Protocols

1 *in vitro* DNA binding tests & Primer Extension

Disclaimer

No warranty is given or assumed; the use of these protocols is entirely at your own risk.

Work should only be performed with appropriate irradiation protection, within authorized laboratories and by trained personnel. Film badges should be used by all personnel working with ^{32}P. If millicurie quantities are manipulated, wrist and finger badges are recommended. Whenever handling ^{32}P on an open bench, the eyes should be shielded with safety glasses. Lucite plastic ($^{1}/_{4}$ inch) is the recommended shielding for working with quantities of ^{32}P up to 10 mCi.

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1.1 PCR labeling

Sambrock, Molecular Cloning edition 3, 17.13 and 9.14

Comment

In this protocol, $\alpha^{32}P$ dCTP and dCTP are both incorporated by chance into the synthesized DNA-strand during PCR at corresponding positions.
Different PCR products differ in DNA sequence and length and, thus, also in the number of C-positions were $\alpha^{32}P$ dCTP and dCTP is incorporated. Therefore, equal labeling of different PCR products must be achieved by the adjustment of the proportion of labeled and unlabeled nucleotides in the labeling reaction. It is recommended to choose an average labeling of 1 label per double-stranded DNA molecule. This is achieved by counting the numbers of C-incorporation sites (I) per double stranded DNA molecule and to adjust the concentration of cold dCTP to be (I) times higher than the concentration of labeled $\alpha^{32}P$ dCTP.

Incorporation sites (I) = No. pos. C+G in forward strand between Oligonucleotide positions + No. pos. G in forward strand within Oligonucleotide positions

Incorp. sites in double stranded PCR prod. / average No of labels per molecule = dNTP / $\alpha^{32}P$ dNTP

Material

dDTP Mix (2,5mM dATP, 2,5mM dTTP, 2,5mM dGTP)
dCTP (0,25mM)
Forward Oligo-Nucleotide (10µM)
Reverse Oligo-Nucleotide (10µM)
MgCl2 (50mM)
10x PCR Buffer (Genecraft)
DNA Polymerase (Biotherm, GeneCraft, 5u/µl)
α32P dCTP (250µCi, GE Healthcare)
Thermocycler (Gene Amp 2700, ABI)
Ethanol
80% ethanol
3M Na Acetate pH.: 5,2
Dry ice
Table top centrifuge
Parafilm
Pasteur pipettes

Protocol

1. Standard PCR reactions are performed in order to adjust amplification conditions for PCR labeling.
2. Labeling PCR is set up as described below and with appropriate cycle conditions.

   Mix

Template DNA (i.e. 1ng 6kb Plasmid)	1,0µl
dDTP [2,5mM]	2,0µl
dCTP [0,25mM]	2,0µl
Forward Oligo [10µM]	1,0µl
Reverse Oligo [10µM]	1,0µl
ddH_2O	x,xµl
$MgCl_2$ [50mM]	1,0µl
10x PCR Buffer	2,5µl
Polymerase [5u/µl]	0,5µl
α^{32}P dCTP [correct ratio to dCTP]	x,xµl
Total volume	25,0µl

   Cycle conditions

93ºC	3min	
93ºC	30sec	30 cycles
An. Temp.	30sec	30 cycles
72ºC	1min	30 cycles
72ºC	7min	
4ºC	∞	

3. The PCR reactions are transferred to 1,5ml reaction tubes. The volume is adjusted to 100µl and 0,5µl are taken off and stored for later scintillation counting.
4. Samples are supplemented with 0,1 volumes (10µl) 3M Na Acetate pH:5,2 and 2,2 volumes 220µl ice-cold ethanol. Precipitation of samples is carried out on dry ice for 30min or overnight at -20ºC. (This step is performed in order to remove non-incorporated α^{32}P dCTP from the samples).
5. Samples are centrifuged in a chilled table top centrifuge at full speed for 25min at 4ºC (tubes are sealed with Parafilm in order to avoid contamination of the rotor). Supernatants are taken off with sterile, elongated Pasteur pipettes. Pellets are washed with 200µl [80%] ethanol and centrifuged for 5min. The supernatant is removed, pellets are left to air-dry and finally, samples are re-suspended in 10µl ddH_2O.
6. Scintillation counting is performed with 1:20 dilutions of labeled PCR products before and after precipitation (protocol 1.5). The fraction of incorporated radioactivity is calculated, as well as the concentration of labeled PCR product.

