted several times until a white precipitate of genomic DNA and proteins is visible. The white precipitate is pelleted by centrifuging at 6000 g for 15 minutes at 4°C. The supernatant containing plasmid DNA is collected without perturbing the pellet and centrifuged a second time to quantitatively remove genomic DNA precipitate. The collected supernatant is then subjected to isopropanol precipitation. The unpurified plasmid DNA solution is mixed with 0.7 volumes of isopropanol. After 5 minutes of incubation at room temperature, precipitated plasmid DNA is recovered with centrifugation at full speed, 16 000 x g, at 4°C for 30 minutes (centrifuge: Heraeus Sepatech Megafuge 1.0 R), the supernatant is removed and discarded. The plasmid DNA pellet is washed with 1 mL 70 % EtOH (4°C) without disturbing the pellet and centrifuged for another 5 minutes at full speed at 4°C and the supernatant is quantitatively removed. The pellet is dried for 5 min at RT and dissolved in 20 µl AD or 1x TE buffer. In some cases this DNA-solution was further purified by an EtOH/NaAc precipitation. The crude preparation of plasmid DNA can be subjected to restriction digest analysis and sequencing but cannot be used for transfection experiments of mammalian cells. In the latter case, the crude DNA solution was purified by Qiagen DNA purification columns.

Ethanol/NaAc Precipitation of Plasmid DNA

Solutions:

- Ethanol 70 %, -20°C
- Ethanol 70 %, 4°C
- NaAc 3 M
- TE buffer: Tris/HCl 10 mM pH 7.5, EDTA 1 mM

The DNA solution or in some cases the restriction digest sample was mixed with 1/10 volume of NaAc, 2.5 volumes of 100 % ethanol (-20°C) and was quickly transferred to -70°C for 5 minutes incubation. Then the plasmid was collected by centrifugation for 30 minutes at full speed, 16 000 x g, 4°C (centrifuge: Heraeus Sepatech Megafuge 1.0 R), the supernatant was removed by suction. The pellet was washed with 1 mL 70 % ethanol (4°C) and again centrifuged for 5 minutes at 14 000 rpm, 4°C and the supernatant was quantitatively removed by suction. The pellet was air-dried for some minutes prior to elution in an appropriate volume of AD or 1x TE buffer (to reach a final concentration of about 1 µg/µl).

Plasmid DNA Preparation by Alkaline Lysis:

Solutions:

- Equilibration buffer (QBT): NaCl 750 mM, MOPS 50 mM pH 7.0, 15 % isopropanol, 0.15 % Triton X-100, stored at room temperature
- Wash buffer (QC): NaCl 1 M, MOPS 50 mM pH 7.0, 15 % isopropanol, stored at room temperature
- Elution buffer (QF): NaCl 1.25 M, Tris/HCl pH 8.5, 15 % isopropanol, stored at room temperature

For midi (maxi) DNA preparations the QIAGEN Plasmid Midi (Maxi) kit was used (Cat.no.12143, 12163). A 250 mL (500 mL) shaking flask filled with 50 mL (100 mL) LB broth containing appropriate selective antibiotics (ampicillin 100 µg/mL, kanamycin 25 µg/mL) were inoculated with the desired transformed single colony of E. coli strain and incubated o/n at 37°C with vigorous shaking at 200 rpm. The cells were harvested by centrifugation at 6000 g for 15 minutes at 4°C (centrifuge: Heraeus model Biofuge Primo R), the supernatant was removed and collected for autoclaving. Leftovers of LB broth can decrease the yield of plasmid DNA, therefore the centrifugation buckets were placed upside down on a paper towel for some minutes in order to remove as much LB broth as possible – remnants of LB on the walls of the tubes were removed by suction. The E. coli pellet was resuspended in 4 mL (10 mL) of Resuspension buffer P1, 4 mL (10 mL) of Lysis buffer (P2) were added and the lysis mix was carefully inverted 5 – 6 times. After 5 minutes incubation time at room temperature 4 mL (10 mL) of Neutralization buffer were added, again the mix was carefully inverted several times until a white precipitate of genomic DNA and proteins were visible and the neutralized lysate was incubated on ice for 20 minutes. The white precipitate was pelleted by centrifuging at 6000 g for 15 minutes at 4°C. The

supernatant containing plasmid DNA was collected without disturbing the pellet and centrifuged a second time 6000 g for 15 minutes at 4°C. In the mean time a Quiagen tip 100 (500) was equilibrated by applying 4 mL (10 mL) of Equilibration buffer on the matrix of the tip, the buffer entered the resin only by gravity flow. The supernatant of the centrifugation was applied on the columns and entered the column by gravity flow. 2 Washing procedures were performed with 10 mL (30 mL) of Wash buffer respectively. Purified plasmid DNA was eluted with 5 mL (15 mL) Elution buffer. After elution DNA was precipitated with 0.7 volumes isopropanol, pelleted by centrifugation at 14 000 rpm for 30 min at 4°C centrifuge: Sorvall RC-5B rotor SA 600), the pellet was air-dried and eluted in 100 µL (500 µl) AD or 1x TE. DNA yield was quantified with an UV spectrophotometer and the plasmid DNA was diluted to a final concentration of 1 µg/µL.

Isopropanol Precipitation of Plasmid DNA

Solutions:

- Isopropanol, room temperature
- Ethanol 70 %, room temperature

The eluted midi (maxi) plasmid DNA solution was mixed with 0.7 volumes of isopropanol (room temperature) and immediately centrifuged for 30 minutes at full speed, 17 000 x g, 4°C (centrifuge: Heraeus Sepatech Megafuge 1.0 R). The supernatant is carefully removed. The DNA pellet is washed with 2 mL (5 mL) 70 % ethanol (room temperature) without disturbing the pellet and another centrifugation step is carried out for 10 minutes at full speed, 4°C, the supernatant is quantitatively removed by suction. The pellet is air dried and dissolved in 100 µL AD. DNA yield is quantified and the plasmid DNA is diluted to a final working concentration of 1 µg/µL.

DNA Quantification

DNA exhibits a specific absorption maximum at 260 nm, which is used for DNA quantification by an UV/VIS spectrophotometer (Pharmacia Biotech Ultrospec 2000). An OD at 260 nm of 1 refers to a DNA amount of 50 µg/mL. An appropriate dilution (1:200 after midi or maxi prep) of the DNA sample is necessary to stay in the linear range (0.1-1) of this quantification method. The purity of DNA concerning the presence of residual proteins can be calculated by the ratio of OD260/OD280. The aromatic amino acid tryptophan exhibits an absorption maximum at 280 nm, which refers to the relative amount of protein in the sample. An OD260/OD280 ratio of 1.8 – 2.0 is desired – which is typical for pure DNA samples resulting from the shoulder of DNA-absorbance at 280 nm.

Restriction Digest

Restriction digest of 20 µL volume: 17 µL of Aqua dest. (nuclease free) are mixed with 2 µL appropriate 10x restriction enzyme buffer, 1 µg (1µg/µL) of DNA added and mixed well. Then 1 µL (10 U) of restriction enzyme (-20°C) are added and again mixed well by swirling and spinning down. After incubation time of 1 hour at 37°C in a water bath (or other appropriate temperature according to manufacturers protocol) DNA fragments can be resolved by agarose gel electrophoresis.

For a double enzyme digest consult the website of the manufacturer and check for appropriate conditions for simultaneous double digest or alternatively digest at the low salt buffer with enzyme 1 first (1 h), then add NaCl to reach the salt concentration of the second enzyme (using e.g. the mixing cross at: http://www.meduniwien.ac.at/user/johannes.schmid/MolarityJava.htm), add enzyme 2 and continue with the digest for another hour.

Ligation

In a microcentrifuge tube prepare the following reaction mixture:

- Linear vector DNA: 5-10 µl (50-400 ng)
- Insert DNA: use a 1:1 up to a 3:1 (or 5 :1) molar ratio of insert DNA to vector DNA
- 10X ligation buffer for T4 DNA Ligase : 2 µl
- 50% PEG 4000 solution (for blunt ends only): 2 µl
- Water, nuclease-free to 20 µl
- T4 DNA Ligase: 0.2-0.4 µl (1-2 u) for sticky ends or 1 µl (5 u) for blunt ends

Vortex the tube and spin down in a microcentrifuge for 3-5 seconds.
Incubate the mixture for 1 hour at 22°C.

After ligation: heat to 65°C for 15 min to destroy DNA-Ligase complexes
(increases transformation efficiency 3-5 fold)
Use the mixture for transformation.

Note
If the yield of ligation product is insufficient, prolong the reaction time (overnight).
DNA can be dissolved in nuclease-free water or TE buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 7.8). An excess of ligation mixture with respect to competent cells may decrease the transformation efficiency.

hints from Fermentas

- Insert : Vector = 5 : 1 (related to pmol ends)
- for 10 ng vector (3 kb) with sticky ends:
  use 1-2 units T4 DNA ligase (10 units for 100 ng)
  for blunt ends: use 5 units for 10 ng (50 units for 100 ng)
- PEG4000 increases the ligase efficiency (but has to be removed for electroporation by 2x chloroform extraction)
- ammonium inhibits the ligase (consider that for PCR products containing $NH4^+$)
- NaCl > 200 mM inhibits the ligase

Agarose Gel Electrophoresis

Solutions:

- TAE 50x stock: Tris base 2 M, 57.1 mL acetic acid, EDTA 50 mM (stock 1 M pH 8), ad 1 L with AD
- Ethidiumbromide 10 mg/mL in aqua dest.
- Running buffer: TAE 1x
- DNA sample buffer 6x:
  (Fermentas:10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60mM EDTA

Typically, 1 % agarose gels are poured by heating up 1 g agarose / 100 mL 1 x TAE until agarose is completely dissolved and no smears are visible. The hot agarose solution is cooled down to about 55°C and 5 µL ethidium bromide solution are added and gently mixed to avoid formation of bubbles. Then, the agarose solution is poured immediately into the gel cast cassette, appropriate combs are placed in the solution and abundant bubbles are pushed away from the slots by pipette tips. After about 30 minutes, polymerization is finished and the agarose gel can be used for electrophoresis. The agarose gel is placed in the electrophoresis chamber (BioRad) with the slots facing the cathode and 1x TAE is added until the gel is sufficiently covered by running buffer. If necessary, the slots are rinsed with running buffer by pipetting up and down some running buffer in order to remove leftovers of agarose slurry. DNA samples are mixed with 6x DNA sample buffer (5:1) and loaded in the slots. After loading, electrophoresis is started immediately in order to prevent diffusion of sample into the surrounding gel. Electrophoresis parameters (power supply BioRad Power PAC 2000): 100 – 120 V for at least 30 minutes depending on the DNA fragment size supposed being resolved. Resolved DNA fragments are visualized under UV transillumination (transilluminator: 260 – 340 nm range) and documented with a BioRad gel doc system. For clonings care is taken that the DNA is not exposed too long to UV light since UV light can irreversibly damage DNA. DNA fragments are excised with a clean razorblade and gel extraction is carried out as described below.

Gel Extraction

Solutions:

- Solubilization buffer (QG)
- Isopropanol
- Wash buffer (PE)
- Elution buffer (EB): Tris/HCl 10 mM pH 8.5

For gel extractions usually the QIAquick gel extraction kit is used (Qiagen, Cat.no. 28704). An excised agarose gel slice is weighed to calculate the approximate gel slice volume (1 g = 1 mL) and transferred into an reaction tube. 3 volumes of solubilization buffer are added and incubated in a 50°C

waterbath until the gel piece is completely dissolved. 1 gel volume isopropanol is added and mixed. The solution is then loaded on the column and centrifuged for 1 minute at full speed (centrifuge: Eppendorf centrifuge 5415 D), the flow through is discarded. 750 µL of wash buffer are pipetted to the column and centrifuged under the same conditions as above, the flow through is discarded and the column is placed in a new microreaction tube. For elution of extracted DNA, 20 µL of elution buffer are directly applied on the matrix, incubated for 1 minute at room temperature and centrifugation as above is carried out. As an alternative to the Qiagen gel extraction kit, sometimes GenElute columns (Sigma, cat. no. # 5-6500) are used, which are equilibrated by loading of 100 µl 1x TE buffer, followed by brief centrifugation (1 min at full speed), addition of the gel slice to the column and centrifugation at full speed for 10 min. The resulting flow through (about 40 µl) contains about 90 % of the DNA, while the agarose remains in the column.

PCR: Polymerase Chain Reaction

Preparative PCR (e.g. for Cloning)

Solutions:

- AD nuclease free
- dNTP mix, each nucleotide 2 mM: stock solutions 100 mM
- Forward and reverse primers 10 pmol/µL in AD respectively
- 10x Pfu puffer (or Taq buffer)
- Template DNA
- Pfu polymerase 2.5 U/µL – or Taq polymerase 2.5 U/µL (Pfu is used for cloning purposes due to the higher fidelity of this DNA polymerase; Taq polymerase is used for analytical PCR's)

For a 100 µL PCR reaction, following solutions are mixed in a thin walled reaction tube (BioRad): 85 µL AD, 10 µL Pfu buffer 10x, 1 µL dNTP mix 2 mM, 1 µL forward primer 10 pmol/µL, 1 µL reverse primer 10 pmol/µL, 500 ng (1 µL) template DNA, 1 µL Pfu polymerase (2.5 U). Handling and pipetting of PCR steps are carried out with gloves in order to prevent contaminations. The reaction tubes arere placed in the hot (94°) PCR block (Perkin Elmer Gene Amp PCR System 2400) and the following program is used:

step 1 10 minutes 94°C (denaturation)
step 2 1 minute 94°C (denaturation)
step 3 1 minute 56°C or other appropriate annealing temperature – about 2-4 °C below the calculated melting temperature (annealing)
step 4 1.5 minutes/ 1kb 72°C (elongation)
step 5 5 minutes 68°C
step 6 ∞ 4°C (cooling)

30 cycles of steps 2-4 are carried out. After finished PCR, 6x DNA sample buffer is added to the PCR samples to inactivate the polymerase. Amplified PCR products are resolved by 1 % agarose gel electrophoresis.

PCR-cloning

1. PCR with Pfu-Polymerase (has higher accuracy than Taq)

for 100 µl:

A.dest. 85 µl

10x Pfu-Buffer 10 µl

dNTP´s (20 mM) 1 µl (ad 200 µM)

Template-DNA 1 µl (50 ng)

Primer forward 1 µl (10 pmol, about 100 ng)

Primer reverse 1 µl (10 pmol, about 100 ng)

Pfu-Polymerase 1 µl (2.5 - 3 units)

94°C 1 min

94°C 1 min

annealing temp. 1 min

75°C 1 min/kb Insert

68°C 5 min

4°C unlimited

Take 10 µl of the PCR-Mix, add DNA-Buffer (2 µl 6x conc.) and run an agarose gel (100 V, 30 min) - the PCR-product should be clearly visible.

2. Phenol/chloroform extraction and ethanol precipitation

The aqueous phase of the PCR-Mix (under the paraffin layer) is transferred to a new tube (90µl), an equal volume phenol (or phenol:chloroform:isoamylalcohol=25:24:1) is added (90 µl) and the sample is vortexed for about 10 sec, followed by centrifugation (14000 rpm, Eppendorf Centrifuge, 3 min).

The aqueous phase (upper layer) is carefully taken up with a pipette (using the “screwing mode” for the last few µl). The white material between the two phases should not be taken up. An equal volume chloroform (or chloroform:isoamylalcohol=24:1) is added, followed by vortexing for 10 sec and centrifugation at full speed for 10 sec. The upper phase is again taken up carefully and transferred to a new tube.

The volume of the final DNA-solution should be measured with the pipette and the DNA precipitated by mixing with 1/10 volume 3M Na-acetate and 2.5 volumes absolute ethanol. The solution should be cooled to -70°C for 5 min and the DNA pelleted by centrifugation (14000 rpm, 30 min at 4°C). The supernatant is removed, the pellet is washed with 100 µl 70% EtOH and centrifuged again for 4 min (14000 rpm, 4°C). The washing solution is removed, the pellet is dried for 5 min at r.t. and resolved in 10 µl TE-buffer (by resuspending with a pipette and warming to 58°C for 5 min).

If possible, the amount of DNA is quantified by photometry (1 µl in 300 µl A.dest.).

A 260 x 15 = µg/µl (for dilution of 1:300 and dsDNA)

3. Restriction digest and electrophoresis

1 - 2 µg of the vector DNA is digested with the restriction enzymes of choice using the appropriate buffer system). Usually a 20 µl solution is prepared (2 µl of 10x buffer, 1 - 2 µl of DNA and A. dest. up to 20 µl). If two enzymes are used, which require different buffers (e.g. L and H buffer), you can start with the low salt buffer and just 1 enzyme (1 µl enzyme, 10 units, 37°C for 1 h), followed by addition of NaCl to obtain the higher salt concentration, addition of the second enzyme and incubation for an additional hour at 37°C.

If you use two different restriction enzymes for cloning (generating incompatible DNA-ends), it is not necessary to dephosphorylate the vector. If you use just one enzyme (or enzymes with compatible ends), you have to dephosphorylate the vector after the restriction digest (by adding 1/10 volume of 10x CIP-buffer and 1 µl of CIP (calf intestine phosphatase) and incubation at 37°C for 15 min. This is necessary to prevent religation of the vector (and a high background in empty vectors after transformation). The phosphatase has to be inactivated afterwards by addition of EDTA (1 µl 0.5 M) and heating to 68°C (for 10 min). Phosphatase treatment usually decreases the efficiency of ligation considerably.

The PCR-product should be subjected to restriction digests as the vector (use an appropriate amount of DNA - e.g. 1 - 2 µg, as well). For the final ligation, you have to consider the molar amount of insert related to vector. Since, the insert is usually much smaller than the vector (e.g. 1 kb compared to 5 kb), 1 µg of insert means a corresponding higher molar amount (5x more than 1 µg of a 5 kb vector).

After restriction digest (and dephosphorylation of the vector) the samples are either extracted with phenol/chloroform and precipitated with ethanol as described above, or subjected to electrophoresis. (Use low melting agarose, if you want to purify the DNA from the cut gel slices by heating and phenol/chloroform extraction, or normal agarose if you want to use GenElute columns from Sigma or similar stuff to obtain the DNA from the gel.).

Electrophoresis is done at 100 V for about 30 min (for normal agarose) or at 80V for 1 h (for low melting agarose).

After electrophoresis, DNA-bands of linearized vectors or PCR-fragments are cut from the gel (as close to the DNA as possible) preventing longer UV-exposure (which damages the DNA).

Gel slices can be transferred to GenElute columns (equilibrated with 100 µl TE and centrifuged at full speed for 5 sec), followed by centrifugation at 14000 rpm for 10 min at r.t., which results in the generation of about 40 µl of DNA solution with about 80 - 90% of the original DNA amount. Alternatively, the gel slices are transferred to Eppendorf tubes, centrifuged briefly (to estimate the volume of the gel), covered with an equal amount of TE, heated to 65°C for 5 min, cooled for 30 sec (at r.t.) and extracted with an equal volume of phenol/chloroform as described above.

4. Ligation

1 µl 10x ligase-buffer, linearized (dephosphorylated) vector (about 100 - 300 ng), insert (about 50 - 100 ng, depending on length) and A. dest (ad 10 µl) are mixed and ligase is added (e.g. from BioLabs, 0.5 - 1 µl). The molarity of the insert should be between 1 - 2 times the molarity of the vector . The ligation mix is incubated at 16°C over night (or alternatively: 4 h at r.t.). The ligase buffer contains ATP (prevent repeated freeze-thawing; you can aliquot the buffer and store it at -70°C for good results).

5. Transformation:

Transform 2 µl of the ligation mix (either conventionally or by electro-transformation: 1.9 kV, 25 µF, 200 , 4-5 msec), recover for 1 h at 37°C in 1 ml SOC medium, centrifuge at 3000 rpm for 10 min, decant the supernatant, suspend the pellet in the residual fluid and streak out on appropriate plates.

