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„Epigenetic control of TLR4-mediated gene expression induced by bacteria and bacterial cell wall components in human intestinal epithelial cells“

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List of Abbreviations

Caco-2-cells: human endothelial colorectal adenocarcinoma cell line

CD40: cluster of differentiation 40

CFU: colony-forming unit

c-Jun: early response transcription factor

DNMT: DNA methyltransferase

ERK: extracellular signal-regulated kinase

FBS: foetal bovine serum

GAPDH: glycerinaldehyd-3-phosphate dehydrogenase

GM-CSF: granulocyte macrophage colony-stimulating factor

HDACi: histone deacetylase inhibitor

HUVECs: human umbilical vein endothelial cells

IECs: intestinal epithelial cells

IL-2, -17, -23: interleukin-2, -17, -23

JNK: c-Jun N-terminal-kinase

LD: *Lactobacillus delbrueckii*

LGG: *Lactobacillus rhamnosus GG*

LPS: lipopolysaccharide

MAPK: mitogen activated protein kinase

NFκB: nuclear factor kappa B

PBS: phosphate buffered saline

PPARγ: peroxisome proliferator activated receptor γ

PSQ: pyrosequencing

q-PCR: quantitative real-time polymerase chain reaction

RT-PCR: reverse transcription polymerase chain reaction

SCFA: short-chain fatty acid

SE: standard error

SP1: specificity protein 1

THP-1: human acute monocytic leukemia cell line

TLR: toll-like receptor

TNFα: tumor necrosis factor α

Zusammenfassung

Hintergrund: Kommensale Bakterien und bakterielle Zellwandbestandteile können über diverse intrazelluläre Signalwege komplexe Zellreaktionen hervorrufen. Beispielsweise scheinen LPS und probiotische Bakterien DNA-Methylierung zu modifizieren und somit Veränderungen der Genexpression mittels epigenetischer Mechanismen zu steuern.

Methoden: In der vorliegenden *in vitro* Studie wurden intestinale Epithelzellen (Caco-II-Zellen) mit LPS, Flagellin, *Lactobacillus rhamnosus GG* (LGG) und *Lactobacillus delbrueckii* (LD) stimuliert und daraufhin die mRNA Level in den Entzündungsprozess involvierter Gene (TNFα, TLR4, p38) und miRNAs (146a, 155) mit Hilfe von q-PCR gemessen. Des Weiteren wurde die DNA-Methylierung von 4 CpG loci in der TNFα-Promotorregion und 4 CpGs des TLR4 Exons mittels Pyrosequenzierung quantitativ bestimmt.

Ergebnisse: Stimulation mit LPS, Flagellin, LGG und LD resultierte jeweils in einer Abnahme der mRNA Level von TNFα und TLR4, während p38 mRNA leicht anstieg. Die Behandlung mit Flagellin löste eine 4,42±0,51-fache (p=0,002) Zunahme der miRNA-146a aus, hatte aber keinen Einfluss auf die miRNA-155. Die DNA-Methylierung der vier analysierten TNFα CpG loci bewegte sich zwischen 70-90%. Signifikante Veränderungen der TNFα-Methylierung wurden durch LGG nach 12 Stdn. (+1,07%±0,24, p=0,048), LD nach 24 Stdn. (+1,54%±0,3, p=0,035) und LPS nach 72 Stdn. (-0,58%±0,05, p=0,007) verursacht. TLR4-Methylierungen schwankten zwischen 10-45% in den unterschiedlichen Zellexperimenten. CpG 4 zeigte eine signifikante Zunahme nach 12 Stdn. Behandlung mit LPS (+2,00%±0,42, p=0,041), CpG 3 eine Abnahme nach 72 Stdn. Behandlung mit LGG (-1,77%±0,29, p=0,027) und CpG 2 eine Abnahme nach 12 Stdn. LPS Behandlung (-1,62%±0,26, p=0,024). 12 stündige Stimulation der Zellen mit LGG führte bei TNFα zu einer Zunahme in Methylierung bei gleichzeitiger Abnahme der mRNA Level.

Schlussfolgerung: Die Resultate dieser Studie lassen darauf schließen, dass die epigenetische Regulation von TNFα und TLR4 zu der Spezifität der inflammatorischen Reaktionen beiträgt, welche durch Bakterien und deren Zellwandbestandteile ausgelöst werden.

Abstract

Background: Commensal bacterial strains and bacterial cell wall components are proposed to induce differential cell responses regulated by intracellular signalling pathways. Epigenetic modulation of gene expression via DNA methylation was recently discussed to be influenced by LPS and probiotic bacteria.

Methods: We analysed the expression of inflammation-relevant genes (TNFα, TLR4, p38) and miRNAs (146a, 155) in caco-II-cells upon stimulation with LPS, flagellin, *Lactobacillus rhamnosus GG* (LGG) and *Lactobacillus delbrueckii* (LD) by measuring mRNA levels with q-PCR. Furthermore, DNA methylation of four CpG loci in the TNFα promoter region and four CpGs of the TLR4 exon was measured by using bisulfite-converted DNA for pyrosequencing analysis.

Results: LPS, flagellin, LGG and LD each decreased mRNA levels of TNFα and TLR4, while p38 mRNA was slightly increased by each treatment. Flagellin induced miRNA-146a expression by 4.42-fold±0.51 (p=0.002), but did not alter miRNA-155. Methylation of four analysed TNFα CpG loci ranged between 70-90% in un-stimulated cells. Significant changes in TNFα methylation over all four CpGs were caused by LGG after 12h of stimulation (+1.07%±0.24, p=0.048), LD after 24h (+1.54%±0.3, p=0.035) and LPS after 72h (-0.58%±0.05, p=0.007). TLR4 methylation ranged between 10 and 45% in different CpGs and cell culture experiments. CpG 4 was significantly increased by 12h LPS treatment (+2.00%±0.42, p=0.041), CpG 3 was decreased after 72h LGG treatment (-1.77%±0.29, p=0.027) and CpG 2 was also decreased after 12h LPS treatment (-1.62%±0.26, p=0.024). LGG treatment for 12h showed an increase in methylation (+1.07%±0.24, p=0.048) and a decrease in mRNA expression (0.73-fold±0.23, p=0.008) of TNFα.

Conclusion: The results indicate, that epigenetic regulation of TNFα and TLR4 contributes to the specificity of inflammatory reactions induced by bacteria and their cell wall components.

Publication draft

Introduction

Commensal probiotic bacteria and bacterial cell wall components are known to exhibit species- and strain-specific properties on intracellular signalling pathways, when encountering intestinal cells, in particular epithelial (IECs) and gut-associated immune cells [1-3]. Especially, probiotics have drawn the attention because they seem to provide strain-specific beneficial health effects on their host via various mechanisms of action [4-8].

It is essential for the intestinal cells to discriminate between pathogens and non-pathogenic bacteria, like commensals and probiotics, in order to promote the fine-tuning of the immune response by avoiding excessive or insufficient reactions. This determination involves recognition by Toll-like receptors (TLR), which subsequently activate specific intracellular signalling pathways and finally result in pro- or anti-inflammatory immune responses. The gram-negative bacterial cell wall components lipopolysaccharide (LPS) and flagellin act as ligands to TLR4 and TLR5 respectively and trigger pro-inflammatory signalling [9, 10] but also tolerance [11]. TLR2 recognizes gram-positive bacteria by their lipoproteins, while forming heterodimers with either TLR1 or TLR6 [12-14]. Although also acting via TLR signalling, probiotic bacteria are rather associated with anti-inflammatory immune responses and even seem to be able to extenuate already persisting inflammation. Models for experimental colitis indicate, that TLR2, 4 and 9 are required for certain probiotics to execute their anti-inflammatory effects [15, 16]. *Lactobacillus casei* and also a mix of DNA from 8 probiotic strains (VSL3) prevent the nuclear translocation of the inflammatory transcription factor NFκB (nuclear factor κB) by stabilising its inhibitor IκB in the cytoplasm, and thereby obviating the transcription of pro-inflammatory cytokines, e.g. TNFα (tumour necrosis factor α) [17, 18]. Another mechanism demonstrated for *Bacteroides thetaiotaomicron* induces a PPAR-γ (peroxisome proliferator activated receptor γ) dependent export of the NFκB subunit RelA/p65 from the nucleus and thus also decelerates cytokine production [19].

Recently new insight into the regulation of immune reactions to bacterial cell wall components and probiotic bacteria came from the field of epigenetics. Small, non-coding RNA molecules affect gene expression post-transcriptionally by mRNA cleavage or translational repression [20]. Previous results also from our laboratory showed, that dendritic cells react to co-incubation with *Lactobacillus rhamnosus GG* (LGG) by modifying the expression of two miRNAs (miR-146a and miR-155) [21]. In another recent study microarray data implicated, that LPS treatment of mouse macrophages leads to differential expression of seven microRNAs. Five microRNAs (miR-196b, miR-196c, miR-146a, miR-155 and miR-222) were up-regulated and two (miR27a* and miR-532-5p) down-regulated after 6 hours of stimulation with LPS [22]. Furthermore, bioinformatic approaches identified about 1000 candidate target genes of these miRNAs, mostly indicating functions in the apoptosis process and inflammatory responses [22]. Stimulus-dependent miRNA expression patterns were observed upon stimulation of primary human monocytes with different pro-inflammatory components providing indications to the theory, that miRNAs act in functional groups [23]. The wide range of involved miRNAs and target genes illustrates the importance of negative post-transcriptional regulation of inflammatory gene expression in the fine-tuning of immune responses.

There is evidence, that also other epigenetic mechanisms might be influenced by the engagement of bacterial products into host inflammatory signalling processes, for example via modifications of chromatin structure. The acetylation of specific sites on histones is associated with transcriptionally active chromatin, while histone deacetylation leads to the silencing of gene expression [24]. Commensal and probiotic bacteria ferment dietary compounds, which results in the production of short-chain fatty acid (SCFA) metabolites, such as butyrate, acetate and propionate. These SCFAs act as histone deacetylase inhibitors (HDACi) and thus epigenetically manipulate gene expression in an enhancing direction [25, 26]. Furthermore, results from other studies imply an involvement of chromatin remodelling in the kinetics of inflammatory immune responses. Especially activation of late immune response genes, but not genes of the early primary response, seems to be influenced by modifications on the chromatin structure [27, 28].

Histone modification and miRNAs have been linked to DNA methylation [29, 30], which is also associated with gene regulation. In particular hypermethylation appears to be connected to decreased transcriptional activity and vice versa [31], whereat this processes depend on the location of the methylated areas in context to the transcribed gene. Recent studies implicate this mechanism as a possibly critical point for differential contribution of bacterial strains to inflammatory gene regulation. CpG methylation was linked to stimulus-dependent gene activation or repression [32, 33]. The DNA methylation of the cytokine TNFα is influenced by LPS stimulation in various cells [34]. Furthermore, it was shown, that LPS-induced expression of the cytokines IL-17, IL-23 can be diminished by probiotic treatments in connection with DNA methylation enhancement, as well as decreased histone acetylation and nuclear translocation of NFκB [35]. As a response to LPS stimulation, the enhancement of TLR4 promoter methylation goes hand in hand with a repression of gene transcription [36]. Commensal bacteria additionally seem to have an influence on these effects, because the methylation level of the TLR4 gene is significantly lower in germ-free mice compared to conventional mice [37].

As DNA methylation of TNFα and TLR4 seems to be regulated in a stimulus-dependent manner, we analysed the effects of two bacterial cell wall components (LPS and flagellin) and two heat-inactivated probiotic strains (*Lactobacillus rhamnosus GG* and *Lactobacillus delbrueckii*) respectively on DNA methylation status of four CpG loci in the TNFα promoter and the TLR4 exon. The two lactic acid bacteria strains were chosen, because they showed immunomodulatory properties in past studies [38].

Material and Methods

Cell culture:

Caco-2-cells were grown in DMEM medium (high glucose 4.5 g/l, with L-Glutamin) supplemented with 10% heat inactivated foetal bovine serum (FBS), 0.5% sodium pyruvate and 1% penicillin/streptomycin at 37° C and 5% CO_2 and passaged every 2-3 days with acutase. For stimulation experiments the Caco-2-cells were cultured on 6-well-plates containing approximately $3x10^5$ cells per well. The cell medium was refreshed every second day and stimulatory agencies were applied at 70-80% confluency. All reagents and plastic labware used for cell culture were purchased from PAA laboratories (Pasching, Austria).

Bacteria and bacterial cell wall components:

Two probiotic strains *Lactobacillus rhamnosus GG* ATCC 53103 and *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 (LGC standards GmbH, Wesel, Germany) were autoclaved at 120°C for 30 minutes, washed three times with PBS (PAA laboratories, Pasching, Austria) and resuspended in cell culture medium prior to stimulation of Caco-2-cells. Commercially available purified bacterial cell wall component LPS (*Escherichia coli* 026:B6, L 2654) was purchased from Sigma-Aldriche Handels GmbH (Vienna, Austria) and flagellin (*S. typhimurium*, FLA-ST) from InvivoGen (San Diego, USA). In the stimulation experiments administered concentrations are listed in Table 1.

Table 1: Administered concentrations of bacteria and bacterial cell wall components in the stimulation experiments

Bacterial Treatment	Concentration	Ratio: Bacterial cell/Caco-cell
Lactobacillus rhamnosus	$5x10^7$ CFU/ml	50:1
Lactobacillus delbrueckii	$1x10^7$ CFU/ml	10:1
LPS	10 µg/ml	
Flagellin	1 µg/ml	

Stimulation experiments:

Bacterial components were added each to separate wells with 70-80% confluent Caco-2-cells (approximately 10^6 cells/well) and incubated for 12h, 24h, 48h and 72h at 37°C and 5% CO_2. For every time point untreated controls were carried along. Cells were harvested and residual medium was washed away with PBS (phosphate buffered saline). 12h samples were divided into two aliquots for DNA- and RNA-extraction. 24h, 48h and 72h samples were only used for DNA-extraction. The stimulations were carried out in duplicates in at least three independent experiments.