1.2 Oligo End-labeling

Material

Optikinase, 10xbuffer (USB, GE Healthcare)
Optikinase, (USB, GE Healthcare)
γ32P ATP (6000 Ci / mmol) (GE Healthcare)
Oligonucleotide (~ 20-25nc)
Yeast tRNA (10mg/ml)
Vortex
Whatman papers (S&S GB002) 0,5cm x 0,5cm
Scintillation counting vials
Scintillation counting liquid (EcoScint)
Ammonium Acetate (5M)
Ethanol
Ethanol (80%)
Elongated Pasteur Pipettes
Heat-bath or thermocycler
Protection shields

Protocol

1. The reaction mix is prepared:

Mix	
ddH_2O	17,5µl
Oligo [10µM]	1,5µl
10x Buffer	2,5µl
Optikinase (USB) [10u/µl]	2,5µl
γ^{32}P ATP [6000 Ci / mmol]	1,0µl
Total volume	25,0µl

2. The reaction is set to incubate for 30min at 37ºC and inactivated by heat incubation for 10 min at 65ºC.
3. For precipitation, 22µl [5M] NH_4-Acetate and 1µl [10mg/ml] yeast tRNA is added to each reaction mix. Samples are well homogenized and 0,5µl is taken off and stored at -20ºC for scintillation counting. Finally, 290µl ice-cold ethanol is added and the solution is mixed carefully on a vortex. Precipitation is carried out for 30min on dry ice or overnight at -20ºC.
4. Samples are centrifuged in a chilled table top centrifuge at full speed for 25min at 4ºC (seal tubes with Parafilm in order to avoid contamination of the rotor). Supernatants are taken off with sterile, elongated Pasteur pipettes. Pellets are washed with 200µl [80%] ethanol and centrifuged for another 5min. The supernatant is removed and pellets are left to air-dry. Finally, samples are re-suspended in 10µl ddH_2O.
5. Scintillation counting is performed with 1:20 dilutions of labeling products before and after precipitation (protocol 1.5). The fraction of incorporated radioactivity is calculated, as well as the concentration of labeled oligo-nucleotide.

1.3 PCR with End-labeled Oligonucleotides

Material

dNTP Mix (2,5mM dATP, 2,5mM dTTP, 2,5mM dGTP, 2,5mM dCTP)
End-labeled Oligo-Nucleotide (10-20pmol)
Reverse Oligo-Nucleotide (10µM)
MgCl2 (50mM)
10x PCR Buffer (Genecraft)
DNA Polymerase (Biotherm, GeneCraft, 5u/µl)
Thermocycler (Gene Amp 2700, ABI)
Protection shields

Protocol

1. End-labeled oligonucleotide is supplemented with standard PCR reagents:

Mix	
End-labeled Oligo [10-20pmol]	9,5µl
ddH_2O	7,5µl
Complementary ("cold") Oligo [10µM]	1,0µl
Template DNA (i.e. 1ng of a 6kb Plasmid)	1,0µl
dNTP [2,5mM]	2,0µl
$MgCl_2$ [50mM]	1,0µl
10x PCR Buffer	2,5µl
Polymerase [5u/µl]	0,5µl
Total volume	25,0µl

Cycle program		
93ºC	3min	
93ºC	30sec	30 cycles
Tan	30sec	
72ºC	1min	
72ºC	7min	
4ºC	∞	

2. PCR products are directly loaded on a preparative acrylamide gel (protocol 1.4)

1.4 PCR product Purification (Preparative Gel)

Material

20cm x 20cm x 1mm Acrylamide gel (1x TBE), 8mm well combs
30 % Acrylamide: (29 Acrylamide : 1 Bis-Acrylamide)
5x TBE Buffer
TEMED
Ammonium Persulfate (APS)
Acrylamide Gel electrophoresis apparatus (Gel: 200mmx200mmx1mm)
Gele-Electrophoresis Power supply
2X Binding buffer (65mM HEPES pH.: 7.6, 0,1mM EDTA, 12,5mM MgCl2, 1mM DTT, 10% Glycerol, 100mM KCl)*
Loading Buffer (containing Bromphenol Blue and Xylene Cyanole)
X-ray exposure holder (KODAK, Sigma E-9010)
X-ray film (GE Healthcare, Hyperfilm)
Radioactive ink (Commercial ink + 32P agent, 5-10 x10 cps / 5µl ink, Geiger counter (x10) at 20cm)
Transparent tape
Saran wrap
Scalpel
Spatula
Syringe, 50ml
Dialysis tube
Dialysis clamps
Horizontal Agarose gel electrophoresis tank
1xTBE
Phenol: Chloroform (1:1)
Chloroform: Isoamylic alcohol (24 :1)
Ethanol
80% ethanol
3M Na Acetate pH.: 5,2
Dry ice

