Gene expression changes triggered by exposure of Haemophilus influenzae to Novobiocin or Ciprofloxacin:

Combined transcription and translation analysis

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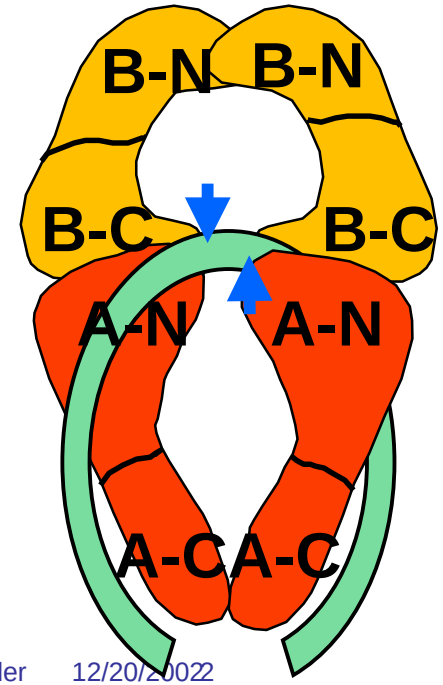
GENE DATA

Some properties of *E.coli* DNA gyrase

	Subunit A	Subunit B
Gene	*gyrA* 2625 bp	*gyrB* 2412 bp
Mol. wt.	96,887 (875 aa)	89,893 (804 aa)
Major role	Breakage and reunion of DNA	ATPase activity
Drug interactions	Target of quinolones	Target of coumarins



DNA Gyrase: mol. wt. = 373,560
Active enzyme A_2B_2 tetramer

GENE DATA

Goal of the study:
Gene expression profiling

- Target: *Haemophilus Influenzae*
- Antibiotics: Ciprofloxacin (Quinolones)
  Novobiocin (Coumarins)
- Both antibiotics inhibit DNA gyrase but by different mechanisms

GENE DATA

Mode of action of Novobiocin

- Novobiocin inhibits ATPase activity of DNA gyrase
  → compensation by overexpression of gyrase

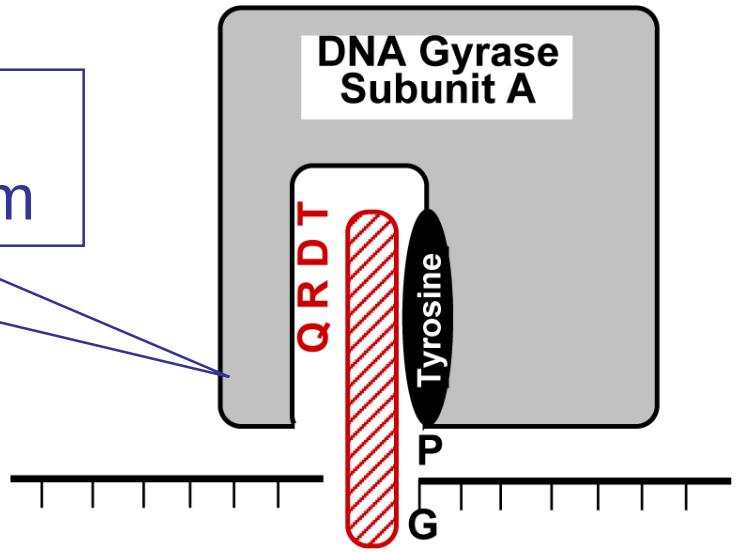
- Novobiocin affects steady-state supercoiling level of DNA

- Transcription of many genes is sensitive to DNA supercoiling
  → Global pattern of gene expression changes
  in a complex way

Mode of action of Ciprofloxacin

- Ciprofloxacin builds with the enzyme and DNA a stable ternary complex
  → DNA damaged
  → repair by inducing the SOS-repair system

Induction of the
RecA (SOS) DNA repair system



GENE DATA

Cell cultures

- Bacterial cultures in minimal medium with a reduced methionine concentration (0.6 µM) to an OD_{600} of 0.4
- Novobiocin: 0, 12.5 and 125 µg/ml
- Ciprofloxacin: 0, 30 and 300 µg/ml
- Time points: 10, 30 and 60 min
- To an aliquot L-[^{35}S]Methionine (>37 TBq/mmol) added and incubation continued for 2 min
- Cells rapidly chilled on ice, harvested by centrifugation, frozen in liquid nitrogen and kept at –80°C

Isolation of total RNA

- Incubation with preheated hot phenol for 5’ at 60 °C
- Addition of preheated NAES buffer (1% SDS) for 5’ at 60 °C
- Cooled on ice and phase separated
- Additional phenol extractions until interface is clean
- Isopropanol precipitation
- Resuspended in DEPC-water followed by Qiagen Midi “clean up”
- DNase treatment for 15’ at 37 °C
- Precipitation, resuspension and quantification by E_{260}

Labeling of random primed sscDNA

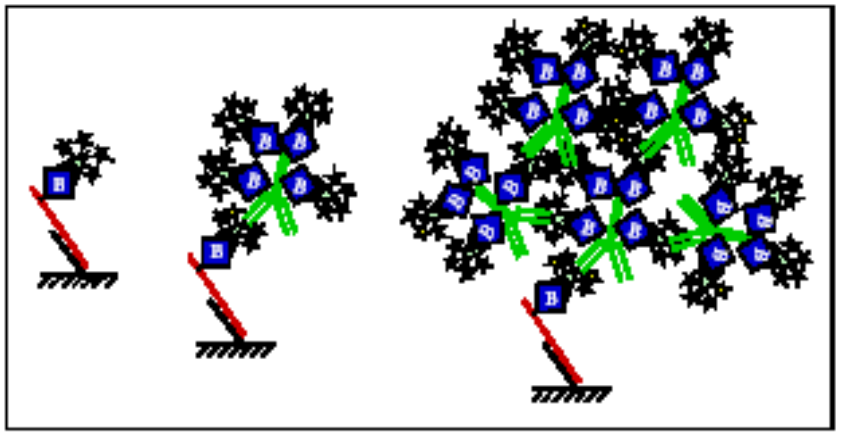
- Labeling reaction overnight at 37 °C containing
  - RNA < 0.5 µg/µl
  - Random hexamers (hexamers / RNA = ¼)
  - Reverse transcriptase (100 units / µg RNA)
  - Nucleotide mix (dCTP, cGTP, dTTP, dATP)
  - Biotinylated dATP
- NaOH 30’ at 60 °C
- Neutralized, precipitated, resuspended
- 2% agarose gels
  - Single strand cDNA sizes should range from 50 to 500 bases

Fragmentation and hybridization

- Column purification of sscDNA
- Fragmentation in smallest volume possible in Tris-Acetate, pH 8.1, KOAce, MgOAce for 40 min at 95 °C
- Centrifuged through 0.22 µm filter units
- Hybridized on prehybridized DNA chips overnight at 40 °C 60 rpm containing
  - fragmented cDNA
  - fragmented yeast RNA
  - acetylated BSA

Staining and washing

- Washing under stringent and non-stringent conditions
- Staining with streptavidin-R-phycoerythrin (SAPE) in the presence of acetylated BSA
- Incubation with biotinylated-anti-streptavidin
- Amplification with streptavidin-R-phycoerythrin
- Washing under non-stringent conditions