Quantitative promoter methylation analysis by DNA-Pyrosequencing:

DNA from all samples was extracted with QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's handbook for cultured cells. DNA concentration and 260:280 ratio were measured by Picodrop (Pico100, Picodrop Limited, U.K.). The extracted DNA was bisulfite-converted using the EpiTectBisulfite Kit (Qiagen, Hilden, Germany). PCR reactions for TLR4 and TNFα were performed with specific primers (Table 2). PCR was performed in a total volume of 25 µl reaction mixtures containing the following: 12.5 µl PyroMark 2x PCR Master Mix (Qiagen, Hilden, Germany), 2.5 µl CoralLoad Concentrate 10x, 5 pmol/reaction of each primer and a.) for TLR4 5-10 ng, b.) for TNFα 50-60 ng bisulfite-converted DNA. PCR conditions are as given in Table 3. 5 µl of the PCR reactions were used for 2% agarose gel electrophoresis, 20 µl were employed to the subsequent pyrosequencing with the PyroMark Q24 (Qiagen, Hilden, Germany). DNA methylation level of a region of the TNFα promoter (from -211 to -132bp; EMBL: NM000594; Figure 1) and TLR4 exon (from +215 to +250bp; EMBL: NM138554.3; Figure 2) was analysed for four CpG sites each (TNFα: -131bp, -158bp, -175bp, -181bp; TLR4: +217bp, +225bp, +234bp, +241bp) by the PyroMark Q24 using the PyroMark Q24 software 2.0.6 (Qiagen, Hilden, Germany). Only CpG sites that passed the program's quality check were included in the statistical analysis. As an internal control the sequencing assay contained non-CpG cytosines as controls to intercept inefficient bisulfite-conversion.

Table 2: Primer sequences

Primer		Sequence 5'-3'	Application
TNFα	forward	TTAAAAGAAATGGAGGTAATAG	PSQ
	reverse	Biotin-CCCATAAACTCATCTAAAAAAAA	PSQ
	sequencing	GTGAGGGGTATTTTTGA	PSQ
TLR4	forward	GGTTAGAGGAAGAGAAGATATTAGTGA	PSQ
	reverse	Biotin-TAACTCCAACCACATACCTCCA	PSQ
	sequencing	ATATTAGTGTTTTAGAAATTG	PSQ
TNFα	forward	AAGAGGGAGAGAAGCAACTACAGA	q-PCR
	reverse	GGTGGAGCCGTGGGTCAG	q-PCR
TLR4	forward	AAGCCGAAAGGTGATTGTTG	q-PCR
	reverse	CTGAGCAGGGTCTTCTCCAC	q-PCR
p38	forward	ACTCAGATGCCGAAGATGAAC	q-PCR
	reverse	GTGCTCAGGACTCCATCTCT	q-PCR
GAPDH	forward	CGACCACTTTGTCAAGCTCA	q-PCR
	reverse	AGGGGAGATTCAGTGTGGTG	q-PCR

Table 3: PCR conditions

PCR	Step	Time	Temperature	Cycles
Pyromark PCR	Initial	15 min	95°C	
	Denaturation	30 sec	94°C	45
	Annealing	45 sec (TLR4) 60 sec (TNFα)	59°C (TLR4) 50°C (TNFα)	
	Extension	45 sec (TLR4) 60 sec (TNFα)	72°C	
	Final	10 min	72°C	
q-PCR (mRNA)	Initial	10 min	95°C	
	Denaturation	30 sec	95°C	40
	Annealing	40 sec	58°C	
	Extension	40 sec	72°C	
	Melting curve	15 sec	95°C	
		60 sec	60°C	
		15 sec	95°C	
p-PCR (miRNA)	Initial	10 min	95°C	
	Denaturation	15 sec	95°C	40
	Annealing/Ext.	60 sec	60°C	

Figure 1: Analysed CpG loci in the TNFα promoter sequence (EMBL: NM000594). The analysed sequence (underlined) is shown relative to the transcription start of the TNFα gene. Blue lines indicate the positions of the PCR primers and the violet line stands for the sequencing primer. The four analysed CpGs (-131bp, -158bp, -175bp, -181bp) are accentuated by fat latters.

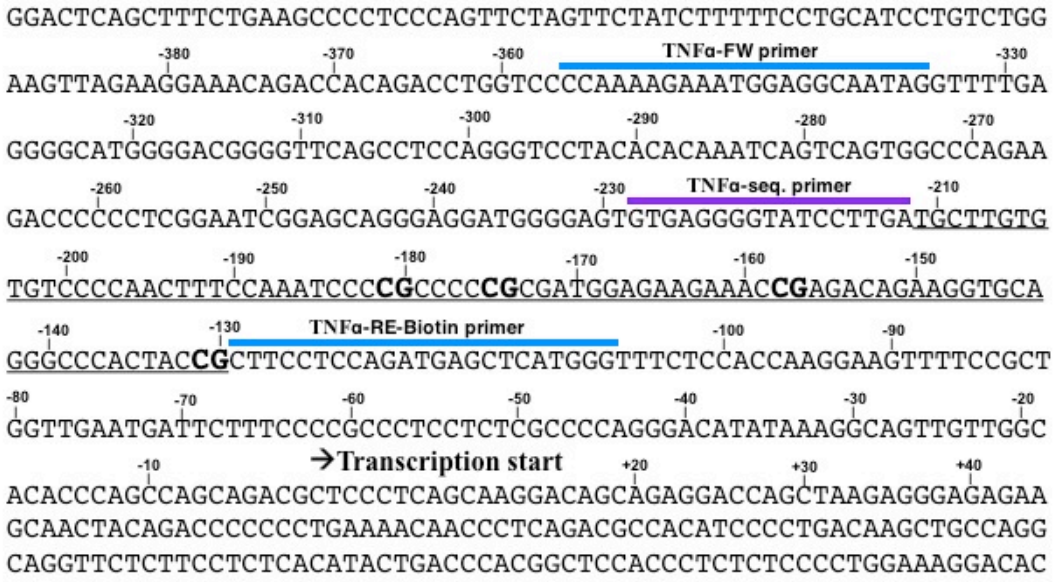
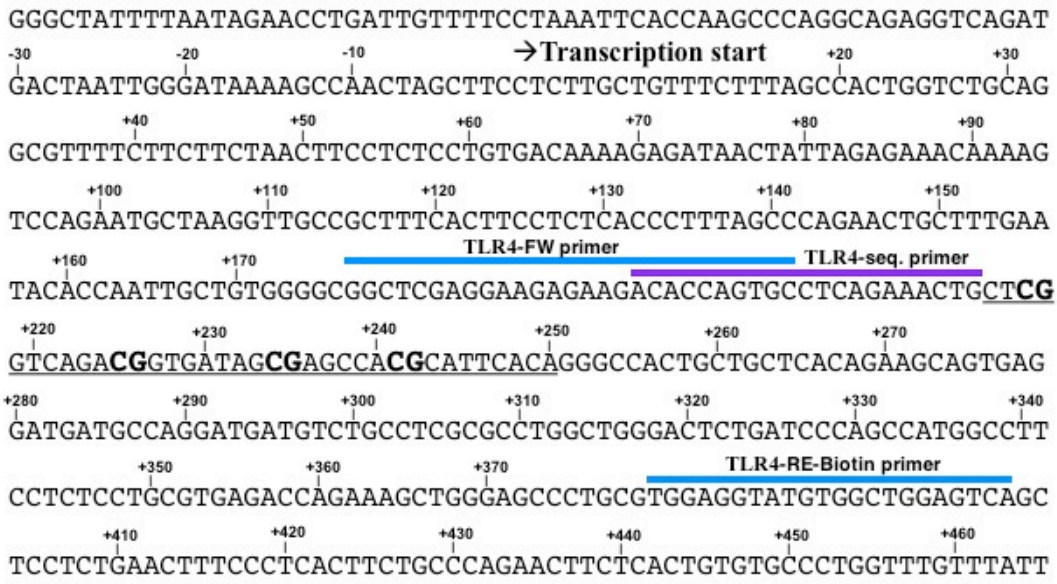


Figure 2: Analysed CpG loci in the TLR4 exon (EMBL: NM138554.3). The analysed sequence (underlined) is shown relative to the transcription start of the TLR4 gene. Blue lines indicate the positions of the PCR primers and the violet line stands for the sequencing primer. The four analysed CpGs (+217bp, +225bp, +234bp, +241bp) are accentuated by fat latters.



mRNA expression:

Total RNA was extracted from all 12h samples using the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) and reverse transcription was performed with the Phusion® RT-PCR Kit (Finnzymes, Vantaa, Finland) according to the manufacturers' instructions. Quantitative mRNA analysis of TLR4, TNFα, p38 and GAPDH (endogenous control) was carried out on the StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) using a SYBR Green master mix. A total volume of 10 µl per reaction contained 5 µl SensiMix™ SYBR No-Rox (Bioline, Germany), 0.5 pmol/µl primers (Table 2) and 1 µl cDNA. The cycling conditions are listed in Table 3 and were followed by melting curve analysis, in order to detect unspecific products. Each reaction was performed in duplicates.

miRNA expression:

For quantitative analysis of miRNA expression total RNA was extracted from 12h samples with the mirVana™ miRNA Isolation Kit (Ambion, Austin, TX) and reversely transcribed by using specific miRNA primers from the TaqMan® MicroRNA Assay (Applied Biosystems, Foster City, CA) for miRNA-146a, -155 and -U66 (endogenous control) and reagents from the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative miRNA analysis was carried out on the StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA) using a TaqMan approach. A total volume of 20 µl per reaction contained 10 µl TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 1 µl TaqMan MicroRNA Assay (forward and reverse primers in addition to a specific miRNA probe for fluorescence detection) and 1.33 µl cDNA. The cycling conditions are listed in Table 3. Each reaction was performed in triplicates.

Statistical analysis:

Quantitative methylation data was analysed by the PyroMark Q24 software 2.0.6 (Qiagen, Hilden, Germany) and the measured methylation levels are expressed as percentage. Quantitative PCR data analysis was obtained with the StepOne software 2.1 (Applied Biosystems, Foster City, CA). Measured mRNA and miRNA levels are expressed as Ct-values, which were further calculated with the comparative ΔΔCt method by normalising the treated samples and the untreated controls to the housekeeping gene (GAPDH and miRNA-U66). Mean fold changes over control are set by the $2^{-\Delta\Delta Ct}$-value. Statistical analysis and graphical presentation of methylation and expression data was obtained by SPSS 17.0 (IBM, Armonok, NY) using Kolmogorow-Smirnow test for normality distributions and student's t-test for determination of significant differences between treated samples and untreated controls. All data are shown as mean±1SE. Expression data is presented as mean fold change over the untreated control ($2^{-\Delta\Delta Ct}$). Methylation data is portrayed as difference of percentage to the untreated control. Results with $p<0.05$ (*) were considered as significant.

Results

TLR4, TNFα, p38 mRNA expression:

12h stimulation with flagellin, LPS, and all cell components of heat-inactivated probiotics *Lactobacillus rhamnosus GG* (LGG) and *Lactobacillus delbrueckii* (LD) significantly down-regulated the amount of TLR4 and TNFα mRNAs compared to the untreated control (Figure 3, Table 4). p38 was up-regulated by all treatments, but no significance was observed due to high standard deviations.

Figure 3: Effects of bacterial treatments on TLR4, TNFα, p38 mRNA expression. Caco-II-cells were stimulated with flagellin, LPS, LGG or LD for 12h and mRNA levels of (**A**) TLR4, (**B**) TNFα and (**C**) p38 were measured by q-PCR. Results represent the mean fold change (±SE indicated by error bars) over an untreated control. Statistical significance is indicated by asterisk.

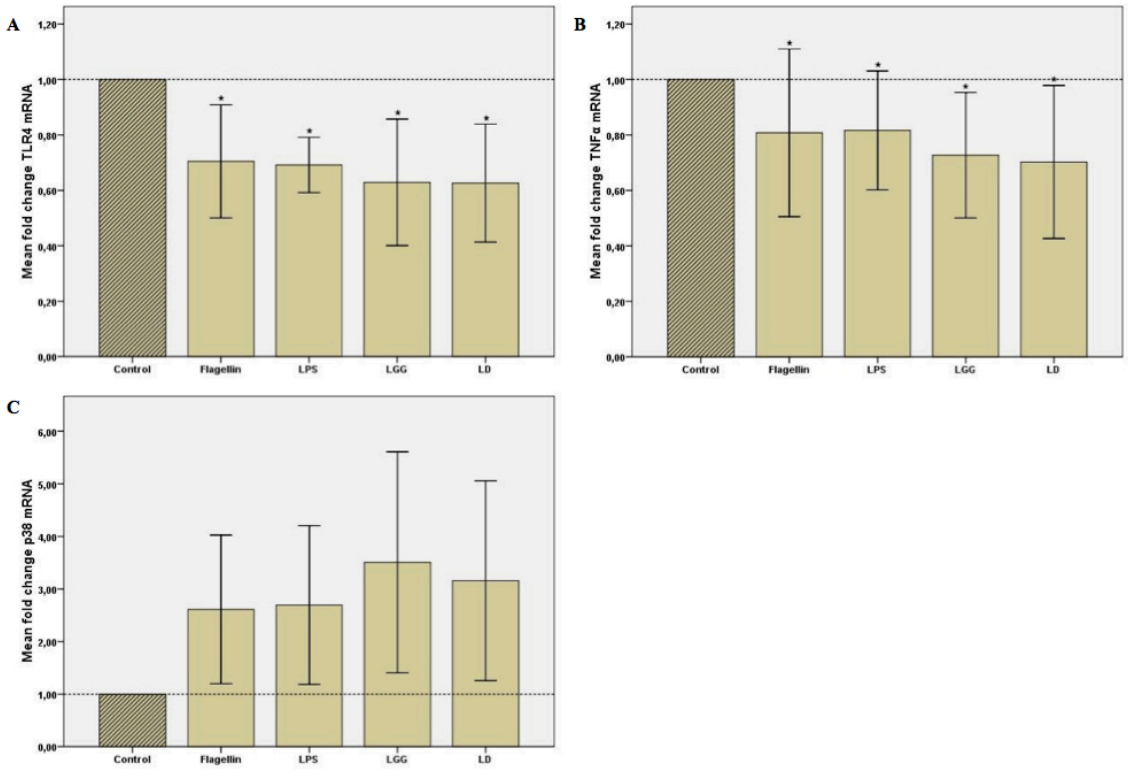


Table 4: Statistical analysis of TLR4, TNFα, p38 mRNA expression

		TLR4	TNFα	p38
Flagellin	mean $2^{-\Delta\Delta Ct}$	**0.71***	**0.81***	2.61
	SE	0.20	0.30	1.41
	p-value	0.003	0.025	0.128
LPS	mean $2^{-\Delta\Delta Ct}$	**0.69***	**0.82***	2.69
	SE	0.10	0.21	1.51
	p-value	0.004	0.044	0.115
LGG	mean $2^{-\Delta\Delta Ct}$	**0.63***	**0.73***	3.51
	SE	0.23	0.23	2.10
	p-value	0.001	0.008	0.063
LD	mean $2^{-\Delta\Delta Ct}$	**0.63***	**0.70***	3.16
	SE	0.21	0.28	1.90
	p-value	0.006	0.008	0.085

miRNA-146a & miRNA-155 expression:

Expression of miRNA-146a and -155 in consequence to LPS treatment were reported in previous studies [21-23]. Figure 4 and Table 5 represent our results of the quantitative expression analysis of miRNA-146a and -155 after 12h of flagellin treatment compared to an untreated control. The miRNA-146a was induced to 4.42±0.51 fold (p=0.002), while miRNA-155 (1.08-fold±0.03, p=0.566) was not affected by flagellin treatment.