* Heberlein, U., England, B., and Tjian, R. (1985) Cell 41, 965-977

Protocol

1. The Labeling-PCR reactions are loaded with one volume 2x EMSA Buffer on a 5% acrylamide gel (20cm x 20cm x 1mm, 1x TBE, 8mm well combs).

Gel		
ddH_2O	31,07ml	
5x TBE Buffer	10,00ml	
30% Acrylamide	8,33ml	
APS [100mg/ml]	0,50ml	(Our APS is very old, better try with less)
TEMED	0,10ml	
Total volume	50,00ml	

1. A maximum of 25µl PCR product is loaded per well. Standard Loading buffer (with dyes Bromphenol blue and Xylene Cyanole) is loaded aside into a free well.
2. Gel electrophoresis is carried out in 1xTBE at 200V for 1,5 – 2h.

3. Dialysis tube is prepared in the meantime. Dialysis tube is boiled for 10min in 0,5mM EDTA (12-15cm per sample). The tube is closed on one side with a dialysis clamp, filled up with autoclaved ddH_2O and closed by twisting the tube. The tube is further dried on the outside with clean paper tissue and checked for possible holes with another dry and fresh paper tissue (we have never found any hole … but just in case). The tube is rinsed once in 1xTBE and left at 4ºC until needed (can be stored for weeks).
4. After gel electrophoresis, the lower buffer tank is usually strongly contaminated with radioactivity. Contaminated buffer is transferred into the radioactive liquid waste with a 50ml syringe. The glass plates with the el are recovered from the tank and remaining buffer is poured out of the sample wells.
   The following steps have to be done carefully and well protected in order to avoid unnecessary exposure to radioactivity.
5. The notched glass plate is removed and the gel is subsequently covered with a layer of saran wrap. Two stripes of transparent tape are placed at the sides of the gel on top of the saran wrap. Asymmetric radioactive marks are painted onto the stripes with radioactive ink (Commercial ink should be supplemented with 32P to give a final radioactivity of 5-10 x10 cps / 5µl ink, at 20cm distance). The dried ink marks are covered with another layer of tape. The gel is transferred to an X-ray exposure holder and exposed to a X-ray film for 15min at RT.
6. The film is developed and aligned to the radioactive ink marks on the gel. The film is fixed with tape and PCR product is labeled with a marker on the back side glass plate of the gel.
7. The film is removed and gel slices with PCR product are cut out from the gel with a clean scalpel (saran wrap is removed only locally, where necessary).
8. PCR product is recovered from the gel slices by eletro-elution. Gel slices are transferred to previously prepared dialysis tubes (6-7cm length), filled with 400µl of 1x TBE. Air bubbles are removed; the tubes are closed with clamps and put into a horizontal gel electrophoresis tank. Electro-elution is carried out at 115V for 2-3hours.
9. Liquids are transferred from the tubes into 1,5ml tubes. One volume Phenol: chloroform is added to each sample, mixed until a cloudy suspension is obtained and centrifuged for 5min at 12000rpm in a table top centrifuge at room temperature. The upper phase is recovered and extraction is repeated once with one volume of chloroform.
10. Sample DNA is precipitated with addition of 0,1 volume of [3M] Na Acetate pH 5,2 and 2,2 volumes of ice cold [100%] ethanol, as described before.
11. Pellets are re-suspended in 20µl ddH_2O. Scintillation counting is done with 0,5µl of purification product (protocol 1.5) in order to determine the amount of purified PCR product.