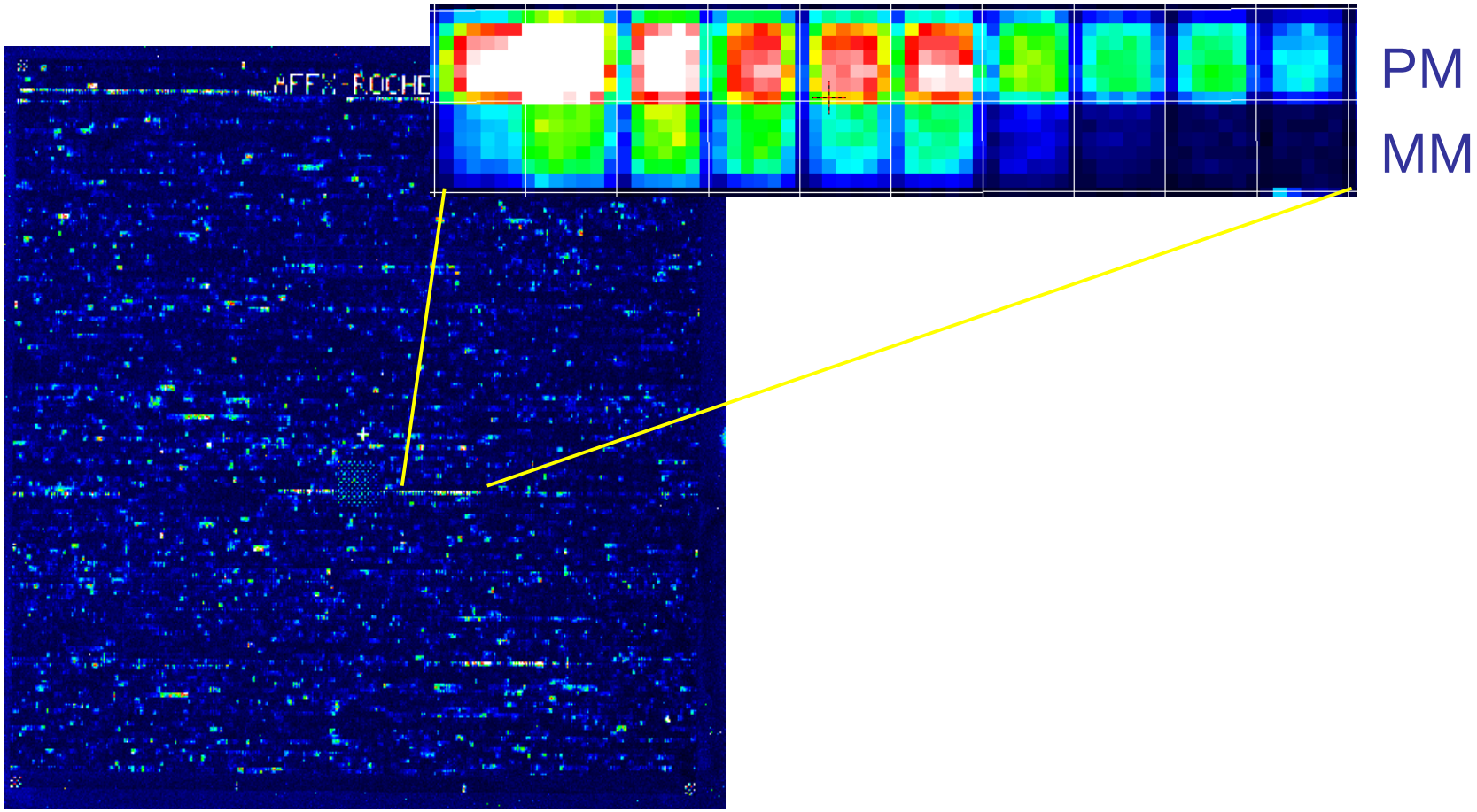


GENE DATA

DNA microarray

- High-density microarray contains oligonucleotides for
  - ca. 2000 genes from the bacterium *Streptococcus pneumoniae* and for
  - ca. 1800 genes from *Haemophilus influenzae*
- 25mer oligonucleotides for a specific gene usually include 25 probe pairs (PM and MM) and at least 20 probe pairs for very short genes
- In addition microarray contains many
  - control genes
  - sequence information from intergenic regions
  - genes coding for ribosomal and transfer RNA

DNA microarray



GENE DATA

2D - PAGE

- Cells lysed in 8 M urea, 4 % CHAPS, 40 mM of Tris base, 65 mM of 1,4-dithioerythritol and 2% ampholytes
- Aliquots of the supernatants containing 4 x 10^6 cpm of radioactivity loaded onto 3-10 non-linear pH gradient strips at the basic end
- Strips equilibrated and loaded onto vertical 12% polyacrylamide slab gels
- Parallel samples (one sample per time point and concentration and their corresponding control) run on parallel gels (same batch of strips, same isoeletric focusing run, same batch of gels for SDS-PAGE and same SDS-PAGE run)
- Only pairs of gels that had been obtained under identical conditions considered for analysis

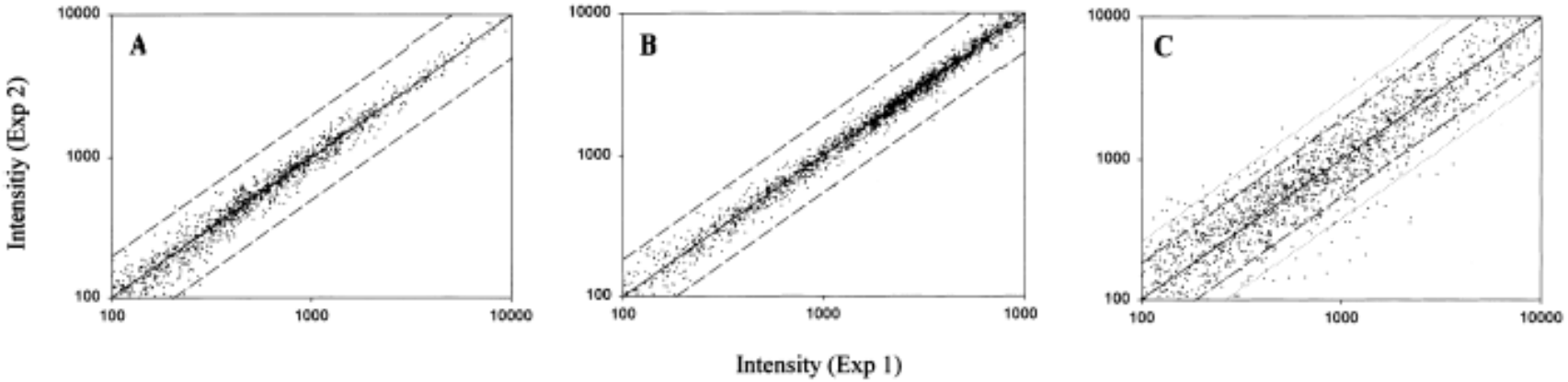
Data analysis

- Spot intensities normalized
  (sum of all spot intensities equal for all gels)
- Significance of results estimated using t-test for paired samples
  (p-values < 0.05, changes considered as significant)

- Microarray hybridization intensities processed using Affymetrix GeneChip algorithm
- Analysis and clustering performed with
  - Microsoft Access
  - SAS Enterprise Miner
  - GeneData Expressionist

GENE DATA

Reproducibility



A: Hybridization results obtained from the same RNA but independently reverse transcribed and hybridized

B: Hybridization results obtained from independently isolated, reverse transcribed and hybridized RNA from same cultures

C: Hybridization results obtained from RNA isolated from different cultures grown under same conditions

Solid line = ideal 1:1 ratio; dashed line a factor of two; broken line a factor of three between the two measurements

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Reproducibility of mRNA quantification

Sample Preparation	Experiment	NF [a]	Increased or decreased [b]	Fold Change >2 or <-2
Same culture, same RNA preparation, independent labeling and hybridization	1	0.98	1	0
	2	1.27	29	6[c]
	3	0.66	7	0
Same culture, independent RNA preparation, labeling, and hybridization	1	0.88	32	7
	2	1.12	3	1
	3	1.34	13	1
	4	1.70	3	0
Different cultures		1.22	333	61

[a] NF = normalization factor
[b] Transcripts called increased or decreased according to the Affymetrix GeneChip software
[c] Fold Changes between –2.1 and –2.5
[d] Genes total = 1961

