Techniques for epigenetic analysis

How to apply them to human and epidemiology studies

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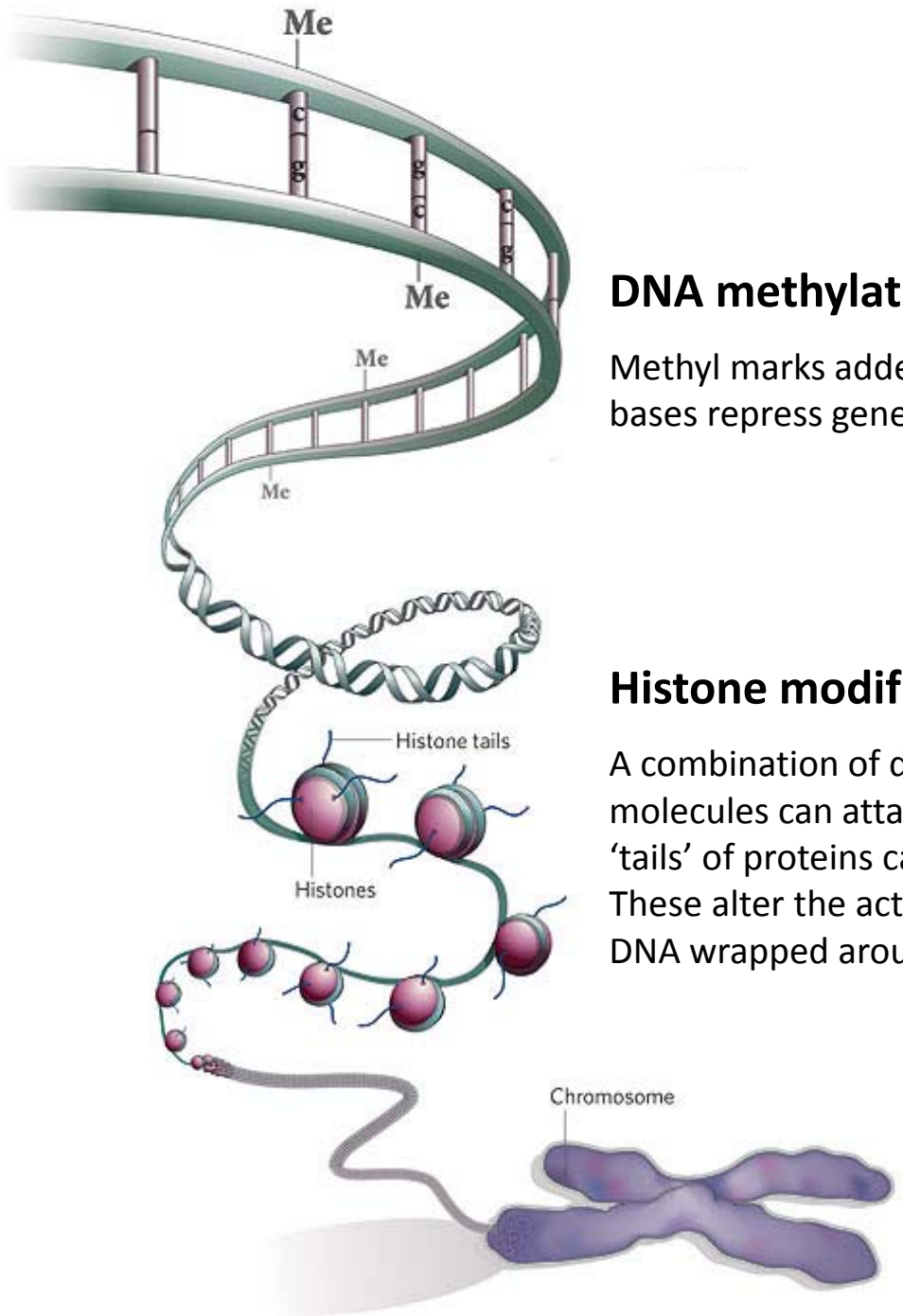
How to detect epigenetic marks?

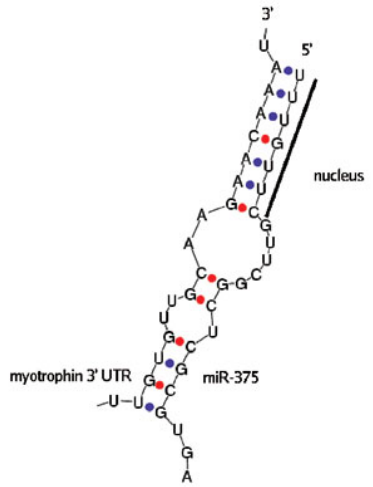


DNA methylation

Methyl marks added to certain DNA bases repress gene activity

Histone modifications

A combination of different molecules can attach to the 'tails' of proteins called histones. These alter the activity of the DNA wrapped around them

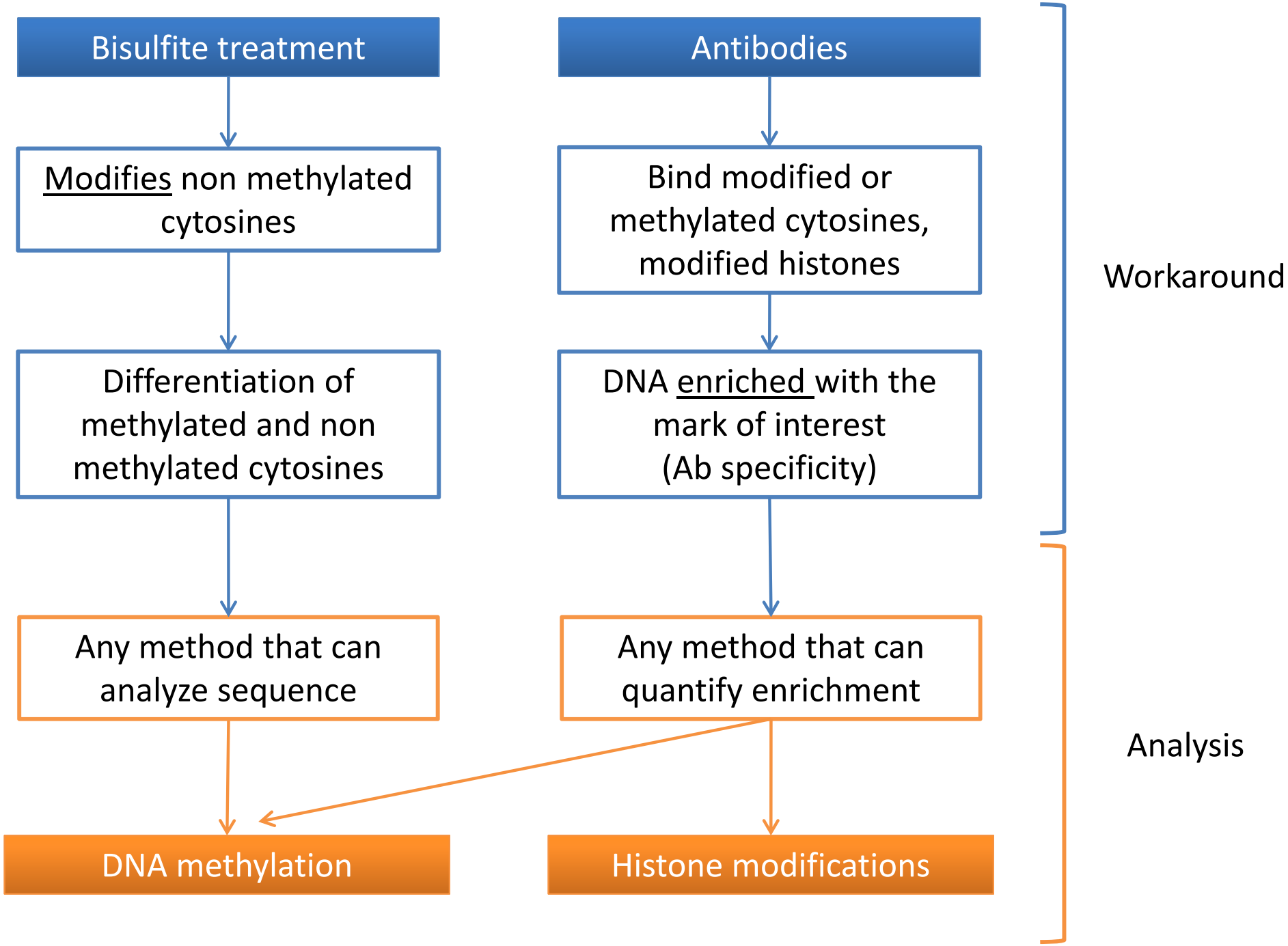


microRNAs (snRNAs)

