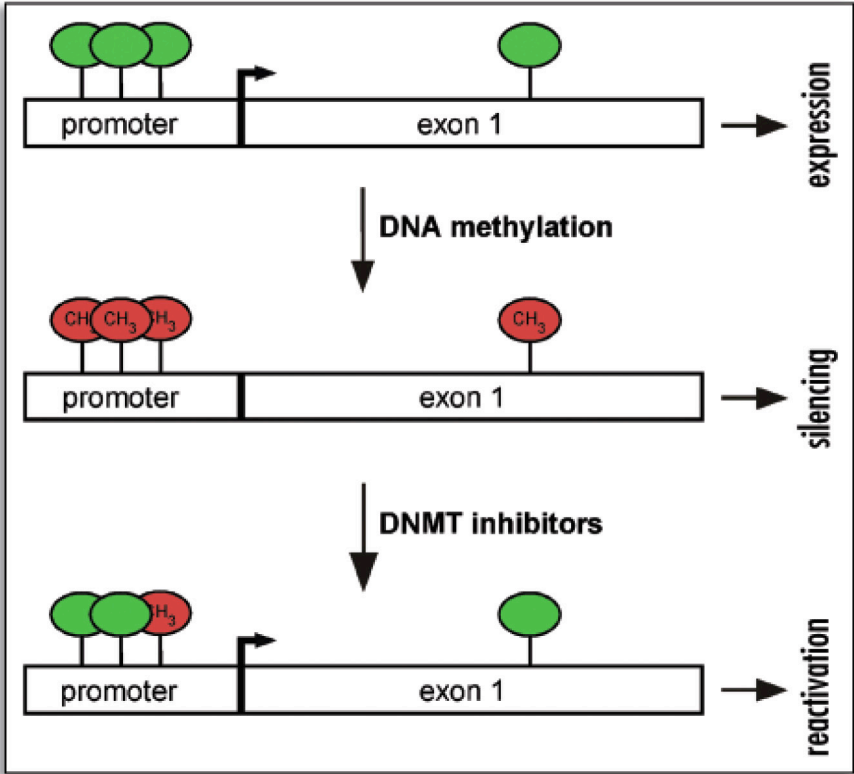


**Part III: Epigenetics – Detection and experimental modulation of DNA methylation in human cancer cells**

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<b>Monday</b>	<b>Tuesday</b>	<b>Wednesday</b>	<b>Thursday</b>	<b>Friday</b>
DK, BB	DK, CS	CS, BW	BW, MM	CS, BW
Preparation of protein extracts	Western blot ctd.	Bisulfite conversion ctd.	COBRA ctd.	COBRA ctd.
SDS-PAGE	Preparation of genomic DNA	Preparation of RNA	cDNA synthesis	RT-PCR ctd
Western blot	Bisulfite conversion	COBRA	RT-PCR	Clean up
	Seminar 1			Seminar 2

## Seminar 1

Jones PA, Baylin SB (2002). The fundamental role of epigenetic events in cancer.

Nature Reviews Genetics 3:415-28 (review).

Weber M, Davies JJ, Wittig D, Oakeley EJ, Haase M, Lam WL, Schubeler D. (2005).

Chromosome-wide and promoter-specific analyses identify sites of differential DNA methylation in normal and transformed human cells. Nature Genetics 37:853-62.

## Seminar 2

Lyko F, Brown R. (2005). DNA methyltransferase inhibitors and the development of epigenetic cancer therapies. Journal of the National Cancer Institute 97:1498-506 (review).

Brueckner B, Stresemann C, Kuner R, Mund C, Musch T, Meister M, Sultmann H, Lyko F (2007). The human let-7a-3 locus contains an epigenetically regulated microRNA gene with oncogenic function. Cancer Res. 67:1-5.

## 1. Preparation of protein extracts

- Seed  $1 \times 10^6$  HCT116 cells/flask ( $25\text{cm}^2$ ) in 6 ml medium (McCoy, 10% FCS, 1% Pen/Strep, 1% L-Glu), 2 flasks per time point, incubate over night at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$

- On the next day, add 500 nM 2'-deoxy-5-azacytidine (DAC) to the medium and incubate for one day at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$

1.1. Scrape the cells from the flask bottom and transfer the cell suspension in a 15 ml Falcon tube

1.2. Centrifuge 5 min at 800 rpm

1.3. Resuspend the cells in 1 ml ice-cold PBS, transfer suspension in 1.5 ml tube

1.4. Centrifuge for 5min at 800 rpm,  $4^\circ\text{C}$ , remove the supernatant and resuspend the cell pellet in 200  $\mu\text{l}$  cold PBS