Figure 4: Effects of flagellin treatment on miRNA-146a and -155 expression.
Caco-II-cells were stimulated with flagellin for 12h and miRNA-146a and -155 levels were measured by q-PCR. Results represent the mean fold change (±SE indicated by error bars) over an untreated control. Statistical significance is indicated by asterisk.

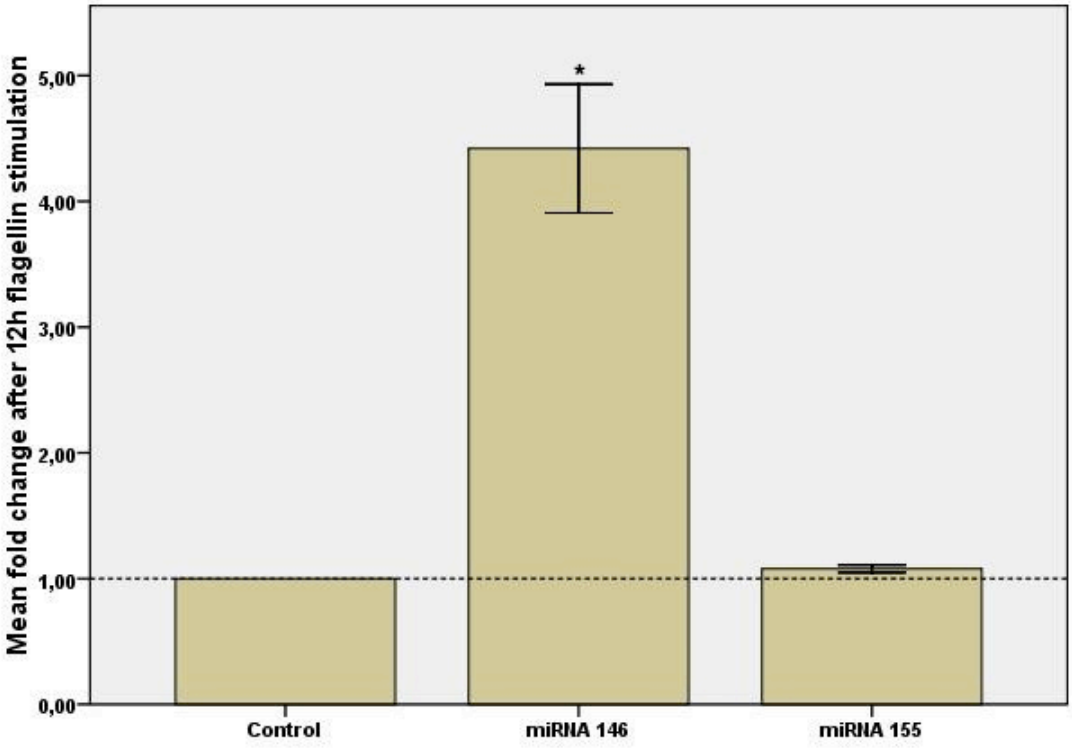


Table 5: Statistical analysis of miRNA-146a and -155 expression

	mean $2^{-\Delta\Delta Ct}$	SE	p-value
miRNA-146a	**4.42***	0.51	0.002
miRNA-155	1.08	0.03	0.566

TNFα-methylation:

In order to observe possible effects of bacterial components and probiotic strains on DNA methylation a 72 bp long sequence of the TNFα promoter region including 5 CpG sites was investigated. The middle CpG site was excluded from further analysis, because it did not pass the Pyromark Q24 software's quality check and only numbered CpGs were analysed further (Figure 5). Inserted control points (dispensation position 4, 13, 18, 26, 44, 52, 60, 66, 70) indicate an efficient bisulfite-conversion reaction (Figure 5). The analysed area was highly methylated with percentages between 70 to 90%, depending on the CpG position.

Caco-2-cells stimulated with LPS, LGG or LD for 12h, 24h, 48h or 72h showed changes in DNA-methylation status for the tested TNFα region compared to the untreated controls. The mean of all four CpGs (Figure 6, Table 6) revealed significant changes caused by LGG after 12h (+1.07%±0.24, p=0.048), LD after 24h (+1.54%±0.3, p=0.035) and LPS after 72h (-0.58%±0.05, p=0.007). CpG sites 3 and 4 appeared to be preferentially altered by treatments, whereas CpG 1 remained mainly unchanged (Figure 7, Table 6).

Additionally we used the EMBOSS transcription factor prediction to detect possible transcription factor binding sites within the analysed sequence. This approach uncovered potential binding sites for the transcription factors SP1, c-Jun and c-ETS-2 (Figure 11B). SP1 binding site includes CpG 1, which was not significantly altered by the applied treatments. c-Jun binding site includes CpG 4, which was significantly altered by some of the applied treatments, such as LPS after 12h (+0.45%±0.05, p=0.01) and 72h (-0.71%±0.14, p=0.035) and LGG after 72h (+1.88%±0.26, p=0.019).

Figure 5: Pyrogram of the analysed TNFα promoter region.
Selected CpGs (1-4 indicated by blue columns) within the TNFα promoter region were analysed by the Pyrosequencing technology and one example of a pyrogram from the Pyromark Q24 software is shown. The middle CpG site was excluded from further analysis because it did not pass the software's quality check. Inserted control points (dispensation position 4, 13, 18, 26, 44, 52, 60, 66, 70) are represented by yellow columns.

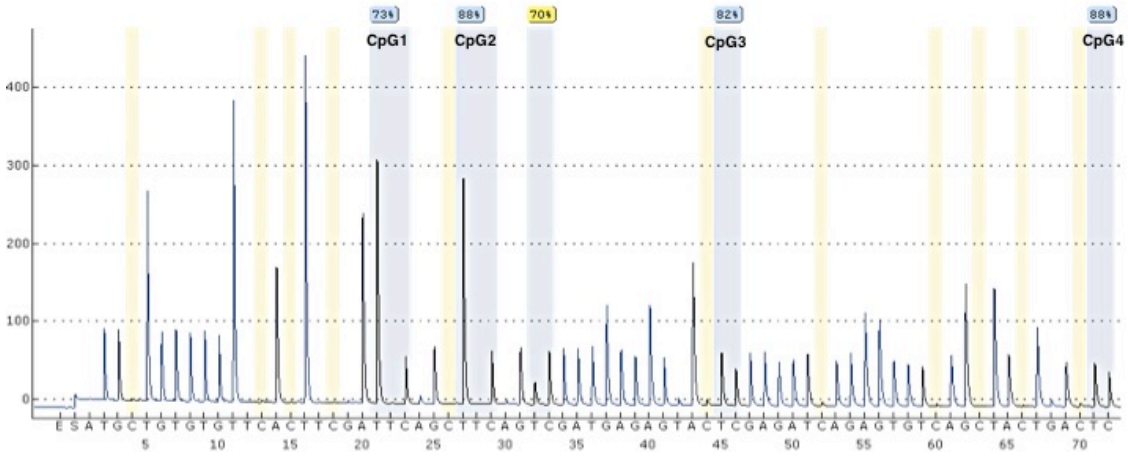


Figure 6: Effects of bacterial treatments on TNFα DNA methylation over the mean of all four CpG loci.
Caco-II-cells were stimulated with LPS, LGG or LD for 12h, 24h, 48h and 72h and DNA methylation of 4 selected CpGs was measured by pyrosequencing. Data represents the mean % difference in methylation (±SE indicated by error bars) to an untreated control (treated – untreated) of the mean of all 4 CpGs. Statistical significance is indicated by asterisk.

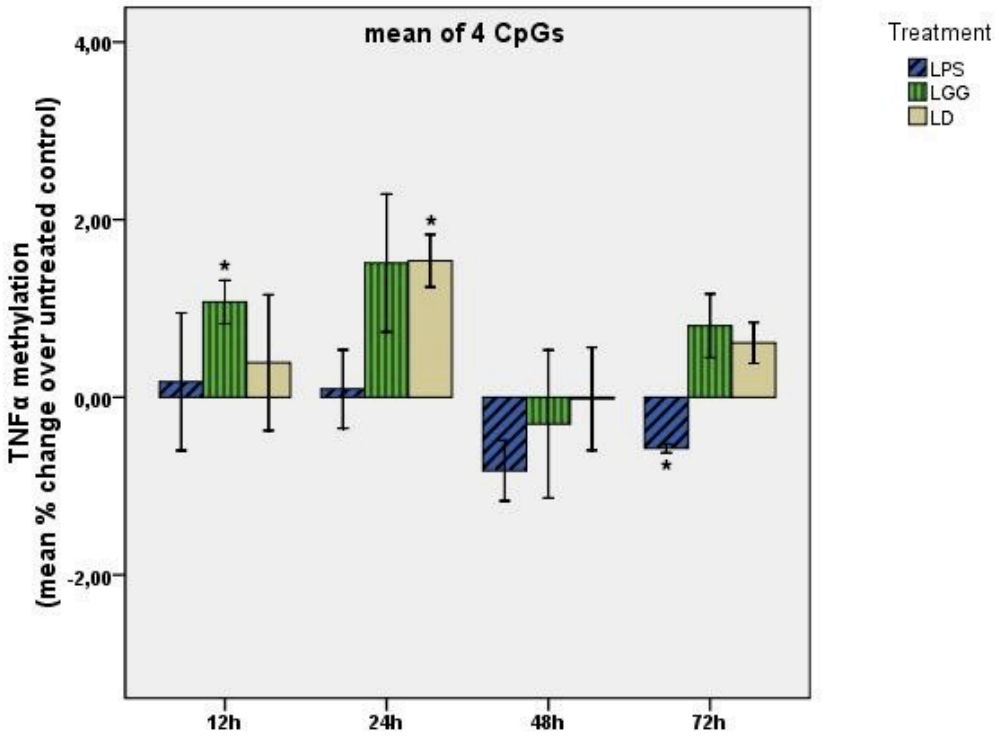


Figure 7: Effects of bacterial treatments on TNFα DNA methylation of each of the four CpG loci.
Caco-II-cells were stimulated with LPS, LGG or LD for 12h, 24h, 48h and 72h and DNA methylation of 4 selected CpGs was measured by pyrosequencing. Data represents the mean % difference in methylation (±SE indicated by error bars) to an untreated control (treated – untreated) of CpG 1-4. Statistical significance is indicated by asterisk.

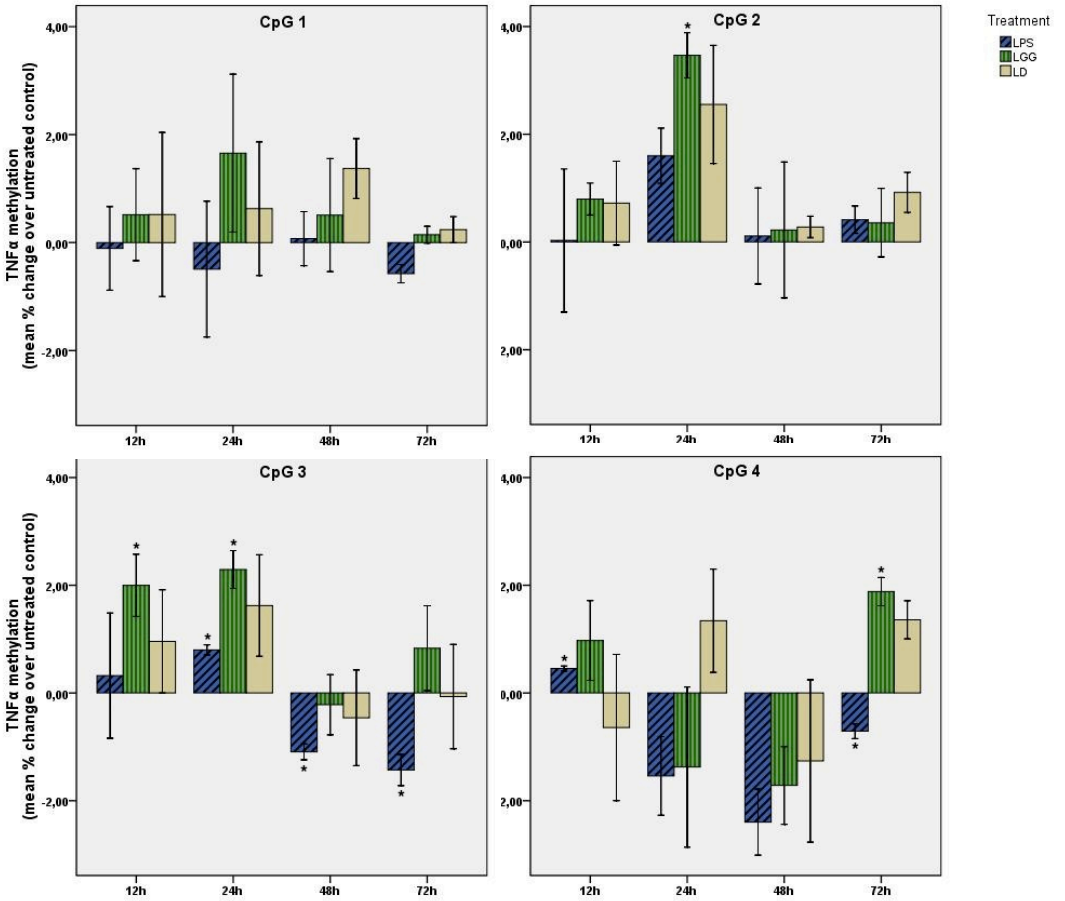


Table 6: Statistical analysis of TNFα DNA methylation

TNFα: % methylation change (treated-untreated)							
Treatment	Time of incubation		CpG 1	CpG 2	CpG 3	CpG 4	mean of 4 CpGs
LPS	12h	mean	-0.11%	+0.03%	+0.32%	**+0.45%***	+0.17%
		SE	0.77	1.33	1.16	0.05	0.78
		p-value	0.902	0.985	0.807	0.01	0.843
	24h	mean	-0.49%	+1.60%	**+0.80%***	-1.54%	+0.09%
		SE	1.26	0.51	0.09	0.73	0.44
		p-value	0.733	0.089	0.013	0.169	0.854
	48h	mean	+0.07%	+0.11%	**-1.09%***	-2.40%	-0.83%
		SE	0.50	0.89	0.15	0.61	0.34
		p-value	0.897	0.912	0.018	0.06	0.136
	72h	mean	-0.58%	+0.41%	**-1.43%***	**-0.71%***	**-0.58%***
		SE	0.17	0.25	0.29	0.14	0.05
		p-value	0.078	0.246	0.039	0.035	0.007
LGG	12h	mean	+0.52%	+0.80%	+2.00%	+0.98%	**+1.07%***
		SE	0.85	0.30	0.58	0.74	0.24
		p-value	0.606	0.116	0.074	0.318	0.048
	24h	mean	+1.66%	**+3.47%***	**+2.29%***	-1.38%	+1.51%
		SE	1.46	0.42	0.35	1.49	0.78
		p-value	0.375	0.014	0.023	0.453	0.191
	48h	mean	+0.51%	+0.22%	-0.22%	-1.72%	-0.30%
		SE	1.05	1.26	0.56	0.72	0.83
		p-value	0.674	0.876	0.734	0.141	0.753
	72h	mean	+0.15%	+0.36%	+0.83%	**+1.88%***	+0.81%
		SE	0.16	0.64	0.79	0.26	0.36
		p-value	0.447	0.63	0.402	0.019	0.155
LD	12h	mean	+0.52%	+0.72%	+0.96%	-0.64%	+0.39%
		SE	1.52	0.78	0.96	1.36	0.76
		p-value	0.79	0.524	0.5	0.719	0.7
	24h	mean	+0.63%	+2.55%	+1.62%	+1.34%	**+1.54%***
		SE	1.24	1.10	0.94	0.96	0.30
		p-value	0.662	0.145	0.227	0.296	0.035
	48h	mean	+1.37%	+0.28%	-0.46%	-1.26%	-0.02%
		SE	0.56	0.20	0.89	1.51	0.58
		p-value	0.132	0.297	0.656	0.491	0.978
	72h	mean	+0.24%	+0.92%	-0.07%	+1.36%	+0.61%
		SE	0.24	0.37	0.97	0.35	0.23
		p-value	0.428	0.132	0.951	0.062	0.116

TLR4-methylation:

For the TLR4 quantitative DNA-methylation analysis a 36 bp long sequence of the exon containing 4 CpG sites was investigated. Inserted control points (dispensation position 6, 28) indicate an efficient bisulfite-conversion (Figure 8). Methylation status of the analysed area was variable between cell experiments, ranging from approximately 10-20% in the first experiment to 35-45% in the last.