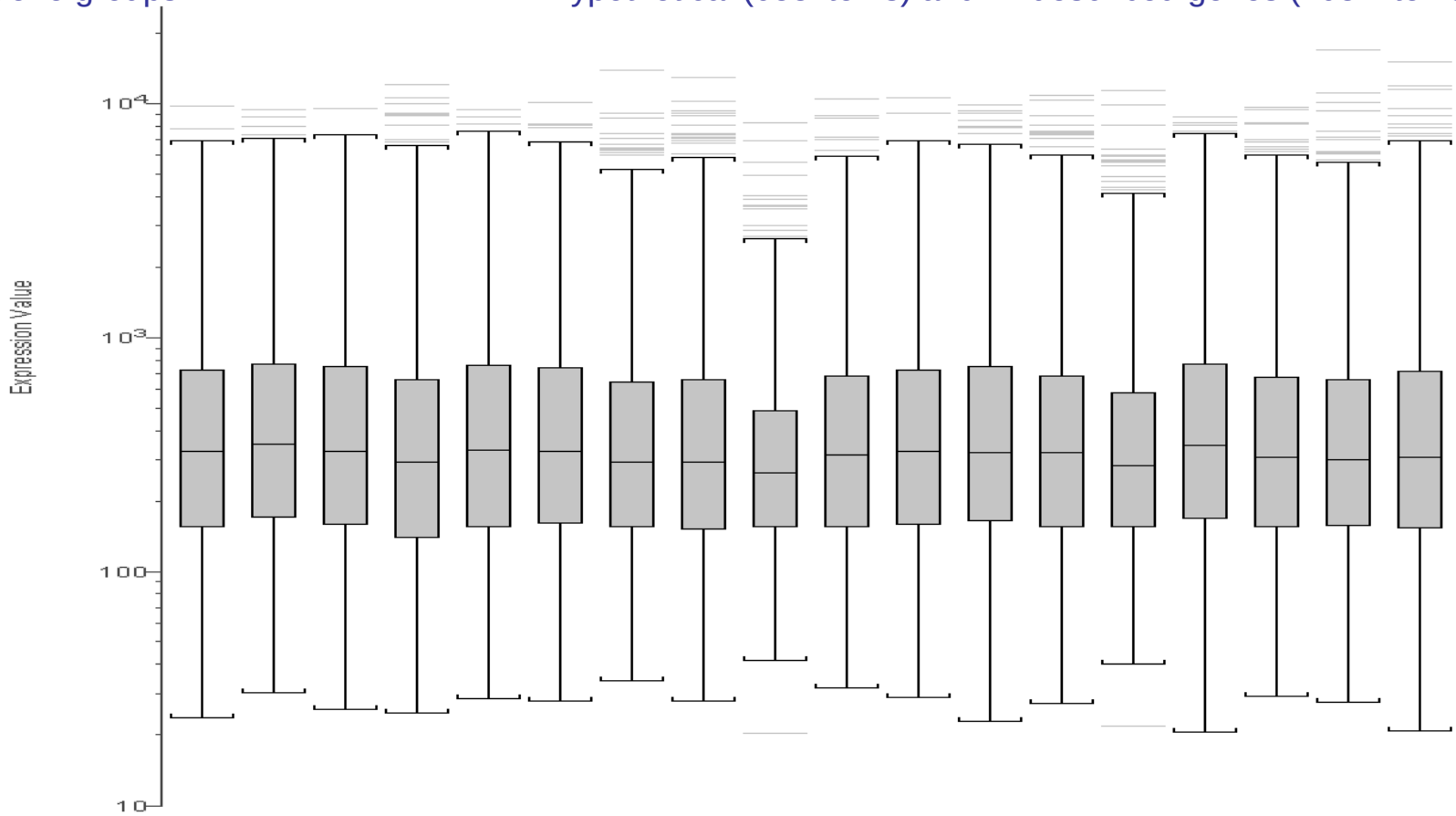
GENE DATA

Data condensation and aggregation

- Normalization
- Filter by valid value proportion > 0.5
- Filter by high variance
- Separate groups by Kruskal-Wallis test (ranking test)
- Clustering (SOM, hierarchical clustering, k-Means)
- Principal components analysis

Boxplot Ciprofloxacin

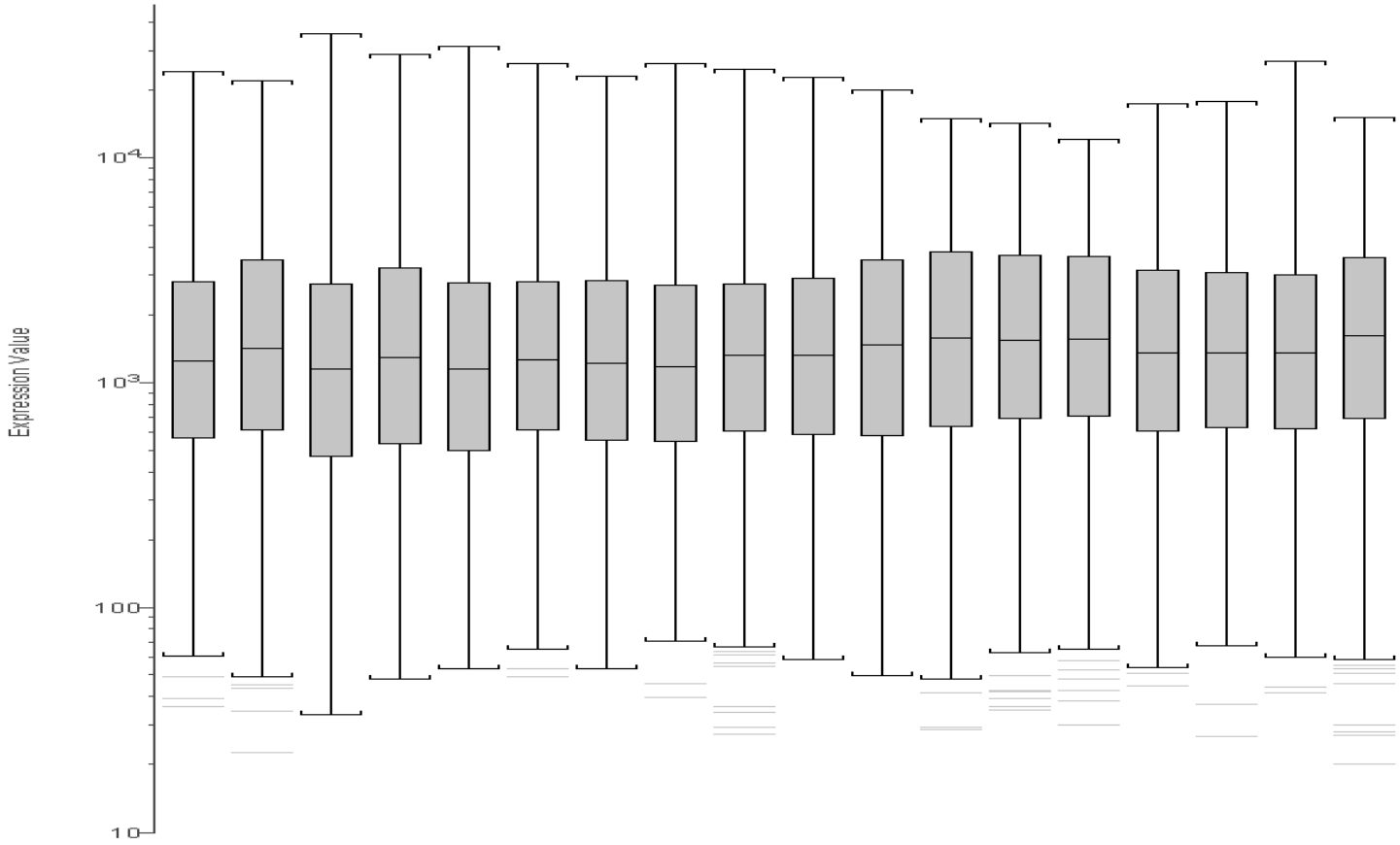
Expression value filtering:	minimum = 20, only present values
Normalization:	logarithmic mean, reference value = 407
Gene groups:	HI hypothetical (658 items) and HI described genes (1037 items)



GENE DATA

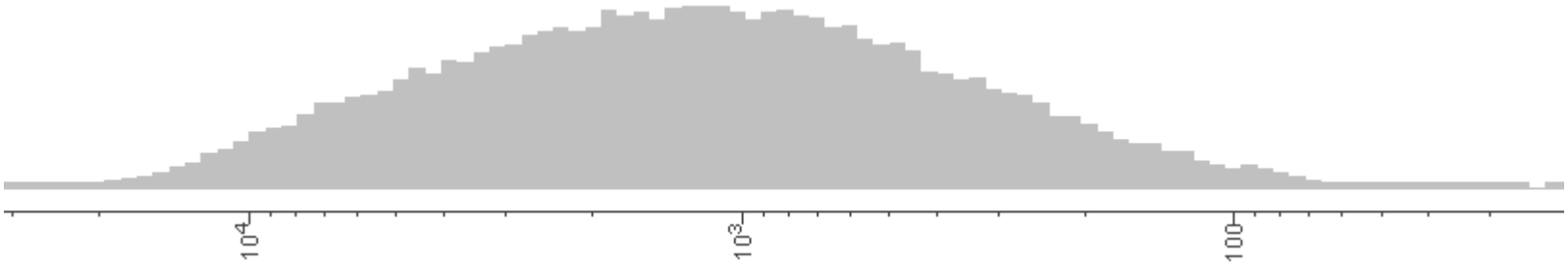
Boxplot Novobiocin

Expression value filtering:	minimum = 20, only present values
Normalization:	logarithmic mean, reference value = 1215
Gene groups:	HI hypothetical (658 items) and HI described genes (1037 items)