Small non coding RNAs that cause mRNA degradation or impair translation into protein

DNA methylation & histone modification analysis

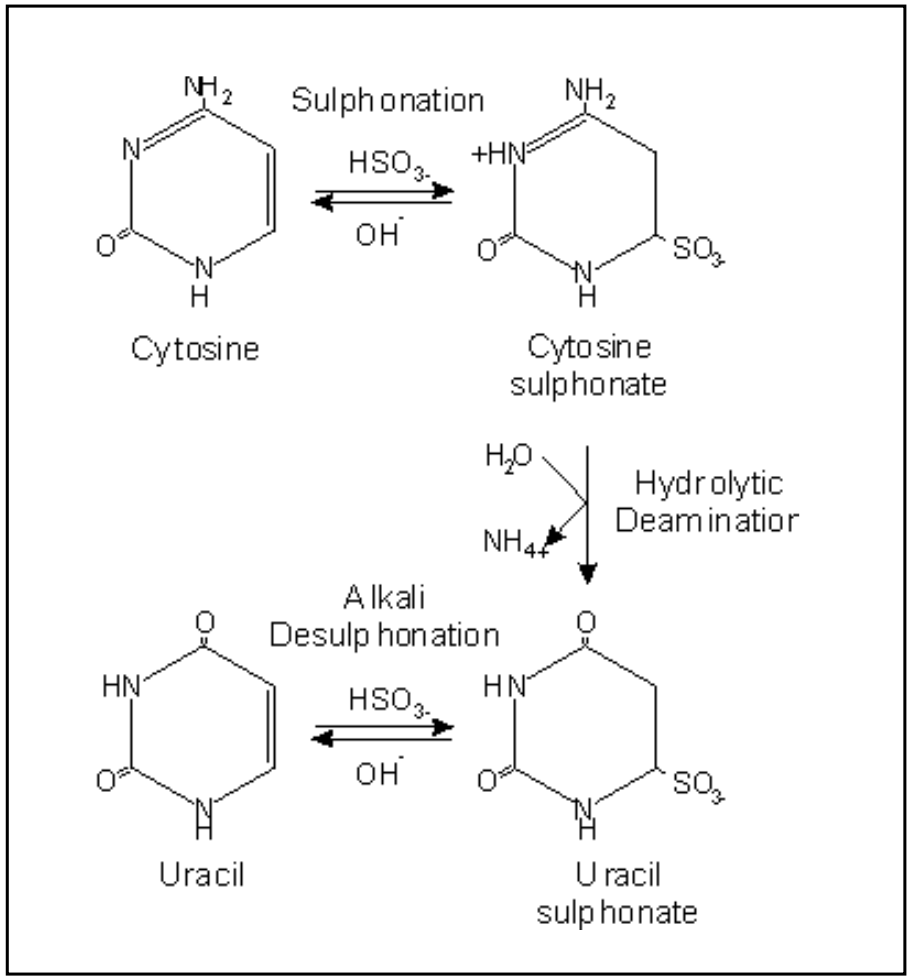
- Molecular biology: largely related to genetics
  - methods that analyze the DNA sequence
- Epigenetic marks: do not modify the underlying DNA sequence
- Workarounds:
  - Bisulfite treatment
  - Antibody-based methods (or alternatively methyl-binding proteins)


Bisulfite treatment
Antibodies
Modifies non methylated cytosines
Bind modified or methylated cytosines, modified histones
Differentiation of methylated and non methylated cytosines
DNA enriched with the mark of interest (Ab specificity)
Any method that can analyze sequence
Any method that can quantify enrichment
DNA methylation
Histone modifications
Workaround
Analysis

Some Lab Nomenclature

- DNA Methylation
  - Gene-specific analysis
    - How much methylation at or nearby a candidate gene
  - Global methylation content
    - How much methylation in a test DNA, regardless of the position
  - Genome-wide scans
    - Microarrays, Next Generation Sequencing
- Histone Modifications
  - Gene-specific analysis
  - Global modification content
  - Genome-wide scans

Bisulfite modification of DNA



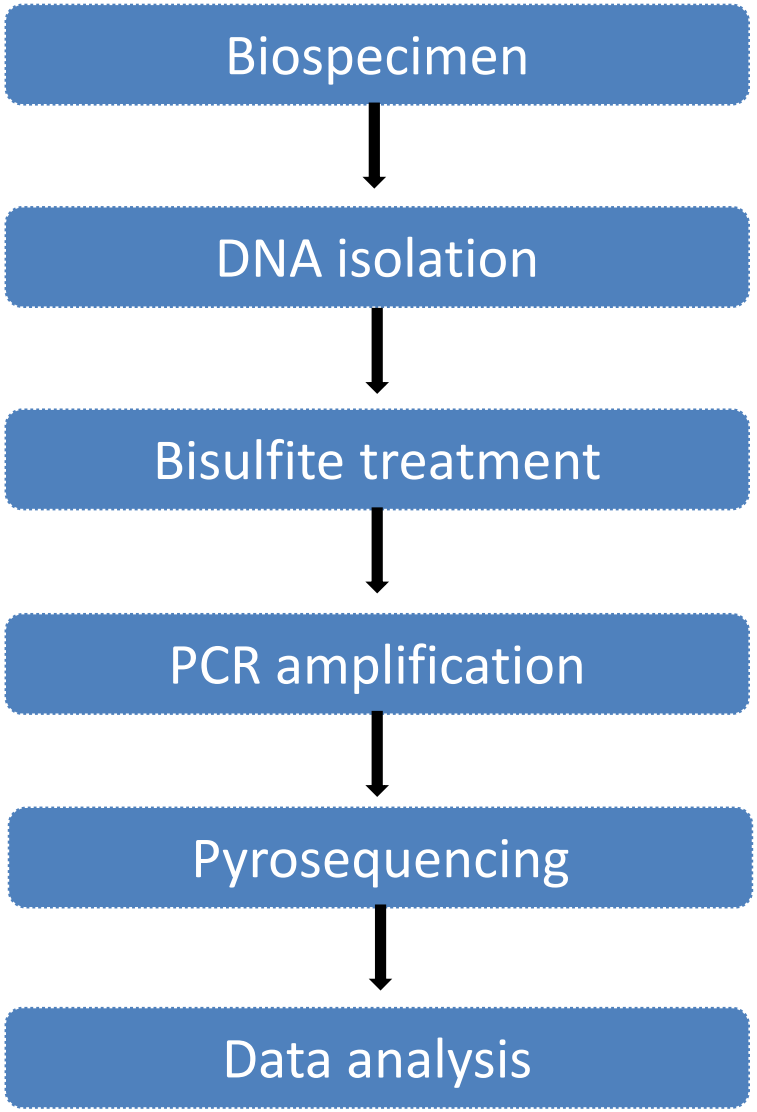
- Prior to PCR, DNA is treated with sodium bisulfite
- Non-methylated C is permanently modified to U
- In PCR, U and T are equivalent

DNA methylation methods

- DNA Methylation Techniques
  - Gene-specific analysis
    - Qualitative
    - Quantitative
  - Global methylation content
    - How much methylation in a test DNA, regardless of the position
  - High-coverage methods
    - Genome-wide scans, Arrays