Caco-2-cells stimulated with LPS, LGG or LD for 12h, 24h, 48h or 72h showed almost no significant changes in DNA-methylation status of the tested TLR4 region compared to the untreated controls. The mean of all four CpGs (Figure 9, Table 7) revealed no significant changes. Overall no different effect between the probiotics and LPS could be determined. Only at the 72h time point all treatments seemed to exert methylation decreasing properties. Figure 10 and table 7 show, that CpG 4 was significantly increased by LPS treatment for 12h (+2.00%±0.42, p=0.041). After 72h of LGG treatment CpG 3 was decreased (-1.77%±0.29, p=0.027) and LPS treatment showed diminished CpG 2 methylation (-1.62%±0.26, p=0.024). No CpG was altered preferentially.

In general, no correlation between mRNA expression and methylation levels of TLR4 and TNFα could be found (Table 8). Only LGG treatment significantly influenced both, increasing DNA-methylation of 4 CpGs (+1.07%±0.24, p=0.048) in the TNFα promoter region and down-regulation of TNFα mRNA expression (0.73-fold±0.23, p=0.008).

The EMBOSS transcription factor prediction showed binding sites for transcription factors ER-alpha and c-Myb, whereas ER-α is partially overlapping with CpG 1 and c-Myb with both CpG 1 and 2 (Figure 11A).

Figure 8: Pyrogram of the analysed region of the TLR4 exon.
Selected CpGs (1-4 indicated by blue columns) within the TLR4 exon were analysed by the Pyrosequencing technology and one example of a Pyrogram from the Pyromark Q24 software is shown. Inserted control points (dispensation positions 6 and 28) are represented by yellow columns.

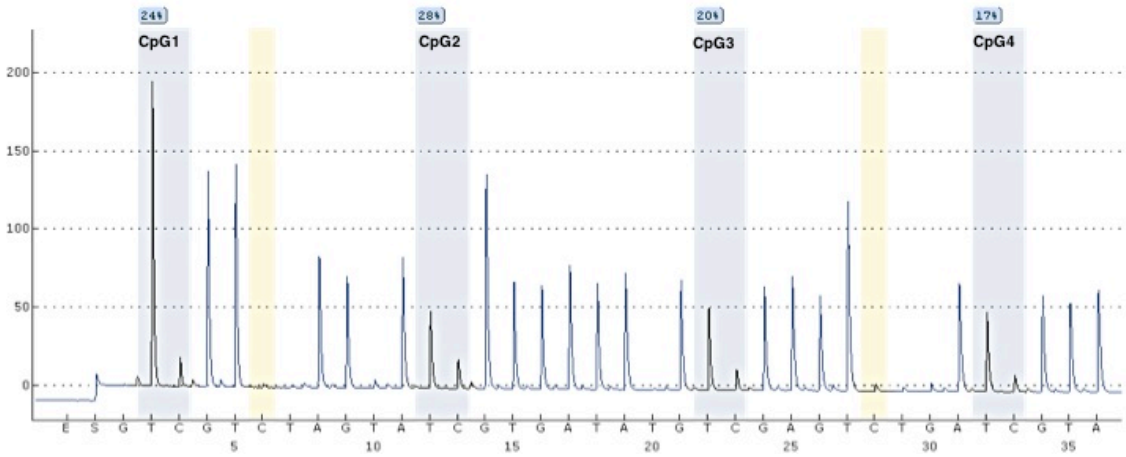


Figure 9: Effects of bacterial treatments on TLR4 DNA methylation over the mean of all four CpG loci.
Caco-II-cells were stimulated with LPS, LGG or LD for 12h, 24h, 48h and 72h and DNA methylation of 4 selected CpGs was measured by pyrosequencing. Data represents the mean % difference in methylation (±SE indicated by error bars) to an untreated control (treated – untreated) of the mean of all 4 CpGs. Statistical significance is indicated by asterisk.

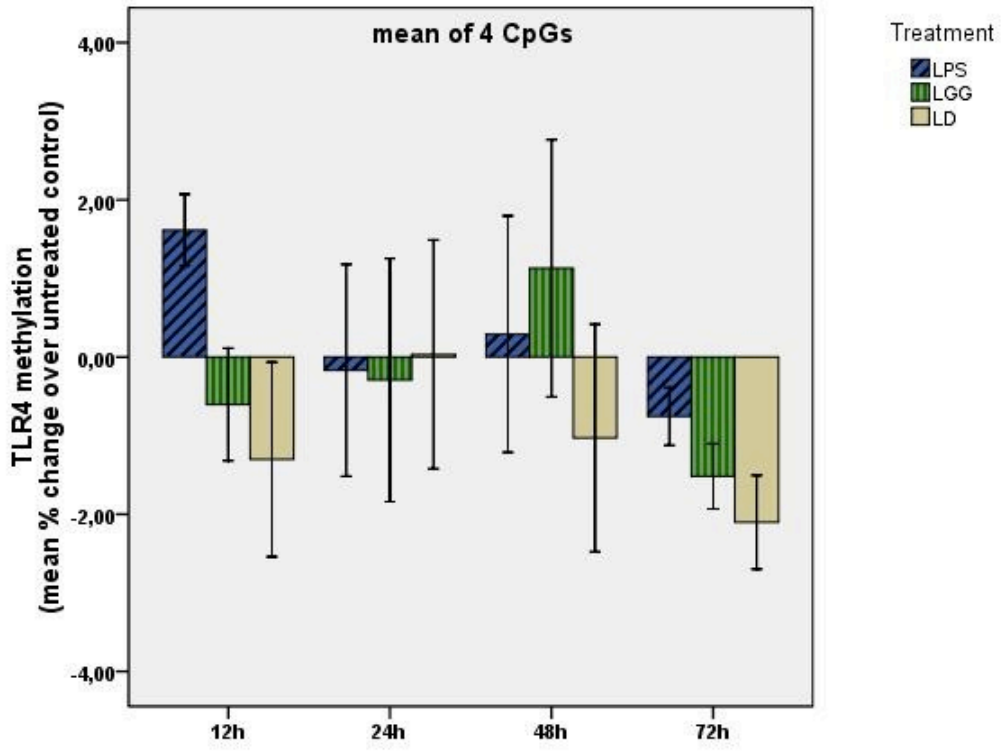


Figure 10: Effects of bacterial treatments on TLR4 DNA methylation of each of the four CpG loci.

Caco-II-cells were stimulated with LPS, LGG or LD for 12h, 24h, 48h and 72h and DNA methylation of 4 selected CpGs was measured by pyrosequencing. Data represents the mean % difference in methylation (±SE indicated by error bars) to an untreated control (treated – untreated) of CpG 1-4. Statistical significance is indicated by asterisk.

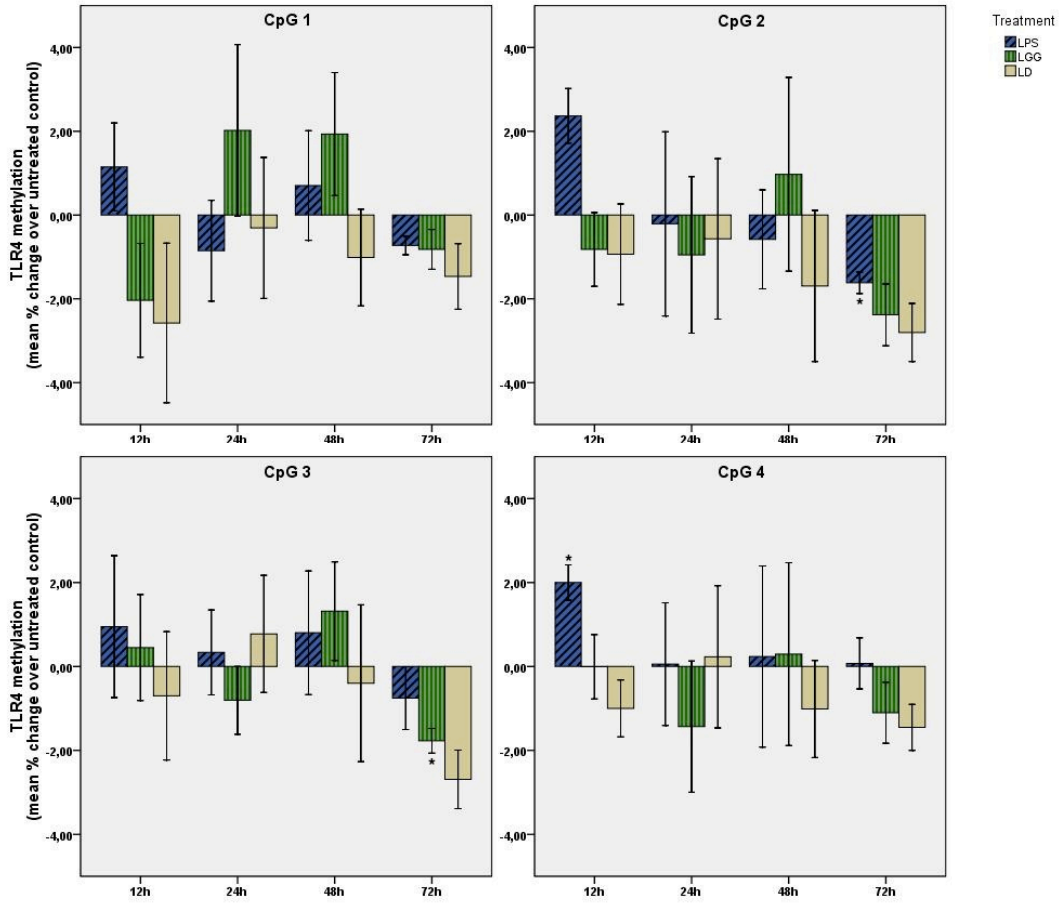


Table 7: Statistical analysis of TLR4 DNA methylation

TLR4: % methylation difference (treated-untreated)							
Treatment	Time of incubation		CpG 1	CpG 2	CpG 3	CpG 4	mean of 4 CpGs
LPS	12h	mean	+1.15%	+2.37%	+0.95%	**+2.00%***	+1.62%
		SE	1.05	0.65	1.69	0.42	0,46
		p-value	0.388	0.068	0.631	0.041	0.071
	24h	mean	-0.85%	-0.21%	0.34%	0.06%	-0.17%
		SE	1.20	2.20	1.01	1.46	1.35
		p-value	0.553	0.933	0.772	0.973	0.913
	48h	mean	+0.71%	-0.58%	+0.80%	+0.24%	+0.29%
		SE	1.31	1.18	1.47	2.16	1.51
		p-value	0.644	0.672	0.64	0.923	0.864
	72h	mean	-0.73%	**-1.62%***	-0.76%	+0.07%	-0.76%
		SE	0.22	0.26	0.75	0.61	0.37
		p-value	0.082	0.024	0.419	0.915	0.175
LGG	12h	mean	-2.04%	-0.82%	+0.45%	-0.01%	-0.60%
		SE	1.36	0.88	1.26	0.77	0.72
		p-value	0.273	0.449	0.756	0.994	0.489
	24h	mean	+2.02%	-0.95%	-0.81%	-1.43%	-0.29%
		SE	2.05	1.87	0.81	1.56	1.55
		p-value	0.428	0.661	0.423	0.456	0.868
	48h	mean	+1.93%	+0.97%	+1.32%	+0.30%	+1.13%
		SE	1.47	2.31	1.17	2.18	1.64
		p-value	0.318	0.715	0.378	0.905	0.561
	72h	mean	-0.82%	-2.38%	**-1.77%***	-1.10%	-1.52%
		SE	0.47	0.74	0.29	0.73	0.41
		p-value	0.226	0.084	0.027	0.269	0.067
LD	12h	mean	-2.58%	-0.93%	-0.70%	-1.00%	-1.30%
		SE	1.91	1.20	1.53	0.68	1.24
		p-value	0.309	0.518	0.692	0.278	0.403
	24h	mean	-0.31%	-0.57%	+0.78%	+0.23%	+0.03%
		SE	1.68	1.91	1.40	1.69	1.46
		p-value	0.872	0.795	0.634	0.904	0.984
	48h	mean	-1.01%	-1.69%	-0.40%	-1.01%	-1.03%
		SE	1.15	1.80	1.87	1.16	1.45
		p-value	0.473	0.447	0.85	0.473	0.55
	72h	mean	-1.47%	-2.80%	-2.69%	-1.45%	-2.10%
		SE	0.78	0.70	0.70	0.55	0.60
		p-value	0.202	0.056	0.061	0.118	0.072

Table 8: Mean % difference in DNA methylation of 4 CpGs versus mRNA expression of TLR4 and TNFα

Gene	Treatment (12h)	Methylation of 4 CpGs (mean % difference to control)	mRNA expression (mean $2^{-\Delta\Delta Ct}$)
TLR4	LPS	+1.62%	**0.69***
	LGG	-0.60%	**0.63***
	LD	-1.30%	**0.63***
TNFα	LPS	+0.17%	**0.82***
	LGG	**+1.07%***	**0.73***
	LD	+0.39%	**0.70***

Figure 11: Potential transcription factor binding sites in the analysed TNFα and TLR4 sequences

The transcription factors located by the EMBOSS algorithm are shown. Yellow bands indicate the length of the analysed regions of (**A**) TLR4 and (**B**) TNFα. Orange bands stand for potential transcription factor binding sites. By pyrosequencing analysed CpG loci are indicated by 'Y' within the sequence.