1.5 Scintillation counting

Material

Scintillation counting vials (Pony vial, 5ml)
Scintillation counting liquid (EcoScint H, National Diagnostics)
Blotting Paper (GB002, Schleicher & Schuell)
Liquid Scintillation Analyzer (TRI-CARB 2900 TR, Packard)

Protocol

1. Suitable sample dilutions are made (1:20 to undiluted).
2. Scintillation counting vials (Pony vial 5ml) are filled with 3ml of Scintillation counting liquid (EcoScint).
3. Blotting paper (GB002) is cut into small pieces of 0,5 x 0,5cm^2 and 1µl of test sample is pipetted onto it. The filter paper sheets are left to dry and then are transferred into the scintillation counting vials.
4. Radioactivity [cpm] is measured as direct ^{32}P counting, using a scintillation counting apparatus. Incorporation ratios or concentration of labeled DNA are calculated:

<u>Determination of counts [cpm]</u>

1. Determination of dpm is possible if the efficiency of the counting apparatus is known, if not it is assumed that cpm equals dpm.
2. The amount of radioactive DNA product is quantified with the below stated relationships:

a. 1 nCi = 2200dpm

b. A_0: Radioactivity of the labeling substrate [Ci / mmol] at activity day (i.e. 6000 Ci / mmol, labeled on the vial)

Determination of A_t:

$A_t = A_0 \text{ x } \exp(-t \text{ x } \lambda)$;

$T_{1/2[32P]} = 14{,}5d = \tau \ln 2 \Rightarrow \tau = 14{,}5d / \ln 2 \approx 20{,}92$ [d]

$\lambda = 1/\tau \Rightarrow \lambda \approx 1/ 20{,}92 \, [d^{-1}] = 0{,}047793103 \, [d^{-1}]$

$\mathbf{A_t \, [Ci /mmol] = A_0 \, [Ci /mmol] \text{ x } \exp(-t \, [d] \text{ x } 0{,}047793103 \, [d^{-1}])}$

⇨ **xxx dpm / 2200[dpm/nCi] = xxx/2200nCi**

⇨ **xxx/2200nCi / A_t [nCi / ftmol] = XXX [ftmol] labeling product**

1.6 Preparation of G+A DNA standard ladder

Material

End-Labeled DNA fragment [10ftmol/µl]
Formic Acid 1M, pH 2,(pH adjusted with Piperidine)
Piperidine 1M
Speed vac (Heto)
Dry ice
Formamide Gel Loading Buffer (80% Formamide, 10mM NaOH, 1mM EDTA, 1mg/ml Xylene Cyanole, 1mg/ml Bromphenol blue)
Teflon tape
Dry-Bath

Protocol

1. The end-labeled DNA fragment is incubated in 0,1M formic acid for 1h at 20ºC. The reaction mixture is prepared in a 1,5ml tube:

Mix	
ddH2O	7µl
End-labeled DNA fragment [10ftmol/µl]	2µl
Formic Acid [1M, pH: 2]	1µl
Total volume	10µl

2. Samples are frozen in dry ice and left to dry in the speed vac for at least 20min. Pellets are re-suspended in 10µl ddH_2O and freezing and drying is repeated.
3. Dried samples are re-suspended in 100µl (1M) Piperidine (in a chemical hood) and the tubes are sealed with Teflon tape. Reactions are heat incubated for 30min at 90ºC in a Dry-Bath.
4. The samples are let to cool down to room temperature and are frozen with dry ice and applied to the speed vac for 2h.
5. Dried samples are re-suspended in 10µl ddH_2O, frozen and dried as described in step 2. This step is repeated three times.
6. Finally, samples are re-suspended in 10µl formamide gel loading buffer and stored at -20ºC until used.

1.7 Preparation and handling of Acrylamide Sequencing gels

Material

Scintillation counting vials
Scintillation counting liquid
40% Acrylamide (19:1 Acrylamide : Bis-Acrylamide)
Urea
5xTBE Buffer
Ammonium Persulfate (APS)
TEMED
Acrylamide Gel electrophoresis apparatus (Gel: 50 cm x 35 cm x 0,05cm, Owl Scientific)
Repel-Silane
Gel-Electrophoresis Power supply (Sigma, P2000B)
1x TBE Buffer
0,5xTBE Buffer
gel loading tips (T-0906, Sigma)
Marker-pen
Scalpel
Spatula (thin)
Blotting Paper (GB002, Schleicher & Schuell)
Saran wrap
Gel drying device
X-ray exposure cassette
X-ray film (GE Healthcare, Hyperfilm)
Formamide Gel Loading Buffer (80% Formamide, 10mM NaOH, 1mM EDTA, 1mg/ml Xylene Cyanole, 1mg/ml Bromphenol blue)

Protocol

1. The glass plates of the sequencing gel are cleaned with water and ethanol. The notched plate must be well impregnated with Repel-Silane (very important for later gel recovery), the gel spacers are aligned and the glass plates are assembled (with hydrophobic lubricant between spacer and glass plate. The plates are then fixed with metal clamps.
2. The acrylamide gel is prepared. Urea is weighted in a 200ml beaker and acrylamide, 5xTBE, APS and 10ml ddH_2O are added. The solution is mixed on a magnetic agitator with a short heat pulse in order to dissolve the urea. The volume of the acrylamide solution is adjusted with ddH_2O to 85ml and TEMED is added. The gel is poured into the glass plate device with a 50ml syringe. Bubbles are removed by moving the solution inside the glass plates while pouring, or afterwards with a thin plastic shaft.