Gene-Specific Analysis

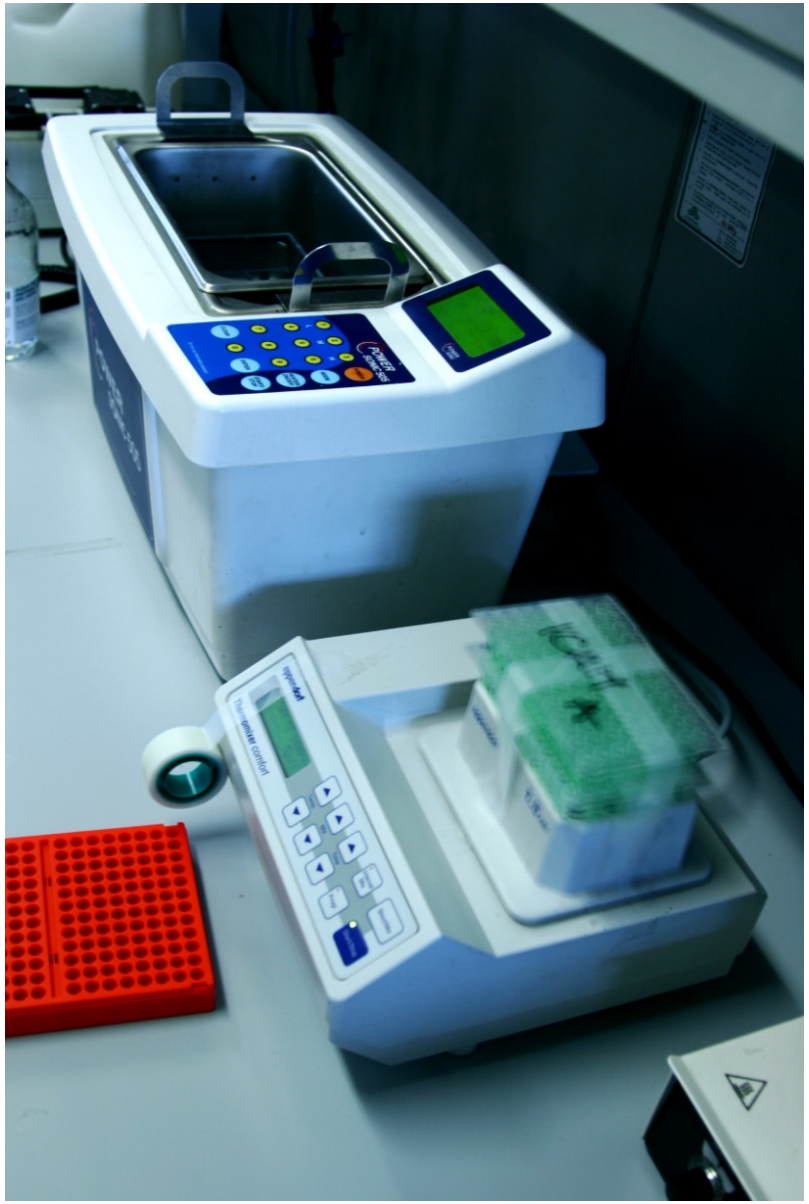
Workflow for DNA methylation analysis by bisulfite-pyrosequencing

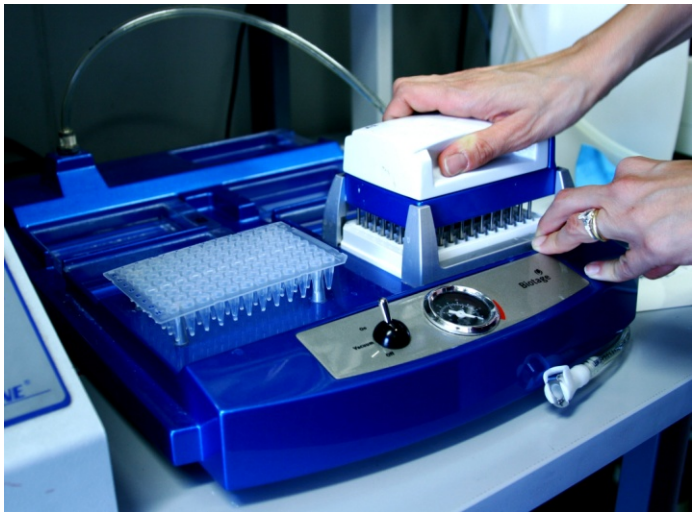


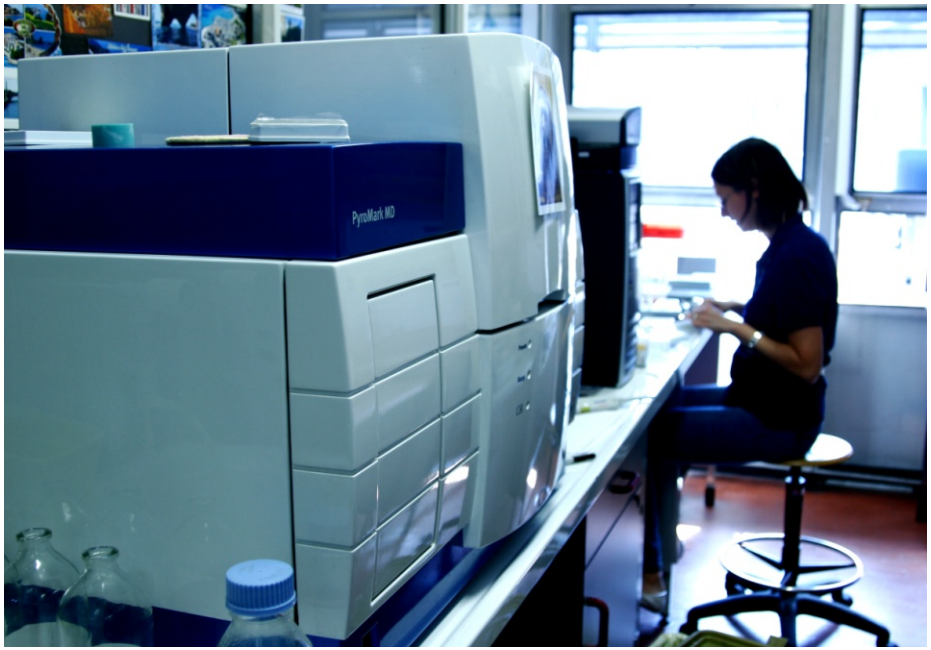
$$\%5mC = \frac{\text{methylated C}}{\text{(methylated C) +(unmethylated C)}} \times 100\%$$