Discussion

The present study aimed to address gastrointestinal bacteria in the regulation of epigenetic mechanisms in an inflammatory context. The heat-inactivated gram-positive bacterial strains *Lactobacillus rhamnosus GG* (LGG) and *Lactobacillus delbrueckii* (LD) and the gram-negative bacterial cell wall components LPS and flagellin were used as stimulants for Caco-2-cells, a model cell line of human intestinal epithelial cells. The use of Caco-2-cell lines is widespread and thus allows comparison of experimental results with other studies, but as an *in vitro* model is only entitled to pioneer for further *in vivo* experiments. Usefulness and limitations of Caco-2-cells as a model for intestinal epithelial cells were discussed in the past [39]. As it is not yet completely understood, where exactly the specificity of probiotic interference in inflammatory cell signalling comes from, we chose to use the approach to apply non-living instead of live probiotic bacteria, in order to rule out possible influence of bacterial metabolites and concentrate exclusively on cell components. Additionally, it is of interest to determine, whether probiotics are able to exert their health improving properties also in the non-living condition, because of reports about potential risks accompanying administration of live organisms to certain populations, especially in sick patients [40] or very young individuals [41]. Although *in vivo* studies show higher activity of live compared to dead probiotics [42, 43], in vitro experiments indicate similar effects of both [44-46].

TNFα, TLR4, p38 mRNA expression:

Flagellin and LPS activate pro-inflammatory signalling by induction of IκB degradation and NFκB nuclear translocation [47], while probiotics seem to be able to diminish this effect, by IκB stabilisation [17, 18]. In this study, we could not see significant strain-specific influences in mRNA expression of TNFα, TLR4 and p38 after 12h of stimulation in Caco-2-cells. Different kinetics of mRNA expression might be responsible for strain-specific differences, and therefore were not detected at the chosen time point. TLR4 up-regulation by LPS treatment was demonstrated to display fast time-dependent kinetics under 60 min after induction in epithelial cells [48]. We observed a reduction of TNFα mRNA by commensal and probiotic bacteria, which was reported before and possibly reflects an anti-inflammatory effect [49]. mRNA levels of p38 were elevated by probiotics same as bacterial cell wall components in our data. The

phosphorylation of p38 MAPK was shown before to be enhanced upon treatment with probiotic bacteria and its inhibition leads to decreased production of chemokines and cytokines in intestinal epithelial cells indicating the importance of this signalling pathway in the regulation of immune responses [50].

miRNA-146a and -155 expression:

Posttranscriptional regulation of gene expression via miRNAs seems to play a key role in immune and inflammatory pathways [51]. Recently, results from our laboratory showed down-regulatory effects of *Lactobacillus rhamnosus GG* treatment on miRNA-146a in dendritic cells, while miRNA-155 expression was induced [21]. LPS treatment enhanced miRNA-146a and miRNA-155 expression, which was also found by another group to be the case in RAW264.7 macrophage cells [22]. In this study, we additionally report up-regulation of flagellin-induced miRNA-146a expression after 12h of incubation, but no alteration of miRNA-155 in our intestinal epithelial cell model. Similar results for miRNA-146a induction upon flagellin stimulation were also detected before at earlier time points in primary monocytes [23]. These results suggest that miRNA-146a and miRNA-155 play different roles in gut-bacteria associated signalling. Furthermore, miRNA-146 was suggested to operate via a negative feedback regulation loop in the control of TLR and cytokine signalling [52]. The observed up-regulation of miRNA-146a was accompanied by a down-regulation of TNFα mRNA after 12h of flagellin stimulation in our experiments. LPS stimulation seems to exhibit similar effects in alveolar macrophages of rats [53]. In the context of endotoxin tolerance miRNA-146a was also linked to transcription repression of pro-inflammatory genes [54, 55].

TNFα- methylation:

Genome-wide DNA methylome mapping suggests the involvement of DNA methylation of immuno-active gene regions in the epigenetic dys-regulation of biological pathways that participate in the pathogenesis of inflammation-associated diseases, such as ulcerative colitis [56], psoriasis [57] and rheumatoid arthritis [58]. However, the stimulants and mechanisms causing these changes are poorly understood. In this study, pyrosequencing was chosen for DNA methylation measurement, because of the high accuracy of the method and therefore its ability to detect even small up- or

down-regulations in DNA methylation. Our results from an intestinal epithelial cell model suggest that LPS and the two probiotic bacteria differentially induce changes in the DNA methylation status of four tested CpGs in the promoter region of the pro-inflammatory cytokine TNFα in a time-dependent manner. CpGs 3 and 4 were favourably altered in DNA methyltion, whereas CpG 1 remained mostly static. Different responsiveness of CpGs in certain regions for DNA methylation are under discussion to be involved in regulation of gene expression [59]. The mean percentage of all 4 CpGs was up-regulated at early time points by LGG (12h) and LD (24h), but down-regulated by LPS at late time points (72h).

Time-dependent interference in regulation mechanisms of gene expression might provide an explanation for strain- and species-specific properties of probiotic actions during inflammatory responses. Different effects of *Lactobacilli* strains on TNFα cytokine production were explored in immune cell models [60, 61], as well as intestinal epithelial cells [62]. Same as pathogens, probiotics modulate immune responses via cell signalling pathways, but cause diveres outcomes of inflammatory gene expression patterns [63]. The variety of patterns induced by different signalling approaches might explain observed strain-specific effects. Past studies reported, that some *Lactobacillus* strains engage into inflammatory signalling by inhibition of NFκB-signalling through stabilization of IκBα [17], others by their ability to activate MAP kinases [64], by suppression of pro-inflammatory cytokines via c-Jun [65] or by modulation of TLR2/TLR4 expression through an ERK-dependent pathway [66]. Expression of TLR9 mRNA is induced by both, *Lactobacillus rhamnosus GG* and *Lactobacillus plantarum,* in an inflammtory backround, but more strongly by LGG [67]. Variing inflammatory gene expression by probiotic strains might be due to this wide range in fine differences of affected signalling pathways, which probably also reflects on the epigenetic gene regulating machinery.

Our results suggest an early (12h, 24h) influence in TNFα DNA methylation of the gram-positive strains in an up-reguating direction, while LPS engages later (72h) into these processes in a down-regulating direction. A diminished LPS-induced expression of IL-17, IL-23 and CD40 was reported after treatment with *Lactobacillus rhamnosus GG* and *Bifidobacterium breve* being paralleled by DNA methylation enhancement, as well as decreased histone acetylation and nuclear translocation of NFκB [35]. Our

results show a similar effect on TNFα DNA methylation after 12h of treatment with LGG accompanied by a down-regulation of TNFα mRNA. DNA methylation has an influence on NFκB binding affinity and thus might alter the expression of NFκB regulated genes [68].

DNA demethylation after LPS treatment, was also reported before in other cell systems. Murine macrophage cells (RAW263.7) showed DNA demethylation at the GM-CSF and IL-2 promoter in a time-dependent manner after LPS stimulation [69]. In human monocytes (THP-1) LPS treatment caused DNA demethylation that correlated with enhanced TNFα production [34]. Responsible mechanisms for DNA demethylation are not yet fully unterstood, but were linked to down-regulated expression of DNA methyltransferases (DNMT1 and DNMT3a) due to activation of immune cells [70] or to age-dependent hypomethyation of T-cells [71]. mRNA expression can be regulated over different epigenetic mechanisms, that adjust each other. Methyl CpG binding proteins recruit histone deacetylases upon docking onto methylated DNA [72, 73]. The other way around, *de novo* DNA methylation was linked to the binding of DNA methyltransferases to H3 lysine 9 methyltransferases [74].

TLR4-methylation

Intestinal epithelial cells are continously exposed to LPS from gram-negative commensal bacteria, but have evolved a hyporesponsiveness to this bacterial cell wall component by expressing low levels of TLR4 and its co-receptor MD-2, in order to avoid chronic inflammation [75]. The repressed transcriptional activity was demonstrated to be due to epigenetic silencing via DNA methylation and histone deacetylation mechanisms at the MD-2 gene [36], as well as at the TLR4 gene [37]. Intestinal commensal bacteria seem to be involved in the maintenance of DNA methylation in large intestinal IECs of mice because germ-free mice show significantly lower methylation of the TLR4 gene, than conventional mice [37]. Our experiments did not display any significant differences in DNA methylation at the TLR4 promoter between LPS or probiotic treatment and untreated samples. We detected quite high overall differences in methylation percentage between independent cell experiments ranging from approximately 15% to 40%. This effect was stimulus-independent and might be due to the differentiation status of the cells, but could not be observed for

TNFα methylation. However, CpG 4 alone was significantly up-regulated by LPS treatment for 12h, simultaneous to down-regulation of TLR4 mRNA levels, which might be explained as an effect of epigenetic control. Nevertheless, TLR4 levels were also down-regulated by both probiotic treatments, but no significant change in DNA methylation was visible, although it was consistent over all four tested CpG positions. DNA-methylation-independent alteration in mRNA expression were also found in one *in vitro* study with human umbilical vein endothelial cells (HUVECs) showing increased TLR2 mRNA expression under inflammatory conditions, but no alteration in the DNA methylation pattern of the TLR2 promoter [76]. Positive correlations between transcriptional activity and DNA methylation were reported by genome-wide DNA methylome mapping studies [56, 57, 77], but remain unexplained by current understanding.

Transcription factor binding sites

The EMBOSS transcription factor prediction revealed a potential binding site of c-Jun transcription factor in the TNFα promotor region overlapping the analysed CpG 4. The DNA methylation at this position was significantly up-regulated by LGG and down-regulated by LPS treatment of caco-II-cells after 72h, suggesting reciprocal regulatory effects, which might manifest in differential binding affinity of the c-Jun transcription factor. Ets2 transcription factors can interact with c-Jun [78] and are also present in our analysed sequence. Amongst other cell processes c-Jun is involved in inflammatory responses and gets activated by MAP kinases such as JNK (c-Jun N-terminal-kinase), p38 and ERK [79, 80]. The effects of bacteria and their cell wall components on DNA methylation at CpGs, which overlap with transcription factor binding sites in promoter regions of inflammation-relevant genes, might play a role in the regulation of expression patterns and pro- or anti-inflammatory responses.

Conclusions

In conclusion, results from the present study suggest an important epigenetic control, which involves DNA methylation in the regulation of inflammatory molecules in an intestinal epithelial cell line. Bacterial cell wall components and commensal or probiotic strains might exhibit differences in the epigenetic control. LGG induces significant up-

regulation in DNA methylation of the TNFα promoter region and down-regulation of TNFα gene expression.

Protocols

Cell culture

Cell culture medium mixture:

- 450 ml DMEM medium (high glucose 4.5 g/l, with L-Glutamin)
- 50 ml heat inactivated foetal bovine serum
- 1 ml sodium pyruvate
- 5 ml penicillin/streptomycin

Passaging of cells:

- Remove the old medium, but be careful not to touch the cells, which are attached to one side of the bottle
- Add 2 ml PBS and slightly move the bottles in order to wash the cells
- Remove PBS
- Add 1 ml acutase and incubate for 10 min at 37°C (cells get detached from the bottle's surface
- Prepare a new bottles with 8 ml medium
- Add 4 ml medium to the old bottle and slightly shake it until all cells are detached from the bottle's surface
- Add 1 ml of the medium with the floating cells to the new bottle with 8 ml medium in it and put it in the incubator for re-growing of the cells
- Use the residual cells for the plating

Plating of cells:

- Transfer the residual cells to a centrifuge tube
- Centrifuge for 5 min at 600 g and 4°C
- Remove supernatant by not disturbing the cell pellet
- Resuspend the pellet in 1 ml PBS
- Count the number of cells contained in the cell suspension with the Countess and note live and viability
- Prepare 6-well-plates by adding 3 ml medium to each well

- Plate about 300 000 cells per well
- Put 6-well-plates in the incubator (37°C, 5% CO_2) and start stimulation experiments at 70-80% confluency

RNA-extraction: *mir*Vana™ miRNA Isolation Kit

Prepare the Wash Solutions

Add 21 ml ethanol to miRNA Wash Solution 1:

Add 21 ml of ACS grade 100% ethanol to the bottle labelled miRNA Wash Soln 1. Mix well. Place a check mark in the empty box on the label to indicate that ethanol has been added.

Add 40 ml ethanol to Wash Solution 2/3:

Add 40 ml of ACS grade 100% ethanol to the bottle labeled Wash Solution 2/3. Mix well. Place a check mark in the empty box on the label to indicate that the ethanol has been added.

Note: A precipitate may form in the Wash Solution 2/3 bottle over the next several days as excess EDTA falls out of solution. Simply leave these crystals in the bottle when removing Wash Solution for use.

Equipment preparation

Lab bench and pipettors:

Before working with RNA, it is always a good idea to clean the lab bench, and pipettors with an RNase decontamination solution (for example, Ambion RNaseZap. Solution).

Gloves and RNase-free technique:

Wear laboratory gloves at all times during this procedure and change them frequently. They will protect you from the reagents, and they will protect the RNA from nucleases that are present on your skin. Use RNase-free pipette tips to handle the wash solutions and the Elution Solution, and avoid putting used tips into the kit reagents.

Preparing equipment:

The equipment used for tissue disruption/homogenization should be washed well with detergent and rinsed thoroughly. Baking is unnecessary, because the Lysis/Binding Solution will inactivate most RNase contamination. If samples are to be ground in a mortar and pestle, prechill the equipment in dry ice or liquid nitrogen.

Sample Type and Amount

Sample type:

This procedure is designed for small scale RNA isolation from plant and animal tissue or cultured cells, bacteria, yeast, viral particles, or enzyme reactions. It can be used with fresh or frozen cultured mammalian cells, or cells stored in RNAlater or RNAlater-ICE. It can also be used with fresh, frozen, or RNAlater-stored animal tissues.

Sample amount:

Samples of 10^2–10^7 cultured eukaryotic cells or 0.5–250 mg of tissue can be processed per prep. However, if the initial lysate volume exceeds 300 µl, the samples will need to be processed in multiple loads due to limitations of filter capacity.

Cell Lysis and Tissue Disruption

1. Collect 10^2–10^7 cells or 0.5–250 mg tissue; wash cells in cold PBS:

- Cultured mammalian cells: ideally cells in culture should be processed fresh (i.e. not frozen). If you need to store cells before RNA isolation, they can be stored in RNAlater, or they can be pelleted, frozen in liquid nitrogen, and stored at –70°C or colder.