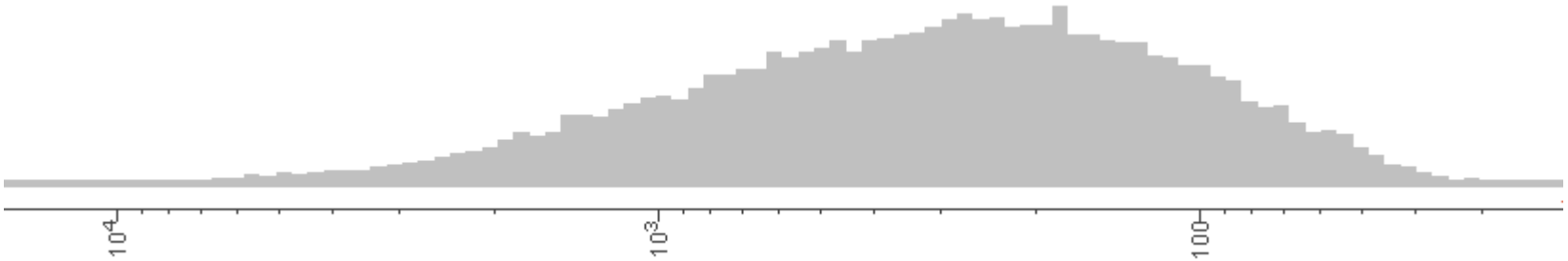


GENE DATA

Distribution of intensities



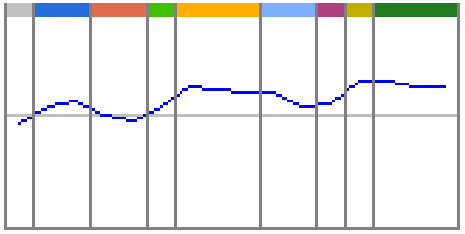
Novobiocin



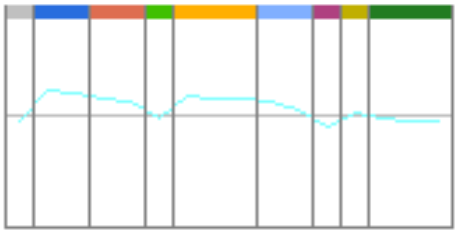
Ciprofloxacin

GENE DATA

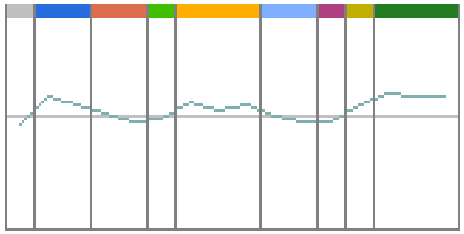
Ciprofloxacin: Clusters of up- or downregulated genes



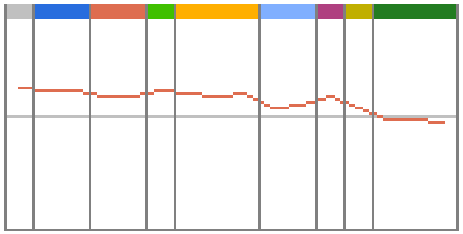
16 genes



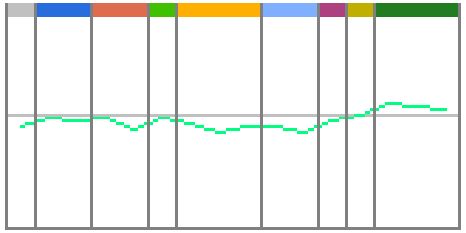
78 genes



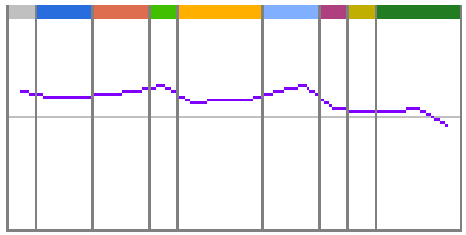
62 genes



54 genes



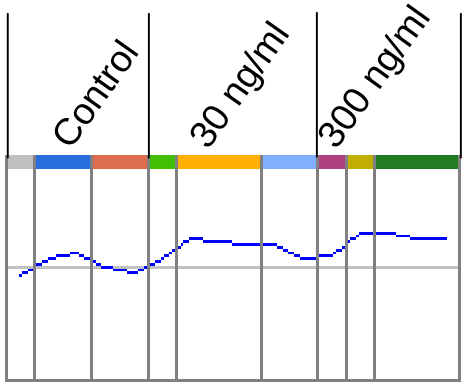
35 genes



27 genes

GENE DATA

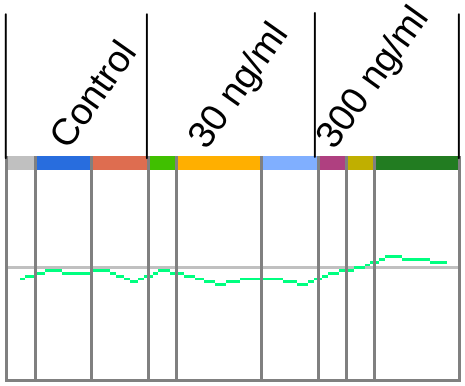
Ciprofloxacin: Upregulated genes at low and high concentration



	Gene product	Fold change highest value (protein)
	Conserved hypothetical protein	3
recn	DNA repair protein	12
uvra	Excinuclease ABC subunit A	4
ssb	Single-stranded DNA binding protein	5 (NC)
ruvb	Holliday junction DNA helicase	3
ruva	Holliday junction DNA helicase	4
gyrb	DNA gyrase, subunit B	2
reca	RecA recombinase	6
lexa	LexA repressor	12
dapf	Diaminopimelate epimerase	5
mutb	DNA helicase II	3
mfd	Transcription-repair coupling factor	3
impa	ImpA protein	20
pgk	Phosphoglycerate kinase	NC (3)

GENE DATA

Ciprofloxacin: Upregulated genes at high concentration



	Gene product	Fold change Highest value (protein)
dnak	Heat shock protein 70	11
napa	Neutrophil activating protein	9 (3.4)
gmk	Guanylate kinase	9
	Oxidoreductase	6
holc	DNA polymerase III, Chi subunit	6
glpq	Glycerophosphoryl diester phosphodiesterase	5
nlpb	Lipoprotein-34	5
merp	Mercuric transport protein periplasmic component precursor	5
pyrr	Pyrimidine operon regulatory protein	5 (NC)
merp	Mercury scavenger protein	5
tig	Trigger factor	5 (2)
tesb	Acyl-Coa thioesterase II	5
rsga	Ferritin like protein	4
pepp	Aminopeptidase P	4
oapa	Cell envelope protein	4
rpoz	RNA polymerase omega subunit	4
purr	Purine nucleotide synthesis repressor protein	4
clpx	ATP-dependent protease ATPase subunit	4 (NC)

GENE DATA

Ciprofloxacin: Downregulated genes at high concentration

	Gene Description	Fold Change highest value (protein)
asna	Aspartate--ammonia ligase	-39 (NC)
dha	Glutamate dehydrogenase	-25
artq	Arginine transport system permease protein	-22
mui	I protein	-14
groel	Heat shock protein	-13
asnc	Regulatory protein	-12
ppc	Phosphoenolpyruvate carboxylase	-11
infa	Initiation factor IF-1	-10
gltp	Proton glutamate symport protein	-10
lamb	Lactam utilization protein	-9
asd	Aspartate-semialdehyde dehydrogenase	-9
arsc	ARSC protein	-9
folc	Folylpolyglutamate synthase/Dihydrofolate synthase	-8
tsf	Elongation factor EF-ts	-8
lctp	L-Lactate permease	-8
dead	ATP-dependent RNA helicase	-8
hflc	Lambda cII stability-governing protein	-7
frua	Fructose-permease IIbc component	-7
pfka	6-phosphofructokinase	-7
bett	High-affinity choline transport protein	-7
mena	Menaquinone biosynthesis protein	-7
serc	Phosphoserine aminotransferase	-7

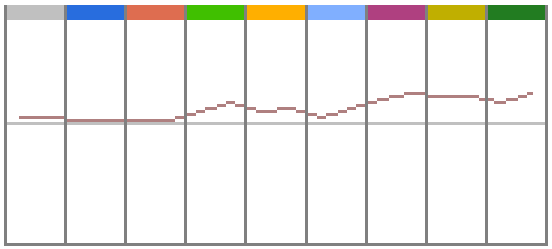
	Gene Description	Fold Change highest value (protein)
rbsb	Periplasmic ribose-binding protein	-7
brab	Branched chain AA transport system II carrier protein	-7
pepe	Peptidase E	-6
tehb	Tellurite resistance protein	-6
glk	Glucose kinase	-6
dppa	Heme-binding lipoprotein	-6
gmha	Phosphoheptose isomerase	-5
uraa	Uracil permease	-5
merp	Mercury scavenger protein	-5
fruk	1-phosphofructokinase	-5
trpg	Anthranilate synthase component II	-5
dsbb	Oxido-reductase	-5
glpr	Glycerol-3-phosphate regulon repressor	-5
thrb	Homoserine kinase	-5 (NC)
thra	Aspartokinase I / Homoserine dehydrogenase I	-5 (NC)
cyse	Serine acetyltransferase	-5
p14	p14 protein	-5
meng	Menaquinone biosynthesis protein	-5
vapa	Virulence associated protein A	-5
metx	S-adenosylmethionine synthetase 2	-5 (-1.9)
topa	Topoisomerase I	-4