Abbreviations:
%5mC, percentage of 5-methylcytosine;
C, cytosines

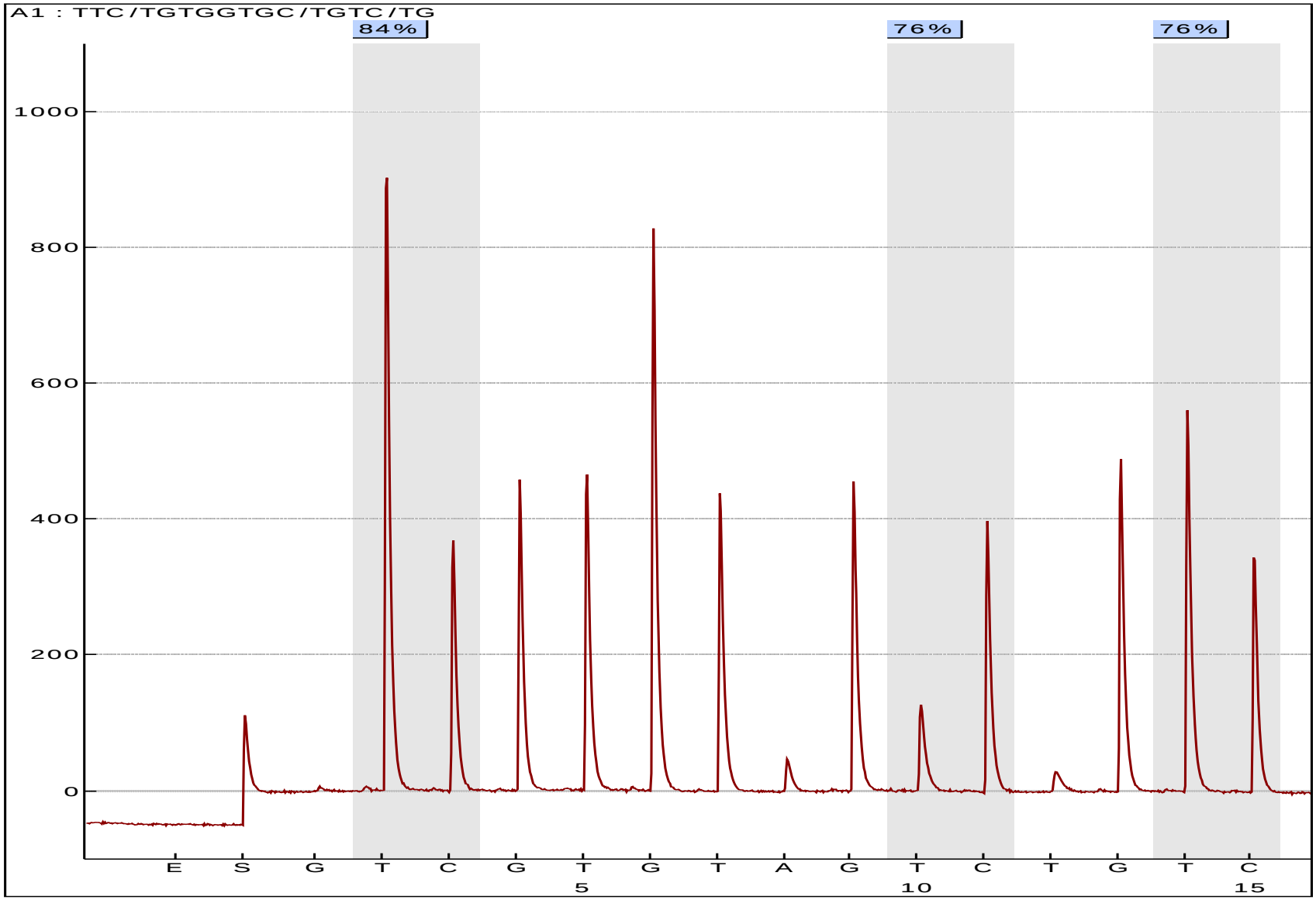
Pyrosequencing







Pyrogram


A1 : TTC/TGTGGTGC/TGTC/TG
84%
76%
76%
1000
800
600
400
200
0
E S G T C G T G T A G T C T G T C
5
10
15

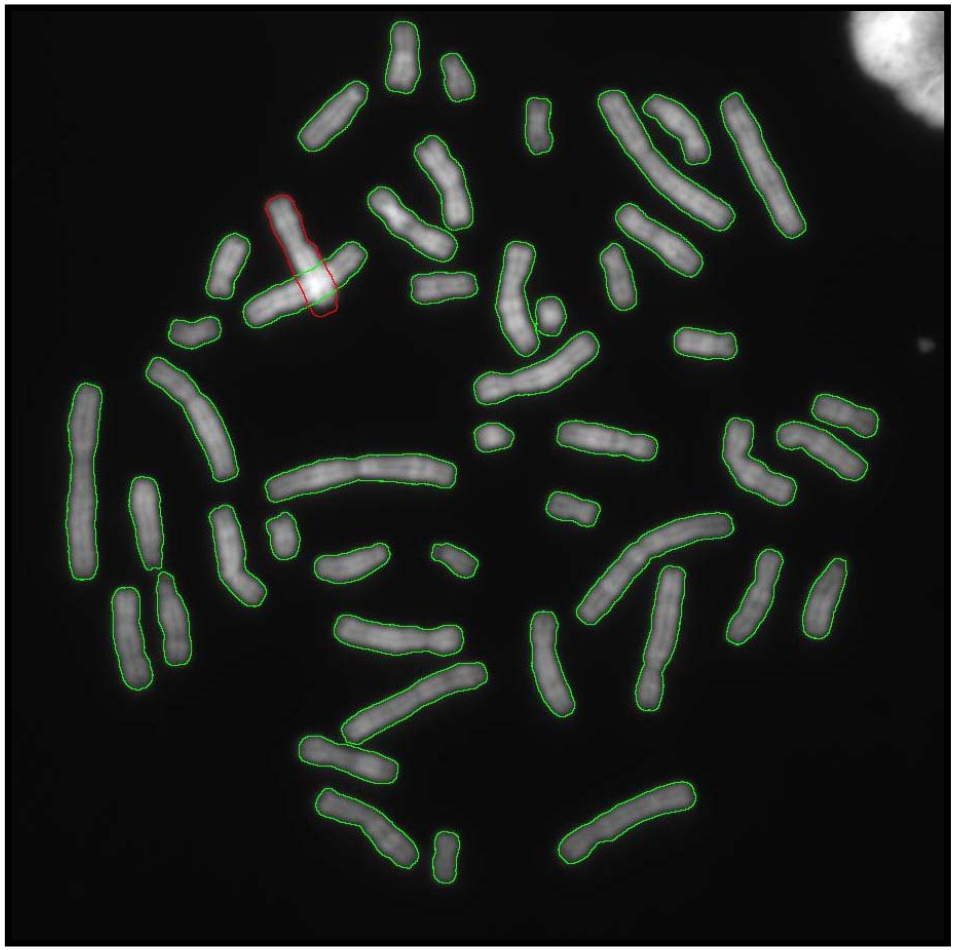
Pyrosequencing analysis

- Provides overview of the the methylation pattern
- Allows for the measurement of the methylation percentage of individual CpG dinucletodes
- Flexibility in sequencing primer position to analyze any CpG sites you like

Pyrosequencing & other methods

- Pyrosequencing
  - Pro: Highly quantitative, single site resolution
  - Cons: dedicated equipment
- Methylation Specific PCR
  - Pro: inexpensive and easy to perform
  - Cons: qualitative/semiquantitative, no single site resolution
- Real-time PCR
  - Pro: equipment easily accessible
  - Cons: low precision, no single site resolution
- Maldi-TOF (Sequenom Mass Array)
  - Pro: Quantitative, single site resolution, extended sequence (amplicon)
  - Cons: dedicated equipment (high costs), high costs/gene

DNA Methylation Content



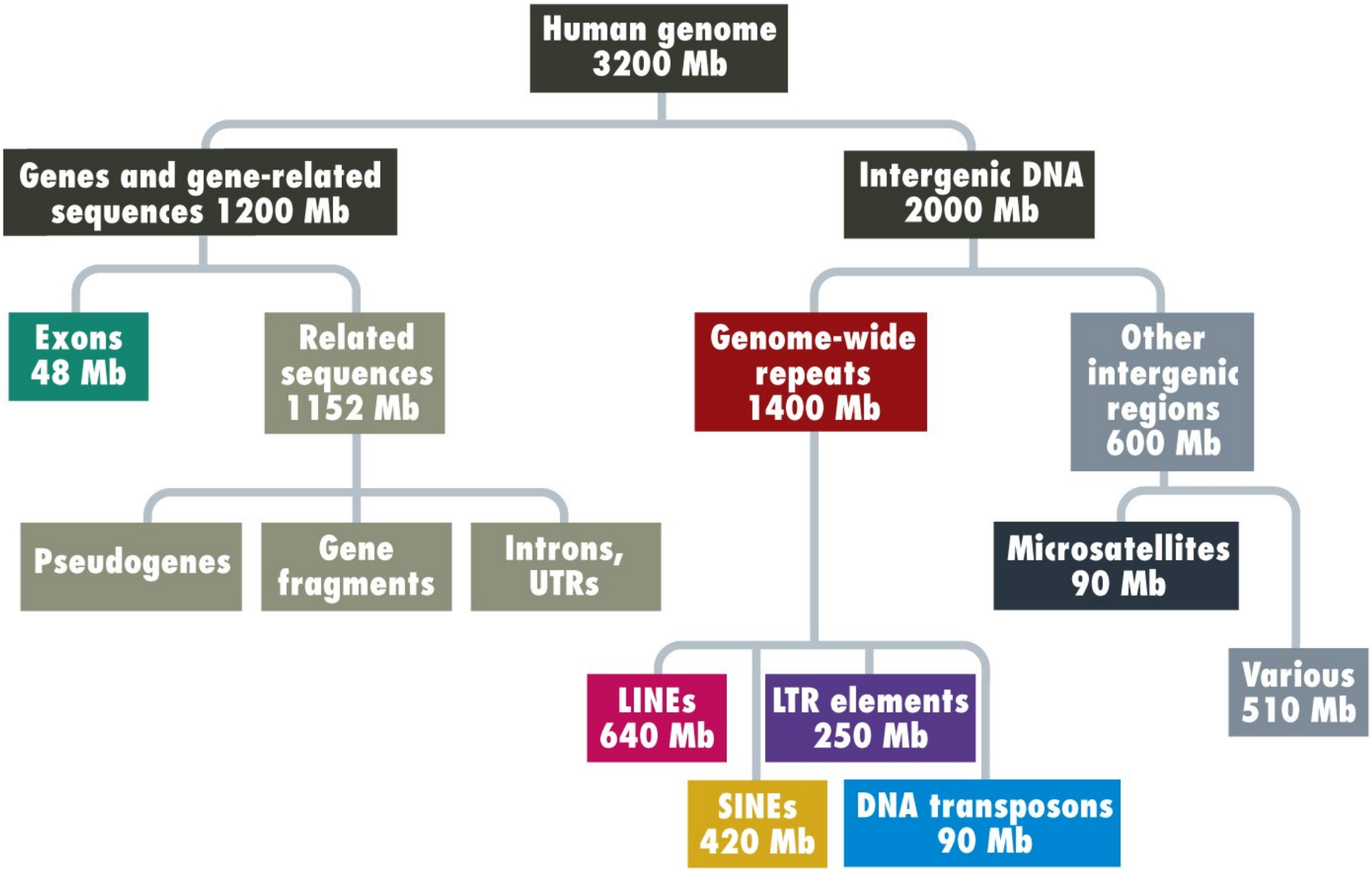


Figure 7.13 *Genomes 3* (© Garland Science 2007)