  Suspension cells: Count the cells, then pellet 10^2–10^7 cells at low speed, and discard the culture medium. Wash the cells by resuspending in ~1 ml PBS, and repelleting. Place the washed cells on ice.

  Adherent cells: do one of the following

  • Aspirate and discard the culture medium, and rinse with PBS. Place the culture plate on ice.

  • Trypsinize cells to detach them and count. Then inactivate the trypsin, pellet the cells, and discard the supernatant (following the method employed in your lab for the cell type). Wash the cells by gently resuspending in ~1 ml PBS, and pelleting at low speed. Place the cells on ice.
- Tissue samples: for good yield of intact RNA, it is very important to obtain tissue quickly and to limit the time between obtaining tissue samples and inactivating RNases in step c below.

  a. Harvest tissue and remove as much extraneous material as possible, for example remove adipose tissue from heart, and remove gall bladder from liver.

Perfuse the tissue with cold PBS, if desired, to eliminate some of the red blood cells.

b. If necessary, quickly cut the tissue into pieces small enough for either storage or disruption. Weigh the tissue sample (for samples to be stored in RNAlater, this can be done later).

c. Inactivate RNases by one of the following methods:

• Drop the sample into RNAlater—tissue must be cut to ≤0.5 cm in at least one dimension for good penetration of the RNAlater.

• Disrupt the sample in Lysis/Binding Buffer as described in step Fresh (unfrozen) tissue.

• Freeze the sample in liquid nitrogen—tissue pieces must be small enough to freeze in a few seconds. When the liquid nitrogen stops churning, it indicates that the tissue is completely frozen. Once frozen, remove the tissue from the liquid nitrogen and store it in an airtight container at –70°C or colder.

2. Disrupt samples in 300–600 µl Lysis/Binding Buffer

- Cultured cells:

  a. Remove the PBS wash or the RNAlater, and add 300–600 µl Lysis/Binding Solution for 100–10^7 cells. Cells will lyse immediately upon exposure to the Lysis/Binding Solution. Use the low end of the range (~300 µl) for small numbers of cells (hundreds), and use closer to 600 µl when isolating RNA from larger numbers of cells (thousands–millions). For adherent cells lysed directly in the culture plate, collect the lysate with a rubber spatula, and pipet it into a tube.

  b. Vortex or pipet vigorously to completely lyse the cells and to obtain a homogenous lysate. Cell cultures typically do not require mechanical homogenization; however, it will not damage the RNA. Large frozen cell pellets (i.e. more than about 10^7 cells) may need to be ground to a powder as described for frozen tissue samples to isolate high quality RNA. Alternatively, samples may be transitioned to –20°C in RNAlater-ICE (P/N AM7030) and processed as described in step 2.a above.

  c. Proceed to section Organic Extraction.

- Yeast or bacterial cultures: use disruption techniques appropriate for yeast and bacterial cultures, which may require vigorous mechanical disruption.

- Solid tissue stored in Ambion RNAlater Solution, or transitioned to –20°C in RNAlater-ICE Solution: samples in RNAlater and RNAlater-ICE solution can usually be homogenized by following the instructions for fresh tissue (below). Extremely tough/fibrous tissues in RNAlater may need to be frozen and pulverized according to the instructions for frozen tissue in order to achieve good cell disruption. Blot excess liquid from samples, and weigh them before following the instructions for fresh tissue below.
- Fresh (unfrozen) tissue

  Important: Use at least a 1:10 ratio (w/v) of tissue to Lysis/Binding Buffer for all tissues.

  a. Measure or estimate the weight of the sample.

  b. Aliquot 10 volumes per tissue mass of Lysis/Binding Buffer into a homogenization vessel on ice (e.g. aliquot 1 mL Lysis/Binding Buffer for 0.1 g tissue). Keeping the sample cold, thoroughly disrupt the tissue in Lysis/Binding Buffer using a motorized rotor-stator homogenizer. A ground-glass homogenizer or a plastic pestle can be used for small pieces (≤10 mg) of soft tissue. Homogenize until all visible clumps are dispersed.

  c. Proceed to section Organic Extraction.
- Frozen tissue, and extremely hard tissues (Frozen tissue transitioned to –20°C in RNAlater-ICE: process as for fresh tissue). Once the tissue has been removed from the –70°C freezer, it is important to process it immediately without even partial thawing. This is necessary because as cells thaw, ice crystals rupture both interior and exterior cellular compartments, releasing RNases.

  a. Measure or estimate the weight of the sample.

  b. Place 10 volumes of Lysis/Binding Buffer per tissue mass into a plastic weigh boat or tube on ice. We suggest using a weigh boat because it is easier to transfer frozen powdered tissue to a weigh boat than to a tube of Lysis/Binding Buffer.

  c. Grind frozen tissue to a powder with liquid nitrogen in a prechilled mortar and pestle sitting in a bed of dry ice.

  d. Using a prechilled metal spatula, scrape the powdered tissue into the Lysis/Binding Buffer, and mix rapidly

  e. Transfer the mixture to a vessel for homogenization and process the mixture

to homogeneity, i.e. until all visible clumps are dispersed. If available, use a motorized rotor-stator homogenizer (e.g. Polytron).

f. Proceed to section Organic Extraction below.

Organic Extraction

Include this organic extraction both for total RNA purification and for purification of small RNAs.

1. Add 1/10 volume of miRNA Homogenate Additive, incubate 10 min on ice

- Add 1/10 volume of miRNA Homogenate Additive to the cell or tissue lysate (or homogenate), and mix well by vortexing or inverting the tube several times. For example, if the lysate volume is 300 µl, add 30 µl miRNA Homogenate Additive.
- Leave the mixture on ice for 10 min.

2. Extract with a volume of Acid-Phenol: Chloroform equal to the initial lysate volume

- Add a volume of Acid-Phenol:Chloroform that is equal to the lysate volume before addition of the miRNA Homogenate Additive. For example, if the original lysate volume was 300 µl, add 300 µl Acid-Phenol:Chloroform.
- *Important:* Be sure to withdraw from the bottom phase in the bottle of Acid-Phenol:Chloroform, because the upper phase consists of an aqueous buffer.
- Vortex for 30–60 sec to mix.
- Centrifuge for 5 min at maximum speed (10,000 x g) at room temperature to separate the aqueous and organic phases. After centrifugation, the interphase should be compact; if it is not, repeat the centrifugation.

3. Recover the aqueous phase; transfer the aqueous phase to a fresh tube

- Carefully remove the aqueous (upper) phase without disturbing the lower phase, and transfer it to a fresh tube. Note the volume removed.

Final RNA Isolation

Choice of elution solution

At the end of this procedure, RNA can be eluted in either nuclease-free water or in the Elution Solution provided with the kit. Elution Solution is nuclease-free 0.1 mM EDTA,

if this could interfere with your application, elute in nuclease-free water instead.

Pre-heat eluent to 95°C

Preheat Elution Solution or nuclease-free water to 95°C for use in eluting the RNA from the filter at the end of the procedure.

100% ethanol must be at room temperature

If the 100% ethanol you plan to use for this procedure is stored cold, warm it to room temperature before starting the Final RNA Isolation.

Total RNA Isolation Procedure

1. Add 1.25 volumes 100% ethanol, and mix thoroughly

Add 1.25 volumes of room temperature 100% ethanol to the aqueous phase (e.g. if 300 µl was recovered in the Organic Extraction step, add 375 µl ethanol).

2. Pass the lysate/ethanol mixture through a Filter Cartridge

- For each sample, place a Filter Cartridge into one of the Collection Tubes supplied.
- Pipet the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. Up to 700 µl can be applied to a Filter Cartridge at a time, for samples larger than this, apply the mixture in successive applications to the same filter.
- Centrifuge for ~15 sec to pass the mixture through the filter. Centrifuge at RCF 10.000 x g (typically 10.000 rpm). Spinning harder than this may damage the filters. Alternatively, vacuum pressure may be used to pass samples through the filter.
- Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter. Reuse the Collection Tube for the washing steps.

3. Wash the filter with 700 µl miRNA Wash Solution 1

Apply 700 µl miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~5–10 sec or use a vacuum to pull the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

4. Wash the filter twice with 500 µl Wash Solution 2/3

- Apply 500 µl Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.

- Repeat with a second 500 µl aliquot of Wash Solution 2/3.
- After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min to remove residual fluid from the filter.

5. Elute RNA with 100 µl 95°C Elution Solution or Nuclease-free Water

Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µl of pre-heated (95°C) Elution Solution or nuclease-free water to the center of the filter, and close the cap. Spin for ~20–30 sec at maximum speed to recover the RNA. Collect the eluate (which contains the RNA) and store it at –20°C or below.

Reverse Transcription mRNA: Finnzymes Phusion® RT-PCR Kit

Guidelines for reverse transcription

- Use gloves and RNase-free plasticware to prevent RNase contamination.
- Prepare premixes to avoid pipetting very small volumes.
- Pipet all components on ice.
- Reaction volume in cDNA synthesis is 20 µl.
- Use up to 1 µg of RNA template. The minimum amount depends on both the template and the primers used.
- Recommended primer amounts in a 20 µl reaction:
    - 100 ng oligo(dT) primers (can be increased up to 1 µg) or
    - 50 ng random primers (may require optimization) or
    - 5 pmol (2–10 pmol) gene-specific primers.
- When determining the amount of RNA template, the expression level of the target RNA molecule should be considered, as it affects the subsequent PCR step. The volume of the cDNA reaction mixture used as a source for template in PCR should not exceed 10 % of the final PCR reaction volume. A high RNA concentration in the PCR may also inhibit the reaction.

Procedure

It is recommended that control reactions be performed in parallel with all experiments. Control RNA and primers are provided with the kit.

1. Thaw template RNA, 10x RT buffer, dNTPs and primers. Mix the individual solutions to assure homogeneity and centrifuge briefly before pipetting.

2. Combine the following components in reaction tubes

- Template RNA x µl (up to 1 µg)
- 10 mM dNTP mix 1 µl
- Oligo(dT) primer 1 µl
- RNase-free H2O Add to 10 µl

3. Incubate at 65°C for 5 minutes to predenature the RNA.

4. Place the reaction tubes on ice and add to each tube

- 10x RT buffer 2 µl
- RT enzyme mix 2 µl
- RNase-free H2O 6 µl

5. Program a thermal cycler as outlined in the table below.

Step	Temperature	Time
Primer extension	25°C	10 min
cDNA synthesis	40°C	30 min
Reaction termination	85°C	5 min
Cooling of the sample	4°C	Hold

6. Place the tubes in the cycler and start the program.

miRNA Reverse Transcription and PCR-Amplification: TaqMan® MicroRNA Assays

Reverse Transcription

RNA Template Guidelines

For the optimal performance of the TaqMan MicroRNA Reverse Transcription Kit and of TaqMan MicroRNA Assays, Applied Biosystems recommends using RNA with the following characteristics:

- Free of inhibitors of reverse transcription and PCR
- Dissolved in PCR-compatible buffer
- Free of RNase activity
- Nondenatured
- *Important:* Do not denature the RNA. Denaturation of the RNA may reduce the yield of cDNA for some miRNA targets.

Per Reaction Input Amount of Total RNA

Use 1 to 10 ng of total RNA per 15- µl RT reaction.

Preparing the RT Reaction Master Mix

Prepare RT master mix using the TaqMan MicroRNA Reverse Transcription Kit components before preparing the reaction.

CHEMICAL HAZARD

10× RT Buffer may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Reverse Transcription Procedure

1. Allow the kit components to thaw on ice.

2. In a polypropylene tube, prepare the RT master mix by scaling the volumes listed below to the desired number of RT reactions. Applied Biosystems recommends adding 10 to 20% overage to account for pipetting losses.

Important: This procedure assumes that you are quantifying miRNA from a single total RNA sample.

Component	Master Mix Volume/ 15-µL Reaction[a]
100mM dNTPs (with dTTP)	0.15
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00
10X Reverse Transcription Buffer	1.50
RNase Inhibitor, 20U/µL	0.19
Nuclease-free water	4.16
Total	7.00

a. Each 15-µL RT reaction consists of 7 µL master mix, 3 µL primer, and 5 µL RNA sample.

3. Mix gently. Centrifuge to bring solution to the bottom of the tube.

4. Place the RT master mix on ice until you prepare the microRNA reaction.

5. For each 15-µl RT reaction, combine RT master mix (from step 2) with total RNA in the ratio of: 7 µl RT master mix to 5 µl total RNA

For example, combine 7.7 µl of RT master mix with 5.5 µl of total RNA. Remember to include the same proportion of excess volume of total RNA that you did for the RT master mix. In this example, a 10% excess volume was included for both RT master mix and total RNA.

Note: Applied Biosystems recommends that you use 1 to 10 ng of total RNA per reaction.

6. Mix gently. Centrifuge to bring the solution to the bottom of the tube.

Important: Do not exceed 2000 RPM or 5 minutes when centrifuging.

7. Before opening the RT Primer tubes, thaw the tubes on ice and mix by vortexing, then centrifuge them.

8. For each 15-µl RT reaction, dispense 12.0 µl of RT master mix containing total RNA (from step 5) into a 0.2-ml polypropylene reaction tube. (This is the RT reaction tube.)

Note: Alternatively, you may dispense into a single well of a 96-well reaction plate.

9. Transfer 3 µl of RT primer (tube labeled RT Primer) from each assay set into the corresponding RT reaction tube or plate well.

10. Seal the tube and mix gently. Centrifuge to bring solution to the bottom of the tube.

11. Incubate the tube on ice for 5 min and keep on ice until you are ready to load the thermal cycler.

12. Leaving the thermal cycler in the 9600 Emulation mode (default), use the following parameter values to program the thermal cycler:

Step Type	Time (min)	Temperature (°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

13. Set the reaction volume to 15.0 µl.

14. Load the reaction tube or plate into the thermal cycler.

15. Start the reverse transcription run.

PCR Amplification

Reagent Preparation Guidelines

Following these guidelines ensures optimal PCR performance:

- Keep all TaqMan MicroRNA Assays protected from light, in the freezer, until you are ready to use them. Excessive exposure to light may affect the fluorescent probes.
- Prior to use, mix the TaqMan Universal PCR Master Mix thoroughly by swirling the bottle.
- Prepare the PCR reaction mix before transferring to the reaction plate for thermal cycling and fluorescence analysis.

PCR Reaction Components

Applied Biosystems recommends performing four PCR replicates per RT reaction. The recommended reaction volume is 20 µl. Prepare the plate so that each PCR reaction contains the components as listed in the following table.

Component	Volume (µL) / 20-µL Reaction
TaqMan MicroRNA Assay (20X)	1.00
Product from RT reaction (Minimum 1:15 Dilution)	1.33
TaqMan 2X Universal PCR Master Mix, No AmpErase UNG[a]	10.00
Nuclease-free water	7.67
Total Volume	**20**

a. For optimal performance of TaqMan MicroRNA Assays, Applied Biosystems strongly recommends that you use Applied Biosystems TaqMan 2X Universal PCR Master Mix, No AmpErase UNG.

