**Topic**

- Arabidopsis thaliana WRKY11, WRKY50, WRKY33, ZIP63, BPC2 binding to DNA
- human p53 tumor suppressor protein
tested different settings of the ELISA-procedure with purified NFkB and crude-cell extracts
- human 6D3 fragment of HLTF, quantification of protein-DNA interaction at equilibrium, detection of [1] DNA bound to immobilised protein vs. protein retained by immobilised DNA
- human p53 binding to DNA, crude- vs. purified extracts, temperature-sensitive p53 mutant
- human 6D3 fragment of HLTF, quantification of protein-DNA interaction at equilibrium, detection of [1] DNA bound to immobilised protein vs. protein retained by immobilised DNA
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- active human papillomavirus type (HPV 16) E2 protein binding to DNA

**Protein Expression**

- pET-DEST42 (Invitrogen) ind: coli, induction with IPTG
- MC9-7 human tumor cell line, induction with UV light
- human fibroblast cells
- human p53 tumor suppressor protein tested different settings of the ELISA-procedure with purified NFkB and crude-cell extracts
- purified extracts, temperature-sensitive p53 mutant

**Protein Extraction**

- sonication; control pET-DEST42 no insert; extraction buffer (4 mM HEPES, 100 mM KCl, 8% glycerol, 0.2% BSA without Biotin, 0.4 mM DTT, protease inhibitors without EDTA (Roche))
extraction buffer (20 mM HEPES pH 7.6, 0.5M NaCl, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA pH 8.0, 0.1% NP-40, 1 mM DTT, protease and phosphatase inhibitors)
extraction buffer (20 mM HEPES pH7.5, 0.35 M NaCl, 20% glycerol, 15 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.1 mM EGTA, protease inhibitor)
sonication; extraction buffer (150 mM NaCl, 16 mM NaH₂PO₄, 4 mM NaH₂PO₄, < 1% Triton-X-100); purification
- freezing; extraction buffer (20 mM HEPES pH7.9, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.4 M KCl, 1 mM PMSE, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 20% glycerol)
- sonication; control pEX-GST;HPV16 E2 in E. coli, induction with IPTG

**DNA Preparation**

- ~30 bp long DNA; 5’ end biotinylation in annealing buffer (40 mM Tris/HCl pH 7.5, 20 mM MgCl₂, 30 mM NaCl); 2 pmol ds-bio DNA/well in TBS-T
- 500 ng ds-bio DNA per reaction
- 122 bp long DNA by PCR, 5’ end biotinylation
- 2 pmol ds-bio DNA/well in PBS + 0.1% Tween20; quantification of fixed DNA by Picogreen Assay (Molecular Probes, OR)
- annealing of 30 bp long DNA - 10 bp 5’ end overhang; filling overhang with Klenow Fragment and dNTPs with 11-biotin-UTP; 7-122 bp long DNA by PCR, 5’ end biotinylatio
- annealing of 27 bp long DNA results in sticky ends - ligation; biotinylation; 10 µg ds-bio DNA/well

**DNA-ELISA Procedure**

- Streptavidin-coated microplates 96 well (Thermo Scientific®) - DNA immobilisation
- Streptavidin conjugated alcaline phosphatase primary against E2, secondary with peroxidase
- Blocking Solution 5% non-fat dried milk (Roth) in TBS-T or antibody related blocking solution (Qiagen) (antibody dependent)
- Primary mouse anti-p53 antibody in protein-binding buffer, secondary with horseradish peroxidase
- streptavidin conjugated alkaline phosphatase (Dako) coated with anti-mouse or anti-rat IgG - immobilisation of antibody bound p53 protein incubated with ds-bio DNA
- MaxiSorb Immuno plates (NUNC) coated with Streptavidin - DNA immobilisation

**Protein Binding Buffer**

- extraction buffer or TBS-T (protein dependent)
- 5 mM Tris, 0.5 mM EDTA, 50 mM KCl (pH 7.8)
- 4 mM HEPES pH 7.5, 100 mM KCl, 8% glycerol, 5 mM DTT, 0.2% BSA, 0.016% poly(ADP)
- PBS
- 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.05% glycerol, 1% NP40, 5 mM DTT, 0.1-1µg poly(dI:dC)
- protein extraction buffer

**Wash Steps**

- TBS-T or PBS-T
- PBS + 0.1% Tween20 (140 mM NaCl, 3.5 mM KCl, 4 mM NaPO₄)
- PBS + 0.1% Tween20
- PBS; 0.1% NP40 in 20 mM Tris-HCl pH 7.5
- PBS-T

**Blocking Solution**

- 5% non-fat dried milk (Roth) in TBS-T or antibody related blocking solution (Qiagen) (antibody dependent)
- 5% BSA in PBS
- no extra step
- 5% skim milk (Defco) [1]
- 20% non-fat dry milk, 1mg/ml heat-denat salmon sperm DNA

**Antibodies for Detection of Protein-DNA-Interaction**

- anti-His conjugated with horseradish peroxidase (Qiagen)
- primary mouse anti-p53 antibody in protein-binding buffer, secondary with horseradish peroxidase
- primary rabbit anti-NFkB antibody (1:1000 in 10 mM pBS pH 7.4, 50 mM NaCl, 1% non-fat dried milk), secondary with horseradish peroxidase
- streptavidin- and biotin-substrated horseradish peroxidase or primary against GST, secondary with horseradish peroxidase
- streptavidin conjugated alkaline phosphatase (Dako) coated with anti-mouse or anti-rat IgG - immobilisation of antibody bound p53 protein incubated with ds-bio DNA
- primary against E2, secondary with peroxidase (Dako/spotter)

**Signal Detection**

- 4 µg OPD (Sigma), 0.001% (v/v) H₂O₂ in 6 ml CP-buffer (10 mM NaHPO₄, 100 mM citric acid, pH 5); after 20 min stop with 1:1 M HCl, 492 nm with 650 nm reference
- ABTS (Chemicon International), 405 nm
- Tetramethylbenzidine (Bio source), 450 nm with 655 nm reference, avoiding of saturation
- ABC detection kit (Dako), 492 nm [1]
- 1mg/ml p-nitrophenyl phosphate in 10mM diethanolamine pH 9.8, 0.5mM MgCl₂, 405 nm
- 4 µg OPD, 0.03% H₂O₂ in 10 ml 0.2M phosphate-citrate buffer pH 5; after 20 min stop with 1:1 H₂SO₄, 490 nm

**Results**

- applicable for any (plant) transcription factor or DNA-binding protein/domain
- linker of 5 bp before and after 20 bp long core DNA sequence or 58 bp long promoter sequence
- DNA-ELISA 10-fold more sensitive than EMSA, competition of protein-DNA interactions with non-bio-ds DNA possible
- DNA-ELISA 10-fold more sensitive than EMSA; ELISA-procedure [1] is more sensitive than [2], because of sterical reasons