Global Methylation

- Most methylation in repeated elements:
  - LINE-1 elements: >500K/haploid genome
  - Alu elements: >1,100K/haploid genome
- LINE-1/Alu methylation is correlated with global content (Weisenberger, 2005)
- Function of repeated elements?:
  - Chromosomal structure
  - Repeat sequence transcription
  - miRNA

How to confuse one reader's mind

- Global DNA methylation
- Genomic methylation content
- Genome-wide methylation content
- Genome-wide methylation
- Global cell methylation

Lower Global Methylation

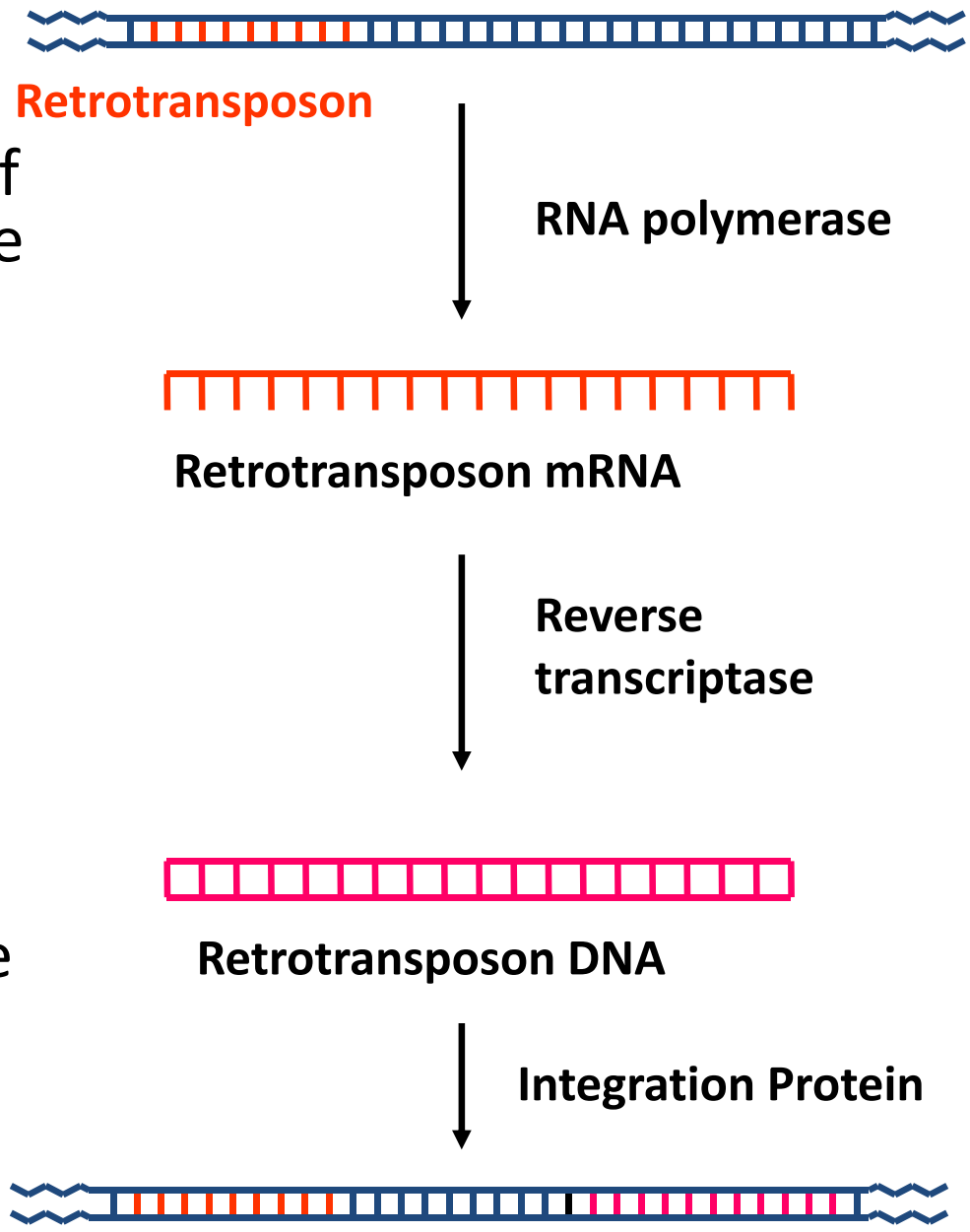
- Tissue DNA
  - Cancer (Feinberg & Vogelstein, 1982)
  - Atherosclerotic lesions (Hiltunen, 2002)
- Blood DNA
  - Cancer (Hsiung, 2007)
  - Cardiovascular Disease (Castro, 2003)
  - Folate deficiency (Choi, 2005)
  - Inflammatory states (Stenvinkel, 2007)
  - Aging (Fuke 2004)
  - Environmental Exposures (Chanda 2005, Bollati 2007, Rusiecki 2008, Baccarelli, 2009)

Analysis of Global Methylation Content

- Direct measurement of methyl group content
  - High-Performance liquid chromatography (Ehlrich M, et al., Nucl Ac Res 1982)
  - GC/MS (Rossella F et al., Rapid Comm MS 2009)
  - Immunostaining with anti-5mC
  - Digestion with methylation sensitive enzymes (*MspI, HpaII)*
- Estimated in repeated elements
  - Based on Pyrosequencing (Yang et al. Nucl Ac Res 2004)
  - Based on Methylight (Weisenberger et al. Nucl Ac Res 2005)

Retrotransposons

- Transposition: Movement of gene from one chromosome to another or movement from one site to another; does not require homology
- Transposons: mobile genetic elements that enable genes to move between non-similar sites
- Retrotransposition: Creates genetic diversity
- Retrotransposons: Replicate and move to other sites on DNA through an RNA intermediate



What do we measure in repeated elements

- A few CpG sites in a specific repeated element sequence
- The sequence (and the CpG sites therein) are repeated throughout one single haploid genome:
  - LINE-1 elements: >500K/haploid genome
  - Alu elements: >1,100K/haploid genome
  - The LINE-1 and Alu assays measure the %mC in those repeated elements, with no distiction about their position in the genome
- Correlation with global content demonstrated only in studies including cancer tissues
  - A marker of global methylation only for cancer tissues?

Genome-wide scans

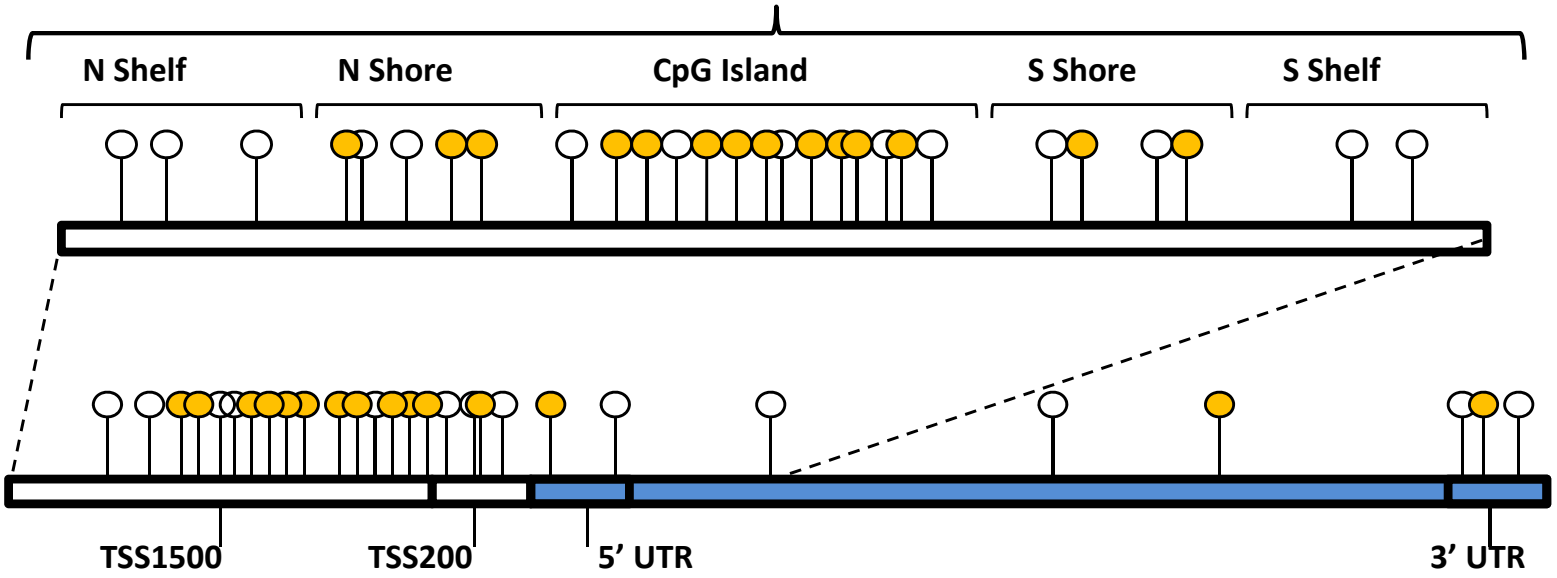
- Microarrays
  - Illumina Infinium for DNA methylation (bisulfite treatment)
    - 484,000 CpG sites (450K, released in early 2011)
    - $400/samples
  - Nimblegen for DNA methylation or Histone Modifications (Ab-based)
    - 2 Million probes
    - >$2000/samples
- Next Generation sequencing
  - Various platforms
  - Bisulfite treatment (DNA methylation)
  - Ab-based (DNA methylation or Histone modifications)
  - $3000/sample for RRBS