<u>Sequencing gel</u>	Urea	35,74g
0,5x TBE	40% Acrylamide	14,90ml
7% Acrylamide	5xTBE	8,5ml
7M Urea	APS [10%]	0,60ml
	ddH2O	up to 85ml
	TEMED	0,05ml
	Total Volume	85,00ml

3. The polymerized gel is assembled with the electrophoresis apparatus. The lower tank is filled with 1,2l 1xTBE and the upper tank is filled with 1l 0,5xTBE. A 30min pre-run is set at 1800V. Afterwards, sample wells are cleaned from urea with a 1000pipetter.
4. The samples, re-suspended in formamide gel-loading buffer, are denatured for 5min at 90ºC and rapidly chilled on ice. Each sample is loaded onto the gel with a gel loading tip. The gel run is set at 1800V for 45min to 3h dependent on the DNA fragment sizes of interest.
5. After the gel run, the electrophoresis apparatus is disassembled and the glass plates with the gel are recovered and left to cool down to room temperature.
6. Often, not all well of the gel were loaded with samples. Thus, the margins of the used part of the gel are labeled with marker on both glass plates. For gel recovery, the glass plates are laid down on the bench with the notched plate up. The notched glass plate is lifted and removed from the gel with a thin spatula or equivalent (the gel should remain attached to the lower glass plate). The used part of the gel is cut out with a sharp scalpel and a sheet of blotting paper (GB002) of suitable size is pressed on top of the gel. The blotting paper with the gel is lifted up and put down with the gel side up. Subsequently, it is covered with saran wrap. The gel is dried in a gel drying device for 2h at 65ºC.
7. The dried sequencing gel is exposed to X-ray film or equivalent.

1.8 Electromobility Shift Assay (EMSA)

Material

2x Binding buffer (65mM HEPES pH.: 7.6, 0,1mM EDTA, 12,5mM MgCl2, 1mM DTT, 10% Glycerol, 100mM KCl)*
Labeled DNA fragment [ftmol/µl]
Purified Protein orCellExtract
Fragmented salmon sperm or Poly(dI-dC)
5xTBE Buffer
0,5xTBE Buffer
30% Acrylamide (29:1 Acrylamide : Bis-Acrylamide)
10% Nonidet P-40 (NP-40)
Ammonium persulfate (APS)
TEMED
Loading Buffer (containing Bromphenol Blue and Xylene Cyanole)
Acrylamide Gel electrophoresis apparatus (Gel: 200mmx200mmx1mm)
Gele-electrophoresis Power supply
Gel loading tips (ART-20G, MβP)
Gel drying device
Blotting Paper (GB002, Schleicher & Schuell)
Saran Wrap
X-ray exposure cassette
X-ray film (GE Healthcare, Hyperfilm)

* Heberlein, U., England, B., and Tjian, R. (1985) Cell 41, 965-977

Protocol

1. The Acrylamide Gel is prepared and assembled for the gel run; electrophoresis buffer is 0,5xTBE.

<u>*EMSA gel*</u>	ddH_2O	35,87ml
0,5x TBE	5xTBE	5,00ml
5% Acrylamide	30% Acrylamide	8,33ml
0,05% NP-40	NP40 [10%]	0,25ml
	APS [10%]	0,50ml
	TEMED	0,05ml
	Total Volume	50,00ml

2. The reaction mixtures are combined into 1,5ml tubes in the strict order, described below. The transcription factor / Cell extract is always added at last. A control sample is prepared without transcription factor / cell extract.