CHEMICAL HAZARD

TaqMan 2× Universal PCR Master Mix, No AmpErase UNG may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

PCR Amplification Procedure

1. Scale the volumes listed below to the appropriate number of RT reactions. Applied Biosystems recommends including four replicates per RT reaction. Prepare on ice.

Reagent	Master Mix Volume for One 20-µL Reaction
TaqMan 2X Universal PCR Master Mix, No AmpErase UNG	10.00
Nuclease-free water	7.67
Total Volume	**17.67**

2. Mix gently. Centrifuge to bring solution to the bottom of the tube.

3. Add 17.67 µl of the PCR master mix/water mixture per 20-µl PCR reaction into a polypropylene tube (the PCR reaction tube), as shown in the following example.

Volume for One 20-µL Reaction	Example: Volume for 4 replicates[a]
17.67	17.67 µL x 4 replicates = 70.68 µL + 8.8 µL excess = 79.48 µL

a. Calculation includes 12.5% extra volume to account for pipetting losses. Keep this extra volume proportional with the extra volume in the next two steps.

4. Transfer 1.0 µl of 20× TaqMan MicroRNA Assay mix (labeled Real Time) into the PCR Reaction tube, as shown in the following example.

Volume for One 20-µL Reaction	Example: Volume for 4 replicates[a]
1.0	1.0 µL x 4 replicates = 4 µL + 0.5 µL excess = 4.5 µL

a. Calculation includes 12.5% extra volume to account for pipetting losses.

5. Transfer 1.33 µl of the RT product from the RT reaction tube into the PCR reaction tube, as shown in the following example.

Volume for One 20-µL Reaction	Example: Volume for 4 replicates[a]
1.33	1.33 µL x 4 replicates = 5.32 µL + 0.68 µL excess = 6.0 µL

a. Calculation includes 12.5% extra volume to account for pipetting losses.

6. Mix gently. Centrifuge to bring solution to the bottom of the plate.

7. Prepare the PCR reaction plate by dispensing 20 µl of the complete PCR master mix (including primer and RT product) into each of four wells.

8. Seal the plate with an optical adhesive cover, then centrifuge the plate briefly to spin down the contents and eliminate any air bubbles.

9. Setting Up the Plate Document: Refer to the appropriate instrument user guide for instructions on how to configure the plate document.

When creating plate documents, use the following parameters:

<table>
<tr><th>Parameter</th><th>Value</th></tr>
<tr><td>Run Mode</td><td>9600 emulation (Default)</td></tr>
<tr><td>Sample Volume</td><td>20 µL</td></tr>
<tr><td>Thermal Cycling Parameters</td><td>
<table>
<tr><th rowspan="3">Step</th><th>AmpliTaq Gold® Enzyme Activation</th><th colspan="2">PCR</th></tr>
<tr><td>HOLD</td><td colspan="2">CYCLE (40 cycles)</td></tr>
<tr><td></td><td>Denature</td><td>Anneal/ Extend</td></tr>
<tr><td>Time</td><td>10 min</td><td>15 sec</td><td>60 sec</td></tr>
<tr><td>Temp (°C)</td><td>95</td><td>95</td><td>60</td></tr>
</table>
</td></tr>
<tr><td>Auto Increment Settings</td><td>Accept default values. (Default is 0.)</td></tr>
<tr><td>Ramp Rate Settings</td><td>Accept default values. (Default is Standard.)</td></tr>
<tr><td>Data Collection</td><td>Accept default values. (Default is 60 °C.)</td></tr>
</table>

10. Run the plate

DNA-Extraction: QIAamp® DNA Mini Kit

Preparation

Important points before starting

- Do not use more than 5 x 10^6 cells (with a normal set of chromosomes).
- All centrifugation steps are carried out at room temperature (15–25°C).
- Use carrier DNA if the sample contains <10,000 genome equivalents.

Things to do before starting

- Heat a water bath or heating block to 56°C.
- Equilibrate Buffer AE or distilled water to room temperature for elution.
- QIAGEN Protease stock solution (store at 2–8°C or –20°C): when using the QIAamp DNA Blood Mini Kit (50), pipet 1.2 ml protease solvent* into the vial containing lyophilized QIAGEN Protease, as indicated on the label. When using the QIAamp DNA Blood Mini Kit (250), pipet 5.5 ml protease solvent into the vial containing lyophilized QIAGEN Protease, as indicated on the label. Dissolved QIAGEN Protease is stable for up to 2 months when stored at 2–8°C. Storage at –20°C is recommended to prolong the life of QIAGEN Protease, but repeated freezing and thawing should be avoided. For this reason, storage of aliquots of QIAGEN Protease is recommended.
- Buffer AL (store at room temperature, 15–25°C): mix Buffer AL thoroughly by shaking before use. Buffer AL is stable for 1 year when stored at room temperature.

  Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.
- Buffer AW1 (store at room temperature, 15–25°C): Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) as indicated on the bottle. Buffer AW1 is stable for 1 year when stored closed at room temperature.
- Buffer AW2* (store at room temperature, 15–25°C): Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96–100%) to Buffer AW2 concentrate as indicated on the bottle. Buffer AW2 is stable for 1 year when stored closed at room temperature.
- Carrier DNA: use carrier DNA (e.g., poly dA, poly dT, poly dA:dT) when the

sample is low-copy (i.e., when <10,000 copies are present). For preparation of DNA from free viral particles in fluids or suspensions (other than urine) using the Blood and Body Fluid protocols, we recommend the addition of 1 µl of an aqueous solution containing 5–10 µg of carrier DNA (e.g., poly dA, poly dT, poly dA:dT) to 200 µl Buffer AL. To ensure binding conditions are optimal, increase the volume of ethanol added at step 6 from 200 µl to 230 µl. Elution should be in 60 µl Buffer AE.

- If a precipitate has formed in Buffer AL, dissolve by incubating at 56°C.

Procedure

1. Harvest cells according to step 1a (for cells grown in suspension) or 1b (for cells grown in a monolayer).

- **1a.** Cells grown in suspension (do not use more than 5 x 10^6 cells with a normal set of chromosomes): Determine the number of cells. Centrifuge the appropriate number of cells for 5 min at 300 x g in a 1.5 ml microcentrifuge tube. Remove the supernatant completely and discard, taking care not to disturb the cell pellet. Continue with step 2.
- **1b.** Cells grown in a monolayer (do not use more than 5 x 10^6 cells with a normal set of chromosomes): Cells grown in a monolayer can be detached from the culture flask by either trypsinization or using a cell scraper.

  To trypsinize cells: Determine the number of cells. Aspirate the medium and wash cells with PBS. Aspirate the PBS, and add 0.10–0.25% trypsin. After cells have detached from the dish or flask, collect them in medium, and transfer the appropriate number of cells (do not use more than 5 x 10^6 cells with a normal set of chromosomes) to a 1.5 ml microcentrifuge tube. Centrifuge for 5 min at 300 x g. Remove the supernatant completely and discard, taking care not to disturb the cell pellet. Continue with step 2.

  Using a cell scraper: Detach cells from the dish or flask. Transfer the appropriate number of cells (do not use more than 5 x 10^6 cells with a normal set of chromosomes) to a 1.5 ml microcentrifuge tube and centrifuge for 5 min at 300 x g. Remove the supernatant completely and discard, taking care not to disturb the cell pellet. Continue with step 2.

2. Resuspend cell pellet in PBS to a final volume of 200 µl and add 20 µl QIAGEN Protease or proteinase K.

3. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.

In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. If the sample volume is larger than 200 µl, increase the amount of QIAGEN Protease (or proteinase K) and Buffer AL proportionally; for example, a 400 µl sample will require 40 µl QIAGEN Protease (or proteinase K) and 400 µl Buffer AL. If sample volumes larger than 400 µl are required, use of QIAamp DNA Blood Midi or Maxi Kits is recommended; these can process up to 2 ml or up to 10 ml of sample, respectively.

Note: Do not add QIAGEN Protease or proteinase K directly to Buffer AL.

4. Incubate at 56°C for 10 min.

DNA yield reaches a maximum after lysis for 10 min at 56°C. Longer incubation times have no effect on yield or quality of the purified DNA.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

If the sample volume is greater than 200 µl, increase the amount of ethanol proportionally; for example, a 400 µl sample will require 400 µl of ethanol.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Close each spin column in order to avoid aerosol formation during centrifugation. Centrifugation is performed at 6000 x g (8000 rpm) in order to reduce noise. Centrifugation at full speed will not affect the yield or purity of the DNA. If the lysate has not completely passed through the column after centrifugation, centrifuge again at higher speed until the QIAamp Mini spin column is empty.

Note: When preparing DNA from buffy coat or lymphocytes, centrifugation at full speed is recommended to avoid clogging.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

It is not necessary to increase the volume of Buffer AW1 if the original sample volume is larger than 200 µl.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µl Buffer AE will increase yields by up to 15%. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield. For samples containing less than 1 µg of DNA, elution in 50 µl Buffer AE or water is recommended. Eluting with 2 x 100 µl instead of 1 x 200 µl does not increase elution efficiency. For long-term storage of DNA, eluting in Buffer AE and storing at –20°C is recommended, since DNA stored in water is subject to acid hydrolysis.

Bisulfite Conversion: EpiTect® Bisulfite Kit

Important Notes

- Yield and size of DNA: The yield of DNA purified after bisulfite conversion depends on the amount of DNA and source of the starting material. Using the standard protocol, the EpiTect Bisulfite Kit is suited for DNA input amounts ranging from 1 ng to 2 µg, with high levels of DNA recovery throughout this range. The size of the template DNA can vary between 500 bp (in laser microdissections) and 30 kb (fresh samples or blood). DNA purified from serum, urine, or FFPE tissue may be <500 bp in length. If purifying bisulfite-treated DNA originating from very small sample amounts or that is very fragmented (e.g., from biopsies or FFPE tissues), we strongly recommend adding carrier RNA to Buffer BL.

  Note: The purified sample will contain considerably more carrier RNA than DNA. Carrier RNA does not influence downstream applications.
- Starting material: The bisulfite conversion also depends on the nature of DNA used as starting material. Genomic DNA should be used for bisulfite treatment without any previous restriction digest step. If working with plasmid DNA, please linearize the DNA first due to the very quick reannealing of the single-stranded DNA after the denaturation step.
- Handling of EpiTect spin columns: Due to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling EpiTect spin columns to avoid crosscontamination between sample preps:
  - Carefully pipet the sample or solution into the EpiTect spin column without wetting the rim of the column. Avoid touching the EpiTect spin column membrane with the pipet tip.
  - Always change pipet tips between liquid transfers. We recommend the use of aerosol-barrier pipet tips.
  - Open one EpiTect spin column at a time, and take care to avoid generating aerosols.
  - Wear gloves throughout the entire procedure. In case of contact between

gloves and sample, change gloves immediately.

- Centrifugation: EpiTect spin columns will fit into most standard 1.5–2 ml microcentrifuge tubes. A set of 2 ml collection tubes is supplied for the dry centrifugation step. If required, additional 2 ml collection tubes can be purchased separately (cat. no. 19201). All centrifugation steps should be carried out at room temperature (15–25°C).
- Processing EpiTect spin columns in a microcentrifuge
  - Always close EpiTect spin columns before placing them in the microcentrifuge.
  - For efficient parallel processing of multiple samples, we recommend filling a rack with the collection tubes into which EpiTect spin columns can be transferred after centrifugation. Collection tubes can be used several times.

Important points before starting

- Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, dissolved Bisulfite Mix can be stored at –20°C for up to 4 weeks without any loss of performance.
- 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.
- Perform all centrifugation steps at room temperature (15–25°C).

Things to do before starting

- Add 30 ml ethanol (96–100%) to Buffer BW and store at room temperature (15–25°C). Invert the bottle several times before starting the procedure.
- Add 27 ml ethanol (96–100%) to Buffer BD and store at 2–8°C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the EpiTect spin column.

- Add 310 µl RNase-free water to the lyophilized carrier RNA (310 µg) to obtain a 1 µg/µl solution. Dissolve the carrier RNA thoroughly by vortexing. When processing 48 samples at once, add the complete volume of dissolved carrier RNA to the bottle of Buffer BL, and check the box on the bottle lid label. If processing fewer samples, split the dissolved carrier RNA into conveniently sized aliquots (e.g., 50 µl) and store at –20°C. Aliquots can be stored for up to 1 year. If fewer than 48 conversions will be performed in a 2-week period, then only make up enough Buffer BL–carrier RNA solution as required (see Table below). Carrier RNA enhances binding of DNA to the EpiTect spin-column membrane, especially if there are very few target molecules in the sample. Carrier RNA is not necessary if >100 ng DNA is used.

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of carrier RNA solution†	6.2 µl	25 µl	50 µl	100 µl	150 µl	310 µl

* The volumes given contain a 10% surplus for pipetting inaccuracies.

† Resulting in a final concentration of 10 µg/ml carrier RNA in Buffer BL.

- Add dissolved carrier RNA to Buffer BL. Calculate the volume of Buffer BL and dissolved carrier RNA required for the number of samples to be processed. If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation. Equilibrate samples and buffers to room temperature.
- Optional: Set a thermomixer, heating block, or heated orbital incubator to 60°C.

Procedure

Bisulfite DNA conversion

1. Thaw DNA to be used in the bisulfite reactions. Dissolve the required number of aliquots of Bisulfite Mix by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.

If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again. Do not place dissolved Bisulfite Mix on ice.

2. Prepare the bisulfite reactions in 200 µl PCR tubes according to the Table below. Add each component in the order listed.

The combined volume of DNA solution and RNase-free water must total 20 µl.

Component	Volume per reaction (µl)
DNA solution (1 ng – 2 µg)	Variable* (maximum 20 µl)
RNase-free water	Variable*
Bisulfite Mix (dissolved), see step 1	85
DNA Protect Buffer	35
Total volume	**140**

* The combined volume of DNA solution and RNase-free water must total 20 µl.

3. Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25°C).

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.

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to the Table below. The complete cycle should take approximately 5 h.

If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available. Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.

Important: Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. It is important to use PCR tubes that close tightly. Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite†	20°C

† Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Cleanup of bisulfite converted DNA

6. Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.

Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

7. Add 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA to each sample. Mix the solutions by vortexing and then centrifuge briefly.

Carrier RNA is not necessary when using >100 ng DNA.

8. Place the necessary number of EpiTect spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube in step 7 into the corresponding EpiTect spin column.

9. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

10. Add 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

11. Add 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25°C).

If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

Important: The bottle containing Buffer BD should be closed immediately after use to avoid acidification from carbon dioxide in the air.

Note: It is important to close the lids of the spin columns before incubation.

12. Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.

13. Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.

14. Repeat step 13 once.

15. Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.