Illumina 450K BeadChip Coverage

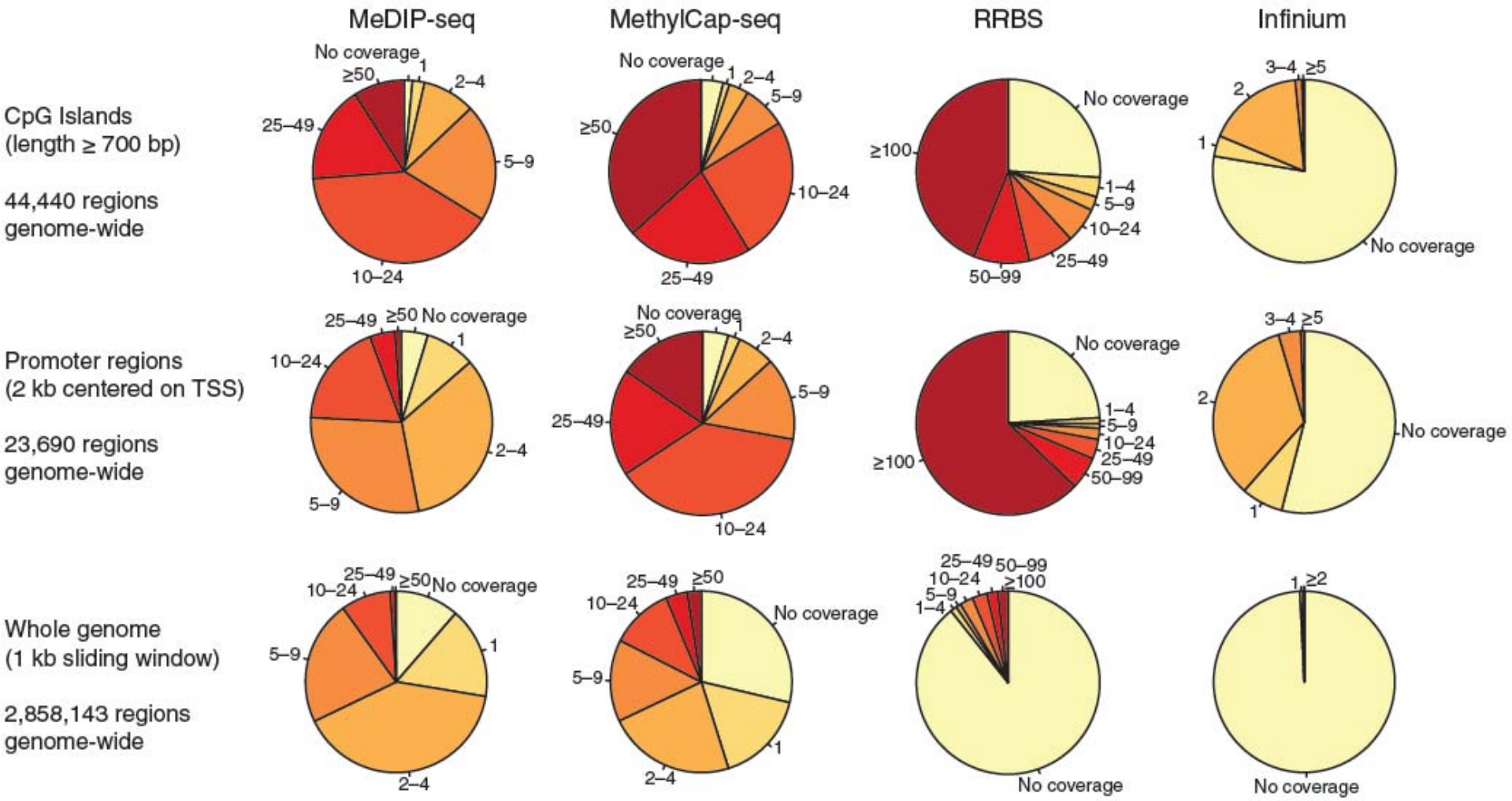
CpG shelves, shores & islands classification (UCSC CpGi annotation)

The 450K BeadChip covers a total of 77,537 CpG Islands and CpG Shores (N+S)

Region Type	Regions	CpG sites covered on 450K BeadChip array	Average # of CpG sites per region
CpG Island	26,153	139,265	5.08
N Shore	25,770	73,508	2.74
S Shore	25,614	71,119	2.66
N Shelf	23,896	49,093	1.97
S Shelf	23,968	48,524	1.94
Remote/Unassigned	-	104,926	-
Total		485,553	