1.5. Freeze in liquid nitrogen, thaw at RT

1.6. Centrifuge for 5 min at 13000 rpm,  $4^\circ\text{C}$ , transfer the supernatant to a new tube, store on ice

1.7. Determine the protein concentration: Bradford-assay

- add 2  $\mu\text{l}$  of the protein extract to 198  $\mu\text{l}$  PBS

- dilute BSA (Stock: 10 mg/ml) standards (200  $\mu\text{g}$ , 100  $\mu\text{g}$ , 50  $\mu\text{g}$ , 25  $\mu\text{g}$ , 12,5  $\mu\text{g}$ ) in 200  $\mu\text{l}$  PBS

- dilute Bradford solution 1:5 in A.dest

- add 800  $\mu\text{l}$  of diluted Bradford solution to 200  $\mu\text{l}$  sample or standard

- measure OD<sub>595nm</sub>, calculate protein concentration

- calculate required volume for a 300  $\mu\text{g}$  sample (max. loading volume is  $\sim 30 \mu\text{l}$ )

1.8. Prepare samples for SDS-polyacrylamide gel electrophoresis

- add an appropriate volume of 5x sample buffer to each sample

- boil for 5min at  $95^\circ\text{C}$ , cool down on ice

- centrifuge for 1min at 13000rpm, store at  $4^\circ\text{C}$

## 2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

2.1. Assemble casting form according to instructions.

2.2. Pour gel

### 8 % Separating gel :

A.dest	4.6 ml
30%Acrylamids mix	2.7 ml
1.5M TrisCl pH 8.8	2.5 ml
10% SDS	0.1 ml
TEMED	0.006 ml
10% APS	0.1 ml

After adding TEMED and APS your gel will polymerize fairly quickly, so do not add these until you are sure you are ready to pour.

Mix the ingredients and pour the solution quickly into your gel casting form. Make sure to leave some room for the stacking gel, usually about 2 centimeters below the bottom of the comb. You can do this by inserting the comb into the dry form, and marking a region below the comb for the height of the stacking gel. Look for bubbles and remove them, then layer the top of the gel with isopropanol. This will remove bubbles at the top of the gel and will ensure this part does not dry out. Wait for about 30 minutes for the gel to polymerize completely. If you always use fresh ammonium persulfate (APS), your gel may polymerize more quickly and reliably.

While waiting, mix the reagents for the stacking gel, but LEAVE OUT the APS and TEMED until you are ready to pour the gel; stacking gels might polymerize VERY quickly!

When the running gel is polymerized completely wash out the isopropanol. Mix in the polymerizing reagents and pour the stacking gel on top of the running gel. Insert your combs and try to avoid bubbles. Allow another 30 min - 1 hour for complete polymerization.

### 5% Stacking gel:

A.dest	2,1 ml
30%Acrylamids mix	0,5 ml
1 M TrisCl pH 6.8	3,8 ml
10% SDS	0.03 ml
10% APS	0.03 ml
TEMED	0.003 ml

2.3. Prepare 1xgel running buffer and 1xblotting buffer, storage at 4°C

2.4. Load samples and a size marker (Pageruler, 10 µl) onto the gel (8%SDS-PAGE)

2.5. Run each gel at 30 mA

### 3. Western Blot

- 3.1. Disassemble gel and equilibrate in 1x transfer buffer for 5 minutes
- 3.2. During equilibration, cut nitrocellulose to the size of the gel (9cm to 6cm) and soak in transfer buffer.
- 3.3. For each gel, cut 2 pieces of 3 MM paper to size of gel and soak in transfer buffer.
- 3.4. Wet pads with transfer buffer.
- 3.5. Assemble transfer unit: (red) pos. pole> black plate> pad> 3 MM> nitrocellulose> gel> paper> pad> white plate> neg. pole (back)
- 3.6. Close the sandwich board and dunk into partially filled transfer chamber.
- 3.7. Put in the cool cartridge and fill chamber to top, but do not let overflow.
- 3.8. Run transfer at 150 volts, 400 mA for 1,5 hours.
- 3.9. Verify transfer by staining with Ponceau S dye.
- 3.10. Block with 20ml 5% blocking buffer (5g milk powder in 100ml PBS + 50 $\mu$ l Tween20) for at least 1 hr at RT or overnight (4°C)
- 3.11. cut blot into two pieces at the 70kDa marker. Add primary antibody (goat anti DNMT1, dilute 1:500 ml in 10ml blocking buffer) to the upper blot, add primary Antibody (mouse anti  $\beta$ -actin, 1:5000, dilute in 10 ml blocking milk) to the lower blot, incubate both blots for 1hr at RT on a shaker
- 3.12. Wash several times with PBS-0.05% Tween (1L + 500 $\mu$ l Tween20) for 1 hr at room temperature
- 3.13. Add secondary antibody (donkey- $\alpha$ -goat or goat- $\alpha$ -mouse, 1:10.000 diluted in blocking buffer, incubate for 1hr at RT
- 3.14. wash for 1hr in PBS-0.05% Tween at RT
- 3.15. Mix 2ml of ECL solution1 with 2ml ECL solution2 and incubate the membrane for 1min, expose membrane to X-ray film.