16. Recommended: Place the spin columns with open lids into clean 1.5 ml microcentrifuge tubes (not provided) and incubate the spin columns for 5 min at 56°C in a heating block.

This step enables evaporation of any remaining liquid.

17. Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Dispense 20 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 x g (12,000 rpm).

To increase the yield of DNA in the eluate, dispense an additional 20 µl Buffer EB to the center of each membrane, and centrifuge for 1 min at maximum speed. If the purified DNA is to be stored for up to 24 h, we recommend storage at 2–8°C. For storage longer than 24 h, we recommend storage at –20°C. At –20°C, DNA converted and purified using the EpiTect Bisulfite Kit can be stored for at least 3 years without decrease of quality or conversion.

PCR Amplification of bisulfite-converted DNA optimized for Pyrosequencing® analysis: Pyromark PCR

Important points before starting

- One primer must be biotinylated at its 5' end in order to prepare a single-stranded PCR product for use in the subsequent Pyrosequencing procedure. We recommend HPLC or an equivalent procedure to purify the biotinylated primer.
- For primer design we recommend use of PyroMark Assay Design Software 2.0.
- The optimal PCR amplicon length for Pyrosequencing is between 80 and 200 bp, although products up to 500 bp might work well for Pyrosequencing assays on genomic DNA.
- HotStarTaq DNA Polymerase requires an activation step of 15 min at 95°C (see step 6 of this protocol).
- PyroMark PCR Master Mix provides a final concentration of 1.5 mM MgCl2 in the final reaction mix, which will produce satisfactory results in most cases. However, if a higher Mg2+ concentration is required, use of the 25 mM MgCl2 provided in the kit is recommended.
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize crosscontamination.

Procedure

1. Thaw the PyroMark PCR Master Mix, CoralLoad Concentrate, primer solutions, and 25 mM MgCl2 (if required) at room temperature or on ice.

It is important to mix the solutions before use in order to avoid localized concentrations of salt.

2. Set up the reaction according to the Table below.

It is not necessary to keep reaction vessels on ice since HotStarTaq DNA Polymerase is inactive at room temperature.

Component	Volume/reaction	Final concentration
Reaction mix		
PyroMark PCR Master Mix, 2x	12.5 µl	Contains HotStarTaq DNA Polymerase, 1x PyroMark PCR Buffer,* and dNTPs
CoralLoad Concentrate, 10x	2.5 µl	1x
25 mM $MgCl_2$ (optional)	Variable, see Table 2	See Table 2
Primer A	Variable	0.2 µM†
Primer B	Variable	0.2 µM†
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	≤ 500 ng/reaction‡ or 10–20 ng bisulfite converted DNA
Total volume	25 µl	

Note: If other reaction volumes are used, reduce the amount of each component accordingly.

* Contains 1.5 mM $MgCl_2$.

† Final primer concentration in the PCR reaction of 0.2 µM is normally optimal.

Note: The Mg2+ concentration in the PyroMark Reaction Buffer will produce satisfactory results in most cases. However, in some cases, reactions may be improved by increasing the final Mg2+ concentration according to the Table below.

Final Mg^{2+} concentration in reaction (mM)	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Required volume of 25 mM $MgCl_2$ per reaction (µl):	0	0.5	1	1.5	2	2.5	3	3.5

3. Gently pipet the master mix up and down for thorough mixing and dispense appropriate volumes into PCR tubes.

4. Add template DNA (≤ 500 ng/reaction) to the individual PCR tubes.

We recommend 10 ng human genomic DNA or 10–20 ng bisulfite converted DNA.

5. When using a thermal cycler with a heated lid, do not use mineral oil. Proceed

directly to step 6. Otherwise, overlay with approximately 100 µl mineral oil.

6. Program the thermal cycler according to the list below.

Note: Each PCR program must start with an initial heat activation step at 95°C for 15 min.

			Additional comments
Initial PCR activation step	**15 min**	**95°C**	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling:			
Denaturation	**30 s**	**94°C**	
Annealing*	**30 s**	**60°C**	For genomic DNA
		56°C	For bisulfite converted DNA
Extension	**30 s**	**72°C**	
Number of cycles	**45**		
Final extension	**10 min**	**72°C**	

* Recommended annealing temperature when using PyroMark Assay Design Software 2.0. In all other cases 5°C below the calculated *Tm* of the primers is a suitable temperature to start with. An annealing temperature that gives the highest specificity for the desired PCR product should be used.

7. Place the PCR tubes in the thermal cycler and start the cycling program.

Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

8. Use 5–20 µl of a 25 µl PCR for subsequent Pyrosequencing analysis.

Note: In most cases 5–10 µl of the PCR product gives satisfactory Pyrosequencing results when using the PyroMark MD instrument, 10–20 µl when using the PyroMark Q24 instrument, and 20 µl when using the PyroMark ID instrument. Adjust the amount of PCR product according to the instructions in the user manual of the specific instrument if required. We recommend checking your PCR product prior to Pyrosequencing analysis, e.g., by fast analysis on the QIAxcel® or by agarose gel analysis. PCR products can be directly loaded onto an agarose gel without prior addition of a PCR loading buffer and gel tracking dyes when using CoralLoad Concentrate. CoralLoad Concentrate contains a gel loading reagent and gel tracking dyes. Refer to

the Table below to identify the dyes according to migration distance and agarose gel percentage and type.

Note: Due to the high viscosity of the solution, apply the solution slowly into the wells of the agarose gel.

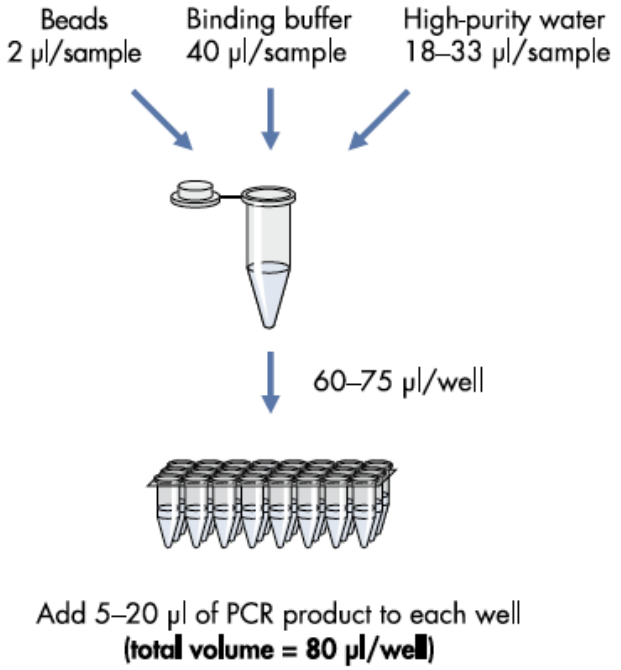
%TAE (TBE) agarose gel	Red dye	Orange dye
0.8	500 bp (270 bp)	~80 bp (<10 bp)
1.0	300 bp (220 bp)	~40 bp (<10 bp)
1.5	250 bp (120 bp)	~20 bp (<10 bp)
2.0	100 bp (110 bp)	<10 bp (<10 bp)
3.0	50 bp (100 bp)	<10 bp (<10 bp)

Pyrosequencing: PyroMark® Q24 Vacuum Workstation

Pyrosequencing Procedure

1. Make a master mix according to the flowchart below.

Note: Before pipetting, gently shake the bottle of streptavidin-coated Sepharose® beads to ensure a homogenous suspension.



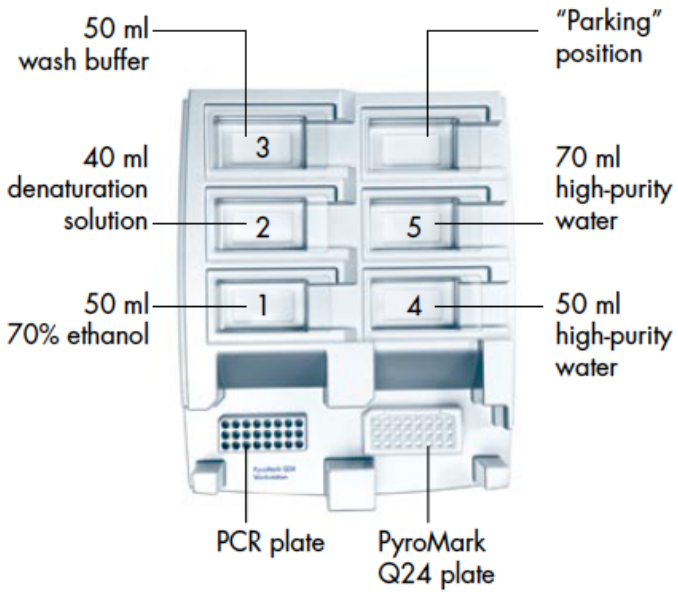
2. Depending on the sample volume, dispense 60–75 µl master mix into each necessary well of a PCR plate to give a total volume of 80 µl per well.

3. Add 5–20 µl PCR product to each well.

4. Seal the wells with strip caps and agitate the PCR plate at 1400 rpm for 5–10 min at room temperature (15–25°C) using an orbital shaker.

5. Dilute the sequencing primers to 0.3 µM with PyroMark Annealing Buffer (cat. no. 979009), and dispense 25 µl into each necessary well of a PyroMark Q24 Plate. Position the plate on the workstation.

6. Fill the workstation troughs according to the diagram below.



7. Start the pump and apply vacuum to the tool by opening the switch. Flush the filter probes with high-purity water (Milli-Q® 18.2 MW x cm or equivalent) in trough 5. Refill the trough with fresh high-purity water for use in step 16.

8. Position the PCR plate on the workstation. Ensure that both plates are in the same orientation as when the samples were loaded.

9. With the vacuum switch ON, lower the vacuum tool into the wells of the PCR plate for 15 s to capture the beads with PCR product.

10. With the vacuum ON, flush the tool with 70% ethanol (trough 1) for 5 s.

11. With vacuum ON, flush the tool with denaturation solution (trough 2) for 5 s.

12. With vacuum ON, flush the tool with wash buffer (trough 3) for 10 s.

13. With vacuum ON, raise the vacuum tool to beyond 90° vertical for 5 s.

14. Align the vacuum tool with the PyroMark Q24 Plate and switch the vacuum OFF. Lower the vacuum tool into the wells and gently shake from side to side to release the beads.

15. With the vacuum OFF, agitate the vacuum tool in high-purity water (trough 4) for 10 s.

16. Switch the vacuum ON and flush the filter probes with high-purity water (trough 5) for 5 s.

17. Raise the vacuum tool to beyond 90° vertical for 5 s, then switch the vacuum OFF and store the tool in the "Parking" position.

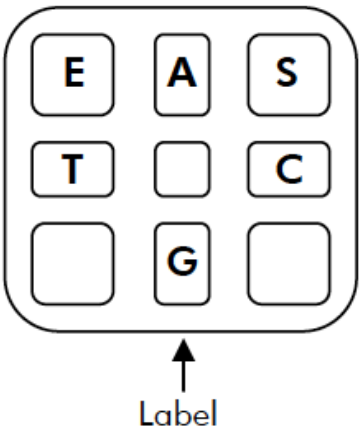
18. Place the PyroMark Q24 Plate in a prewarmed PyroMark Q24 Plate Holder.

Heat the Pyrosequencing samples on a heating block at 80°C for 2 minutes.

19. Remove the plate from the holder and allow the samples to cool to room temperature (15–25°C) for at least 5 minutes. The cooled plate can now be processed in the PyroMark Q24.

20. Load the PyroMark Gold Q24 Reagents into the PyroMark Q24 Cartridge

- The Pre Run information report, found in the "Tools" menu at run setup, provides information about the volume of nucleotides, enzyme mixture, and substrate mixture needed for a specific run.
- Open the PyroMark Gold Q24 Reagents box and remove the vials containing lyophilized enzyme and substrate mixtures, and the vials containing nucleotides.
- Dissolve the lyophilized enzyme and substrate mixtures in 620 µl each of high-purity water (Milli-Q 18.2 MΩ x cm or equivalent, filtered through a 0.22 µm filter).
- Mix by swirling the vial gently. Do not vortex! In order to ensure that the mixture is fully dissolved, leave it at room temperature (15–25°C) for 5–10 min. Make sure that the solution is not turbid before filling the PyroMark Q24 Cartridge. If the reagents are not to be used immediately, place the reagent vials on ice or in a refrigerator.
- Allow the reagents and the PyroMark Q24 Cartridge to reach ambient temperature (20–25°C).
- Place the PyroMark Q24 Cartridge with the label facing you.
- Pipet the reagents into the PyroMark Q24 Cartridge according to the Figure below. Make sure that no air bubbles are transferred from the pipet to the catridge.



- Switch on the PyroMark Q24 instrument. The power switch is located at the rear of the instrument.
- Open the cartridge gate and insert the filled PyroMark Q24 Cartridge with the label facing you. Push the cartridge in fully and then push it down.
- Ensure that the cartridge is properly inserted and close the gate. Refer to the PyroMark Q24 User Manual for more information.
- Open the plate-holding frame, and place the PyroMark Q24 plate on the heating block.
- Close the plate-holding frame and the instrument lid.
- Insert the USB memory stick (containing the run file) into the USB port at the front of the instrument. Do not remove the USB port before the run is finished.
- Select “Run” in the main menu and press “OK”.
- Select the run file. To view the contents of a folder, select the folder and press “Select”. To go back to the previous view, press “Back”.
- When the run file is selected, press “Select” to start the run.
- When the run is finished and the instrument confirms that the run file has been saved to the USB memory stick, press “Close”.
- Remove the USB memory stick.
- Open the instrument lid.
- Open the cartridge gate and remove the PyroMark Q24 Cartridge by lifting it up and pulling it out.
- Close the gate.
- Discard solutions remaining in the PyroMark Q24 Cartridge.

- Rinse the PyroMark Q24 Cartridge 4 times with high-purity water.
- Spray the outside of the needles with high-purity water.
- To rinse the needles, fill the compartments completely with highpurity water and hold the cartridge over a sink while pressing firmly on top of each compartment with a finger (wear gloves). Check that the needle is clear. A jet of water should come straight out of the tip of the needle.
- When all needles have been rinsed and checked, discard the water and let the PyroMark Q24 Cartridge dry on a lint-free tissue.
- When the PyroMark Q24 Cartridge is dry, store it in a dust-free place.

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10/2006 – 10/2012	**Universität Wien** *Study of Biology (Main subject: Anthropology – Human genetics)*	Vienna, Austria
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INTERNSHIP

04/2011 – 06/2011	**University of Vienna, Dept. of Nutritional Sciences** *End-point and real-time PCR methods, Cell-culture of Caco-II-cells and dendritic cells, Pyrosequencing, statistical analysis of data with Excel and SPSS* *Supervisor: Univ.-Doz. Dr. Alexander G. Haslberger*	Vienna, Austria

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