Bacterial colony PCR

(to test for successful cloning and presence of inserts)

- Mix a single colony in 20 µl A. dest
- Heat to 95°C for 10 min (to lyse cells and release plasmids)
- Spin at 14000 g for 2 min
- Take 2 µl per PCR

PCR Mastermix (20x)

	Per sample	For 20 samples
10x buffer (incl. Mg)	2 µl	40
2 mM dNTPs	2 µl	40
Primer 1	0.4 µl (40 ng or 4 pmol)	8
Primer 2	0.4 µl	8
A.dest	12.7 µl	254
Taq (Roche)	0.5 µl (0.5 u)	10
Supernat from colony	2 µl	

PCR: A) 3 min 94°; B) 35 cycles: 30 sec 94°, 30 sec 52°, 45 sec 72°; C) 68° 5 min

Realtime PCR

… will be filled soon by Bastian

Northern Blotting

1. Extraction of RNA

    1.1. Incubation of cells under the appropriate conditions (usually on petri-dishes with 10 cm diameter)

    1.2. Complete removal of the media with a sterile pasteur pipette linked to the vacuum pump: after the first removal of the media lean the petri-dishes nearly vertically against a support (for a short time) so that the rest of the media can be collected from the bottom (in the laminar flow).

    1.3. Add 1 ml of TriZol Reagent (Gibco: 15596-018) to the petri-dish and cover quickly the whole area (by shaking) - use sterile tip (if possible with a filter included).
    This step should be done in the fume cupboard because of the phenol of the reagent.
    If you want to prevent any potential contamination with RNases, you can do it in the laminar flow, too.

    1.4. Extract the cells by repeated pipetting of the solution over the whole area (the solution usually gets a little bit turbid) and transfer the extract to a sterile eppendorf tube.

2. Preparation of total RNA
    according to the protocol provided by Gibco.

3. Quantification of the RNA
    The final pellet is dried for 10 min in the laminar flow and dissolved in 22 µl of nuclease-free distilled water by heating to 56°C for 30 min. 2 µl of the solution are diluted with 500 µl of distilled water and the A_{260} and A_{280} values are determined using quartz cuvettes (switch on the reader at least 1 h before in order to warm up the lamp).
    40 µg RNA/ml have an $A_{260} = 1$
    µg RNA/ml = measured A_{260} x 250 (dilution factor) x 40; thus: A_{260} x 10 = µg RNA/µl
    The ratio A_{260}/A_{280} should be 1.8 - 2.0 for clean RNA solutions.

4. Dot Blot (otional):
    Calculate the amount of RNA solution to give 5 µg of RNA (for higher sensitivity: 10 µg).
    The volume has to be 3µl or less. Pipette the corresponding volume into a fresh sterile eppendorf tube and add nuclease-free water to give a total of 3 µl. Centrifuge the tubes to get a drop at the bottom of the tube. Then directly pipette 3 µl onto a dry Hybond-N^+ membrane (you can draw a grid with 1 cm squares onto the membrane using a pencil and apply the drops in the middle of each square). Let the membrane dry for 10 min and then wet it a little bit from below using 5x SSC buffer. Immobilise the RNA on the membrane by UV-crosslinking (Stratagene Crosslinker - Dorian Bevec lab: Auto-crosslink with setting of 1200). Store the blot in Saran wrap at -20°C.

5. Electrophoresis in denaturing Agarose-Gels

5x MOPS: 41.2 g 3-(N-morpholino-)-propanesulfonic acid
800 ml 50 mM Na-Acetate solution (4.1 g/l in nuclease-free water)
10 ml 0.5 M EDTA solution
adjust the pH to 7.0 with 2 N NaOH
adjust the volume to 1 l with nuclease-free water
autoclave the buffer and store at R.T. protected from light (wrapped in foil)

Preparation of the gel (1% Agarose)

1.5 g Agarose (Pharmacia, NA-Agarose) are suspended in 93 g of nuclease-free water (fresh bottle!) and heated for 3 min at full power (850 W) in the microwave oven. Shake a little bit to dissolve completely; weigh the amount of water that is lost due to evaporation and fill up to the original weight (if necessary heat again to dissolve completely and check the weight again).
Add 30 ml 5x MOPS buffer, 27 ml 37% formaldehyde (2.2 M) and 7.5 µl ethidiumbromide solution (10 mg/ml) - mix and try to prevent air bubbles.
The gel is poured into the gel-bed (this should be completely horizontal - check with bubble of spirit level) and the sample comb is put in (the sample comb is usually stored in ethanol to prevent RNase contamination and dried in the laminar flow before use). Polymerisation of the gel is allowed for 30 min and then the gel is transferred to the electrophoresis apparatus (filled with 1 l of 1xMOPS - diluted from 5x MOPS with nuclease-free water). The surface of the gel should be covered. The sample comb is carefully removed.

Preparation of RNA-samples for electrophoresis

10 µg total RNA (calculated volume)
2.5 µl 5x MOPS
3.5 µl 37% formaldehyde
10 µl formamide

are combined in an eppendorf tube, briefly centrifuged and the RNA is denatured by heating to 56°C for 15 min. After a brief cooling on ice, 2 µl of 10x loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenolblue, 0.4% xylenecyanol) are added and the samples are again briefly centrifuged.

Electrophoresis

The samples are mixed with loading buffer (at the bottom of the tube after centrifugation) by

repeated pipetting. Then they are carefully filled into the sample pockets of the gel. Electrophoresis is carried out at 20V over night or at 100V for 3 h (RNA migrates towards the plus-pole). The bromophenolblue should migrate about 8 cm, before ending the run. The gel is checked on the UV-monitor and a picture is taken with the Polaroid camera (put a ruler to the gel). Two bands should be visible (28S and 18S rRNA). Partly RNase-digested samples migrate faster.

6. Capillary Blot

The gel is marked on the right lower edge, removed from the electrophoresis container and submersed in 0.05 N NaOH for 20 min (with some shaking). This is important for the transfer of RNA larger than 2.5 kb.

Wash the gel with nuclease-free water.

Shake the gel for 45 min in 20x SSC-buffer.

A tray is filled with 5x SSC, and a glass plate is put on the tray. Two Whatman 3MM filters (11 cm x ca. 40 cm) are wetted with the 5x SSC and laid over the pate so that both ends are in the buffer. Air bubbles between the plate and the filters are removed by rolling a sterile pipette on the filter. The gel is put on the wet filters with the upper side down (transfer is more efficient in this way; besides, the orientation of the samples on the blot is then equal to the orientation on the gel). Air bubbles between gel and filters have to be removed with the pipette !!!! A pre-cut Hybond-N^+ membrane (10 cm x 14 cm, dry) is put exactly on the gel (the membranes becomes wet when it touches the gel).

2 Whatman 3MM filters (10 cm x 14 cm) are wetted in the 5x SSC buffer and laid on the Hybond-membrane. Air bubbles are removed again. (If the pre-cut 10 cm x 13 cm Whatman filters are used, the gel and the Hybond membrane have to be cut to this size; in this case just 13 instead of 15 samples can be applied to the gel).

Parafilm is put exactly to the edges of the gel, in order to prevent contact between the wet filters below the gel with the filters above the gel.

A stack of dry Whatman filters (height: 5 - 8 cm) is laid on top, followed by a glass plate and a 500 g weight (bottle). The capillary transfer from the gel to the membrane should be carried out for 18 - 24 h. Afterwards, the transfer is checked under UV-light. There should be no bands visible on the gel, but only on the Hybond-membrane (a picture can be taken with the Polaroid camera)

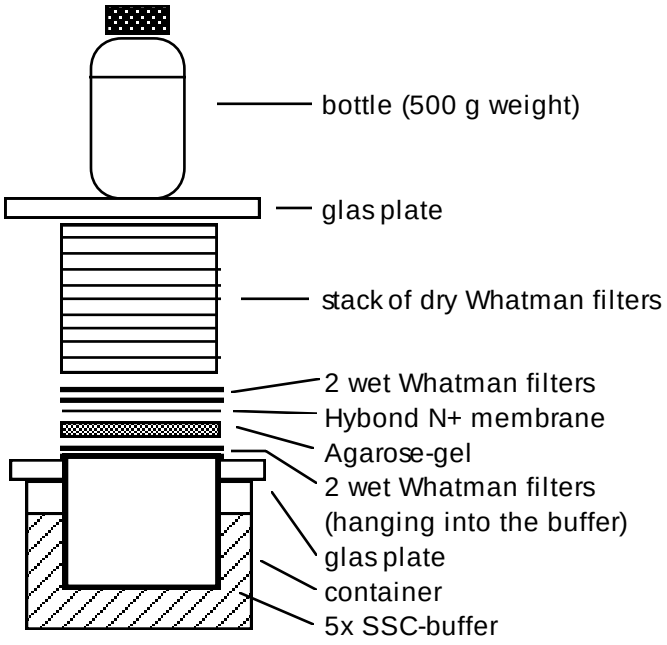


Fig. 1: Capillary Blot

The RNA is immobilised on the membrane by UV-crosslinking (Exposure to 120 mJ; Bevec´s lab: Stratagene Auto-Crosslink set to 1200 units).

7. Methyleneblue staining of the Blot (optional)

Wash the membrane for 10 min in 3% HAc (under nuclease-free conditions)
Stain for 30sec - 1 min with 0.04% methyleneblue/0.5 M Na-acetate pH5.2
Destain with nuclease-free dist. water until the background is nearly white.

8. Pre-hybridisation

Reagents:

50x Denhardt´s:	10 g Ficoll (Sigma F-9378)
	10 g Polyvinylpyrrolidone (Sigma PVP-10)
	10 g BSA (Sigma A-7906)
	ad 1000 ml with nuclease-free water

Pre-hybridisation solution:

5x SSC	(25 ml 20x SSC)
5x Denhardt´s	(10 ml 50x Denhardt´s)
20 mM Na-phosphate	(20 ml 1 M Na-phosphate buffer pH7.0)
7% SDS	(35 ml 20% SDS)
	(10 ml nuclease-free water)
	(stored in aliquots at -20°C)

The pre-hybridisation solution is pre-warmed to 65°C before use.

Hybridisation solution
= pre-hybridisation solution containing 10% dextransulfate (10 g/100 ml)
(stored in aliquots at -20°C).

For the pre-hybridisation, the blots (wet) are put into the hybridisation tubes (not more than 2 blots per tube) with the RNA facing inside, and 10 ml of pre-warmed pre-hybridisation solution are added, as well as:

100 µl Poly-A(10 mg/ml; final concentration 100 µg/ml)
100 µl sonicated fish sperm DNA (10 mg/ml; final concentration: 100 µg/ml)
(for 10 ml = for one tube)

The blots are pre-hybridised for 4 h at 65°C (2.5 h are sufficient, too) under continuous rolling of the tube.
1 h before the end of the pre-hybridisation, the hybridisation solution is pre-warmed to 65°C and the radioactive labelling of the oligo is carried out.

9. ^{32}P-labelling of the oligonucleotide (with Terminal deoxynucleotidyl-Transferase)

1 µl Oligonucleotide solution (10 ng/µl) = 10 ng
2 µl 5x TdT reaction buffer
1 µl $CoCl_2$ solution (10 mM)
1 µl TdT enzyme (included in the TdT-Kit: Boehringer Nr. 220582)
5 µl (□-^{32}P) dATP (50 µCi) (Amersham, Redivue)

Mix by centrifugation and incubate for 1 h at 37°C
In the meantime prepare NICK spin column (Pharmacia) for the purification of the labelled oligo: put the column in vertical position, let the gel settle and remove the lids. Let the column drain and

apply 1 ml TE-buffer, let it drain again and apply 2 ml TE-buffer. Let the column drain again, put into a centrifugation tube and centrifuge it at 500 g for 4 min using a counterbalance (2000 rpm in the Heraeus centrifuge). Remove the water that was spun off, and put a small cryotube (1.5 ml) into the centrifugation tube, followed by the column. (see Fig. 2).

After the incubation of the oligonucleotide with (α-^{32}P) dATP and TdT, add 100 µl of TE-buffer containing bromophenolblue and mix shortly. Briefly centrifuge the sample and apply it to the prepared NICK spin column (the solution is taken up by the semi-dry gel). Centrifuge the column under exactly the same conditions as before (500 g, 4 min). Low molecular compounds like bromophenolblue or unbound (α-^{32}P) dATP remain in the gel, whereas high molecular compounds as the (α-^{32}P) dATP-labelled oligonucleotide are spun into the cryotube.

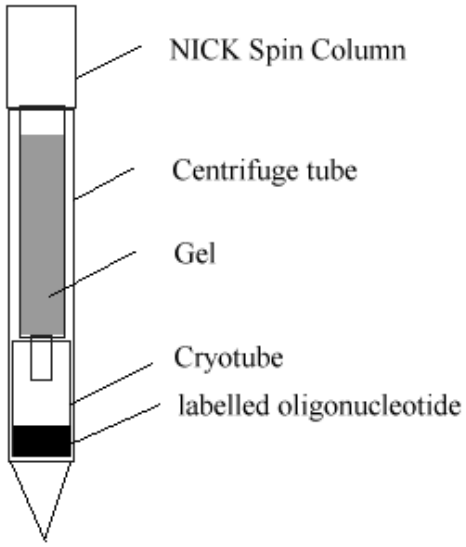


Fig. 2

10. Hybridisation

The pre-hybridisation solution is poured off and 10 ml pre-warmed hybridisation solution is added, as well as 100 µl Poly-A solution and
100 µl sonicated fish sperm DNA
Finally the labelled oligonucleotide is added, and the tube is transferred to the hybridisation oven. Hybridisation is carried out over night at 65°C with continuous rolling of the tube.

11. Post-hybridisation washing

After the hybridisation, the radioactive solution is poured onto some paper towels on a bench coat and can be thrown to the solid radioactive waste. The membranes are removed from the tubes into

a plastic container and washed with continuous shaking under appropriate conditions in a water bath. Two or three washing solutions are used, usually at 65°C for 20 min, each.

Oligo-Wash 1:
150 ml 20x SSC
100 ml Na-phosphate buffer pH7.0
100 ml 50x Denhardt´s
400 ml nuclease-free water
250 ml 20% SDS (at last)

Oligo-Wash 2:
50 ml 20x SSC
900 ml nuclease-free water
50 ml 20% SDS (at last) = 1x SSC/1% SDS (Na^+-concentration: 0.225 M)

Oligo-Wash 3 (optional):
10 ml 20X SSC
980 ml nuclease-free water
10 ml 20% SDS = 0.2x SSC/0.2% SDS (Na^+-concentration: 0.045 M)
The washing solutions are pre-warmed to the appropriate temperature (usually 65°C)
For each oligonucleotide the melting temperature has to be calculated for the different washing solutions according to the formula:

T_m = 81.5 + 16.6 log (Na^+-concentration) + 0.41 (%GC) - 600/N - 0.63 (formamide%)

%GC.... percentage of G and C in the oligo
N ..... length of the oligo (number of bases)
(in our procedure there is no formamide included)

The last washing step should be about 10°C below the calculated melting temperature.
For oligonucleotides in the range of 30 bases, oligo-wash 1 and 2 are usually sufficient, for oligos in the range of 60 bases a third wash is recommendable. Either the temperature or the sodium concentration has to be adjusted in a way that the applied temperature is 10°C below the melting point. After the use, the first washing solution has to be poured into the container for liquid radioactive waste, the second and the third washing solution can be poured into the decontamination sink. The membranes are wrapped in Saran wrap and either measured on the InstantImager detector or exposed to film (Kodak X-OMAT or BioMax). The film cassette is stored at -70°C until development of the film.

12. Dehybridisation:

After evaluation of the northern blot, the membrane can be stripped in reprobed. The stripping is carried out by washing in 0.1% SDS at 80°C for 20 - 30 min. The next probing starts with the pre-hybridisation.

Protein Methods

Cytosolic Cell Extract Preparation

Solutions:

- Nonidet P-40 (NP 40)
- Protease inhibitor cocktail (1 tablet Complete™ protease inhibitor, Roche) in 1 mL PBS makes 50x stock solution): pepstatin, aprotinin, leupeptin, phosphoramidon
- Lysis buffer: PBS def. containing 0.5 % (v/v) NP 40 and protease inhibitors (when the protein concentration had to be determined by Coomassie staining the NP-40 concentration was decreased to 0.2% to reduce interference with the protein quantification).
- Proteasome activity lysis buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 150 mM, $MgCl_2$ 10 mM, ATP 5 mM (freshly added prior to use), 0.2 % NP 40
- Adenosintriphosphate (ATP) 100 mM pH 7.4 in PBS complete (PBS deficient containing additionally $CaCl_2$ 1mM, $MgCl_2$ 0.5 mM

Typically cells are lysed with 0.2 % NP 40, protease inhibitors in PBS. Incubation of some minutes is enough to achieve effective lysis. Destruction of the cytoplasm membrane is checked with the inverse microscope (10 x magnification), intact nuclei are visible whereas the cytoplasmic membranes disappear. After lysis, cell extracts are centrifuged for 15 minutes at full speed, 17 000 x g at 4°C (centrifuge: Heraeus Sepatech Megafuge 1.0 R) to get rid of cytoplasm membrane parts and nuclei.

Protein Quantification (Bradford)

Solutions:

- BSA 1 µg/µL in PBS
- BioRad 1:5 Coomassie Reagent diluted in AD

96-well format: Generation of BSA standard curve: 0 – 5 µg of BSA respectively are pipetted in a well respectively. Cell extract samples are diluted e.g. 1:10 and an appropriate amount is used for quantification in order to stay in the linear range of the assay. 150 µL of diluted BioRad Coomassie Reagent (5x) are added to each well and incubated for some minutes. Absorbance at 595 nm is then measured with an Elisa reader (SLT Lab instruments 340 ATTC). The BSA standard curve is evaluated in MS Excel. According to the equation after linear regression analysis (for the linear range, usually between 0 and 4 µg protein), protein amounts are calculated.

Immunoprecipitation

Solutions:

- 1 % BSA in PBS
- PBS def., 4°C
- Agarose beads: αflag agarose beads (M2-αflag affinity matrix, Sigma, Cat #A-1012), αIKK1/2 agarose beads (Santa Cruz)

Preparation: An appropriate amount of agarose beads with covalently bound antibody (about 15 µl of a 50 % slurry are necessary for one immunoprecipitation) are washed twice with 1 mL PBS (4°C) and centrifuged for 1 minute at full speed in a table top centrifuge (centrifuge: Heraeus Sepatech Megafuge 1.0 R). Blocking: the agarose beads are suspended in 1 mL 1 % BSA in PBS solution and incubated while rotating for 1 hour at 4°C, in order to block unspecific protein binding sites and therefore to increase the specificity. The beads are centrifuged as above and the supernatant is removed. Immunoprecipitation: cell extract and PBS (4°C) are added to the blocked beads and PBS (4°C) is added to a final volume of 800 µL and incubated while rotating for 1 hour at 4°C. Washing procedure: 4x washing with PBS (4°C) and centrifugation are performed as described above. After the third time washing the resuspended beads are transferred to a new reaction tube in order to prevent that proteins that bound unspecifically to the tube are eluted by SDS-buffer. 20 µL of 1x SDS sample buffer are pipetted to the beads and proteins are detached from the beads by heating to 95°C for 5 minutes. After centrifugation for 3 minutes at full speed in a table top centrifuge, the supernatant is used for SDS PAGE (or stored at -80°C for later electrophoresis and Western blotting).