**Recipes for buffers:**

10xbuffers are diluted to 1xbuffer with A.dest

**Tris-glycine SDS-polyacrylamide gel running buffer (10X)**

Tris base	30.3	gram (g)
Glycine	144	gram (g)
SDS	10	gram (g)
ddH2O to	1	litre (l)
Total volume	1	litre (l)

**Tris-glycine SDS-polyacrylamide gel loading buffer (2X)**

1M Tris-HCl (pH6.8)	1.6	millilitre (ml)
10% SDS	4	millilitre (ml)
Glycerol (100%)	2	millilitre (ml)
B-mercaptoethanol	1	millilitre (ml)
Bromophenol blue	4	milligram (mg)
ddH2O	1.4	millilitre (ml)
Total volume	10	millilitre (ml)

**Blotting buffer (10X)**

Boric acid	15,46	gram (g)
EDTA	7,4	gram (g)
ddH2O to	1	Litre (l)
Adjust pH to 8.3 (~6ml 10M NaOH)		

**For 1X blotting buffer add 0.1 g DTT, store buffer on ice!**

**PBS (10X)**

NaCl	80	Gram (g)
KCl	2	Gram (g)
Na <sub>2</sub> H <sub>2</sub> PO <sub>4</sub>	26.8	Gram (g)
KH <sub>2</sub> PO <sub>4</sub>	2.4	Gram (g)
ddH2O to	1	litre (l)

Adjust pH to 7.4 with HCl

#### 4. Preparation of genomic DNA

- Centrifuge the cells (maximum  $5 \times 10^6$ ) for 5 min at 300 g (1000 rpm)

4.1. Resuspend the pellet in 200  $\mu$ l PBS, add 4  $\mu$ l RNaseA (10 mg/ml), incubate 10 min at RT

4.2. Add 20  $\mu$ l proteinase K solution.

4.3. Add 200  $\mu$ l Buffer AL Mix thoroughly by vortexing, and incubate at 70°C for 10 min. **It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.**

4.4. Add 200  $\mu$ l ethanol to the sample, and mix thoroughly by vortexing. **It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.**

4.5. Pipet the mixture from step 3 into the spin column placed in a 2 ml collection tube. Centrifuge at 6000 g (8000 rpm) for 1 min. Discard flow-through and collection tube.

4.6. Place the spin column in a new 2 ml collection tube, add 500  $\mu$ l Buffer AW1, and centrifuge for 1 min at 6000 g (8000 rpm). Discard flow-through and collection tube.

4.7. Place the spin column in a new 2 ml collection tube, add 500  $\mu$ l Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube. **It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions.**

4.8. Place the spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 100  $\mu$ l Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 5 min, and then centrifuge for 1 min at 6000 g (8000 rpm) to elute.

4.9. Repeat elution once as described in step 7.

4.10. Determine DNA concentration at 260nm.

#### 5. Bisulfite DNA conversion

5.1. Thaw DNA to be used in the bisulfite reaction. Dissolve the Bisulfite Mix by adding 800  $\mu$ l RNase-free water to each aliquot. **Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min. Note: Do not place dissolved Bisulfite Mix on ice.**

5.2. Prepare bisulfite reactions in 200  $\mu$ l PCR tubes according to the Table below. Add each component in the order listed. **Note: The combined volume of DNA solution and RNase-free water must total 20  $\mu$ l.**

Component	Volume per reaction ( $\mu$ l)
DNA solution (1 $\mu$ g)	Variable*(maximum 20)
RNase-free water	Variable*
Bisulfite Mix (dissolved), see step 1	85
DNA Protect Buffer	35
<b>Total volume</b>	<b>140</b>

\* The combined volume of DNA solution and RNase-free water must total 20  $\mu$ l.

5.3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store tubes at room temperature. Note: The DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

5.4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to the Table below.