GENE DATA

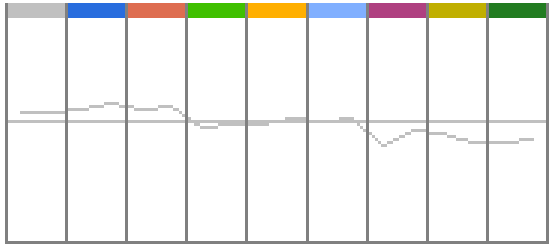
Ciprofloxacin: Changes at the protein level at high concentration

	Gene product	Fold change protein (mRNA)	
		10 min	60 min
ssb	Single-stranded DNA binding protein	2.0 (5)	1.5 (5)
hslv	HslUV operon heat shock protein	3.6 (NC)	3.2 (NC)
	Conserved hypothetical protein	-2.5 (NC)	-1.5 (-3)
tsf	Elongation factor EF-Ts	-2.2 (NC)	-3.2 (-8)
glys	Glycyl-tRNA synthetase beta chain	NC (NC)	3.4 (2)
deoc	Deoxyribose aldolase	1.4 (NC)	2.7 (2)
metx	S-Adenosylmethionine synthetase 2	-1.3 (NC)	-1.9 (-5)
ribh	Riboflavin synthase, beta chain	2.7 (NC)	1.7 (2)
napa	Neutrophil activating protein	1.7 (NC)	3.4 (9)

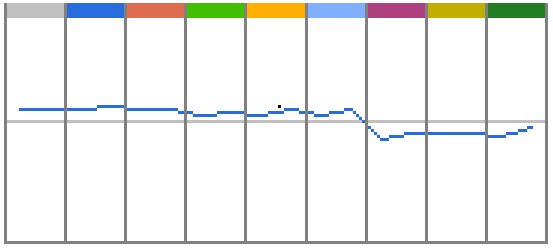
Novobiocin: Clusters of up- or downregulated genes



54 Genes



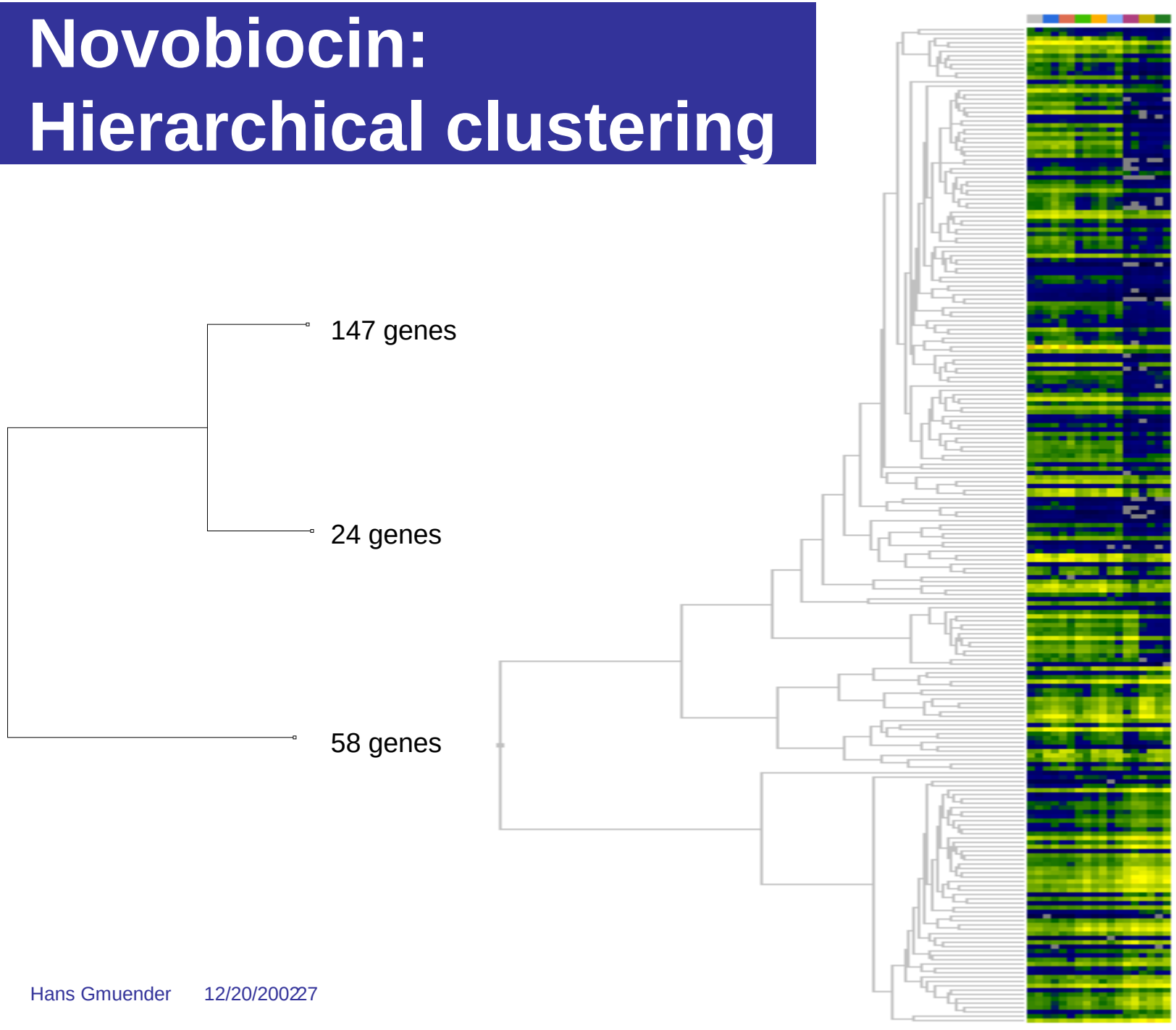
26 Genes



109 Genes

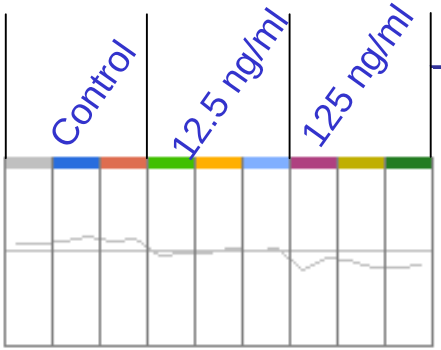
GENE DATA

Novobiocin: Hierarchical clustering



GENE DATA

Novobiocin: Downregulated genes at low and high concentration

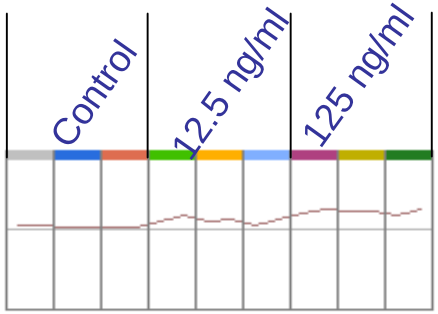


	Gene product	Fold change highest value (protein)
	Conserved hypothetical protein	-15
	Conserved hypothetical protein	-13
	Conserved hypothetical protein	-12
gltp	Proton glutamate symport protein	-7
glpr	Glycerol-3-phosphate regulon repressor	-6
topa	Topoisomerase I	-6
frr	Ribosome releasing factor	-5 (-3.1)
	Glucose kinase	-5
riba	GTP cyclohydrolase II	-4
pdxh	Pyridoxamine phosphate oxidase	-4
prtc	Collagenase	-4
met2	Homoserine acetyltransferase	-4
potd	Spermidine/putrescine-binding periplasmic protein precursor	-4
merp	Mercury scavenger protein	-4

GENE DATA

Novobiocin: Upregulated genes at low and high concentration

	Gene product	Fold Change highest value (protein)
rffe	UDP-N-Acetylglucosamine epimerase	6
mdl	Multidrug resistance protein	5
lktb	Leukotoxin secretion ATP-binding protein	5
hype	Hydrogenase gene region	5
map	Methionine aminopeptidase	4 (3.2)
rfbp	Undecaprenyl-phosphate galactosephosphotransferase	4
htrh	Htra-like protein	4
nusb	N utilization substance protein B	4
mlga	Virulence plasmid protein	4
	fmu/fmv gene product	4
recf	DNA/ATP binding protein	4