Co-Immunoprecipitation for the Detection of Protein Interactions

1. Transfection of cells with tagged proteins (one 6-well of CHO or HeLa cells is sufficient for one sample).
2. Preparation of extracts:
   2.1. 1 d after transfection: wash cells with PBS
   2.2. Lysis with 500 µl/well Lysis-Buffer + Protease Inhibitors: 15 min at 4°C.
   Buffer: 0.5% NP40, 50 mM Tris/HCl pH 7.5, 1 mM EDTA, 150 mM NaCl.
   Protease Inhibitors: 10 µg/ml Aprotinin, 20 µg/ml Phosphoramidon, 40 µg/ml Pefabloc, 1 µg/ml Leupeptin, 1 µg/ml Pepstatin (from 1000x stock solutions, Boehringer Protease Inhibitor set).The lysis is suited for cytosolic proteins and membrane proteins. Nuclei remain intact (you can leave the nuclei on the plate when you take off the supernatant).
   2.3. Spin the extracts for 15 – 30 min at 14 krpm, 4°C (HeLas: 15 min, CHO: 30 min)
   2.4. Keep the supernatant and adjust the NaCl-concentration (150 mM – 1000 mM depending on the strength of interaction; start in the range of 150 – 250 mM, increase the concentration if you want to increase the stringency)
3. Co-Immunoprecipitation
   3.1. Take 400 µl of extract for IP (keep about 30 µl extract for direct western analysis).
   Use flat-top tubes (the visibility of the pellet is better in these tubes) Add 400 µl Lysis-Buffer/250 mM NaCl (without NP40 > final concentration: 0.25%). Add beads (15 µl anti-flag-M2-Agarose, Sigma A-1205; alternatives: other antibodies directly coupled to CNBr-activated Sepharose; Protein A- or Protein G-Agarose: the later will give more unspecific binding). Rotate extracts + beads for 2 h at 4°C.
   3.2. Spin for 30 sec at 14 krpm 4°C. Take off the supernatant, add 1 ml of lysis buffer/250 mM NaCl/without NP40 and invert tubes several times (do not vortex). Repeat this washing step.
   3.3. Suspend the beads in 1 ml cold PBS and transfer the suspension to a new tube.
   Spin 30 sec at 14 krpm, 4°C, take off the supernatant and repeat this washing step. Final centrifugation: 1 min at 14 krpm, 4°C. Remove the supernatant and suspend the beads in SDS-PAGE buffer (30 µl). Incubate for 5 min at 95°C and pellet the beads for 2 min at 14 krpm.
4. SDS-PAGE
5. Western Blot: if possible use HRP-conjugated primary antibodies (anti-HA-HRP from Boehringer, anti-myc-HRP from Invitrogen). This gives much lower background of unspecific bands (Ig light chain …).

Denaturing SDS-PAGE

Solutions:

- Solution A: 1.25 M Tris/HCl pH 8.8, 0.4 % SDS
- Solution B: 30 % Acrylamide/N', N'-Bismethyleneacrylamide (29:1) in H_2O
- Solution A Stack (ASt): 0.5 M Tris/HCl pH 6.8, 0.4 % SDS, phenol red
- Ammoniumpersulfate (APS) 10 % (w/v) in AD
- N, N, N', N'- Tetra-ethylethylene-diamine (TEMED)
- Butanol
- 1x SDS running buffer: Tris base 25 mM, glycine 250 mM, 0.1 %SDS
- 6x protein loading dye: Tris/HCl 750 mM pH 6.8, 60 mM DTT, 12 % SDS, 60 % glycerol anhydrous, bromophenol blue

- prestained protein marker: Benchmark™ (INVITROGEN)

For 2 SDS-PAGE gels (6 cm x 9 cm x 0.015 cm) 10 mL of separating gel are prepared. The following table contains the volumes of solutions according to 7.5 %, 10 % and 12.5 % SDS-PAGE gels. H_2O, solution A and solution B are mixed (preventing air bubbles), then APS and TEMED are added in order to start polymerization. The separating gel solution is mixed briefly and is filled in the prepared gel cast system (BioRad Mini Gel system), about 5 cm high (to about 0.5 – 1 cm below the position of the bottom of the sample comb). A layer of butanol is pipetted on top to exclude oxygen from the surface since the presence of oxygen disables polymerization. After about 30 minutes polymerization is finished, the butanol layer is poured off and the stacking gel is layered on top of the separation gel, immediately (to prevent drying of the gel).

For 2 stacking gels 10 mL are prepared. According to the next table H_2O, solution Ast and solution B

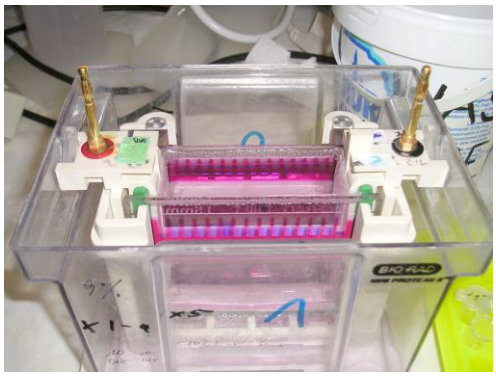


Figure 3. 1: BioRad PAGE electrophoresis apparatus

separating gel SDS PAGE

solution	**7.5%**	**10%**	**12.5%**
H_2O [mL]	4.85	4	3.2
sol. A [mL]	2.5	25	2.5
sol. B [mL]	2.5	3.4	4.2
APS 10% [µL]	50	50	50
TEMED [µL]	10	10	10

are mixed, and then APS and TEMED are added in order to start polymerization. The solution is pipetted on the separating gel to the upper edge of the glass plates (until overload, bubbles are swept away like this) and then the combs are placed between the glass plates avoiding air bubbles. Again, polymerization is finished after 30 minutes, the SDS PAGE gel is then ready for use.

stack SDS PAGE	
Solution	**4 %**
H_2O [mL]	6.1
sol. Ast [mL]	2.5
sol. B [mL]	1.3
APS 10 % [µL]	50
TEMED [µL]	15

Protein samples are mixed with 6x protein loading dye to a final concentration of 1x protein loading dye. Samples are boiled for 5 minutes at 95°C, and then briefly centrifuged for 2 minutes at full speed in an eppendorf table top centrifuge. After polymerization, the gel sandwich is taken out of the gel cast

and placed in the electrophoresis tank, adding 1x running SDS running buffer in the inner and outer chamber, the combs are removed and the slots are cleansed from half-polymerized gel pieces by pipetting up and down some running buffer. Then protein samples and prestained protein marker are loaded on the gel. Electrophoresis is started immediately to prevent diffusion of the samples into the surrounding gel area. Electrophoresis parameters: 30 mA per gel for about 1 hour, depending on how big the resolved proteins were.

	For final concentration of gel (% T):					
	Separating gel (10 ml)				**Stack gel (10 ml)**	stack gel 5 ml
	7%	**10%**	**12,5 %**	**15%**	**5%**	
30% Acrylamide-bis solution 29:1 (A)	2.33	3.33	4.17	5	1.67	0.835
4x Separation buffer **1.5 M Tris/HCl pH 8.8**	2.5	2.5	2.5	2.5		0
4x Stacking buffer **0.5 M Tris/HCl pH 6.8 + phenol red**					2.5	1.25 0
aqua dest.	5	4	3.2	2.4	5.7	2.85
SDS (10 %)	0.1	0.1	0.1	0.1	0.1	0.05
TEMED	0.015	0.015	0.015	0.015	0.015	0.0075
APS (10%)	0.03	0.03	0.03	0.03	0.03	0.015
total ml	9.98	9.98	10.02	10.05	10.02	
Acryl in (A)	0.699	0.999	1.251	1.5	0.501	
Acryl %	7.01	10.02	12.49	14.93	5.00	

SDS-sample buffer

	final conc	6x		**6X Sample Buffer (with DTT)**	
Tris/HCl pH6.8	0.125	0.75		**1** M Tris-Cl (pH 6.8)	2.4 ml
Glycerol	10%	60%		SDS	0.96 g
SDS	2%	12%		Glycerol	4.8 ml
DTT		739	mg	DTT	7.39 mg
Bromophenol blue		4.8	mg	Bromophenol Blue	4.8 mg

Do not adjust the volume.

Reduction: either by 2% β-Mercaptoethanol
(final conc., equals about 255 mM SH-groups, would mean about 125 mM DTT with 2 SH groups

STRIPPING BUFFER (2% SDS, 62.5mM TRIS pH6.8, 100mM Beta-mercaptoethanol (bME)

Reagent	ml for 500ml	ml for 100 ml
20% SDS	50	10
1M TRIS pH6.8	31.25	6.25
H2O	418.75	83.75
****ADD THE bME FRESH BEFORE USE****		
beta-Mercapto	3.55	0.71

Incubate the membrane for 15 - 30 min at 50ºc submerged in stripping buffer.

Electro Transfer Blotting on PVDF Membrane

Solutions

- 1x SDS western transfer buffer (1L): 25 mM glycine (2.9 g), 50 mM Tris base (5.8 g), 20 % methanol (200 mL), 0.4 % SDS, aqua dest. ad 1L, pH should be 8.0
- Methanol 100 %

Activation of PVDF membrane (Millipore, Immobilon Cat. No. #IPVH20200): in order to make the PVDF membrane hydrophilic it has to be soaked in methanol 100 % for 15 seconds, then rinsed in aqua dest. for 2 minutes and equilibrated in Western transfer buffer for at least 3 minutes. The blotting sandwich is built as follows: one blotting sponge lying on blotting cassette soaked in western transfer buffer, two 3MM whatman filterpapers soaked in western transfer buffer, the activated PVDF membrane, the polyacrylamide gel, two 3MM whatman filterpapers soaked in western transfer buffer, one blotting sponge soaked in western transfer buffer. After each layer possible bubbles are removed by rolling a plastic pipet on top of the current layer. The blotting cassette (BioRad) is then closed and placed in the transfer chamber (orientation overview: cathode – / gel / membrane / anode +). Blotting parameters: 150 mA per blotting cassette/gel for 2 hours.

Western Blot

Solutions:

- Ponceau S solution: 0.1 % (w/v) Ponceau S in 5 % (v/v) acetic acid
- Blocking solution: 5 % skimmed milk powder in PBS def.,0.1 % Tween20
- Wash solution: PBS def. 0.1 % Tween20
- Antibody solution: primary or secondary antibody diluted in 1 % skimmed milk powder in PBS def., 0.1 % Tween20
- Primary antibodies: αIKK2 (1:500, mouse IMGENEX), αJAB1/CSN5 (1:10 000, rabbit, Santa Cruz), αflag tag M2 (1:2000, mouse, Sigma), αNEMO/IKKγ (1:500, rabbit, Santa Cruz), α HA tag (1:1000), αIKK1 (1:500, Santa Cruz)

- Secondary antibodies coupled with horseradish peroxidase (HRP) αmouse (1:5000, Amersham-Pharmacia), α rabbit (1:5000, Amersham-Pharmacia)
- NaN_3 solution 20 % (w/v) 200x in AD
- ECL: (Amersham), ECL plus (Amersham)
- Pierce Super signal West pico (Cat. No. #34080)
- Pierce Super signal West femto (Cat. No. #34095)

The PVDF membrane is taken out of the transfer sandwich, possible gel leftovers sticking on the membrane are removed and the membrane is incubated in Ponceau S solution while gently shaking for 5 - 10 minutes. After this, the Ponceau S solution is removed and collected for reuse, the membrane is destained by rinsing with AD until the bands can be clearly seen. At this point, desired lanes can be separated by cutting and trimming the membrane with a sharp scalpel. The stained membrane is scanned on a regular computer-scanner to assess equal protein loading (respectively for normalization purposes). Then the membrane is incubated in blocking solution for 30 minutes while gentle shaking to block unspecific binding sites. Appropriate primary antibodies are diluted in 5 – 10 mL antibody solution in a 50 mL Falcon tube. In order to cover the membrane evenly with antibody solution, the membrane is placed on the inner wall of the 50 mL Falcon tube after the blocking step with the protein side facing the inner side and the antibody solution and incubated on a rotor for 1 hour at 4°C. For reuse the antibody solution is supplemented with NaN_3 20 % (1:200) to a final concentration of 0.1 % NaN_3 to prevent bacterial growth and stored at 4°C. The membrane is rinsed twice shortly with about 20 mL wash solution, then washed twice with about 100 mL wash solution for 5 minutes and washed once with about 100 mL for 15 minutes. After the last washing step, the antibody solution containing the secondary antibody is incubated under the same conditions as above for 1 hour. The washing protocol is the same (2 x rinsing, 2 x 5 minutes, 1 x 15 minutes). Detection of immunoblotted proteins is done by chemiluminescence reaction with substrate solutions provided by Pierce or Amersham-Pharmacia. The membrane is incubated with the substrate solutions according to the manufacturer's protocol. (ECL: (amersham) mixing ratio of solution 1 and 2 1:1 incubation time 1 minute, ECL plus mixing ratio of solution A and B 1:40, incubation 5 minutes, Pierce Super signal West pico: mixing ratio of solution 1 and 2 1:1 incubation time 5 minutes, Pierce Super signal West femto mixing ratio of solution 1 and 2 1:1 incubation time 5 minutes). The membrane is taken out of the solution and is placed in a plastic wrap avoiding wrinkles of the wrap. Then excess of substrate solution was removed by streaking from the outside with a paper towel. Depending on signal intensity the blot is exposed to Kodak X-OMAT or BioMax films or a CCD-camera based detection system (LumiImager, Roche) is used for detection of chemiluminescence. The distances of the protein markers from the top of the separation gel are measured and used for generating a standard curve of the molecular weights (using the correlation between the log of the MW and the migration distance; calculations and regression analysis are done with MS Excel).

Silver Staining of PAGE Gels

Solutions

- Fixing solution: 50 % ethanol, 10 % glacial acetic acid, ad 100 % with aqua dest.
- Incubating solution (1L): 30 % ethanol, sodiumthiosulfate anhydrous 2g, sodiumacetat anhydrous 34 g, fill up to 1L with aqua dest. Before use add 125 µL of glutaraldehyde/50 mL incubating solution.
- Silvernitrate solution (1L): $AgNO_3$ 1 g, dissolved in 1L aqua dest.. Before use add 10 µL of formaldehyde/50 mL of silver nitrate solution.
- Developing solution (1L): Na_2CO_3 anhydrous 25 g, dissolved in 1L aqua dest.. Before use add 10 µL of formaldehyde/50 mL of developing solution.
- Stop solution (1L): sodium-EDTA 15.78 g dissolved in 1L aqua dest..

After electrophoresis, the polyacrylamide gel is taken out of the casting sandwich and placed in a clean glass beaker filled with fixing solution. All following steps are carried out while gently shaking. The gel has to be incubated with the fixing solution for 30 minutes. After fixation an appropriate amount of incubating solution including glutaraldehyde (the gel has to be at least covered by liquid) is prepared and added to the gel, followed by incubation for 15 minutes, discarding the fixing solution and washing with aqua dest. 3x for 5 minutes and 10 minutes incubation in silvernitrate solution including formaldehyde. The silvernitrate solution is collected (special waste). Developing is carried out by incubating the gel in developing solution including formaldehyde until the desired intensity of protein staining is reached, followed by discarding of developing solution and adding stop solution. The gel should incubate for at least 1 hour in the stop solution. Afterwards the gel can be stored in aqua dest. or dried with vacuum.

Stripping of PVDF Membranes

Solutions

- Stripping buffer: 62.5 mM Tris/HCl pH 7.0, 2 % (w/w) SDS, 0.7 % mercaptoethanol

For reprobing PVDF membranes the previous antibody has to be removed by a socalled stripping procedure. An appropriate amount of stripping buffer (about 25 ml per blot) is prewarmed to 50°C, the membrane is laid on the inner wall of a hybridization tube and together with the stripping buffer it is incubated under rotation at 50°C in a hybridization oven for 30 minutes. After stripping, the membrane is washed twice for 10 min in PBS/0.5% Tween 20 and blocked again (by incubation in 5 % milk powder in PBS-Tween). It is then ready for the next immunoblotting.

Proteasome Purification by Immunoprecipitation

Solutions:

- Proteasome activity lysis buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 150 mM, $MgCl_2$ 10 mM, ATP 5 mM pH 7.4 (freshly added prior to use), 0.2 % NP 40
- Proteasome wash buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 150 mM, $MgCl_2$ 10 mM, ATP 5 mM (freshly added prior to use)
- Proteasome elution buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, KCl 2 M, $MgCl_2$ 10 mM, ATP 5 mM pH 7.4 (freshly added prior to use)
- α20S beads: antibody against subunit α4 coupled to agarose beads (Affinity Ltd, UK, PW 9005, Batch Z04562)
- NaN_3 10 mM in PBS def.

A blue p1000 tip was plugged with a p200 filter, then 0.5 mL of αsubunit α4 coupled to agarose beads were placed on the filter. The generated small column is equilibrated with 3 x 1 mL proteasome wash buffer by gravity flow. 293 or HeLa cells are lysed with proteasome activity lysis buffer and precleared as described above under “cell extract generation”. The cell extract is loaded on the column by gravity flow, the column is washed with 3 x 1 mL physiological proteasome wash buffer. Elution is performed with 3 x 1 mL high salt proteasome elution buffer. Elution fractions are collected and investigated for proteasome activity with the fluorogenic substrate Suc-LLVY-AMC. The proteasome fractions are either dialysed or TCA precipitated. The column is reequilibrated with proteasome wash buffer and stored in PBS def. containing NaN_3 to prevent bacterial growth.

Proteasome Activity Assay

Solutions

- Fluorogenic peptide solution: Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) 1 mg/mL in N,N – Dimethylformamide (100x)
- Proteasome activity buffer: Tris/HCl 10 mM pH 7.4, KCl 140 mM, $MgCl_2$ 5 mM, ATP 5 mM

10 µL of cell extract are mixed with 90 µL proteasome activity buffer and 1 µL of Suc-LLVY-AMC solution and vortexed well. With a 1 mL syringe the sample is injected bubblefree into the fluorescence detector (model: FP-920 Jasco Inc, Japan). The measuring parameters of the acquisition method are: Time scan for 5 min at an excitation wavelength of 380 nm and emission wavelength of 440 nm with data acquisition every 5 seconds. The gain is set to 10 (2^{nd} stage of 4 logarithmic amplification steps) and the emission bandwidth is set to 18 nm slit. Due to proteasome activity, the fluorescent molecule AMC (7-amino-4-methyl coumarin) is released from the non-fluorescent peptide substrate Suc-LLVY-AMC resulting in a linear increase of fluorescence over time with the slope being proportional to proteasome activity. The resulting graph of proteasome activity is then exported to MS Excel and evaluated. The slope of the fluorescence increase is calculated by linear regression and normalized by total protein amount to determine the normalized proteasome activity. It has to be noted that the

fluorogenic peptide substrate is cleaved by proteasomes independent from ubiquitination and most likely also independent from the proteasome activator complex.

Proteasome Activity Overlay Assay

Solutions:

- Fluorogenic peptide solution: Suc-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC) 1 mg/mL in N,N – Dimethylfomamide (100x)
- Proteasome activity buffer: Tris/HCl 10 mM pH 7.4, KCl 140 mM, $MgCl_2$ 5 mM, ATP 5 mM

After electrophoresis, the native 4 % PAGE gel is carefully taken out of the gel sandwich and placed on a plastic wrap. About 5 mL of overlay solution containing 5 mL proteasome activity buffer and 50 µL of Suc-LLVY-AMC solution arere spread evenly over the surface of the native gel and the gel is soaked for about 10 minutes. Fluorogenic peptide activity is detected with the Sybr green filter of the LumiImager™ (Roche) detection system.

Dialysis

Solutions:

- Dialysis buffer: Tris/HCl 10 mM pH 7.4, 10 % (v/v) glycerol, NaCl 100 mM, $MgCl_2$ 10 mM, ATP 5 mM pH 7.4 (freshly added prior to use)

Samples with salt concentrations of 2 M are transferred to a reaction tube, the lid was removed and instead a dialysis membrane (cut off) is fixed. This small dialysis chamber was fixed up side down on the wall of a big glass beaker filled with about 300 mL dialysis buffer. Dialysis was carried out o/n at 4°C.

Glycerol Gradient Centrifugation

Preparing: a glycerol gradient of 35 – 80% glycerol is used. Glycerol dilutions from 35 - 80% are prepared in 5 % steps. Thick walled centrifugation tubes are placed upright in a rack over night at –80°C. For pouring the gradient, 120 µL of each glycerol dilution are pipetted in the centrifugation tube starting with the heaviest (80% glycerol). After each dilution the rack is placed back at –80°C for 2 minutes until the layer is either very viscous or frozen. Then the next layer is pipetted on top and so forth. With that procedure a very sharp gradient can be created. On top of the last frozen layer the sample of 120 µL are pipetted. Then all tubes are balanced on an analysis balance.
Centrifugation parameters (Beckmann Ultracentrifuge Optima TLX): 40000 rpm, 4°C, vacuum, 16 hours. After centrifugation, the gradient was fractionated manually with a Gilson p200 120 µL steps each.