Mix

1. ddH_2O	to 16µl
2. 2x Binding Buffer	8µl
3. Unspecific DNA (Salmon sperm [100-400ng] or Poly(dI-dC))	0,5-2µl
4. Labeled DNA fragment [35-70ftmol]	0,5-2µl
5. Purified transcription factor [50-200ng] / cell extract	0,5-2µl
Total Volume	16µl

3. Each reaction is left at room temperature for 15min and then loaded quickly onto the gel with Gel loading tips (ART-20G, MβP). Loading Buffer (with dyes Bromphenol blue and Xylene Cyanole) is loaded aside in a spare well (in order to control sample migration during the gel run). The gel is run for 4min at 400V and for 1,5-2h at 200V.
4. The gel is recovered and radioactively contaminated buffer is discarded into the liquid radioactive waste.
5. The notched glass plate of the gel is lifted and removed. A sheet of blotting paper (SS GB002) is pressed firmly on top of the acrylamide gel. The gel with paper sheet is lifted up from the lower glass plate, turned over and put down with the gel side up. The gel is covered with saran wrap and dried for 2h in a gel drying device.
6. Finally, the gel is exposed to an X-ray film (overnight, without intensifying screens) or equivalent.

1.9 DNase I Footprinting

Material

DNase I Dilution Buffer (10mM MgCl2, 20mM CaCl2)
DNase I
Stop Solution (0,3 % SDS, 15 mM EDTA, 600 mM Na Acetate, 250 µg/ml yeast tRNA, 15µg/ml Proteinase K)
Labeled DNA fragment [3ftmol/µl]
Proteinase [K 10mg/ml]
Yeast tRNA [10mg/ml]
Dry bath
Phenol: Chloroform (1:1)
Chloroform: Isoamylic alcohol (24 :1)
Ethanol
80% ethanol
3M Na Acetate pH.: 5,2
Dry ice
Formamide Gel Loading Buffer (80% Formamide, 1mM EDTA, 1mg/ml Xylene Cyanole, 1mg/ml Bromphenol blue)

Comment

This procedure is based on the protocol of Heberlein et. al (1985). The DNase I stop solution has been changed and no competitor DNA is employed. Amounts of purified transcription factors / cell extracts have to be adjusted empirically by EMSA or footprint trials. DNase I concentrations should be optimized in trial digestions of end-labeled DNA under experimental conditions. An even ladder of DNA fragments should be obtained.

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Protocol

1. DNAse I is diluted in DNase I dilution buffer to 0,05-0,3ng/µl.
2. The End-labeled DNA fragment is diluted in ddH_2O to 3ftmol/µl and (if used) purified transcription factor is diluted to 10-50ng/µl in protein specific buffer.
3. The reaction mix for Protein-DNA binding is combined in the strict order:

Mix	
1. ddH_2O	to 20µl
2. 2x Binding Buffer	10µl
4. Labeled DNA fragment [3ftmol/µl]	2µl
5. Purified transcription factor [10-50ng/µl] / cell extract	0,5-3µl
Total Volume	20µl

Several samples with different protein concentrations (and later DNase I concentrations) are prepared. Additionally, negative control reactions and a mock

reaction are prepared that lack purified transcription factor / cell extract. Reactions are left to incubate at room temperature for 20min:

4. Each sample is supplemented one by one with 20µl of diluted DNAse I and is gently mixed. Digestion is carried out for 30sec. Subsequently, 40µl stop solution (containing proteinase K) is added, the sample is well homogenized and left at 56ºC for 5-10min (total volume: 120µl). The mock reaction is supplemented with DNase I dilution buffer without DNase I.
5. Samples are extracted once with one volume phenol:chloroform, as described before. Supernatants are precipitated with 300µl ice cold ethanol, overnight at -20ºC or for 30min on dry ice.
6. Dried pellets are re-suspended in 6µl formamide gel loading buffer. Finally, 1µl of each sample is used for scintillation counting (protocol 1.5) and equal amounts of radioactivity are loaded on a 7% sequencing gel (protocol 1.7). Usually, 10000-15000cpm of each sample is loaded. A G + A chemical sequencing DNA ladder is loaded aside (see Maxam & Gilbert sequencing).