	Gene product	Fold Change highest value (protein)
rpod	RNA polymerase sigma-70 factor	3
def	Polypeptide deformylase	3
pth	Peptidyl-tRNS hydrolase	3
hemx	Uroporphyrinogen iii methylase	3
dang	DNA primase	3
fabh	beta-ketoacyl-ACP synthase III	3
fumc	Fumarate hydratase class II	3
gyrb	DNA gyrase, subunit B	3
plsb	Glycerol-3-phosphate acyltransferase	3
ftse	Cell division ATP-binding protein	3
pgk	Phosphoglycerate kinase	3
uvrb	Excinuclease ABC subunit B	3
gyra	DBA gyrase, subunit A	3
ribh	Riboflavin synthase, beta chain	3
menc	O-succinylbenzoate-Coa synthase	3
nlpd	Lipoprotein	3
purr	Purine nucleotide synthesis repressor protein	3
ftsy	Cell division protein	3
fabd	Malonyl Coa-acyl carrier protein transacylase	3
dnaa	Chromosomal replication initiator protein	2
ftsx	Cell division membrane protein	2
dcd	Deoxycytidine triphosphate deaminase	2
metg	Methionyl-tRNA synthetase	2
nrda	Ribonucleoside-diphosphate reductase, alpha chain	2
dnab	Replicative DNA helicase	2

GENE DATA

Novobiocin: Changes at the protein level at high concentration

Gene product	Fold Change protein (mRNA)		
	10 min	30 min	60 min
Oxidoreductase	-3.1 (NC)	-3.5 (NC)	-1.7 (NC)
Aspartokinase I / Homoserine dehydrogenase I	-2.6 (-3.4)	-1.3 (NC)	-2.0 (-2.0)
Adhesin B precursor	-261.8 (NC)	-12.8 (-2.8)	-34.1 (-2.7)
Inorganic pyrophosphatase	-2.1 (1.2)	-3.3 (NC)	-5.4 (NC)
Periplasmic ribose-binding protein	-1.8 (-2.2)	-2.5 (-3.9)	-3.5 (-1.9)
Fructose-bisphosphate aldolase	2.0 (1.8)	2.1 (1.6)	1.0 (1.9)
Phosphoglycerate kinase	-2.0 (2.2)	-2.1 (2.1)	-2.9 (2.0)
Heat shock protein GroES	-1.9 (-2.3)	-4.4 (-4.6)	-3.5 (-3.7)
Ribosomal protein L9	-3.5 (-1.5)	-2.0 (-1.7)	-3.6 (NC)
Ribosomal protein S6	-4.2 (NC)	-2.1 (NC)	-2.7 (NC)
6-Phosphogluconate dehydrogenase	-5.5 (NC)	-4.4 (NC)	-3.2 (NC)
Conserved hypothetical protein	-3.8 (-2.1)	-3.8 (-2.9)	-4.0 (-2.3)
Ribosome releasing factor	-4.7 (-4.5)	-4.4 (-5.4)	-4.7 (-4.9)
Disulfide oxidoreductase	-2.6 (NC)	-2.0 (-1.9)	-2.4 (-2.1)
Ribosomal protein S2	-2.3 (NC)	-3.8 (-2.4)	-5.2 (-2.2)
Elongation factor EF-Ts	-4.2 (NC)	-5.5 (-3.0)	-2.9 (-2.6)
Glycyl-tRNA synthetase alpha chain	2.5 (NC)	2.9 (1.5)	-1.2 (1.6)
Uracil phosphoribosyltransferase	-3.0 (-2.0)	-3.2 (-2.2)	-3.4 (-2.0)
Hypothetical protein	-4.1 (1.3)	-3.2 (-3.5)	-2.9 (-3.2)
Conserved hypothetical protein	-2.0 (-2.3)	-1.8 (-1.8)	-2.3 (-1.6)
Enoyl- reductase	-8.8 (-2.6)	-7.2 (-2.6)	-6.2 (-2.1)

GENE DATA

Common up- or downregulated genes

138 genes up- or downregulated

ftse	cell division atp-binding protein	upregulated
ftsh	cell division protein	
ftsy	cell division protein	
gyra	dna gyrase, subunit a	upregulated
gyrb	dna gyrase, subunit b	
uvrb	excinuclease abc subunit b	upregulated
rpod	rna polymerase sigma-70 factor	upregulated
rpoe	rna polymerase sigma-e factor	downregulated
uree	urease accessory protein	downregulated
uref	urease accessory protein	
ureg	urease accessory protein	
ureh	urease accessory protein	
ureb	urease, beta subunit	
urea	urease, gamma subunit	

GENE DATA

Conclusions

- High density microarrays yield highly reproducible results
- Transcripts can reproducibly be detected even when present at low concentrations
- Sensitivity and reproducibility of the expression analysis using oligonucleotide chip technology was better than expression analysis using 2D-PAGE
- Expression analysis using the bacterial microarray system or 2D gels can be used to profile the effect of an inhibitor on a cell
- Main challenge is to discover the appropriate concentration and time points

GENE DATA

Conclusions

- Ciprofloxacin and Novobiocin illustrate that response to an antibiotic can yield important information about mode of action
- Both compounds induce expression of DNA gyrase and negatively affect topoisomerase I expression
- Ciprofloxacin mainly stimulates expression of DNA repair systems
- Novobiocin changes expression rates of many genes reflecting the fact that the initiation of transcription for many genes is sensitive to DNA supercoiling
- Changed expression levels also observed for many genes coding for proteins either annotated as “unknown function” or “hypothetical” or for proteins not directly involved in DNA topology or repair

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Stefan Evers (2D-PAGE)

Karin Wernli-Kuratli (DNA microarray)

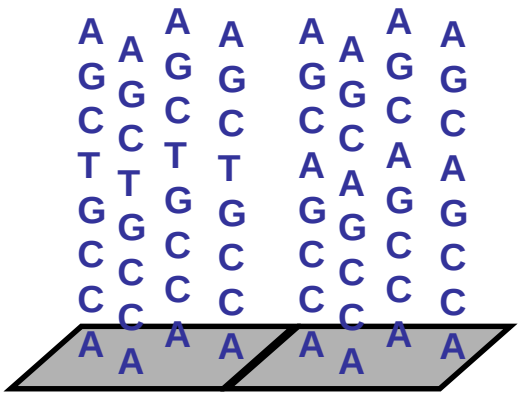
Karin Di Padova (2D-PAGE)