Native PAGE

Solutions:

- Native separating gel buffer / running buffer: 0.18 M Tris/borate pH 8.3, $MgCl_2$ 5 mM, DTT 1 mM, 1 mM ATP
- Acrylamide/N', N'-bismethyleneacrylamide mix: 37.5:1
- Ammoniumpersulfate (APS) 10% w/w
- N, N, N', N'- Tetra-ethylmethylen-diamine (TEMED)
- Adenosintriphosphate (ATP) 100 mM pH 7.4 in PBS complete
- Native loading dye: 50 % glycerol containing 0.0025 % xylene cyanol

Protocol (Glickman et al. 1998a) slightly modified

The native PAGE gels containing 4 % acrylamide/bisacrylamide are poured without a stack, for 2 gels (6 cm x 9 cm x 0.015 cm) 25 mL of separating gel are prepared as follows: 21.3 mL native separating gel buffer and 3.7 mL acrylamide/bisacrylamide mix are mixed thoroughly avoiding bubbles, 250 µL 10 % APS and 25 µL TEMED are added to start polymerization. The solution is poured immediately into the prepared gel cast cassette and the combs are inserted. After 30 minutes when polymerization is finished, the gel sandwich is placed in the electrophoresis tank and running buffer is poured in both electrode chambers. 20 µL samples are mixed with 5 µL of loading dye and loaded on the gel. Electrophoresis parameters: 30 mA for 1 gel. The gel is running for about 2 hours until the blue front eluated into the lower running buffer chamber. In most cases, the native gel is afterwards subjected to a proteasome activity overlay assay.

Trichloro-Acetic Acid Precipitation of Proteins

Solutions:

- TCA 50 % w/w
- Acetone

The samples are mixed with trichloro acetic acid solution (TCA) to a final concentration of 10 % w/w. After incubation for 1 hour at 4°C the samples are centrifuged for 30 minutes at 17.000 x g at 4°C in a tabletop centrifuge. The supernatant is removed and the pellet is washed once with cold acetone – followed by drying for 5 min and uptake in the appropriate buffer (e.g. SDS-PAGE buffer).

Kinase Assays in vitro

Lysis buffer (final conc.): for 20 ml:

20 mM Tris/HCl pH7.5	400 µl 1 M
150 mM NaCl	600 µl 5 M
25 mM β-glycerophosphate	500 µl 1 M
2 mM EDTA	80 µl 0.5 M
2 mM pyrophosphate	400 µl 0.1 M
1 mM orthovanadate	200 µl 0.1 M
1% Triton X-100	2 ml 10%
1 mM DTT	20 µl 1 M
1 mM NaF	20 µl 1 M
A. dest.	15.8 ml

Protease Inhibitors: added before use (Leupeptin, Pepstatin, Pefa-Block) according to stock

Kinase buffer (final conc.): for 20 ml:

20 mM Tris/HCl pH7.5	400 µl 1 M
20 mM β-glycerophosphate	400 µl 1 M
100 µM orthovanadate	20 µl 0.1 M
10 mM $MgCl_2$	200 µl 1 M
50 mM NaCl	200 µl 5 M
1 mM DTT	20 µl 1 M
50 µM ATP	50 µl 20 mM
1 mM NaF	20 µl 1 M
A. dest.	18.7 ml

1. Lyse cells (in 6 wells) with 500 µl per well of Lysis buffer (+ protease inhibitors): 20 min at 4°C.
2. Clear by centrifugation (14000 rpm, 4°C 15 min Eppendorf centrifuge). Save an aliquot (30 µl) for Western blotting.
3. Immunoprecipitate the kinase (e.g. with 10 µl anti-flag affinity matrix beads, Sigma, for flag-tagged transfected kinase; or with appropriate antibody for endogenous kinase + Protein A-Sepharose or directly coupled to agarose): 2h at 4°C (rotating).
4. Wash the beads: 3x with 1 ml PBS (4°C), 1x with 1 ml Kinase buffer (4°C): pellet the beads by centrifugation (14000 rpm, 4°C, 45sec) and remove the supernatant.
5. Prepare Kinase buffer: add $MnCl_2$ to 10 mM (stock: 1 M) and ^{32}P-γ-ATP (5 µCi per sample, usually 1/10 volume, i.e. 1 µl of stock solution for one 10 µl assay) and preincubate at 30°C for 10 min.
6. Add 1 µg substrate: GST-IκB (1 µl) or mutant substrate (as control) to the beads; add 10 µl kinase buffer, mix gently and incubate at 30°C for 30 min (or longer).
7. Stop the reaction by addition of 4x SDS-sample buffer (4 µl) and perform SDS-PAGE with the samples, followed by fixation of the gel (10% methanol, 10% HAc), drying and autoradiography.

For detection with PhastGel: use only 5 µl beads, 5 µl kinase buffer, 0.5 µl substrate and 2 µl 4x SDS-sample buffer: Run a 12.5% PhastGel with 4 µl per sample

Detection of ^{32}P γ-ATP Phosphorylated Proteins

Fixed and silver-stained SDS-PAGE gels are sandwiched between a layer of Whatman filter paper and plastic wrap, the sandwich is placed in the vacuum dryer with the wrap layer facing up. The gel is dried for about 15 minutes at 80°C with vacuum, and then the dried gel is cooled down between two heavy objects to decrease deformation and cracking. The dried gel covered with a plastic wrap is exposed to a phosphor screen o/n or to Kodak films for some days depending on signal intensity. The phosphor screens are scanned with Storm™ equipment (Molecular Dynamics).

Protein-DNA Binding Assays

EMSA

Preparing of cell extracts

Cells (e.g. 293 cells) of one 6-well (10 cm^2, app. 10^6 cells): add 100 µl/well:

1x EMSA lysis buffer (stock sol.: 5x):

- 10 mM Tris/HCl pH 7.5
- 1 mM EDTA
- 5 mM MgCl
- 50 mM KCl
- 1 mM DTT
- 1x protease inhib. (Complete)

- Lysis of cells by 4 freeze/thaw cycles (-80°C/37°C incubator): on the plates,
- check breakage by microscopy
- suspend with pipette and transfer to Eppendorf tubes > 1 additional freeze/thaw cycle
- centrifugation: 14 000 rpm, 4°C, 15 min > take supernatant: measure vol.: approx.75 µl (> can be frozen at -70°C)
- add glycerol to 10% final conc., add KCl to 150 mM (4.2 µl 1 M to 75 µl sample)

Determine protein concentration of the extracts with Bradford reagent:

standards: BSA: 0, 1, 2, 3, 4 µg (µl) in 96well plates
extracts: 1 µl each (or diluted);
+ Biorad Bradford reagens (1:5, 200 µl) > measure OD595 in a microtiter plate reader
expected concentration of extracts: 2 – 3 µg/µl

Annealing of oligos

- equimolar amount of sense and antisense oligo: 400 pmol each (approx. 5 µg): 4 µl
- 20 µl 10x Buffer B (Roche)
- A.dest. ad 200 µl (172 µl)
- heat to 95°C (5 min)
- switch off the thermoblock and let cool down to RT

concentration: 400 pmol/200 µl = 2 pmol/µl

Labeling of annealed oligo with ^{32}P-alpha-dATP with TdT

(Fermentas #EP0161, Terminal deoxynucleotide Transferase)

- 10 µl 5x reaction buffer
- 2.5 µl annealed oligo (10 pmol of 3' termini = 5 pmol ds-oligo)
- 5 µl ^{32}P-alpha-dATP (10 µCi/µl > 50 µCi)
- 2 µl TdT (40 u)
- 30.5 µl A.dest. nuclease free

incubate at 37°C for 15 min
(Stop the reaction by heating to 70°C for 10 min).

Incubation of extracts with oligos and native PAGE

(Electrophoresis on PhastSystem, GE Healthcare, formerly Pharmacia-Amersham: see instruction manual of the manufacturer)

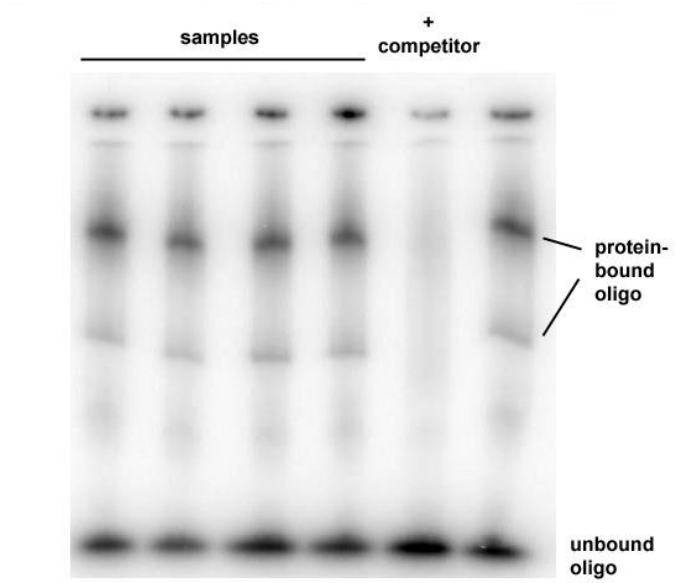
- 2 µl extract,
- 1 µl 1x EMSA lysis buffer (or competitor > 20x molar excess)
- 0.5µl ^{32}P-Oligo
  > incubated 15 min at RT

+ 1 µl 1x EMSA lysis buffer (+ small amount bromphenolblue)

- pipetted into 4 µl sample combs of the PhastSystem
- run samples on 12.5% homogenous Phastgels using native buffer strips

Sample Appl. down at 4.2.	0Vh			
Sample Appl. up at 4.2	2Vh			
Step 4.1. 400 V	10.0 mA	2.5W	15°C	10 Vh
Step 4.2. 400 V	1.0 mA	2.5W	15°C	2 Vh
Step 4.3. 400 V	10.0 mA	2.5W	15°C	140 Vh

Example:



ABCD-Assay (Avidin-Biotin Complex with DNA)

(1) Preparations

Buffer H

	c (stock)	**End-c**	für V = 50 ml	für V = 100 ml
HEPES pH 7,8-7,9	0,5 M	20 mM	2 ml	4 ml
KCl	1 M	50 mM	2,5 ml	5 ml
Glycerol	100 %	20 %	10 ml	20 ml
DTT	1 M	1 mM	50 µl	100 µl
(NaF)				
NP-40	100 %	0,1 %	50 µl	100 µl
			ad ddH_2O	ad ddH_2O

HEPES pH 7,8-7,9:
- 1 M solution use NaOH to adjust pH 7,8-7,9
- sterile filter all solution used
- The stringency of the assay is dependent on KCL concentration: High stringency: 150mM or 250mM KCL
- For endogenous protein 50mM KCL appears to work best

(2) Streptavidin-Agarose

- Re-suspend streptavidin-agarose by vortexing, take out 500 µl (mark with a pen), centrifuge and discard supernatant
- Wash three times with 1000 µl Buffer H (50mM KCL, +5mg/ml BSA), washing: re-suspend, 10min on rotating well (4°C), centrifuge (4°C), discard supernatant
- Add 1 ml Buffer H (+5mg/ml BSA, +10 µl 10mg/ml SS DNA) incubate 24 h on rotating well (4°C)
- wash three times with 1000 µl Buffer H (50mM KCL, without BSA)
- add up to (50mM KCL, without BSA) 500 µl (use mark)

(3) Anneal oligonucleotides

50 µg S-Oligo
50 µg AS-Oligo
10 µl 10x Annealing Buffer
Add to 100 µl with H_2O

10x Annealing Buffer: 0,5 M NaCl
0,2 M Tris pH 7,4

- Incubate on heating block for 5min at 95°C
- Switch of heating block, let sit until block has cooled to 30°C to 40°C
- Store oligos at 4 °C or -20 °C

Example sequence of NFkB oligo (for Pulldown)

NFKB_for	Bio´ GGGAAATTCCCGGAAATTCCCGGAAATTCCCGGAAATTCC
NFKB_rev	Bio´ GGAATTTCCGGGAATTTCCGGGAATTTCCGGGAATTTCCC

(red: one NFKB consensus sequence)

Competitor oligo: same oligo without biotin

(4) Preparation of cell lysate

NETN

	c (stock)	**End-c**	for V = 50 ml	for V = 100 ml
Tris pH 8.0	1 M	10mM	0.5 ml	1 ml
NaCl	5 M	100mM	1 ml	2 ml
EDTA pH 8.0	0.5 M	1mM	100 µl	200 µl
Glycerol	100 %	10 %	5 ml	10 ml
NP-40	100 %	0,5 %	250 µl	
DTT	1 M	1mM	50 µl	500 µl
Protease Inhibitor 25X 40µl/ml (Frisch dazugeben)				100 µl
			ad ddH_2O	ad ddH_2O

Sterile filter NETN solution.

Scrape cells in PBS with cell scraper.
Centrifuge cells: 5´ 1500 rpm

Re-suspend pellet in 1ml NETN (for 75 cm^2 flask)

Sonify:
6 pulses á 2 s, 30% power

centrifuge: 14 000 rpm, 4°C, 15 min

Keep supernatant.

(5) Assay

Pulldown

200 µl Cell Lysate
2 µl biotyniliated oligo (1 µg/µl)
2 µl SS-DNA (10 mg/ml)
200 µl Buffer H

Competitor control

200 µl Cell Lysate
2 µl biotyniliated Oligo (1 µg/µl)
2 µl SS-DNA (10 mg/ml)
200 µl Buffer H
20 µl not biotyniliated oligo (1 µg/µl)

Negative control

200 µl Cell Lysate
2 µl SS-DNA (10 mg/ml)
200 µl Buffer H

Positive/Input control (for WB)
20 µl Cell Lysate

(if cell lysate is less than 200 µl add to 200 µl with Buffer H, successful PD will depend on initial protein concentration vs stringency)

→ incubate 5 min at 37 °C in heating block shaking (1100 rpm)

→ incubate for 1 h on ice

- add 40 µl of prepared streptavidin-agarose

  → incubate 30 min at 4 °C on rotating wheel
- centrifuge at 4°C (13.200rpm, 2 min)
- wash 5 times with 1 ml Buffer H (50 mM, 150mM or 250mM KCl)

  washing: remove supernatant, add 1 ml Buffer H, incubate 10 min at 4 °C on rotating wheel, centrifuge at 4°C (13.200rpm, 2 min)
- after last wash remove **all** remaining Buffer H: you can use the tips for blot loading to avoid sucking in the beads
- add 25 µl 1x Western Blot loading Buffer

  optional: you can freeze your samples at this point
- Blot loading

→ incubate 3 min at 95 °C shaking (1100 rpm)

- Centrifuge (13.200rpm, 2 min), use 20 µl for loading
- for Input control use 20 µl cell lysate + 4 µl loading buffer

Products:

Strepavidin-Agarose (Heidelberg) - Novagen Cat.# 69203-3
Strepavidin-Sepharose (ab HU + MS-275) - GE Healthcare # 17-5113-01

ChIP Protocol

Johannes Schmid Group – Bastian Hoesel

For 75cm^2 Plates containing 15-20 Mio cells
p65 antibody
Sonicator:
Bandelin Sonopuls

Sonotrode: MS73

DAY 1:

A: Preparation of magnetic beads
Perform all steps in an ice bucket or in the cold room at 4°C.

1. Add **56µl re-suspended** magnetic bead slurry **per IP** to a 1.5 ml microfuge tube on ice containing 1 ml PBS with 5mg/ml BSA. Vortex briefly to mix well.

2. Place the microfuge tubes on the magnet and remove supernatants.

3. Resuspend the beads in 1 ml PBS containing 5mg/ml BSA.

4. Repeat Steps **2 and 3 3 times**.

5. Add 1 ml PBS containing 5mg/ml BSA to beads. Add respective antibodys.

6. Gently mix on a rotator platform for 24h at 4°C

7. Wash beads 3 times PBS containing 5mg/ml BSA (steps b-c), resuspending the beads by inverting the tubes during each wash.

8. Add in **110µl PBS** containing 5mg/ml BSA per resuspendend beads amount **(56µl)**

9. Add **100µl** of resuspendend beads **per IP**

DAY 1:
A: Preparation of cells:

1. Wash cells one time with 10ml PBS.

   First step fix: Add 10 mL 2 mM DSG in RT PBS (M_{DSG} = 326.3 mg/mmol). Dissolve DSG in minimum volume of DMSO before adding to PBS.

   500 DMSO for 50mg DSG
   Final conz.: 0,1mg/µl
   64,7 ul for 10ml PBS

   If PBS is cold, DSG will precipitate again; make sure PBS is at ambient temperature before commencing. This step is for crosslinking Proteins.

2. Incubate **45mins** shaking (RT).

3. Wash one time with 10ml PBS.

4. **Second step fix:** Add 540µl 18,5 % PFA directly to 10 ml PBS (1% final concentration).

5. Incubate **15mins** shaking (RT). *This step is for crosslinking Proteins with DNA.*

6. **Quench PFA**: Add 1410µl of 1M Glycin directly to 10 ml of PBS (125mM final concentration)

   Incubate **5mins** shaking (RT).
7. Wash one time with ice-cold 10ml PBS. Scrape cells in ice-cold 10ml PBS supplemented with 1/200 (50 µl) vol. 100 mM PMSF (17.4 mg/ml) in isopropanol, added immediately before use, to each plate of cells. Transfer to 15ml Falcon.

8. **Transfer** cells to ice; Centrifuge for 5 minutes at 2.800 rpm 4°C; carefully remove supernatant.

9. **PAUSE STEP**: Cells can be stored in -80°C.

10. **Resuspend Pellets** in 10 ml Farnham Lysis Buffer containing Protease Inhibitors. Incubate on a rocker for 15 mins in the cold room.

11. **Centrifuge** for 5 minutes at 2800 rpm 4°C. Discard supernatant (be careful not to disturb the pellet).

12. **Resuspend cell** pellet in **1ml RIPA** containing Protease Inhibitors. Resuspension should be done by carefully pipetting up and down to avoid foaming. Incubate for 10 mins on ice.

13. **Sonication of cells**. Sonicate cells on a 4°C water bath in the cold room. Sonication has to be set up. We use a Bandelin Sonopuls with a Sonotrode MS73. 30 x 30s Pulses at 30 % Power. 1 min Pause between pulses.

14. **Centrifuge** at max speed for 10 minutes at 4°C.

15. **Transfer cleared chromatin** to a new tube be careful not to disturb the pellet.

16. Measure **protein concentration** using Bradford Assay and a standard curve using BSA.

17. **PAUSE STEP**: Chromatin can be stored at -80°C up to one year (storing cells appears better).

18. Take **1mg of sonicated cells** per IP **and fill up to 900ml with RIPA Buffer** containing Protease Inhibitors.

19. Remove Input (4%) (36µl) of **each** reaction and store at 4°C.

20. Add 100µl of beads in PBS from step 9 and incubate at 4°C for 24h shaking (cold room).

DAY 2:

1. **Pellet Protein A magnetic beads** with the magnetic separator and remove the supernatant completely

2. Wash the Protein A bead-antibody/chromatin complex by re-suspending the beads in 1 ml each of the cold buffers in the order listed below and incubating for 10 minutes on a rotating platform followed by magnetic clearance and careful removal of the supernatant fraction:

- 1 ml of **Wash Buffer III 5** times containing Protease Inhibitor - 1 ml of **TE Buffer** 1 time containing Protease Inhibitor	Each wash 10 mins at 4°C **(Shaking in the cold room)**

3. **Elute the specific** chromatin from beads by adding 200µl of Elution Buffer. Fill up INPUT samples to 200µl with Elution Buffer (164µl).

4. **Reverse the cross-linking** for all samples with final concentration of 0.3M NaCl by adding 8µL NaCl; Incubate for O/N with shaking at 65°C.