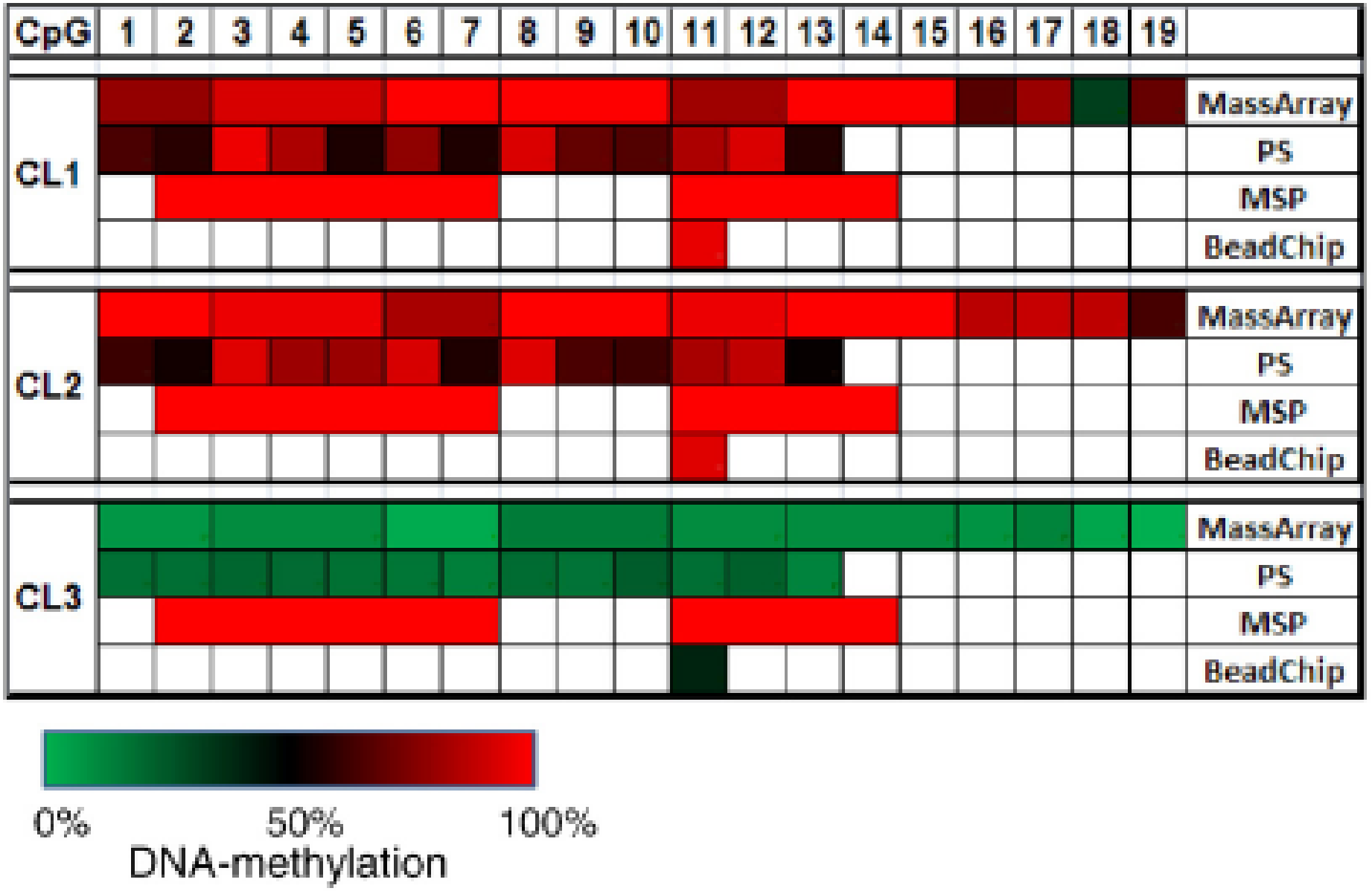


NGS vs Infinium HM27



Infinium: less expensive, most accurate (together with RRBS), easiest for bioinformatic analysis

Bock et al., Nat Genet 2010



MSP: Methylation>25% generated positive results

Ammerpohl O et al. Biochim Biophys Acta. 2009 Sep;1790(9):847-62.

Histone Modification Analysis

- Step 1:
  - Histone Purification & Isolation
- Step 2:
  - Histone Analsyis (Several Methods)
    - ELISA (Enzyme-Linked ImmunoSorbent Assay)
    - ChIP (Chromatin ImmunoPrecipitation)

Types of measure

- ELISA
  - Global genomic content of a certain modification:
- ChIP qPCR
  - Gene specific measure of a certain modification next to a specific gene
- ChIP-on-chip
  - Gene specific measure of a certain modification next to many specific gene at the same time

Examples

- ELISA
    - Histone: H3
    - Modification: Di-Methyl-Histone
    - Position: Lys4 (K4)
- ChIP qPCR
    - Histone: H3
    - Modification: Di-Methyl-Histone
    - Position: Lys4 (K4)
    - Gene: p15
- ChIP-on-chip
    - Histone: H3
    - Modification: Di-Methyl-Histone
    - Position: Lys4 (K4)
    - Genes: all the genes spotted on the chip