Christopher P. Gray

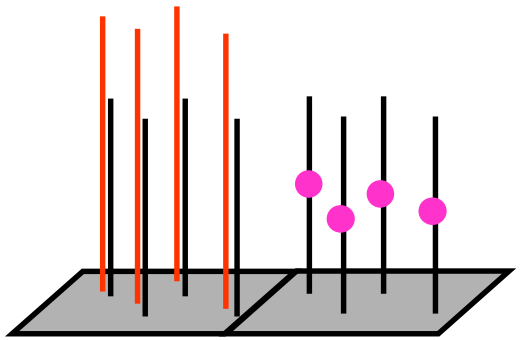
Wolfgang Keck

Genechip probe array

25mers from sense strand



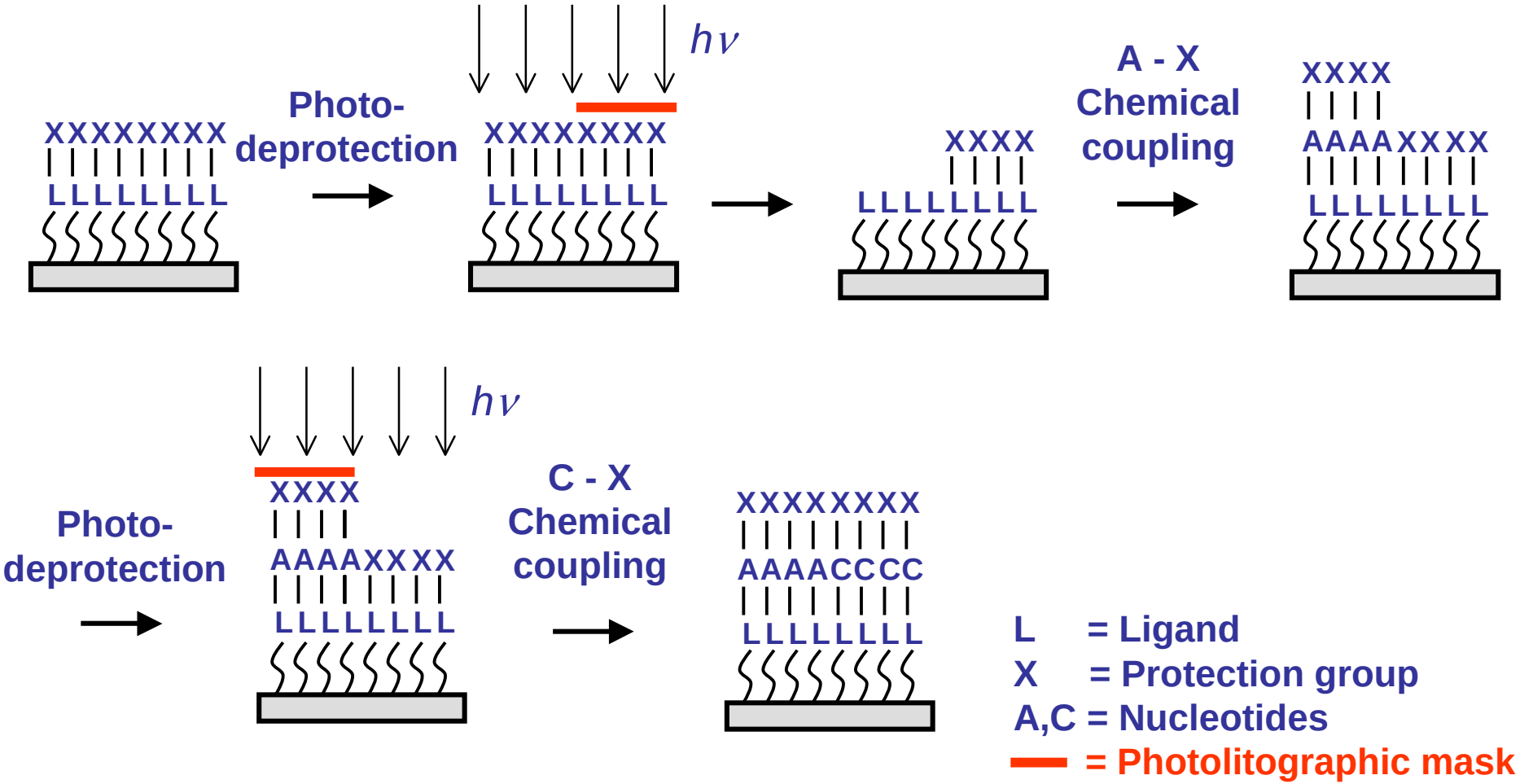
Hybridization of IVT (40º C)



- Wash (high stringency and low stringency)
- Hybridized IVT stained with streptavidin phycoerythrin
- Fluorescent signals detected by scanning confocal microscopy

GENE DATA

Light-directed chemical synthesis



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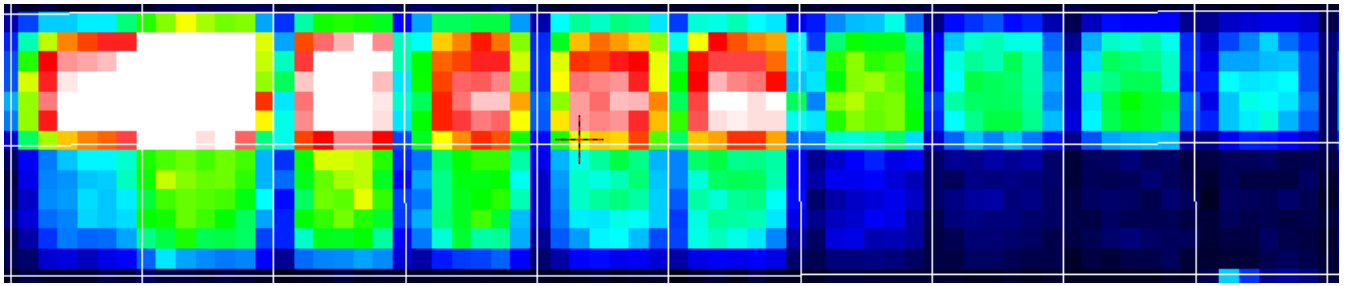
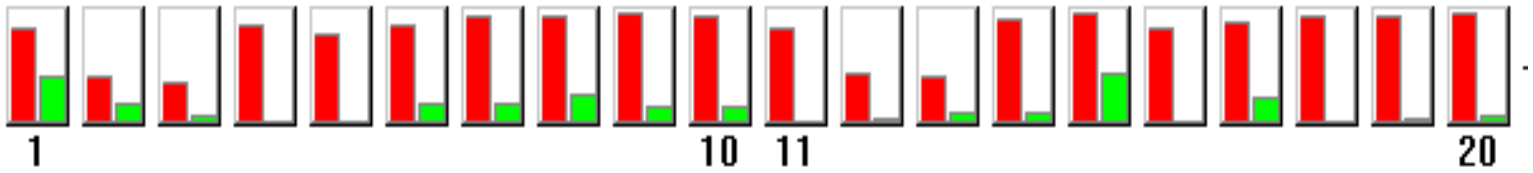
Fluorescent signal detection

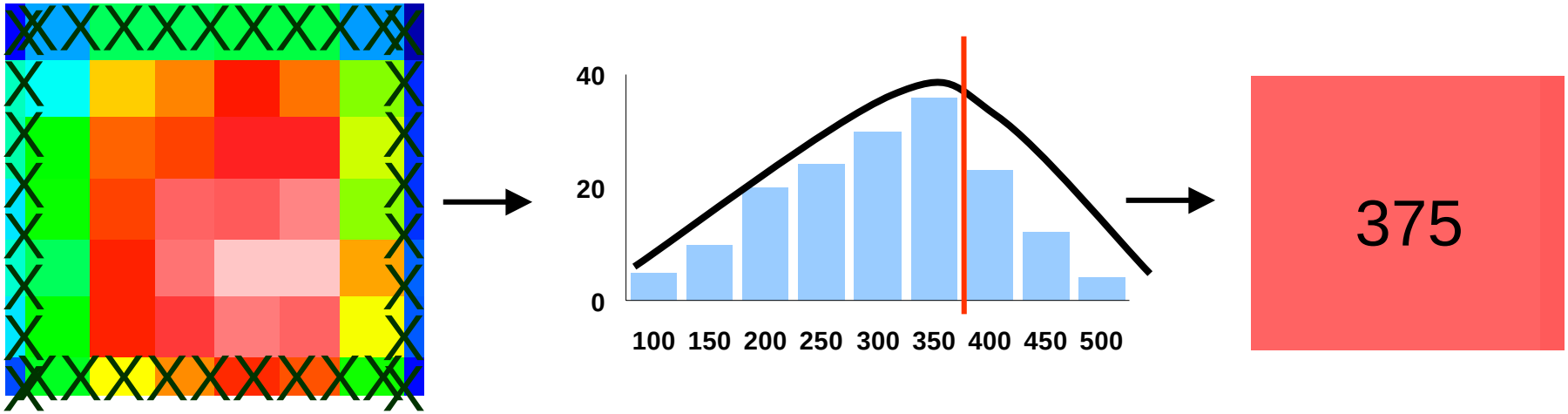


- Space occupied by each specific oligonucleotide sequence is termed a ‘feature’
- Each feature contains ≈ 10^7 25mer oligos
- Fluorescent signal = average of intensities within a feature



GENE DATA

Calculating probe cell averages



- 24 µm x 24 µm
- Array scanned at a resolution of 3 µm
- Creates 8 x 8 pixels for every feature

- Bordering pixels excluded
- Remaining pixel intensity distribution calculated
- Intensity value associated with 75 % of the the distribution used as AVERAGE INTENSITY of the feature

GENE DATA

Background and noise

- Eliminating autofluorescence and nonspecific binding
- Background
  - each chip divided in 4 x 4 zones
  - from each zone 2 % features with lowest Average Intensity (≈ 430 / zone)
  - background subtracted from average intensities of all features within zone
- Noise (Q)
  - Results from small variations in the digitized signal
  - Calculated by using the standard deviations of pixel intensities of the background cells

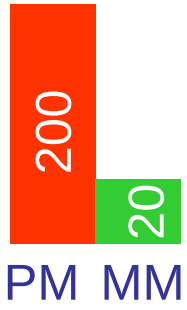
Positive and negative probe pairs

- Statistical Difference Threshold (SDT)
  = Q * SDT multiplier (default = 2 or 4)
- Statistical Ratio Threshold (SRT)
  = 1.5 by default
- For each pair of each gene:
  Difference PM - MM and ratio PM / MM
- If PM - MM > Difference Threshold and PM / MM > Ratio Threshold
  → Position considered positive
- If MM - PM > Difference Threshold and MM / PM > Ratio Threshold
  → Position considered negative

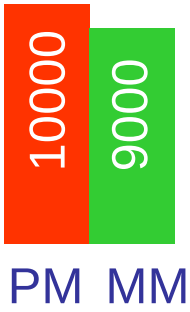
GENE DATA

Examples

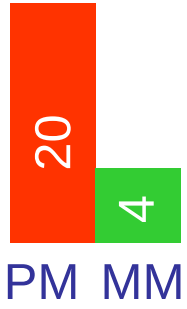
Difference Threshold = 50
Ratio Threshold = 1.5