5. Briefly chill on ice and do a quick spin;

6. Briefly chill on ice do a quick spin and **isolate DNA.**

A. Using Columns (Fermentas)

1. Add 200µl of phenol:chloroform:isoamylalcohol (25:24:1) to samples.
2. Shake tubes gently by hand for 3-4 mins.
3. Centrifuge at fullspeed for 5 mins.
4. Remove aqueous upper layer and transfer to a new tube (~200µl).
5. To IP (for INPUT not necessary) samples add 100µl of water (re-extract organic phase)
6. Shake tubes gently by hand for 3-4 mins.
7. Centrifuge at fullspeed for 5 mins.
8. Pool IP Samples
9. Add 200µl/300µl of binding solution to all samples.
10. Add 200µl/300µl of Isopropanol to all samples (important otherwise no DNA).
11. Apply mixture to column.
12. Centrifuge 60s full speed.
13. Discard flow through.
14. Wash with 700 µl Wash Buffer.
15. Discard flow through.
16. Centrifuge to dry columns for 60s full speed.
17. Discard flow through.
18. Add 100µl of Elution Buffer (opt. prewarm to 55°C) directly to the center of the column; Let stand for 1 minute.
19. Centrifuge for 60s fullspeed.

B. Using Phenol/Chloroform Extraction (work under fumehood!)

1. Add 200µl of phenol:chloroform:isoamylalcohol (25:24:1) to samples.
2. Shake tubes gently by hand for 3-4 mins.
3. Centrifuge at fullspeed for 5 mins.
4. Remove aqueous upper layer and transfer to a new tube (~200µl). Add an equal volume of chloroform.
5. Shake tubes gently by hand for 3-4 mins.
6. Centrifuge at fullspeed for 5 mins.
7. Remove aqueous upper layer (~200µl) and transfer to a new tube.
8. Add 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volumes ice cold ethanol (95%) and mix. For 200µl: 20µl Sodium Acetat, 500µl Ethanol.
9. Precipiate DNA at –20°C for at least 30 minutes up to 2h.
10. Centrifuge at fullspeed for 15 minutes at 4°C. Check tube orientation in the centrifuge.
11. Being careful not to disturbe the pellet, remove the supernatant. Pellet might not be visible.
12. Carefully rinse the pellet once with 500µl with ice-cold 70% ethanol.
13. Centrifuge at fullspeed for 5 minutes at 4°C

14. Being careful not to disturbe the pellet, remove the supernatant. Open the lid of the tube and dry the pellet for ~5 minutes. You can carefully remove remaining liquid with a yellow tip.
15. Resuspend the pellet in 50ul H_2O.

BUFFER´S USED (For ChIP Only! Sterile Filter before Use!)

Hypotonic Lysis Buffer	**Stock**	**50ml**
KCl 10 mM	3 M	167µl
Tris-HCl 20 mM pH 8	1 M	1ml
Glycerol 10%	100 %	5ml
DTT 2 mM	1M	2µl/ml
with additional Protease inhibitor cocktail (25x Roche)		40µl/ml

Farnham Lysis Buffer (Alternative)	**Stock**	**50ml**
5 mM PIPES pH 8.0	1M	250µl
85 mM KCl	3 M	1,42ml
1% NP40	100%	500µl

SDS Lysis Buffer	**Stock**	**50ml**
Tris 50mM ph 8	1 M	2,5ml
EDTA 10mM pH 8	0,5 M	1ml
SDS 1%	10 %	5ml
DTT 1 mM	1M	1µl/ml
with additional Protease inhibitor cocktail (25x Roche)		40µl/ml

RIPA Buffer		**50ml**
50 mM Tris-HCl pH 8.0	1 M	2,5ml
150 mM NaCl	5 M	1,5ml
1% NP-40	10%	500µl
0.5% Sodium Deoxycholate 250mg		
0.1% SDS	10%	500µl
DTT 1 mM	1M	1 µl/ml
with additional Protease inhibitor cocktail (25x Roche)		40 µl/ml

Chip Dilution Buffer	**Stock**	**50ml**
Tris-HCL 16,7 mM ph 8	1 M	835µl
NaCl 167 mM	5 M	1,67ml
EDTA 1,2 mM pH 8	0,5 M	120µl
Triton X-100 1,1 %	10 %	5,5ml
SDS 0,01 %	10 %	50µl
DTT 1 mM	1M	1 µl/ml
with additional Protease inhibitor cocktail (25x Roche)		40 µl/ml

Wash Buffer I (low salt):		**Stock**		**50ml**
EDTA 2 mM	0.5 M		200µl	
Tris-HCl 20 mM pH 8.0	1 M		1ml	
SDS 0.1%	10%		500µl	
Triton X-100 1%		10%		5ml
NaCl 150 mM	5 M		1,5ml	
add PMSF right before use			5µl/ml	

Wash Buffer II (high salt):	**Stock**		**50ml**	
EDTA 2 mM	0.5 M		200µl	
Tris-HCl 20 mM pH 8.0	1 M		1ml	
SDS 0.1 %	10%		500µl	
Triton X-100 1%		10%		5ml
NaCl 500 mM	5M		5ml	
add PMSF right before use			5µl/ml	

Washing Buffer III (LICL):	**Stock**			**50ml**
Tris-HCl 10 mM pH 8.0	1 M			500µl
EDTA 1 mM	0.5 M			100µl
LiCl 250 mM	1 M		12,5ml	
Deoxycholate 1 %			0,5g	
NP-40 1 %			500µl	
add PMSF right before use			5µl/ml	

TE Buffer:	**Stock**		**50ml**	
EDTA 1 mM	0.5 M			100µl
Tris-HCl 10 mM pH=8.0	1 M		500µl	

ddH2O

Elution Buffer

100mM NaHCO3
1% SDS

PBD = PBS + DSG

M_{DSG} = 326.3 mg/mmol
326.3 g/mol
PMSF in isopropanol
10xPBS
1,37M NaCl 80g
27mM KCl 2g
80 mM Na2HPO4 11,4g
20 mM KH2PO4 2,72g

Adjust pH 7.4
Fill up to 1l

BUFFER Stocks

3M KCL (autoclave)
5M NaCL (autoclave)
1M Tris (adjust pH to 8) (autoclave)
0.5 M EDTA (adjust pH to 8) (autoclave)
1M NaHCO3 (Filter)
10% SDS (Filter)
1M LiCl (autoclave)
10% Deoxycholate (Filter)
10% NP40 (Filter)
10% Triton X-100 (Filter)

IgG, from Rabbit Serum I5006 - 10mg
Reconstitute in 10ml 150mM NaCl freeze in aliquots at -20°C

18,5 % PFA (make fresh)
0,92 g PFA
4,8 ml PBS
3 drops 5 M NaOH
dissolve at 70°C adjust pH with
add to 5 ml with PBS

.

Western Blots

Monoklonaler Anti-c-myc von Boehringer (#1667149)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,1% Tween 20: 30 min schütteln lassen
2. Waschen: 2x 2 min PBS def./0,1% Tween 20
3. Inkubation mit 1. Antikörper: monocl. anti-c-myc 1:200 in PBS def./0,1% Tween 20
2 Stunden bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
5. Inkubation mit 2. Antikörper: anti-mouse-Ig-HRP 1:5000 (Amersham NA 931,
Batch 133232) in PBS def./0,1% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit PBS def./0,1% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im
Verhältnis 1:1 gemischt: 5 min inkubieren
8. Exponieren auf Film, bzw. Detektion am LumiImager

Polyklonaler Anti-c-myc Tag 409-420(cat#06-549/Lot#14966)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,1% Tween 20: 30 min schütteln lassen
2. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
3. Inkubation mit 1. Antikörper: polykl. anti-c-myc 1:1000 in 1x PBS def./0,1% Tween
20, 1 Stunde bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
5. Inkubation mit 2. Antikörper: anti-rabbit-Ig-HRP 1:5000 (Amersham NA 934,
Batch 103) in 1x PBS def./0,1% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im
Verhältnis 1:1 gemischt: 5 min inkubieren
8. Exponieren auf Film, bzw. Detektion am LumiImager

Anti-X-Press (#46-0528/Lot#800969)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,1% Tween 20: 30 min schütteln lassen Johannes A. Schmid
2. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
3. Inkubation mit 1. Antikörper: anti-X-Press 1:5000 in 0,1% BSA /1x PBS def./0,1%
Tween 20, 1 Stunde bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
5. Inkubation mit 2. Antikörper: anti-mouse-Ig-HRP 1:5000 (Amersham NA 931,
Batch 133232) in 1x PBS def./0,1% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2
Blot , 2 Lösungen im
Verhältnis 1:1 gemischt: 5 min inkubieren, Exponieren auf Film, bzw. Detektion am LumiImager

Monoklonaler Anti-P-Tag (H902-ascites)

1. Blocken mit 2% Magermilch / 1x PBS def.-0,05% Tween 20: 30 min schütteln lassen
2. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,05% Tween 20
3. Inkubation mit 1. Antikörper: monocl. anti-P-Tag 1:2000 in 0,2% Trockenmilch/1x
PBS def./0,05% Tween 20
über Nacht bei 4 ° C inkubieren (1:1000 verdünnen, wenn man 1 Std. bei RT
inkubiert)
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,05% Tween 20
5. Inkubation mit 2. Antikörper: anti-mouse-Ig-HRP 1:5000 (Amersham NA 931,
Batch 133232) in 1x PBS def./0,05% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,05% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2Blot , 2 Lösungen im
Verhältnis 1:1 gemischt: 5 min inkubieren
8. Exponieren auf Film, bzw. Detektion am LumiImager

Polyklonaler Anti-Flag von Santa Cruz (D-8/ cat#sc807/Lot#B057)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,1% Tween 20: 30 min schütteln lassen Johannes A. Schmid
2. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
3. Inkubation mit 1. Antikörper: polykl. anti-Flag 1:1000 in 1x PBS def./0,1% Tween 20
1 Stunde bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
5. Inkubation mit 2. Antikörper: anti-rabbit-Ig-HRP 1:5000 (Amersham NA 934, Batch 103) in 1x PBS def./0,1% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2Blot , 2 Lösungen im Verhältnis 1:1 gemischt: 5 min inkubieren
8. Exponieren auf Film, bzw. Detektion am LumiImager

M1 Monoklonaler Anti-FLAG M1 von Kodak (cat#13001)

1. Blocken mit 5% Magermilch/50 mM Tris(50 ml 1 M Tris bei 1l)+ 200 mM NaCl(40 ml
5 M NaCl)+ 2 mM CaCl 2 (2 ml 2 M CaCl 2) 1 Stunde schütteln lassen
2. Waschen: 3x kurz 50 mM Tris(50 ml 1 M Tris bei 1l)+ 200 mM NaCl(40 ml 5 M NaCl)+ 2 mM CaCl 2 (2 ml 2 M CaCl 2)
3. Inkubation mit 1. Antikörper: monocl. M1 anti-Flag 1:500 in 50 mM Tris(50 ml 1 M Tris bei 1l)+ 200 mM NaCl(40 ml 5 M NaCl)+ 2 mM CaCl 2 (2 ml 2 M CaCl 2) Inkubation 1 Stunde bei R.T.
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 50 mM Tris(50 ml 1 M Tris bei 1l)+ 200 mM NaCl(40 ml 5 M NaCl)+ 2 mM CaCl 2 (2 ml 2 M CaCl 2)
5. Inkubation mit 2. Antikörper: anti-mouse-Ig-HRP 1:5000 (Amersham NA 931, Batch 133232) in 50 mM Tris(50 ml 1 M Tris bei 1l)+ 200 mM NaCl(40 ml 5 M NaCl)+ 2 mM CaCl 2 (2 ml 2 M CaCl 2) 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 50 mM Tris(50 ml 1 M Tris bei 1l)+ 200 mM NaCl(40 ml 5 M NaCl)+ 2 mM CaCl 2 (2 ml 2 M CaCl 2) Johannes A. Schmid
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im Verhältnis 1:1 gemischt: 5 min inkubieren, Exponieren auf Film, bzw. Detektion am LumiImager

Polyklonaler Anti-IKB- αααα (C-21/cat#sc-371/Lot#A058)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,1% Tween 20: 30 min schütteln lassen
2. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
3. Inkubation mit 1. Antikörper: polykl. anti-IKB- α 1:500 in 1x PBS def./0,1% Tween 20, 1 Stunde bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
5. Inkubation mit 2. Antikörper: anti-rabbit-Ig-HRP 1:5000 (Amersham NA 934, Batch 103) in 1x PBS def./0,1% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im Verhältnis 1:1 gemischt: 5 min inkubieren
8. Exponieren auf Film, bzw. Detektion am LumiImager

Polyklonaler Anti-Traf-1 (H-186/cat#sc-7186/Lot#Do18)

Polyklonaler Anti-Traf-2 (H-249/cat#sc-7187/Lot#Do88)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,05% Tween 20: 30 min schütteln lassen
2. Inkubation mit 1. Antikörper: polykl.cl. anti-Traf 1/2 1:500 in 5% Magermilch/1x PBS def./0,05% Tween 20, 1 Stunde bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
3. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,05% Tween 20
4. Inkubation mit 2. Antikörper: anti-rabbit-Ig-HRP 1:5000 (Amersham NA 934,

Batch 103) in 1x PBS def./0,05% Tween 20: 1 Stunde bei R.T.
5. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,05% Tween 20
6. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im Verhältnis 1:1 gemischt: 5 min inkubieren Johannes A. Schmid

Anti-HA-HRP-konjugiert (Böehringer cat#1667475)

1. Blocken mit 1% BSA / 50 mM Tris/ 200 mM NaCl/ 0,1% Tween 20: 1 Stunde schütteln
2. Inkubation mit 1. Antikörper: anti-HA-HRP 1:1000 in 1% BSA / 50 mM Tris/ 200 mM NaCl/ 0,1% Tween 20, 2 Stunden bei Raumtemperatur oder über Nacht bei 4 ° C inkub.
3. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 50 mM Tris/ 200 mM NaCl/ 0,1% Tween 20
4. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im Verhältnis 1:1 gemischt: 5 min inkubieren
5. Exponieren auf Film, bzw. Detektion am LumiImager

Anti-myc-HRP conjugated (Invitrogen, #46-0709)

1. Block: 5% dry milk in TBS
2. Wash 2x 5 min with TBS/0.05% Tween-20 (TBS/T)
3. Antibody: anti-myc-HRP (1:2000 in blocking buffer): 2 h at r.t. or over night at 4°C
4. Wash 2x 5 min with TBS/T
5. Wash once for 5 min with TBS
6. Detection with Pierce SuperSignal or equivalent

Anti-TAK1 (Santa Cruz, rabbit polyclonal, #sc-7162, Lot: J227)

1. Block: 30 min with 5% dry milk in TBS (or PBS)
2. Wash briefly with TBS
3. 1st antibody: anti-TAK1 (1:500 or 1:1000 in TBS): 1 h at r.t. or over night at 4°C
4. Wash 3 x 5 min with TBS/0.05% Tween-20
5. 2nd antibody: anti-rabbit-Ig-HRP 1:5000 in TBS/T (Amersham NA 934): 1 h at r.t.
6. Wash 3 x 5 min with TBS/0.05% Tween-20
7. Detection with Pierce SuperSignal or equivalent

Anti-FLAG (M2): Monoclonal mouse ab, Sigma F3165, Lot: 68H9255

1. Block with 5% dry milk in TBS (1 h at 37°C)
2. Wash 2x 2 min with TBS
3. 1st antibody: anti-flag M2 (1:1000 in TBS or in blocking buffer): 30 min – 1 h at r.t.
4. Wash 3x 2 min with TBS
5. 2nd antibody: anti-mouse-Ig-HRP (1:5000 in TBS or blocking b., Amersham NA931) 30 min – 1 h at r.t.
6. Wash 3x 15 min with TBS
7. Detection with Pierce SuperSignal …

Polyklonaler Anti-GFP (Clontech.cat#8363-1)

1. Blocken mit 5% Magermilch / 1x PBS def.-0,1% Tween 20: 30 min schütteln lassen
2. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
3. Inkubation mit 1. Antikörper: polykl. anti-GFP 1:1000 in 1x PBS def./0,1% Tween-20 , 1 Stunde bei Raumtemperatur oder über Nacht bei 4 ° C inkubieren
4. Waschen: 2x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
5. Inkubation mit 2. Antikörper: anti-rabbit-Ig-HRP 1:5000 (Amersham NA 934, Batch 103) in 1x PBS def./0,1% Tween 20: 1 Stunde bei R.T.
6. Waschen: 3x kurz, 1x 15 min und 2x 5 min mit 1x PBS def./0,1% Tween 20
7. Detektion mit dem Pierce SuperSignal Reagenz: 125 µl/cm2 Blot , 2 Lösungen im Verhältnis 1:1 gemischt: 5 min inkubieren

Exponieren auf Film, bzw. Detektion am LumiImager

Anti-Phospho-p38

1. Block: 3% milk in PBS/0.1%Tween 20 (PBS/T): 30 min
2. 1st antibody (New England Biolab, Diana Mechtcheriakova) 1:1000 in Block: 1 h at RT
3. Wash: 3x 5 min in PBS/T
4. 2nd antibody: anti-rabbit Ig-HRP 1: 2500 (Amersham): 1 h at r.t.
5. Wash: 3x 5 min in PBS/T
6. Detection with Pierce SuperSignal or equivalent

Microscopy

Confocal laser scanning microscopy

Confocal microscopy differs from conventional light microscopy in the light source, the detection, generation and resolution of acquired images. In a conventional fluorescence microscope the whole specimen is illuminated by a certain excitation wavelength, likewise emitted light is gathered from all planes of the specimen. Visible light cannot be focused in a single plane, therefore not only emitted light from the focal plane but below and above the specimen are detected. This additional light detected results in a blurry image of decreased contrast and resolution, especially for thick specimens. In contrast to conventional light microscopy, in confocal microscopy a defined spot in the focal plane of the specimen is illuminated at a certain time point. Laser light sources of defined excitation wavelengths are used. The laser light is focused by passing through a very small aperture, such as a pinhole or a slit. Furthermore, emitted light from below or above the focal plane is eliminated by preventing passing a second pinhole (see figure 3.6, orange lines). By changing the z-axis distance between specimen and objective lense, the focal plane can be adjusted and so called optical slices of the specimen in X-Y plane can be generated. This technique is a non-invasive approach to investigate both fixed and living cells. Emitted light of an illuminated spot in the focal plane is detected by photomultiplier tubes (PMT), which enhance signal. Series of illuminated PMT outputs are processed to an image.

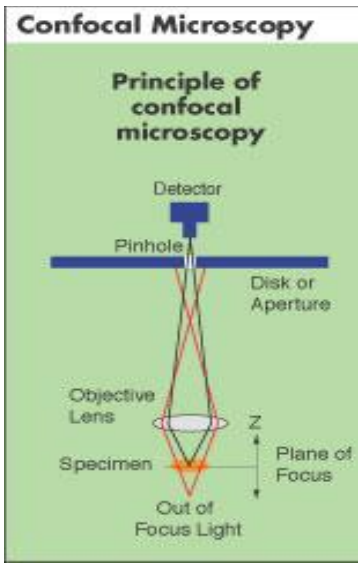


Figure 3. 2: raypath in confocal microscopy

Fluorescence Microscopy of Fixed Cells

Solutions:

- 4 % (w/v) paraformaldehyde pH 7.4 in PBS
- mounting medium: glycerol in PBS (1:6)
- round glass cover slips (∅ 15 mm) , sterile, stored in 70 % ethanol

293 or HeLa cells are passaged and seeded at a density of 500.000 cells/6-well (40 % confluence), each 6-well contains a round sterile glass cover slip. 1 day after lipofectamine or $CaCl_2$ transfection of 293 or HeLa cells with fluorescent fusion proteins, the culture medium is removed, the cells are washed once with PBS def. and about 2 mL 4 % paraformaldehyde solution per 6-well are carefully pipetted to the cells. After about 10 minutes incubation at room temperature the paraformaldehyde solution is removed. The fixed cells are washed with PBS and covered with mounting medium. Then the coverslip is carefully taken out of the 6-well and placed on a glass slide with the cells facing the slide, trying to avoid bubbles. Excess of mounting medium is removed and clear nailpolish is applied

on the edges of the coverslip to seal the fixed sample to prevent drying. Fluorescent microscopy is then performed with the sample.