1.10 Methylation Interference assay

Material

Labeled DNA fragment
Yeast tRNA [10mg/ml]
Dimethyl sulfate (DMS) (Fluka: 41610, Sigma)
DMS reaction Buffer (50mM Na Cacodylate, pH 8, 1mM EDTA, pH 8, stored at 4ºC)
DMS Stop Buffer (1,5M Na Acetate, pH 7, 1 M β-Mercapto Ethanol)
Vortex
0,3M Na Acetate / 0,1mM EDTA (5ml of 3M Na Acetate pH 5.2, 10ml 0f 0,5M EDTA)
5M NaOH (10g NaOH in 50ml, prepared freshly)
2x Binding buffer (65mM HEPES pH.: 7.6, 0,1mM EDTA, 12,5mM MgCl2, 1mM DTT, 10% Glycerol, 100mM KCl)*
Pasteur pipettes
Purified protein or cell extract
Fragmented salmon sperm or Poly(dI-dC)
5xTBE Buffer
0,5xTBE Buffer
30% Acrylamide (29:1 Acrylamide : Bis-Acrylamide)
10% Nonidet P-40 (NP-40)
Ammonium Persulfate (APS)
TEMED
Loading Buffer (containing Bromphenol blue and Xylene Cyanole)
Acrylamide Gel electrophoresis apparatus (Gel: 200mmx200mmx1mm)
Gele-Electrophoresis Power supply
Gel drying device
X-ray exposure cassette
X-ray film (GE Healthcare, Hyperfilm)
Radioactive ink (Commercial ink + radioactive agent, 10-15 cps / 5µl ink, 20cm distance)
Transparent tape
Scalpel
Spatula
Dialysis tube
Dialysis clamps
Horizontal agarose gel electrophoresis tank
0,5xTBE
Phenol: Chloroform (1:1)
Chloroform: Isoamylic alcohol (24:1)
Ethanol
80% ethanol
3M Na Acetate pH.: 5,2
Dry ice
Piperidine 1M
Speed vac (Heto)
Formamide Gel Loading Buffer (80% Formamide, 10mM NaOH, 1mM EDTA, 1mg/ml Xylene-Cyanole, 1mg/ml Bromphenol blue)
Teflon tape
Dry-Bath

Comment

This protocol is a combination of previously described procedures. It involves a DMS treatment of End-labeled DNA that is further used for a preparative EMSA. The retarded and the non-retarded DNA bands are recovered, DNA is purified from the acrylamide gel slices and is finally digested with piperidine, following the G+A DNA chemical sequencing protocol (protocol 1.6).

Protocol

DMS treatment

1. The methylation mix is prepared in a chemical hood:

Mix	
ddH_2O	to 13µl
End-labeled DNA fragment [140ftmol]	1-13µl
DMS reaction Buffer	200µl
DMS	1µl
Total volume	214µl

   Working ain a chemical hood, end-labeled DNA is combined with DMS reaction buffer and is briefly mixed with the vortex. One by one, DMS is added to each sample and the solution is mixed again (vortex 2sec). Incubation is carried out for 60sec at 20ºC (exactly). The reaction is stopped by addition of 40µl DMS stop solution and another sample mix (vortex).
2. DNA is precipitated with addition of 600µl ice-cold ethanol. The sample is left on dry ice for at least 15min (samples can be stored in order to re-combine the sample set) and then centrifuged for 15min at 14.000rpm in a chilled table top centrifuge. Supernatants are removed with elongated Pasteur pipettes and transferred to a fresh 1,5ml tube. If almost non-radioactive, supernatants can be discarded into the DMS liquid waste (5N NaOH). If strong radioactive, the supernatant is re-precipitated on dry ice in order to rescue sample DNA.
3. Pellets are re-suspended briefly (vortex 2sec) in 250µl 0,3M Na Acetate / 0,1mM EDTA and samples are precipitated with addition of 750µl ice-cold ethanol. Precipitation and centrifugation is repeated as stated above and dried pellets are re-suspended in 6µl ddH_2O. Finally, 0,5µl of each sample is used for scintillation counting (protocol 1.5). Usually, the loss of material during DMS treatment is about a 20-30%. Sample are stored at -20ºC.

Preparative EMSA

1. The acrylamide gel is prepared as described in protocol 1.8. Test EMSA experiments should be performed with DMS treated DNA in order to determine optimal binding conditions. Ideally, 50% of DNA is retarded. The preparative EMSA is the up-scaled repetition of the optimal binding test. Samples should be loaded across several adjacent sample wells in case that the sample volume exceeds 70µl.
2. The samples are loaded onto the gel as described before. The gel is run for 4min at 400V and for 1,5-2h at 200V in 0,5xTBE.
3. Meanwhile, dialysis tube is prepared (protocol 1.4).
4. The gel is recovered and radioactively contaminated buffer is discarded into the liquid radioactive waste.
5. The gel is prepared as described in protocol 1.4. with the exception that the asymmetric radioactive marks are made with weaker radioactive ink (10-15 cps / 5µl ink, 20cm distance). The gel is exposed to X-ray film for 4h at 4ºC in darkness.
6. Retarded and non-retarded DNA bands are cut from the gel (protocol 1.4) and DNA is purified by electro-elution, carried out at 120V for 2-3hours (or 60V overnight); followed by phenol: chloroform extraction. Finally, sample DNA is precipitated with Na Acetate and ethanol and washed and air-dried DNA pellets are re-suspended in 100µl 1M piperidine (in the chemical hood).