PM - MM = 180 > DT and
PM / MM = 10.0 > RT
→ Position positive



PM - MM = 1000 > DT and
PM / MM = 1.11 < RT
→ Position not positive



PM - MM = 16 < DT and
PM / MM = 5 > RT
→ Position not positive

GENE DATA

Calculation for each gene

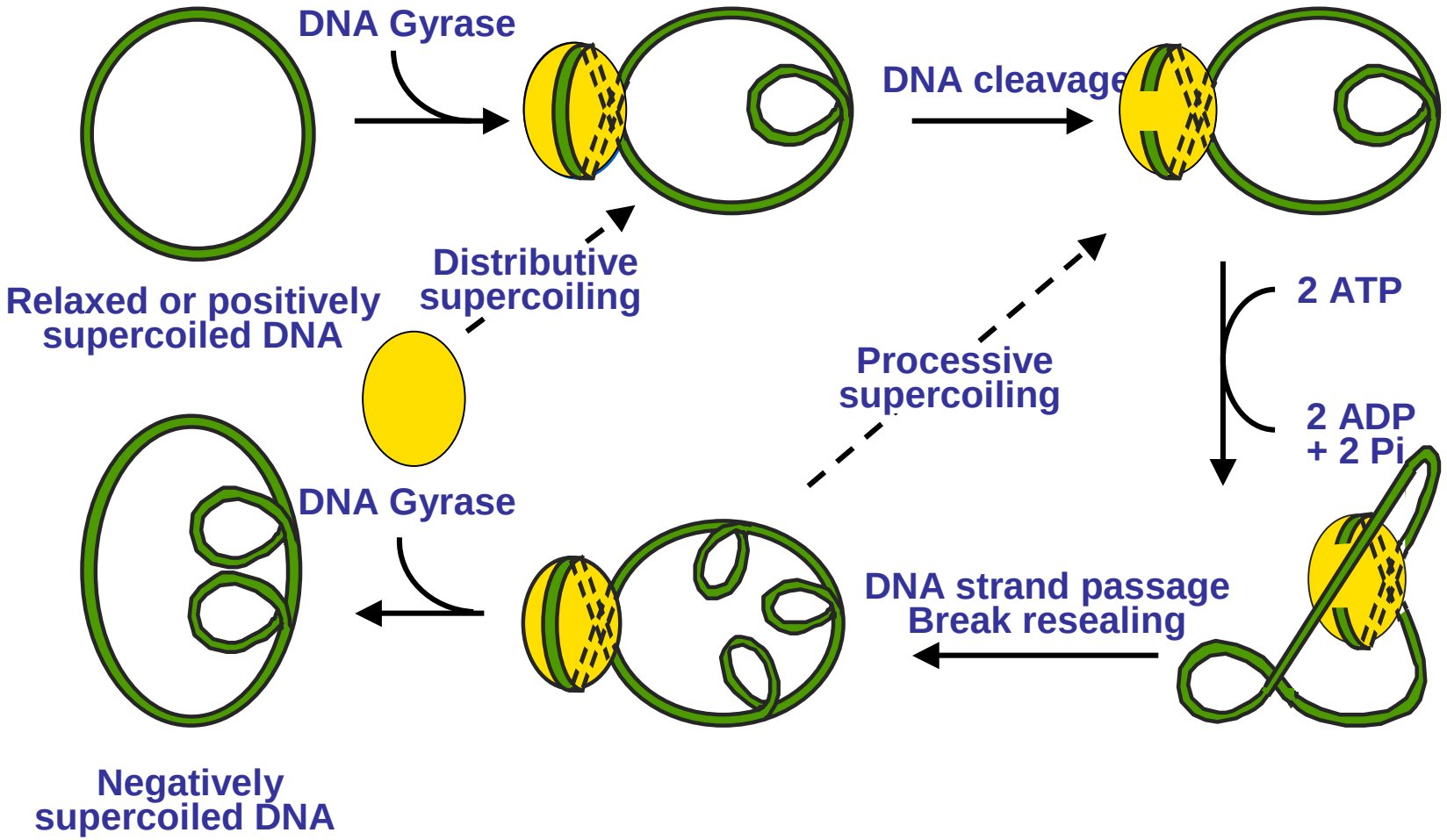
✓ • Positive fraction	# of positives / # of pairs
• Negative fraction	# of negatives / # of pairs
✓ • Average of PM / MM ratios	10x $\sum$ log (PM / MM) / # of pairs in average
✓ • Positive/Negative	# of positives / # of negatives
• Average of intensity differences	$\sum$ (PM - MM) / # of pairs in average

✓ → Decision matrix → Transcript present or absent

Average intensity difference → additional information about the abundance of a transcript

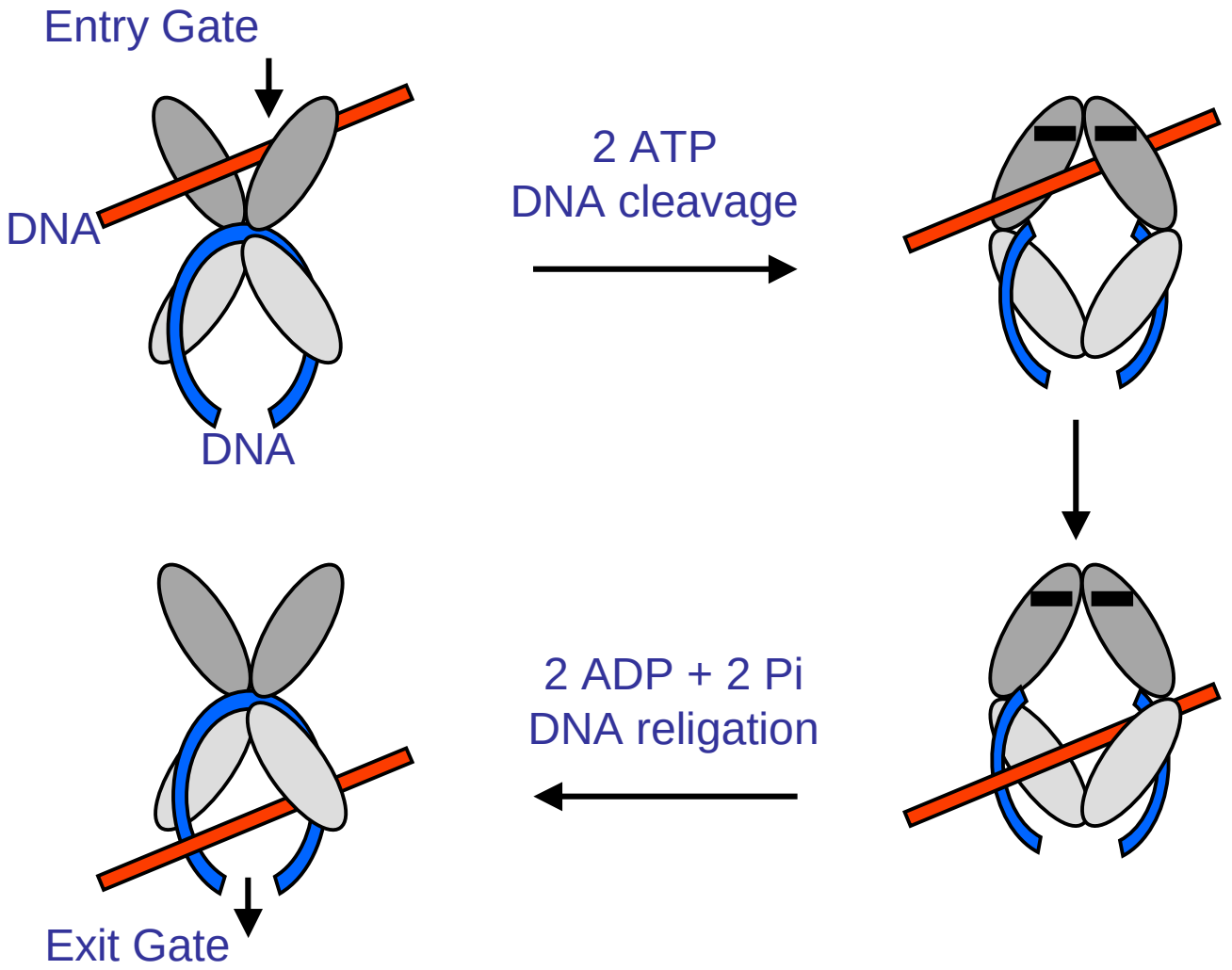
GENE DATA

Mechanism of DNA gyrase



GENE DATA

Mechanistic model for DNA gyrase



GENE DATA