Live Cell Fluorescence Microscopy

- greased aluminum slide

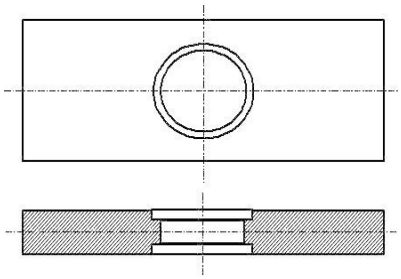


Figure 3. 3: aluminum slide used for live microscopy

293 or HeLa cells are seeded in 6-well plates containing round sterile coverslips (diameter: 15 mm), the next day lipofectamine or $CaCl_2$ transfection is carried out. 1 day after transfection the round coverslips are carefully taken out of the well and pressed on the hole of an aluminum slide (figure 1.7). The edges of the aluminum slides are greased to seal the cover slips. About 70 µL of DMEM complete medium is pipetted on the cells on the coverslip. With a second blank coverslip pressed on the other side of the aluminum slide a chamber filled with medium is created. The aluminum slide is placed on the object table with the cells grown on the coverslip on the bottom.

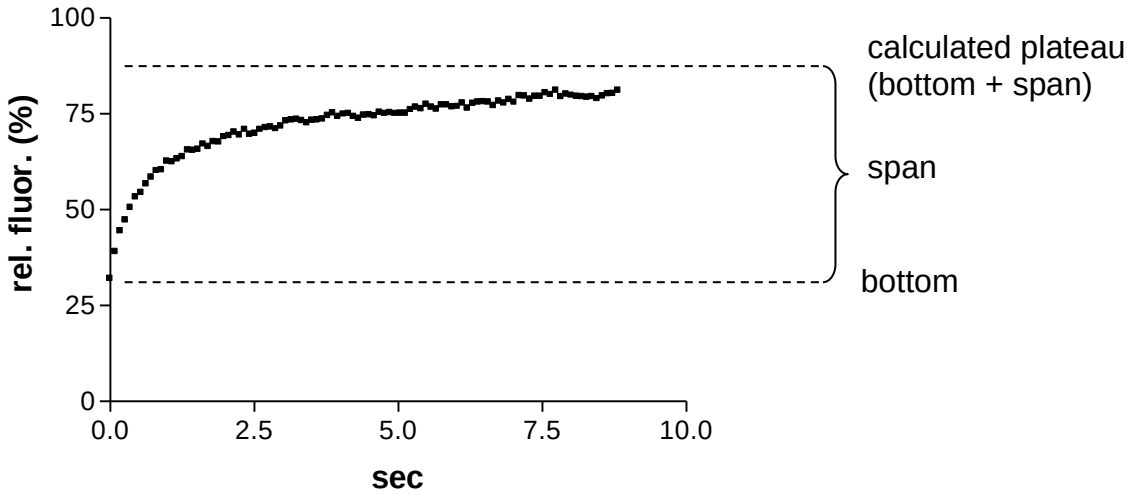
Fluorescence Recovery after Photobleaching (FRAP) on Zeiss LSM510

1. Capture an image of the whole cell before bleaching
2. Define a bleaching / scan region (and maybe in addition another scan region that is not bleached)
3. Perform a time series with 1 scan prebleach, about 70 iterations of bleaching with 100% laser power and then 50-100 scans of the bleach region (and also the non-bleached control region if you specified one)- a good time resolution can only be obtained if just the small bleach region (and maybe the control region) is scanned - and not the whole cell; averaging of 2 or 4 scans reduces the electronic noise and leads to better quantifications.
4. Capture an image of the whole cell after the FRAP time series (with the same conditions as the prebleach image – for calculating the total loss of fluorescence.
5. Using LSM Image Browser (Freeware from Zeiss) export the images and the time series in TIF format (in a new folder for each time series) and record the seconds of the single images (seconds: see values in the slices-view: type first 2 of the series in the Excel sheet and extend the column to the final value)
6. Using NIH-Image (ScionImage for Windows) open the TIF files: measure the mean fluorescence in a control region or for the whole cell for both the prebleach and the postbleach images and calculate the loss of overall fluorescence due to the bleaching in the region of interest.
7. Open all the TIF images of the FRAP time series (using the “open all” command)

8. Measure the bleach/scan-region of all images using the "measure all" command of the Measure Macro (white is zero, black is 255)
   (measure also the mean fluorescence of the control region, if you recorded that)
9. Copy fluorescence raw data from the results window to the corresponding column in the Excel sheet
10. Calculate the difference of mean fluorescence from the background and normalize the fluorescence values to 100% for the initial fluorescence.
11. Divide the percent values by the correction factor calculated from the total loss of fluorescence (e.g. if total fluorescence decreased from 1 to 0.9 then divide the mean fluorescence of the FRAP regions for each time value by 0.9 to compensate for the loss in total fluorescence). A similar compensation can be obtained by normalizing the FRAP fluorescence values to the control scan region that was not bleached. This method also compensates more exactly for the bleaching effect in the course of scanning of the time series (this scanning-dependent bleaching effect is opposed to the recovery of fluorescence in the bleach region due to diffusion of non-bleached molecules in the bleach region). This “dynamic correction” gives a somewhat better estimation of the curve (and the kinetics of the recovery) – but leads in principle to results that are very similar to the curve obtained with the “constant correction factor” (by calculating the total loss in fluorescence based on the intensities of the images that were captured before and after the FRAP-time series)
12. For non-linear regression analysis (curve fit of the data to a single exponential association algorithm): Copy the data to a fitting program (such as GraphPad Prism) and perform the fitting with a “bottom to span” algorithm:

$$y = span \times (1 - e^{-kx}) + bottom$$

Halftime for recovery (diffusion): $t_{1/2} = 0.69 / k$



Fluorescence resonance energy transfer (FRET) microscopy

HeLa, 293 or endothelial cells were seeded on 23 mm round glass coverslips in 6-well plates (4x 10^5 cells per well) and transfected with ECFP-IKK2 and EYFP-IKIP.

One day after transfection, coverslips were mounted on a self-made perfusion chamber and living cells were imaged by fluorescence microscopy using a Nikon Diaphot inverted microscope equipped with a cooled CCD-camera (Kappa, Bad Gleichen, Germany) and filter sets that discriminate between ECFP and EYFP fluorescence (Omega Optical Inc., VT, USA). FRET microscopy was carried out by detecting the increase in donor (ECFP) fluorescence after photodestruction of the acceptor (donor recovery after acceptor photobleaching). For that purpose, cells were imaged with an oil immersion objective and images were captured with the donor filter using a 90% neutral density filter to prevent donor bleaching. This was followed by bleaching of the FRET acceptor (EYFP) with the appropriate filter set in the absence of the neutral density filter using a 100W Mercury lamp for about 45 – 60 sec. Subsequently, the neutral filter was again included into the excitation light path and another image was taken with the ECFP-filter set under the same camera setting as the first one. An increase in the donor-fluorescence intensity was visualized by calculating a ratio image of the ECFP-image before and after acceptor photobleaching using NIH-Image software or the Windows™-equivalent ScionImage (Scion Corporation Inc. Maryland, USA). As alternative to the donor recovery technique, the 3-filter method of FRET microscopy was applied as described (Youvan et al., 1997: Youvan DC, Coleman WJ, Silva CM, et al.: Calibration of fluorescence resonance energy transfer in microscopy using genetically engineered GFP derivatives on nickel chelating beads. Biotechnology et alia 1997, 3:1--18. (= online journal: http://www.et-al.com/); FRET techniques are also reviewed in Schmid & Sitte, 2003: Schmid JA, Sitte HH. Fluorescence resonance energy transfer in the study of cancer pathways.Curr Opin Oncol. 2003 Jan;15(1):55-64.)

Mouse Model Methods

Isolation of Mononuclear Cells from solid tumors

(Julia Pisoni)

Natascha 12.12.09 modified after: T helper 17 cells promote cytotoxic T cell activation in tumor immunity. Martin-Orozco N, Muranski P, Chung Y, Yang XO, Yamazaki T, Lu S, Hwu P, Restifo NP, Overwijk WW, Dong C. Immunity. 2009 Nov 20;31(5):787-98; and (Vremec et al., 2000).

Exp:… …… /……EP……

Need: camera, scissors and pinzette
Petri-dishes, PBS with 2% FCS
Ruler, Nunc-vial, dry-ice

Isolate **draining (inguinal)** and **spleen** per mouse
Take **lungs** in 2ml 10% Formalin, take **blood** for serum, take **prostate/tumor (some for -80°C)**

different FACS analysis due to multi-color staining on Fortessa, spleen - Calibur

Mice: Body weight of the mice in RN
Tumor weight in Lab

mouse weight	+Seminal V	-prostate
93		

1. Measure tumor volume-

prepare tumor from killed mice- kill mice individually take a picture with a ruler!
+/- Seminal vesicles -> all together

2. Cell isolation from tumor

place the tumor in a 6-well plate from the petri-dish containing ice-cold PBS + 2% FCS

Cut the tumor into small pieces (razor blade or small knifes) in a 6 well plate

digest with 1 mg/ml collagenase D for 45 min at 37°C (5 ml / tissue)

number of mice: **1**		**5 ml**
For 10ml:	take 8.55 ml PBS	4,1 ml
	+ 100µl Collagenase (250mg/ml) / (100mg/ml)	50µl / **125µl**
	+ 100µl DNase (100mg/ml) / (20mg/ml)	50µl / **250µl**
	+ 1ml MgCl (for DNase activity 25mM)	500µl
	+ 200µl Serum	100µl
		pre-warm at 37°C

5 min at 37°C with 0.01 EDTA for prevention of DC-T cell aggregates

+ 100µl EDTA (0.5M) per 5ml

put through a 70 µm cell strainer placed in a 6 well dish (back of 1 ml syringe plunger)

Collect the single cell suspension into a 15 ml tube on ice

Wash the cell-strainer with PBS and add to the tube until the solution is clear.

Take small aliquot (250µl) of digested tumor cells for (cell counting) surface staining (CD4 PE, CD8 APC, CD45 FITC, DX-5 PE-Cy7).

Centrifuge for 8-10 min at 1400rpm end: 1 TU samples

Meanwhile **isolate draining LN and spleens with cell strainer**

Prepare the Percoll 30% and 70% in PBS (+Serum) (Sigma P1644-500ML)!

20ml 30% Percoll: 6ml +14ml PBS: number of mice: 5ml x …1.. = …5……..ml

10ml 70% Percoll: 7ml + 3ml PBS: number of mice: 2,5ml x …1.. = …2,5……..ml

- Cells are resuspended in 30% Percoll: 5ml
    - Turn the pipette boy to S (slow)
    - Overlay **very** slowly with the lower (and lighter) 30% Percoll solution containing the cells onto the already provided 70% Percoll 2,5ml
    - Centrifuge (1400rpm, 20min, RT , **NO BRAKE**)
    - Remove the surface – tumor cell debris and fat with 5ml pipette or 1ml Tip
    - Carefully suck off the interphase with mononuclear cells by using the pipette boy and get rid of Percoll contamination by washing
- Transfer into 15ml tubes, wash two times with PBS (+2%FCS)
- Centrifuge (1400rpm, 7min, 4°C)

Split cells: 1/3 for surface and 2/3 for IC (1/3 eBioscience [IC C], 1/3 Biolegend [IC D])

Start with restimulation!! (IC D)

Take 1/3 surface and split again – use 1/3 for Surface B (Calibur) and other 1/3 for Surface A (Fortessa), 1/3 for Surface M (Calibur)

2/3 of cells for IC (1/3 has to be restimulated for 4 hours – proceed on page 5), 1/3 for eBio TF Fortessa staining

Resuspend prostate
in 2,5 ml media (sum 7 tubes)
*** 500µl/tube for IC (Fortessa)**
*** 280µl/tube for others**

Tumor infiltrates isolation modified for colon

(Mario Kuttke)

Materials:

- Icebox
- Biorad Counting chambers
- Eppi for re-genotyping
- Scalpel
- Scissors (sterile)
- Forceps (sterile)
- Petri dish
- 2x PFA for FACS
- MACs beads stuff
- 50ml tubes (4x)
- 15ml tubes (1x)
- 2x Syringe+filter for sterile filtration of DNAse & Collagenase Mix

Reagents:

- Percoll: Sigma P 4937
- 10x PBS, autoclaved
- 1x PBS, autoclaved, 110ml/mouse
- BSA: Sigma A 9418
- 70µm strainer (734-0003 ZELL-SIEBE 70µm WEISS Corning B.V. / 352350)
- RPMI for DNAse and Dissociation solution
- Complete RPMI + (5 mM) EDTA [30ml/mouse]
  → 3ml 0.5M EDTA in 300ml RPMI complete (+15 mg gentamycin + 3 ml PenStrep)
- **Dissociation medium (5ml per tumor):**
  - DNase I (Worthington Biochemicals LS 002139) 5 mg
  - Collagenase P (Roche 11249002001) 12.5mg
  - Collagenase/Dispase (Roche 11097113001) 12.5mg
  - RPMI to 5ml
  - Filter sterilize 0.22µm

- **DNASE SOLUTION (1ml per tumor)**
  - Can be aliquoted and stored at –20°C. Make fresh solution is best!
  - DNase I (Worthington Biochemicals LS 002139) 0.5 mg
  - RPMI to 1 ml
  - filter sterilize on 0.45 micron filter
- **Percoll gradient** (2x if you want to isolate IELs as well)
  - Mix 9 vol Percoll with 1 vol PBS 10x = Percoll 100 4ml (3,6ml Percoll +0,4 ml 10xPBS)
  - Make up Percoll 35 (35% Percoll 100 + 65% PBS) 4ml (1,4ml+2,6ml)
  - Make up Percoll 60 (60% Percoll 100 + 40% PBS) 4ml (2,4ml+1,6ml)
  - Prepare your gradients as follows:
  - Add 4 ml Percoll 35 to a 15 ml tube and underlay with 4ml Percoll 60

Tumor associated macrophage isolation PROCEDURE:

1. Prepare fresh **Digestion medium and DNase Solution** and keep it at room temperature (RT) during the procedure
2. Preheat incubator and EDTA/RPMI to 37°C
3. Collect the colon in a 50 ml tube containing 10 ml PBS and bring it under the tissue culture hood.
4. Aspirate the PBS. Cut the colon longitudinally with scissors and chop into small pieces (into a fine mush) using scissors on the lid of a 50ml tube (or a scalpel on an upturned petri dish). Put pieces from individual colon in 50 ml Falcon tubes and fill up to 10 ml with PBS
5. Invert several times, allow to sediment and discard supernatant (aspirate off)
6. Add Pre-warmed RPMI/EDTA up to 10 ml and incubate 15 minutes @ 37C shaking (shake ~200 rpm), alternatively: incubate in cell incubator and shake every 5 minutes
7. Discard supernatant (SN) (or collect in separate tube if you want to isolate IELs), repeat twice (pool with 1st SNs for IELs> spin down, wash> put on gradient (see below))
8. Add PBS to ~15 ml, invert and incubate for 10 minutes at RT, discard SN (take care to remove everything- residual EDTA inhibits the collagenase!) and add 5 ml of **Digestion medium** in a 50 ml tube.
9. Incubate 20-**60 minutes**, 37^{o}C shaking incubator. During this time, prepare percoll gradiants (60%/35%). 4ml 60% percoll with 4ml 35% percoll overlay in a 15ml tube.
10. Cool centrifuge to 4°C
11. Dissociate the tumor with a 10 ml pipette.
12. Pass the cell suspension through a 70 µm strainer (there will be some chunks remaining in the filter – leave them) in a new 50ml tube. Wash the strainer with 10 ml of PBS.
13. Spin 5 minutes, 300x g, +4^{o}C, **BRAKES ON.**
14. Aspirate the supernatant. Resuspend the cells first by taping the bottom tube with your finger and second by adding 1ml of fresh **DNAse Solution** per tube **(*resuspension is extremely important*)**. Add 4 mls of PBS per tube. SLOWLY lay over the cell suspension on the Percoll gradient using a 5 ml pipette.
15. Spin for 20 minutes, 2000xg, +4^{o}C, **BRAKES OFF**.
16. Harvest the cells.

    a. Aspirate the cells that are located between the 5ml suspension medium and 35% percoll. Usually this cell layer comes easily as one sheet.

    b. With a transfer plastic pipette, harvest the tumor cells / macrophages that settled on the 60% Percoll. Try to remove all the cells, put them in a 50 ml tube, fill up with PBS.

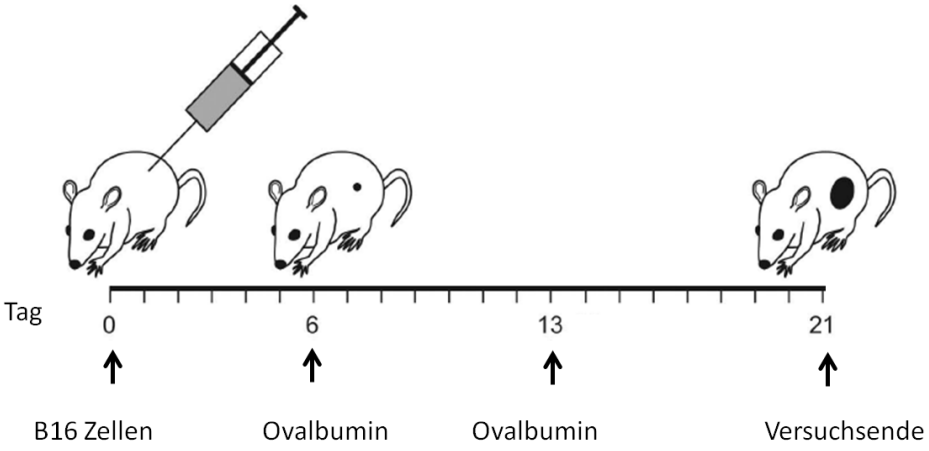
Spin 5 minutes, 300x g, +4^{o}C, **BRAKES ON.**

B16-OVA tumor induction

(Julia Pisoni)

http://www.lgcstandards-atcc.org/products/all/CRL6475.aspx?geo_country=at

B16-OVA Tumormodell zur Ermittlung der Compound-Wirkung auf die Anti-Tumor Immunität: WT-Mäuse werden, unter oraler Verabreichung der Compounds, mit B16 Zellen orthotopisch subcutan in die Flanke inokuliert. Dabei werden die Mäuse mit Isofluran betäubt und $1x10^6$ B16-Zellen werden in 200µl einer 1:2 PBS Matrigel Mischung mit einer Insulin Spritze (23 Gauge Kanüle) injiziert. Nach dem sichtbaren Anwachsen des Tumors (Tag 6) werden die Tiere 2x mit OVA-Antigen vakziniert (Tag 6, Tag 13) und das Wachstum bzw. die Abstossung des Tumors wird entsprechend dokumentiert. Der Versuch wird am Tag 21 beendet bzw. werden Mäuse aus ethischen Gründen vorher euthanasiert wenn der Tumor zu einer schweren Beeinträchtigung führt. Am Versuchsende wird der Tumor für die Histologie aufbereitet sowie die T-Zellen der Tumor Draining Lymphnodes mittels Durchflusszytometrie analysiert.



Exp.: 15.10.2013

➔ **Thaw at least 1- 1,5 week prior to the injection and split at least two times**

1x 10^5 B16-OVA cells injected subcutaneously into de-haired (w) or shaved (m) left flanks of
8-12 week old female/male mice

1 *10^5 in 100 µl

How many mice:
1x: 100.000: ……8…mice… need …$8*10^5$ …… cells in ……800µl
EXTRA: 3x: 1.000.000: ……24…… need …$2,4*10^6$ …… cells in ……2400µl

1. Injection of B16- melanoma cells:

B16 –Ova- Melanoma cells: (modified after Christina 07.01.10)

Growth:

DMEM +++: Medium β-mercaptoethanol. (500ml + 2µl)
Split every second day (except weekend) 1:4 in relation to the area
Use cell culture dishes- not petri dishes- or cell culture flasks to grow in
Cells must be in their exponential growing phase for optimal results
DMEM +

- G418- BC (30.000U/ml) Biochrom: 80µl mikroliter pro 10ml.
- 10% FCS,
- Glutamat
- PenStrep

Harvesting:
Remove DMEM medium
Wash with PBS (2x)
Add 2-3 ml Trypsin (+EDTA Nr……) per flask (4 flasks for 18 mice)
Incubate for 3-5 min at 37°C
Add DMEM+++ medium (serum stops trypsin) and wash 2 times (12000rpm 7min)
(Zellen kleben stark, gut resuspendieren)

Freezing
$5x10^6$ cells/ml
1:10 in DMSO FCS in the Mister cooler -80°C
Defrost fast – put into 37°, wash well with DMEM to get rid of the DMSO and put into a flask.