Piperidine treatment

1. Protocol 1.6 is followed from step 3 on. Dried samples are re-suspended in 100µl 1M Piperidine, are incubated at 90ºC for 30min and speed vac dried. Finally, samples are four times re-suspended in 10µl ddH_2O, frozen and dried.
2. Sample re-suspension is done with formamide gel loading buffer and quantified by scintillation counting (protocol 1.5). Usually 10000-15000cpm of each sample is loaded onto a 7% acrylamide sequencing gel (protocol 1.7).

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1.11 Primer Extension

Material

End-labeled Oligonucleotide (~ 30nc)
Yeast tRNA (10mg/ml)
Whatman papers (S&S GB002) 0,5cmx0,5cm
Scintillation counting vials
Scintillation counting liquid
Hybridization Buffer (200mM PIPES pH.: 6,5-7, 5mM EDTA, 2M NaCl)
Formamide des-ionized
PCR Thermocycler
Superscript III Reverse transcriptase, 10x Buffer, 0,1M DTT (Invitrogen)
dNTP Mix (2,5mM of each Nucleotide)
RNase guard (Amersham)
RNase, DNase free
RNase Dilution buffer (10mM Tris, pH.: 7,5, 15mM NaCl)
Phenol: Chloroform (1:1)
Chloroform: Isoamylic alcohol (24 :1)
Ethanol
80% ethanol
DEPC treated ddH2O
3M Na Acetate pH.: 5,2
Dry ice

Protocol

Hybridization

1. An approximate 10fold molar excess of end-labeled oligo-nucleotide is added to the RNA template. The mixture is precipitated with 0,1 volumes of 3M Na Acetate pH. 5,2 and 2,5 volumes of ice-cold 100% ETOH as described before.
2. Samples are centrifuged at full speed for 25min at 4ºC. The supernatants are removed and pellets are washed once with 100µl 80% ETOH. Centrifugation is repeated for 5min, the supernatant is discarded and pellets are left to air-dry.
3. Samples are re-suspended in 6µl of Hybridization buffer and are further mixed with 24µl formamide (final concentrations: 40mM PIPES, 1mM EDTA, 0,4M NaCl, 80% formamide). For hybridization, samples are heat incubated for 10min at 85ºC and subsequently left overnight at 48ºC (a PCR thermo-cycler is used).
4. Hybridization samples are supplemented with 170µl H_2O DEPC and 400µl ice-cold 100% ETOH. Precipitation is carried out for 30min on dry ice or overnight at -20ºC.
5. Samples are centrifuged and washed as in step 2 and air-dried pellets are re-suspended in 5µl ddH_2O DEPC.

Retro-Transcription

1. The retro transcription mix is prepared:

Mix	
ddH_2O DEPC	9,0µl
Hyb. product	5,0µl
dNTP Mix [2,5mM each]	1,0µl
DTT [0,1m]	1,0µl
5x Buffer	2,0µl
RNase guard	1,0µl
Superscript III	1,0µl
Total Volume	20,0µl

2. Incubation is carried out in a PCR thermocycler for 1 hour at 48ºC.
3. Samples are supplemented with 1µl 0,5M EDTA, pH.: 7,5 and 1µl of 0,1µg/µl RNase (DNase free), (diluted in RNase dilution buffer) and incubation is continued for 30min at 37ºC.
4. The sample volume is adjusted to 150µl with RNase Dilution buffer and samples are extracted once with phenol: chloroform and once with chloroform.
5. Purified samples are supplemented with 0,5µl [10mg/ml] Yeast tRNA and precipitated with 0,1 volumes Na-Acetate and 2,2 volumes ethanol as described before. Air-dried pellets are re-suspended in 5µl Formamide gel loading buffer.
6. The sample concentration is quantified by scintillation counting (protocol 1.5) and 2000-10000cpm is loaded onto a 7% acrylamide sequencing gel (protocol 1.7).