**Bisulfite Conversion Thermal Cycler Conditions**

Step	Time	Temperature
Denaturation	5 min	99°C
Incubation	25 min	60°C
Denaturation	5 min	99°C
Incubation	85 min (1h 25 min)	60°C
Denaturation	5 min	99°C
Incubation	175 min (2h 55 min)	60°C
Hold	Indefinite <sup>†</sup>	20°C

5.5. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation. Bisulfite conversion is carried out over night.

5.6. Centrifuge the PCR tubes containing the bisulfite reactions briefly, then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes. Any precipitants in the solution will not affect the performance or yield of the reaction.

5.7. Add 560  $\mu$ l freshly prepared Buffer BL. Mix the solution by vortexing and centrifuge briefly.

5.8. Place a spin column and collection tube in a suitable rack. Transfer the whole mixture from step 7 into the spin column.

- 5.9. Centrifuge the column at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
- 5.10. Add 500  $\mu$ l Buffer BW (wash buffer) to the spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
- 5.11. Add 500  $\mu$ l Buffer BD (desulfonation buffer) to the spin column, and incubate for 15 min at room temperature. If there are precipitates in Buffer BD, avoid transferring them to the spin column. **Note: It is important to close the lid of the column before incubation.**
- 5.12. Centrifuge the column at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
- 5.13. Add 500  $\mu$ l Buffer BW and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin column back into the collection tube.
- 5.14. Repeat step 13 once.
- 5.15. Place the spin column into a new 2 ml collection tube, and centrifuge the spin column at maximum speed for 1 min to remove any residual liquid.
- 5.16. Place the spin column into a clean 1.5 ml microcentrifuge tube. Add 20  $\mu$ l Buffer EB to the center of the membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 g (12,000 rpm).
- 5.17. Repeat step 16 once and pool the eluates. Store samples on ice!

## 6. Combined bisulfite restriction analysis (COBRA) of *TIMP-3*

6.1. Prepare HOT START-PCR reactions in 200  $\mu$ l PCR tubes according to the Table below. Add each component in the order listed.

Reddy Mix (10x)	2 $\mu$ l
Forward Primer (10 mM)	2 $\mu$ l
Reverse Primer (10 mM)	2 $\mu$ l
dNTPs (10 mM)	2 $\mu$ l
ddH <sub>2</sub> O	9,7 $\mu$ l
DNA Template ( ~ 25ng/ $\mu$ l)	2 $\mu$ l
<b>Total Volume</b>	<b>19,7 <math>\mu</math>l</b>

6.2. Program the thermal cycler according to the Table below.

Program step		
1	95 ° C	3 minutes
Press PAUSE		
Add 0,3µl Thermoprime-Polymerase (HOT START)		
2	95 ° C	30 seconds
3	55 ° C	30 seconds
4	72 ° C	30 seconds
2-4	35x	
5	72 ° C	3 minutes
6	18° C	for ever

6.3. Once the PCR reaction is complete, load the samples on a 2% TBE agarose gel.

6.4. Separate samples for 40 minutes at 120 volts.

6.5. Place gel on a UV transilluminator and excise the TIMP-3 amplicons (301 bp) with a clean, sharp scalpel.

6.6. Add 300 µl of Buffer QG to the gel fragment and incubate at 50°C for 10 min. To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

6.7. Add 100 µl of isopropanol to the sample and mix.

6.8. Place a spin column in a provided 2 ml collection tube. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

6.9. Discard flow-through and place column back in the same collection tube.

6.10. Add 0.5 ml of Buffer QG to column and centrifuge for 1 min.

6.11. To wash, add 0.75 ml of Buffer PE to column and centrifuge for 1 min.

6.12. Discard the flow-through and centrifuge the column for an additional 1 min at 13,000 rpm (~17,900 g).

6.13. Place column into a clean 1.5 ml microcentrifuge tube.

6.14. To elute DNA, add 30 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the column membrane. Incubate at room temperature for 5 min, and then centrifuge the column for 1 min at 13,000 rpm (~17,900 x g).

6.15. Perform Re-PCR: use 2µl of the eluted DNA (step 6.14), repeat steps 6.1.-6-14.

6.16. Set up the amplicon digest reaction as follows (0.2 ml tubes )

30 $\mu$ l	purified DNA fragment
3.6 $\mu$ l	NEB Buffer 2
2 $\mu$ l	BstU I (enzyme)

6.17. Mix the tubes by vortexing and spin the samples in a centrifuge to collect.

6.18. Incubate in a thermal cycler at 65°C for 5 hours and store in the cycler overnight at 18°C.

6.19. Add 4.5  $\mu$ l of the loading buffer to each sample and load on a 3% TBE agarose gel

6.20. Separate sample on gel (60 min, 120 V) and analyze results.

## 7. Preparation of RNA

7.1. Thaw the cell pellets on ice.

7.2. Before starting with the procedure add 10  $\mu$ l  $\beta$ -Mercaptoethanol per 1 ml Buffer RLT.

7.3. Add 350  $\mu$ l Buffer RLT to the cells and vortex to mix.

7.4. Homogenize the sample: Pass the lysate at least 5 times through a 20-gauge needle (0,9 mm diameter) fitted to an RNase-free syringe.