Injection:
Be careful not smaller than 0,4mm needle (27 gauge)
1x 10^5cells in 100µl PBS mix gently every time you take out an aliquot- no air bubbles!
Use matrigel if tumor is subjected to IHC staining!

I.p or (depending on tumor model)
i.v. inject the mice- that have been below the red light for at least 15min.

measure tumor growth every 2nd day with a caliper

Stainings

Immunohistochemistry Stainings (IHC)

Arginase 1 IHC Staining

- 5-10 min 60°C
- Rehydrate (10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH 6, 1h
- Let it cool off, then wash 3x in 1XPBST
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBST
- 10min Avidin block
- Wash 3x in 1XPBST
- 10min. Biotin block
- Wash 3x in 1XPBST
- Surround tissue with DAKO pen
- 7min. Superblock (2 drops)
- Wash 3x in 1XPBST
- 1h mouseblock (2 drops)
- Wash 3x in 1XPBST
- 1° ab (Arg 1) 1h at RT / on at 4°C (1:500 in PBS 1% goat serum)
- Wash 3x in 1XPBST
- 10min. Biotinylated ab
- Wash 3x in 1XPBS (NO TWEEN)
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS (NO TWEEN)
- AEC (20µl substrate +1000µl buffer)
- stop in tab water
- Wash in aqua dest.
- Hemalaun
- stop in tab water
- mount with Aquatex

sc-20150 Arginase I (H-52) rabbit polyclonal IgG Santa Cruz

FoxP3 / IL-17 double IHC Staining

- 30min-1h 60°C
- Rehydrate (3x10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH 9, 1h
- Let it cool off, then wash 3x in 1XPBS
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBS
- 10min Avidin block
- Wash 3x in 1XPBS
- 10min. Biotin block
- Wash 3x in 1XPBS
- 7min. superblock
- Wash 3x in 1XPBS
- 1h mouseblock
- Wash 3x in 1XPBS
- 1° ab (FoxP3) 1h at RT (1:200 in PBS 1% BSA)
- Wash 3x in 1XPBS
- 10min. Biotinylated ab
- Wash 3x in 1XPBS
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS
- AEC
- Wash in aqua dest., then 1XPBS
- Block 1h in 5% goat serum in PBS
- 2° ab (IL-17) 1h at RT or overnight (1:200 in PBS 1% BSA)
- Wash 3x in 1XPBS
- 30min. anti-rabbit biotinylated ab 1:100
- Wash 3x in 1XPBS
- 10.min Streptav.-HRP (ID labs)
- Wash 3x in 1XPBS
- AEC(20µl substrate + 1000µl Buffer)/ histogreen develop, stop in tap water
- Wash in Aqua dest.
- Hemalaun 1:5, 30s, stop in tap water
- Aquatex

Foxp3(F-9)

sc-166212 mouse mono

IL-17 Abcam ab91649

All washing steps in PBS-T (0.1%) until biotinyl. Ab, then only PBS!

Rabbit polyclonal: **anti-rabbit biotinylated ab** 1:100

DAB/Metal Concentrate

Thermo scientific # 34065 (=new cat. Number)

1:10

Histogreen

Linaris #E109

CD3 IHC Staining

- 5-10 min 60°C
- Rehydrate (10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH 6, 1h
- Let it cool off, then wash 3x in 1XPBST
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBST
- 10min Avidin block
- Wash 3x in 1XPBST
- 10min. Biotin block
- Wash 3x in 1XPBST
- Surround tissue with DAKO pen
- 7min. Superblock
- Wash 3x in 1XPBST
- 1h mouseblock
- Wash 3x in 1XPBST
- 1° ab (CD3) 1h at RT / o n at 4°C (1:150-300 in PBS 1% goat serum)
- Wash 3x in 1XPBST
- 10min. Biotinylated ab
- Wash 3x in 1XPBS (NO TWEEN)
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS (NO TWEEN)
- AEC (20µl substrate +1000µl buffer)
- stop in tab water
- Wash in aqua dest.
- Hemalaun
- stop in tab water
- mount with Aquatex

anti-CD3 (Early T Cell Marker), Clone: SP7, Thermo Scientific

CD8a IHC Staining

- 30min-1h 60°C
- Rehydrate (3x10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH9, 1h
- Let it cool off, then wash 3x in 1XPBST
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBST
- 10min Avidin block
- Wash 3x in 1XPBST
- 10min. Biotin block
- Wash 3x in 1XPBST
- 7min. superblock
- Wash 3x in 1XPBST
- 1h mouseblock
- Wash 3x in 1XPBST
- 1° ab 1h at RT (1:100 in PBS 1% BSA)
- Wash 3x in 1XPBST
- 10min. Biotinylated ab
- Wash 3x in 1XPBS
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS
- AEC (20µl substrate + 1000µl Buffer), develop, stop in tap water
- Wash in aqua dest.
- Hemalaun 1:5, 30s, stop in tap water
- Aquatex

All washing steps in PBS-T (0.1%) until biotinyl. Ab, then only PBS!

Rat-anti-mouse CD8a (53-6.7)
Biolegend cat# 100701
Stored at 4°C

CD163 IHC Staining

- 30min-1h 60°C
- Rehydrate (3x10min Xylen, 30s. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH9, 1h
- Let it cool off, then wash 3x in 1XPBS
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBS
- 10min Avidin block
- Wash 3x in 1XPBS
- 10min. Biotin block
- Wash 3x in 1XPBS
- 7min. superblock
- Wash 3x in 1XPBS
- 1h mouseblock
- Wash 3x in 1XPBS
- 1° ab 1h at RT (1:200 in PBS 1% BSA)
- Wash 3x
- 10min. Biotinylated ab
- Wash
- 10.min Streptav.-HRP
- Wash
- AEC (20µl substrate + 1000µl Buffer), develop, stop in tap water
- Wash in aqua dest.
- Hemalaun 1:5, 30s, stop in tap water
- Aquatex

CD163 sc33560 (M-93)
Rabbit polyclonal IgG

All washing steps in PBS-T (0.1%) until biotinyl. Ab, then only PBS!

ERG IHC Staining

- 5-10 min 60°C
- Rehydrate (10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH 9, 1h
- Let it cool off, then wash 3x in 1XPBST
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBST
- 10min Avidin block
- Wash 3x in 1XPBST
- 10min. Biotin block
- Wash 3x in 1XPBST
- Surround tissue with DAKO pen
- 7min. Superblock
- Wash 3x in 1XPBST
- 1h mouseblock
- Wash 3x in 1XPBST
- 1° ab (ERG EP111) 1h at RT / on (1:50eigentlich) in DAKO antibody diluent)
- Wash 3x in 1XPBST
- 10min. Biotinylated ab
- Wash 3x in 1XPBS (NO TWEEN)
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS (NO TWEEN)
- AEC (20µl substrate +1000µl buffer)
- stop in tab water
- Wash in aqua dest.
- Hemalaun
- stop in tab water
- mount with Aquatex

F4/80 IHC Staining

- 30min-1h 60°C
- Rehydrate (3x10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), Aqua dest.
- Antigenretrieval microwave pH6 (3min. 800 W, 15 min 290 W)
- Let it cool off, then wash 3x in 1XPBS
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBS
- 10min Avidin block
- Wash 3x in 1XPBS
- 10min. Biotin block
- Wash 3x in 1XPBS
- 7min. superblock
- Wash 3x in 1XPBS
- 1h mouseblock
- Wash 3x in 1XPBS
- 1° ab overnight (1:100)
- Wash 3x
- 10min. Biotinylated ab
- Wash
- 10.min Streptav.-HRP
- Wash
- AEC (20µl substrate + 1000µl Buffer), develop, stop in tap water
- Wash in aqua dest.
- Hemalaun 1:5, 30s, stop in tap water
- Aquatex

1° rat-anti-F4/80 1:100 in 1% goat serum; 150-200µl per slide

F480 macrophage monoclonal antibody	AbD Serotec	MCA497G

F4/80 staining (double staining with P-Stat3)

- Bake paraffin embedded slides for 1h at 60°C
- Rehydrate: 3x in Xylen, 10 min. each,; then 30sec. each in 100%, 96%, 80%, 70%, 50% EtOH; 5min in dH_2O
- Antigenretrieval: 60min in steamer pH6 (Dako target retrieval solution, Citrate pH6 (10X) Ref.no. S2369
- Wash 3 times in PBS-T
- Block 10. min in 3% H_2O_2 in PBS
- Wash 3x in PBS-T (if u like u can draw a line with a Dako pen on your slide to mark your tissue area)
- Incubate 7. min with superblock
- Wash 3 times in PBS-T
- Block 1h in 5% goat serum in PBS
- No washing, add 1° ab overnight at 4°C
- Wash 3 times in PBS-T
- 2^{nd} ab 10min, wash 3 times in PBS-T
- Streptavidin-HRP 10min (ID labs)
- Wash with PBS
- Develop with AEC Chromogen (drop Chromogen on slide, check under the microscope until the staining is complete 4-7min), stop in tap water, then in Aqua dest.
- If no further ab staining: Hemalaun staining (1:5), coat with Aquatex

1° rat-anti-F4/80 1:100 in 1% goat serum; 150-200µl per slide
F480 macrophage monoclonal antibody AbD Serotec MCA497G

2° anti polyvalent biotinylated antibody (kit from ID labs contains also super block, mouse block) IDST1007 ?
The P-Stat3 staining done by Michi in AKH, with AP, not with HRP system!
P-Stat3 (Y705) Rabbit mAb (D3A7) Cell Signaling #9145L

PYStat3 staining

- Bake paraffin embedded slides for 1h at 60°C
- Rehydrate: 3x in Xylen, 10 min. each,; then 30sec. each in 100%, 96%, 80%, 70%, 50% EtOH; 5min in dH_2O
- Antigenretrieval: 60min in steamer pH9 (Dako target retrieval solution, Tris/EDTA pH9 (10X)
- Wash 3 times in PBS-T
- Block 10. min in 3% H_2O_2 in PBS
- Wash 3x in PBS-T (if u like u can draw a line with a Dako pen on your slide to mark your tissue area)
- Incubate 7. min with superblock
- Wash 3 times in PBS-T
- Block 1h in 5% goat serum in PBS
- No washing, add 1° ab overnight at 4°C
- Wash 3 times in PBS-T
- 2^{nd} ab 10min, wash 3 times in PBS-T
- Streptavidin-HRP 10min (ID labs)
- Wash with PBS

- Develop with AEC Chromogen (drop Chromogen on slide, check under the microscope until the staining is complete 4-7min), stop in tap water, then in Aqua dest.
- If no further ab staining: Hemalaun staining (1:5), coat with Aquatex

2° anti polyvalent biotinylated antibody (kit from ID labs contains also super block, mouse block) IDSTM003

P-Stat3 (Y705) Rabbit mAb (D3A7) Cell Signaling #9145

F4/80+P-Stat3 for IF

- Bake paraffin embedded slides for 1h at 60°C
- Rehydrate: 3x in Xylen, 10 min. each,; then 30sec. each in 100%, 96%, 80%, 70%, 50% EtOH; 5min in dH_2O
- Antigenretrieval: 60min in steamer pH6 (Dako target retrieval solution, Citrate pH6 (10X) Ref.no. S2369
- Wash 3 times in PBS-T
- Block 10. min in 3% H_2O_2 in PBS
- Wash 3x in PBS-T (if u like u can draw a line with a Dako pen on your slide to mark your tissue area)
- Incubate 7. min with superblock
- Wash 3 times in PBS-T
- Block 1h in 5% goat serum in PBS
- No washing, add 1° antibody mix overnight at 4°C
- Wash 3 times in PBS-T
- 2^{nd} antibody mix 30min, wash 3 times in PBS-T
- Wash 3 times in PBS-T
- DAPI (1:50.000) 5min.
- Wash 3 times in PBS-T
- Dako mounting media

1° Ab: 1° rat-anti-F4/80 1:100 in 1% goat serum; 150-200µl per slide
F480 macrophage monoclonal antibody AbD Serotec MCA497G

P-Stat3 (Y705) Rabbit mAb (D3A7) Cell Signaling #9145L

2° Ab: goat anti rat PE (invitrogen A10522) 1:200
goat anti rabbit FITC (invitrogen A11008) 1:200

Total Stat3 H190 (Santa Cruz): 1:80

F4/80+Total Stat3 for IF

- Bake paraffin embedded slides for 1h at 60°C
- Rehydrate: 3x in Xylen, 10 min. each,; then 30sec. each in 100%, 96%, 80%, 70%, 50% EtOH; 5min in dH_2O
- Antigenretrieval: 60min in steamer pH6 (Dako target retrieval solution, Citrate pH6 (10X) Ref.no. S2369
- Wash 3 times in PBS-T
- Block 10. min in 3% H_2O_2 in PBS
- Wash 3x in PBS-T (if u like u can draw a line with a Dako pen on your slide to mark your tissue area)
- Incubate 7. min with superblock
- Wash 3 times in PBS-T

- Block 1h in 5% goat serum in PBS
- No washing, add 1° antibody mix overnight at 4°C
- Wash 3 times in PBS-T
- 2nd antibody mix 30min, wash 3 times in PBS-T
- Wash 3 times in PBS-T
- DAPI (1:50.000) 5min.
- Wash 3 times in PBS-T
- Dako mounting media

1° Ab: 1° rat-anti-F4/80 1:100 in 1% goat serum; 150-200µl per slide
F480 macrophage monoclonal antibody AbD Serotec MCA497G
Total Stat3 H190 (Santa Cruz): 1:80 cat.# sc-7179 1:80

2° Ab: goat anti rat PE (invitrogen A10522) 1:200
goat anti rabbit FITC (invitrogen A11008) 1:200

Total Stat3

- Bake paraffin embedded slides for 1h at 60°C
- Rehydrate: 3x in Xylen, 10 min. each,; then 30sec. each in 100%, 96%, 80%, 70%, 50% EtOH; 5min in dH_2O
- Antigen retrieval: 60min in steamer pH6 (Dako target retrieval solution, Citrate pH6 (10X) Ref.no. S2369
- Wash 3 times in PBS
- Block 10. min in 3% H_2O_2 in PBS
- Wash 3x in PBS (draw a line with a Dako pen on your slide to mark your tissue area)
- Incubate 7. min with superblock
- Wash 3 times in PBS
- Block 1h in mouseblock (ID lab)
- Wash 3x, PBS
- add 1° antibody total Stat3 1:80 in PBS+ 1% BSA overnight at 4°C
- Wash 3 times in PBS
- Biotinylated secondary ab 10min (ID labs)
- Wash PBS 3x
- HRP (ID labs) 10min
- Wash 3x PBS
- Develop with AEC 1ml buffer + 20 microL substrate (stop in tap water, then in Aqua dest.)
- Hemalaun 1:5 (in Aqua dest.), 15-20 sec.

F4/80+Total Stat3

- Bake paraffin embedded slides for 1h at 60°C
- Rehydrate: 3x in Xylen, 10 min. each,; then 30sec. each in 100%, 96%, 80%, 70%, 50% EtOH; 5min in dH_2O
- Antigen retrieval: 60min in steamer pH6 (Dako target retrieval solution, Citrate pH6 (10X) Ref.no. S2369
- Wash 3 times in PBS
- Block 10. min in 3% H_2O_2 in PBS
- Wash 3x in PBS (draw a line with a Dako pen on your slide to mark your tissue area)
- Incubate 7. min with superblock
- Wash 3 times in PBS
- Block 1h in mouseblock (ID lab)
- Wash 3x, PBS
- add 1° antibody F4/80 1:100 in PBS+ 1% BSA overnight at 4°C
- Wash 3 times in PBS

- Biotinylated secondary ab 10min (ID labs)
- Wash PBS 3x
- HRP (ID labs) 10min
- Wash 3x PBS
- Develop with AEC 1ml buffer + 20 microL substrate (stop in tap water), then in Aqua dest.
- Block with 5% goat serum, RT
- Do not wash, add. Total Stat3 ab (1:200 in PBS 1% BSA), 4°C ON
- Wash 3x PBS
- Biotinylated anti rabbit (vectastain) 30min (kit)
- Wash
- HRP (vectastain) 30min
- Wash
- DAB (Thermosc.) (Buffer 4°C, substrate -20°C 1:10)
- Stop in tap water
- Hemalaun 1:5 Merck in aqua dest. (Mayer), 15-20 sec (filter!).
- Rinse in tap water
- In aqua dest
- Aquatex mounting media

F4/80 & Total Stat3

- 1h 60°C
- Rehydrate (3x10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), Aqua dest. 5min.
- Antigenretrieval autoclave 20min. pH6
- Let it cool off, then wash 3x in 1XPBS
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBS
- 7min. superblock
- Wash 3x in 1XPBS
- 1h 5%goat serum in PBS
- 1° ab mix overnight 4°C
- Wash 3 times in PBS-T
- 2nd antibody mix 30min, wash 3 times in PBS-T
- Wash 3 times in PBS-T
- DAPI (1:50.000) 5min.
- Wash 3 times in PBS-T
- Dako mounting media

1° Ab: 1° rat-anti-F4/80 1:100 in 1% goat serum; 150-200µl per slide
F480 macrophage monoclonal antibody AbD Serotec MCA497G
Total Stat3 H190 (Santa Cruz): 1:80 cat.# sc-7179 1:80

2° Ab: goat anti rat PE (invitrogen A10522) 1:200 this time 1:300

goat anti rabbit FITC (invitrogen A11008) 1:200

Gr1 IHC Staining

- 30min-1h 60°C
- Rehydrate (3x10min Xylen, 30sec. 100%, 96%, 80%, 70%, 50%), A. dest.
- Antigenretrieval steamer pH9, 1h
- Let it cool off, then wash 3x in 1XPBS
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBS
- 10min Avidin block
- Wash 3x in 1XPBS
- 10min. Biotin block
- Wash 3x in 1XPBS
- 7min. superblock
- Wash 3x in 1XPBS
- 1h mouseblock
- Wash 3x in 1XPBS
- 1° ab 1h at RT (1:200 in PBS 1% BSA)
- Wash 3x in 1XPBS
- 10min. Biotinylated ab
- Wash 3x in 1XPBS
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS
- AEC (20µl substrate + 1000µl Buffer), develop, stop in tap water
- Wash in aqua dest.
- Hemalaun 1:5, 30s, stop in tap water
- Aquatex

Gr1 Rat anti mouse Ly6B.2

Serotec MCA771GA 1:200

iNOS mouse IgG

BD #610431 1:200

PTEN IHC Staining

- 5-10 min 60°C
- Rehydrate (5 min Xylen, 2 min. 96%, 96%, 1min 80%, 70% A. dest.
- Antigen retrieval (steamer pH 9, 1h)
- Let it cool off, then wash 3x in 1XPBST (0,1% TWEEN)
- Block 10min in 3%H_2O_2 (in PBS)
- Wash 3x in 1XPBST
- 10min Avidin block
- Wash 3x in 1XPBST
- 10min. Biotin block
- Wash 3x in 1XPBST
- Surround tissue with DAKO pen
- 7min. Superblock
- Wash 3x in 1XPBST
- 1° ab (PTEN) 1h at RT / on 4°C (1:200 in PBS 5% goat serum)
- Wash 3x in 1XPBST
- 10min. Biotinylated ab
- Wash 3x in 1XPBS (NO TWEEN)
- 10.min Streptav.-HRP
- Wash 3x in 1XPBS (NO TWEEN)
- AEC (20µl substrate +1000µl buffer)
- stop in tab water
- Wash in aqua dest.
- Hemalaun
- stop in tab water
- mount with Aquatex

In Situ Nick Translation (ISNT) to detect apoptotic cells

1. Make cryo sections of human skin (7 µm thick) and mount them on poly-L-lysine coated cover glasses. Let the sections dry for 30 min at room temperature.
2. Fixation in acetone (precooled) for 10 min at 4°C.
3. Wash in PBS for 5 min at r.t.
4. ISNT-reaction: 100 µl/ cover glass; 40 min at r.t.: amount for 1 ml reaction mixture

3 µM FITC-12-dUTP (Boehringer 1373242):	3.75 µl (0.8 mM stock solution)
(or 3 µM Biotin-16-dUTP)	
3 µM dGTP	7.5 µl (0.4 mM stock)
3 µM dATP	7.5 µl (0.4 mM stock)
3 µM dCTP	7.5 µl (0.4 mM stock)
DNA-Polymerase I (Boehringer 642711)	50 units/ml (10 µl of 5 u/µl stock)
(endonuclease-free)	
10x reaction buffer (50 mM Tris/HCl pH	100 µl
10 mM $MgCl_2$	
0.1 mM DTT)	
A. dest nuclease-free	ad 1 ml

5. Washing with PBS: 3 times for 5 min at r.t.
6. Protein block: 30 min at r.t. with 10% FCS in PBS
7. Incubation with peroxidase-conjugated anti-FITC (Boehringer; 1:25 in block solution; 30 min at 37°C)
8. Washing with PBS: 3 x 5 min at r.t.
9. Metal-enhanced DAB-staining (Pierce, 34065): 100 µl/cover glass: Incubation about 5 - 20 min (r.t.)