7.5. Add 350  $\mu$ l 70% ethanol and mix well by pipetting. **Do not centrifuge.**

7.6. Apply aliquots (up to 700  $\mu$ l) of the sample – including any precipitate that may have formed – successively onto a spin column placed in a 2 ml collection tube. Close the tube gently, and centrifuge for 15 s at 10,000 rpm. Discard flow-through after each centrifugation step.

7.7. Add 700  $\mu$ l Buffer RW1 to the column. Close the tube gently, and centrifuge for 15 s at 10,000 rpm to wash the column. Discard flow-through and collection tube.

7.8. Transfer the column into a new 2 ml collection tube.

7.9. Pipet 500  $\mu$ l Buffer RPE onto the column. Close the tube gently and centrifuge for 15 s at 10,000 rpm to wash the column. Discard flow-through.

7.10. Add another 500  $\mu$ l Buffer RPE to the column and centrifuge for 2 min at 10,000 rpm.

7.11. Place the column in a new 2 ml collection tube and centrifuge at full speed for 1 min to eliminate any residual RPE Buffer from the silica-gel membrane.

7.12. Elution: Transfer the column to a new 1.5 ml eppendorf tube and pipet 50  $\mu$ l RNase-free water directly onto the column silica-gel membrane. Incubate for 2 min at room temperature and then centrifuge for 1 min at 10,000 rpm.

7.13. Determine RNA concentration at 260nm

## 8. cDNA synthesis

8.1. set up reaction in a 0.2-ml tube:

(for 1 reaction):  
1  $\mu$ l 5x Oligo(dT) primer  
x  $\mu$ l total RNA (ca. 1 $\mu$ g)  
2  $\mu$ l dNTP Mix (10 mM)  
add DEPC-treated water up to 12  $\mu$ l

8.2. Denature RNA and primer by incubating at 65°C for 5 min and then place on ice.

8.3. Prepare a mix on ice and vortex gently:

(mix for 1 reaction):  
4  $\mu$ l 5x cDNA Synthesis Buffer (**Vortex for at least 5 s just prior to use!**)  
1  $\mu$ l DTT (0.1 M)  
1  $\mu$ l RNaseOUT™ (40 U/ $\mu$ l)  
1  $\mu$ l DEPC-treated water  
1  $\mu$ l ThermoScript™ RT (15 U/ $\mu$ l)

8.4. Pipet 8  $\mu$ l mix into each reaction tube on ice.

8.5. Perform cDNA synthesis in a thermal cycler

Thermal cycler conditions:  
50°C for 50 min (reverse transcription)  
85°C for 5 min (heat inactivation)  
4°C for ever

8.6. completed reactions will be stored at -20°C overnight.

### 9. RT-PCR for *TIMP-3* and *$\beta$ -Amyloid*

The housekeeping gene  *$\beta$ -Amyloid* will be used as a loading control.

9.1. Set up 2 reactions: For each reaction, pipet 2  $\mu$ l cDNA in a fresh 0,2-ml tube and add:

- 2  $\mu$ l Reddy-Mix (10x)
- 2  $\mu$ l Primer *TIMP-3/  $\beta$ -Amyloid* sense (10pmol/ $\mu$ l)
- 2  $\mu$ l Primer *TIMP-3/  $\beta$ -Amyloid* antisense (10pmol/ $\mu$ l)
- 2  $\mu$ l dNTPs (10 mM)
- 9,7  $\mu$ l ddH<sub>2</sub>O
- 0,3  $\mu$ l Thermoprime polymerase (5U/ $\mu$ l)

9.2. Perform RT-PCR in a thermal cycler. Thermal cycler conditions:

- 95°C for 3 min
- 95°C for 30 s
- 60°C for 30 s
- 72°C for 30 s
- go to step 2 ; 35x
- 72°C for 5 min
- 4°C for ever

9.3. Add 2  $\mu$ l of the loading buffer to each sample and load on a 2% TBE agarose gel.

9.4. Separate sample on gel (40 min, 120 V) and analyze results.