Mounting and conventional microscopy (maybe after hemalaun counterstaining)

Alternative: (if Biotin-16-dUTP was used):

7. Incubation with FITC- or Texas Red conjugated streptavidin (Amersham)
8. Washing with PBS: 3 or 4 times for 5 min at r.t.
9. Mounting and fluorescence or laser scanning microscopy

Immunofluorescence Stainings

Gem stain

Fixation: 5 min with Methanol (-20°C)

1. 3x 5 min mit TBST wash (50mM Tris-HCl pH7.4, 150 mM NaCl, 0.1%Triton)
2. Block: 1 h at RT with 3% BSA in TBS
3. Incubation with 1. AK: anti-Gem (rabbit polyclonal, sc-371 Santa Cruz)
   1:250 (**maybe better: 1:100)** in TBS/3% BSA, over night at 4°C (or 1 h at 37°C).
4. 2x 5 min wash with TBST, 1x 5 min with TBS
5. Incubation with Alexa488 (or FITC or similar) anti-goat 1:2000 in TBS/BSA: 1 h at 37°C
6. 3x 5 min wash with TBST, 1x 5 min with TBS
7. Mounting in PBS/Glycerol (1:7) – sealing with nail polish
   (or mounting with Mowiol or Dako Fluorescent Mounting Fluid)

IκB stain

Fixation: 15 min 4% Paraformaldehyd

8. 3x 5 min mit TBST wash (50mM Tris-HCl pH7.4, 150 mM NaCl, 0.1%Triton)
9. Block: 1 h at RT with 3% BSA in TBS
10. Incubation with 1. AK: anti-IκB (rabbit polyclonal, sc-371 Santa Cruz)
    1:300 in TBS/3% BSA, over night at 4°C (or 1 h at 37°C).
11. 2x 5 min wash with TBST, 1x 5 min with TBS
12. Incubation with Alexa488 goat anti-rabbit 1:2000 in TBS/BSA: 1 h at 37°C
13. 3x 5 min wash with TBST, 1x 5 min with TBS
14. Mounting

p65 (RelA)

1. 3x 5 min mit TBST waschen (50mM Tris-HCl pH7.4, 150 mM NaCl, 0.1%Triton)
2. Block: 1 h bei RT mit 3% BSA in TBS
3. Inkubation mit 1. AK: anti-p65 NF-κB (rabbit polyclonal, sc-109, Santa Cruz)
   1:200 in TBS/3% BSA, über Nacht bei 4°C (oder 1 h bei 37°C).
4. 2x 5 min mit TBST waschen, 1x 5 min mit TBS
5. Inkubation Alexa 488 goat anti-rabbit 1:2000 in TBS/BSA: 1 h bei 37°C
6. 3x 5 min mit TBST waschen, 1x 5 min mit TBS
7. Mounting

NIK

1. 3x 5 min mit TBST waschen (50mM Tris-HCl pH7.4, 150 mM NaCl, 0.1%Triton)
2. Block: 1 h bei RT mit 3% BSA in TBS
3. Inkubation mit 1. AK: anti-NIK (rabbit polyclonal, sc-7211 Santa Cruz)
   1:100 in TBS/3% BSA, über Nacht bei 4°C (oder 1 h bei 37°C).
4. 2x 5 min mit TBST waschen, 1x 5 min mit TBS
5. Inkubation Alexa 488 goat anti-rabbit 1:2000 in TBS/BSA: 1 h bei 37°C
6. 3x 5 min mit TBST waschen, 1x 5 min mit TBS
7. Mounting

Flow Cytometry Methods

BrdU Staining Protocol with DNase

Supplies:
Ethanol 100% USP (highest quality)
FACS Staining Buffer (1XPBS w/ 3% calf serum, 0.05% azide--filtered) —Dilute staining antibodies in Buffer
DNAse (Sigma D-5025, Bovine Pancreas)
RNase (Boehringer, 25 mg bovine pancrease)
Anti-BrdU-FITC (Becton Dickinson or Phoenix Flow)
0.15 M NaCl, 1.5 M NaCl
10% Paraformaldehype (kept as stock in -80°C)
Tween
1M MgCl2
FACS Tubes

Protocol:

Cells in 96 well FACS plate

1. Block with 24G-2
2. Surface stain cells as usual
   -Omit fourth channel labeled antibodies on **all** stains; **EtOH destroys APC**
3. Prepare tubes from which to transfer EtOH drop wise (1.2 ml EtOH on ICE)
4. Resuspend cells from 96 well place with 100 µl 0.15M NaCl (cold)
5. Transfer to FACS tubes ON ICE. Add 400 µl 0.15M NaCl to each tube
6. Vortex at 1/3 speed and add EtOH with pasteur pipette at 1 drop per second.
   This is a critical step... do not add EtOH too quickly
7. Incubate on ice for 30 minutes
8. Spin 10 minutes @ **2000 RPM, 4° C**
9. Dump and shake liquid into waste
10. Using repeat pipetter, squirt 1 ml FACS staining buffer into each tube
11. Spin 10 minutes and dump as before (step 8)
12. Add 1 ml 1% paraformaldehyde + 0.05% Tween 10
    -For 20 ml:
    2.0 ml 10% paraformaldehyde
    10 µl Tween-20
13. Incubate at **room temperature** for 30 minutes
14. Incubate **on ice** for 30 minutes
15. Spin and dump as before (step 8)
    Add 1 ml DNAse (0.15M NaCl + 4.2mM MgCl + 100 Kunitz units/ml DNAse)
    -For 50 ml:

    46.5 mL dH20
    200 ul MgCl2 (1M stock)
    1500 uL NaCl (5M stock)
    100 Kunitz units Dnase (volume depends on activity of batch)
    Incubate for 30 minutes @ 25°

16. Spin 10 min. and dump as before (step 8)
17. Transfer cells from FACS tubes to 96-well plate. Wash once with staining media.
18. Block with 10% rat serum. Incubate 15 minute on ice. Spin and dump as before (step 8: it is critical to spin at high speed once the cells have been fixed with EtoH/ PFA since they become less dense).
19. Add **anti-BrdU-FITC or biotin (1:20 dilution for Phoenix flow).**
20. Pipette up and down to resuspend pellet. Incubate for 30 minutes on ice (or overnight at 4°C).
21. Wash and dump as before. Transfer cells into FACS tubes.

BrdU staining for cell cycle analysis (with HCl treatment)

A total of $3x10^6$ retinoblastoma cells were treated with cytokines, at the concentration noted above, for 72 h in medium with 10% FBS. The cells were then pulsed with 20 µm BrdU (Sigma, St. Louis, MO) for one hour at 37 °C in 10% FBS. The cells were then fixed with cold 95% ethanol, washed with PBS and resuspended in 0.4 mg/ml pepsin in 0.1 N HCl for 30 min at room temperature (RT) to release nuclei. The nuclei were pelleted and incubated with 2 N HCl for 30 min at RT. Following neutralization with 0.1 M $Na_2B_4O_7$, nuclei were pelleted and washed with PBS + 0.5% Tween-20 + 0.1% BSA. Nuclei were then stained with anti-BrdU antibody (Becton-Dickinson, Franklin Lakes, NJ) for 90 min in the dark at RT. The nuclei were then washed with PBS, pelleted and stained with FITC-labeled goat anti-mouse antibody (1:50, Becton-Dickinson) for 30 min at RT in the dark. The nuclei were washed with PBS, incubated with propidium iodide (0.1 mg/ml) and RNAse A (10 µg/ml) overnight at 4 °C, and then analyzed using a FACScalibur machine. Because of extensive aneuploidy, we chose to quantitate only the non-aneuploid portions of the FACS. The portions of the FACS analysis used for gating each population of cells were between 200 and 400 on the X-axis. The percentages, therefore, do not total 100%. However, the percentages total to over 70% for all samples thus represent the majority of cells in the population

FACS Analysis of Endothelial Cells

(Karin Ebner)

.)cell starving: 24h with serumreduced Medium (5% serum)

.)Incubation with e.g. TNF (24h)
for max. stimulation of ICAM-1 we added IL-1ß (20 Units/ml) for the last 24h hours of incubation

.)after incubation wash cells with cold PBS, scrap the cells from the plate in PBS+5%Serum into a tube and put them on ice (no trypsin)

.)centrifugation: 4°C/1.500g/15min

.)cell pellet + first antibody -we incubated the cells for 15 min on ice and shaked the cells during this 15 min a few times (not vortexing)

.)stop the reaction with 1ml PBS+5%Serum, centrifugation: 4°C/1.500g/5min

.)wash cells once in PBS+5%Serum

.)cell pellet + second antibody -we incubated 30 min on ice (sometimes shaking the cells)

.)stop the reaction and wash cells as above, afterwards resuspend the cells in PBS for FACS-analysis

The use of PBS with 5% Serum may sound strange. We used this for a good treatment of the sensitive primary endothelial cells. Could be that this is not necessary.

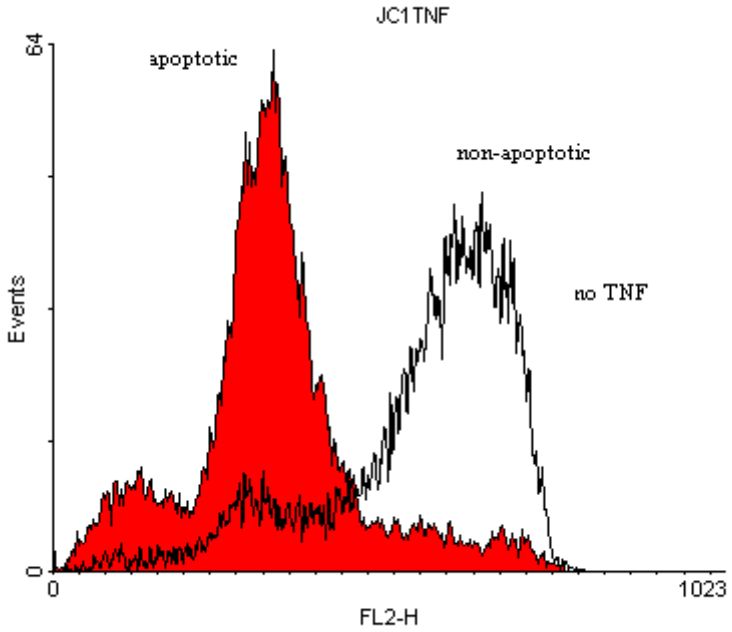
There is also a publication which might be interesting:
KIM JA, Atherioscler Thromb 14: 427-433, 1994

JC-1 stain of apoptotic cells

JC-1 (5, 5´, 6, 6´-tetrachloro-1, 1´, 3, 3´-tetraethylbenzimidazol-carbocyanine iodide) is a lipophilic fluorescent cation that incorporates into the mitochondrial membrane, where it can form aggregates due to the physiological membrane potential of mitochondria. This aggregation changes the fluorescence properties of JC-1 leading to a shift from green to orange fluorescence.
Intact living cells stained with JC-1 therefore exhibit a pronounced orange fluorescence of mitochondria, which is detectable by flow analysis (in the FL-2 channel).
Apoptosis results in a break-down of the mitochondrial membrane potential and a subsequent decrease of the orange fluorescence (and a slight increase of the green fluorescence). By that means, apoptotic cells can be easily distinguished from non-apoptotic cells.
For a review Andrea Cosarizza´s website: http://imoax1.unimo.it/~cossarizza/mo1997/data/A-PAGE13.HTM
JC-1 is prepared as a 1000x stock solution in DMSO (5 mg/ml).
For the staining of adherent cells it is diluted in medium to 5 µg/ml (with vortexing during the dilution to prevent the formation of precipitates); the JC-1 containing medium is added to the cells, followed by incubation for 10 min at 37°C (or RT for 15 min).
Subsequently the cells are washed twice with PBS, trypsinized, suspended in 500 µl PBS and analyzed by flow analysis.
Suspension cells (lymphocytes): suspend 1:1 with 10 µg/ml JC-1 in medium (final conc.: 5 µg/ml)

Approximate detection settings on FACSort:
FL1: 360 V (log)
FL2: 310 V (log)
Compensation : FL1-7% FL2 und FL2-74% FL1
Andere Einstellungen: Zelltyp-spezifisch, bei TF-1 z.B.:
SSC: 336 V lin
FSC: E00 lin 1.0
Threshold: FSC: 52

Example of an analysis:



Cell Cycle Analysis by Propidium Iodide (PI) Staining

Adherent cells:

- trypsinized
- suspended in medium + 10% FCS
- centrifuged (1000 rpm, 5 min)
- Pellet suspended in PBS (1 ml)

Suspension cells:

- Centrifuged (1000 rpm, 5 min)
- Pellet suspended in PBS (1 ml)

Fixation with EtOH:
Pipet cell suspension into 2.5 ml absolute EtOH (final concentration approx. 70%)
(or vortex the suspension at half speed while adding the EtOH) – to prevent clustering of cells during the fixation. Incubate on ice for 15 min (or over night at –20°C).

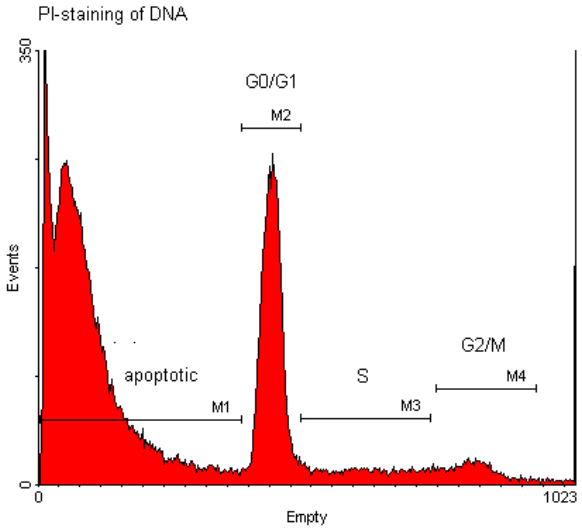
Alternative fixation with paraformaldehyde:
Pipet the 1 ml cell suspension into 3 ml 4% paraformaldehyde and fix for 15 min at r.t.

Staining:

- Pellet the cells at 1500 rpm for 5 min
- Suspend the pellet in 500 µl PI-solution
  in PBS: 50 µg/ml PI from 50x stock solution (2.5 mg/ml)
  0.1 mg/ml RNase A
  0.05% Tritin X-100
  Incubate for 40 min at 37°C
- Add 3 ml of PBS, pellet the cells (1500 rpm, 5 min) and take off the supernatant
- Suspend the pellet in 500 µl PBS for flow analysis
  (you can also leave about 500 µl of the diluted staining solution on the pellet and suspend the cells in this solution > less loss of cells when you take off the sup.) – the rest of the staining solution does not interfere with the flow analysis.

Flow analysis:
Approximate settings (on FACSort):
FL1: 570 V log. (e.g. if you want to detect GFP):
FL2: 470 V linear

Example:



Common Solutions

RIPA Protein lysis buffer

RIPA final concentrations
150mM NaCl
50mM Tris pH7.3
0,1% SDS
1% NP-40
0,5% Natrium-Deoxycholat
1mM Natrium ortho vanadat pH 10 (pre-activated)
1mM EDTA

For 100ml:

5M NaCl	3ml
1M Tris pH7.3	5ml
10% SDS	1ml
NP-40	1ml
5% Na-Deoxycholat	10ml
100mM Natrium-ortho-vanadate	1ml
500mM EDTA	0,2ml
dH2O	78,8ml

- store at 4°C
- Na_4VO_3 might also be added just prior to use
- Add PMSF prior to use
  (1 tablet Roche Complete Mini Protease Inhibitor for 10ml or 40µl 25x PMSF for 1ml)

Pre-Activation of Na_3VO_4 for maximal inhibition of phosphotyrosyl phosphatases

- Prepare 100mM Stock of Na_3VO_4
- Adjust pH to 10.0 with NaOH or HCl. At pH 10 the solution will be yellow.
- Boil the solution until it turns colorless
- Cool to RT
- Readjust the pH to 10.0 and repeat boiling and cooling until the solution remains colorless and the pH stabilizes at 10.0
- Store aliquots at -20°C
- This procedure depolymerizes the vanadate, converting it into a more potent inhibitor of phosphatases

Procedure:

- All steps should be performed on ice!
- Homogenize Sample in RIPA (+Na_3VO_4 +PMSF) or add RIPA to PBS-washed cell layer, incubate 5min and scrape off cells, transfer to 1,5ml tube
- Incubate on ice for 30min for efficient lysis
- Centrifuge 10.000xg 4°C for 10min
- Transfer supernatant to new tube and store at -20°C

TAE Buffer Stock Solution, 50 x

Reagent	Final Concentration	Volume	Mass
Tris base	40 mM		121 g
glacial acetic acid		28.55 ml	
0.5 M EDTA stock, pH 8.0	1 mM	50 ml	
dH_2O		400 ml	

add dH_2O to 500 ml total volume

PBS (1x)

Dulbecco's Phosphate-Buffered Saline (D-PBS) (1X) liquid
Contains no calcium or magnesium.

COMPONENTS	Molecular Weight	Concentration (mg/L)	Molarity (mM)
Potassium Chloride (KCl)	75	200	2.67
Potassium Phosphate monobasic (KH2PO4)	136	200	1.47
Sodium Chloride (NaCl)	58	8000	137.93
Sodium Phosphate dibasic (Na2HPO4-7H2O)	268	2160	8.06

6X SDS-PAGE Sample Buffer (with DTT)

2 M Tris-Cl (pH 6.8)	**2.4 ml**
SDS	**0.96 g**
Glycerol	**4.8 ml**
DTT	**739 mg**
Bromophenol Blue	**4.8 mg**

other variants:

Sample buffer 2 x, 10 ml:
Stacking gel buffer 4 x 2.5 ml
SDS, 10 % (cat.no. 20763 as 10 % solution in water) 4.0 ml
Glycerol (cat.no. 23176) 2.3 ml
2-Mercaptoethanol (cat.no. 28625) 0.5 ml
Bromophenolblue (cat.no. 15375) 1.0 mg

6X Composition		(for 10 mls)
60%	Glycerol	6.0 ml 100% Glycerol
300 mM	Tris (pH 6.8)	3.0 ml 1 M Tris 6.8
12 mM	EDTA	240 ul 0.5 M EDTA
12%	SDS	1.2 g SDS
864 mM	2-mercaptoethanol	600 ul 2-mercaptoethanol
0.05%	bromophenol blue	"pinch" bromophenol blue

SDS-PAGE sample buffer, 4X, pH 6.8

0.25 M Tris
8% SDS
40% Glycerol
0.04% Bromphenol