ChIP qPCR

Formaldeide Cross Link

↓

Cells isolated nuclei

↓

Sonicate

↓

Immunoprecipitate

↓

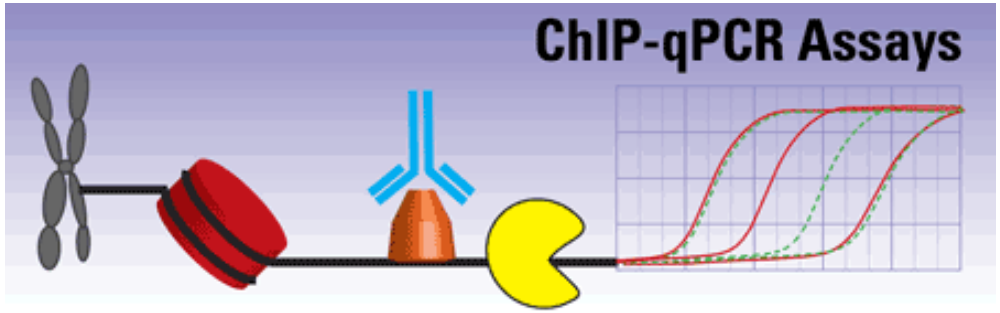
Reverse crosslink

↓

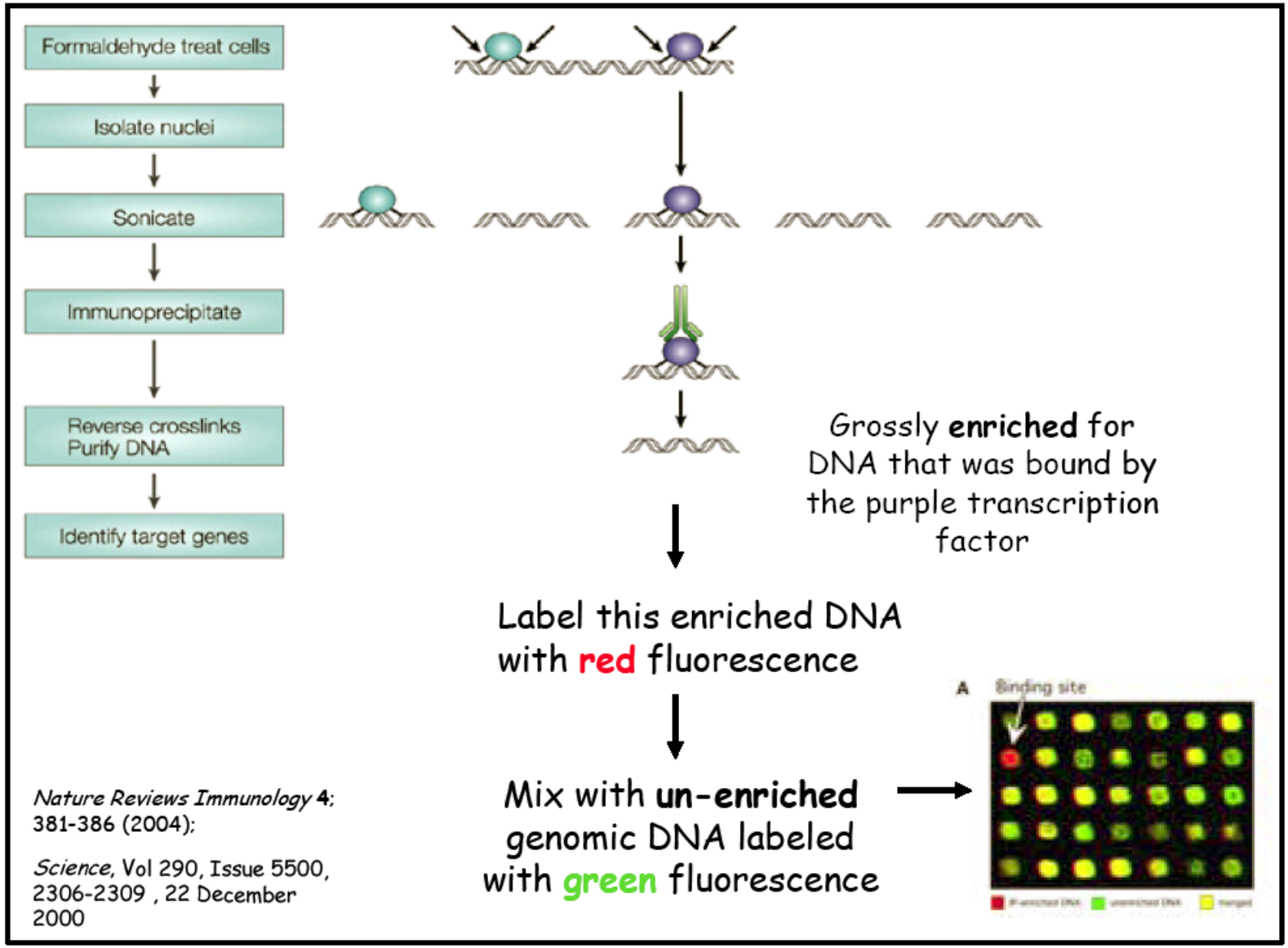
Purify DNA

↓

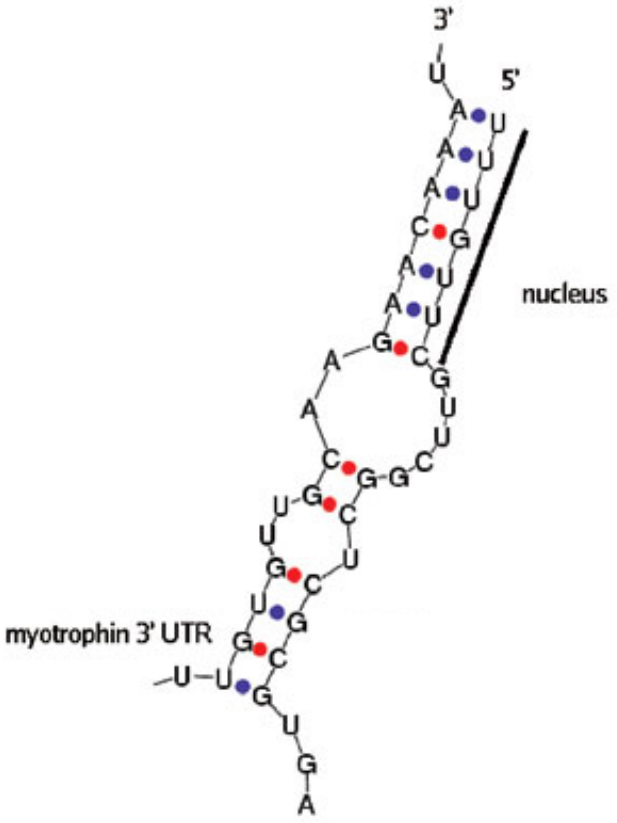
qPCR

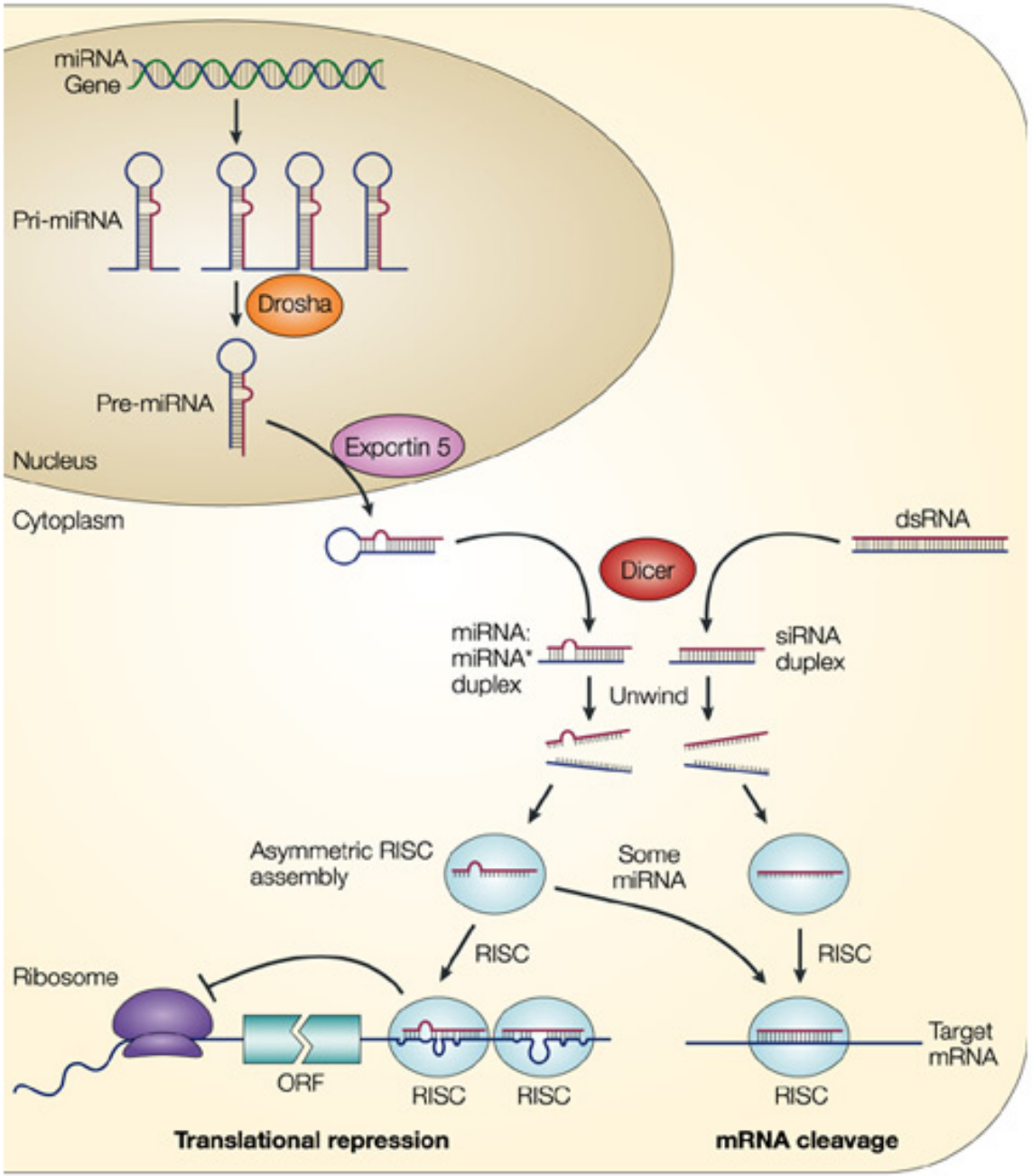


ChIP-on-chip


Formaldehyde treat cells
Isolate nuclei
Sonicate
Immunoprecipitate
Reverse crosslinks
Purify DNA
Identify target genes
Grossly enriched for DNA that was bound by the purple transcription factor
Label this enriched DNA with red fluorescence
Mix with un-enriched genomic DNA labeled with green fluorescence
A
Binding site
Nature Reviews Immunology 4: 381-386 (2004);
Science, Vol 290, Issue 5500, 2306-2309 , 22 December 2000

MiRNA biogenesis and mechanisms of action





He and Hannon, 2004

miRNA analysis

- Sample collection and processing
  - Same methods as for mRNA
  - Isolation of total RNA
- Methods
  - Candidate miRNAs: Real Time PCR
  - Microarrays
  - nCounter Nanostring Analysis
  - Deep Sequencing

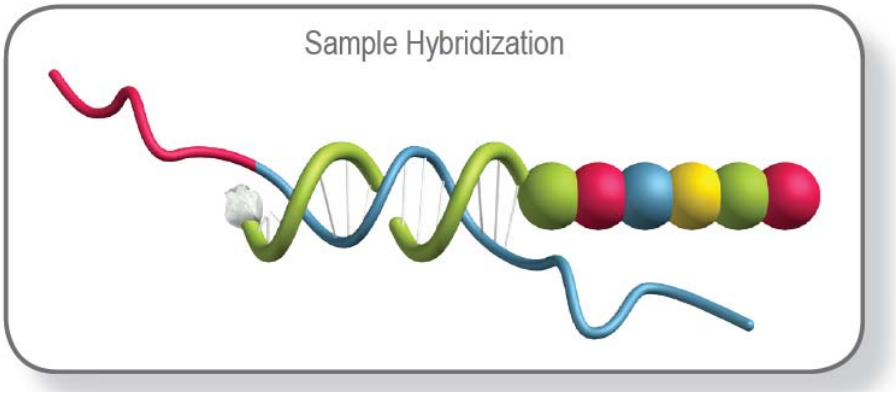
Microarrays for miRNA analysis

	# of measured human miRNAs
ABI TLDA	**673**
Affymetrix	**847**
Agilent	**723**
Exiqon	**732**
Illumina	**1146**

From Susan Hester, EPA

http://www.abrf.org/Other/ABRFMeetings/ABRF2009/ABRF2009%20presentation/r3-b_SHester.pdf

Nanostring nCounter



- Digital counting technology
- Allows for measuring more than 700 miRNAs
- Moderate operating costs
- No need for PCR amplyfication

Next Generation Sequencing

Analysis

Nature Biotechnology **26**, 407 - 415 (2008)
doi:10.1038/nbt1394

Discovering microRNAs from deep sequencing data using miRDeep

Marc R Friedländer[1], Wei Chen[2], Catherine Adamidi[1], Jonas Maaskola[1], Ralf Einspanier[3], Signe Knespel[1] & Nikolaus Rajewsky[1]

The capacity of highly parallel sequencing technologies to detect small RNAs at unprecedented depth suggests their value in systematically identifying microRNAs (miRNAs). However, the identification of miRNAs from the large pool of sequenced transcripts from a single deep sequencing run remains a major challenge. Here, we present an algorithm, miRDeep, which uses a probabilistic model of miRNA biogenesis to score compatibility of the position and frequency of sequenced RNA with the secondary structure of the miRNA precursor. We demonstrate its accuracy and robustness using published *Caenorhabditis elegans* data and data we generated by deep sequencing human and dog RNAs. miRDeep reports altogether ~230 previously unannotated miRNAs, of which four novel *C. elegans* miRNAs are validated by northern blot analysis.

Questions related to study design

- Which tissue can I use?
- How stable are the epigenetic marks within-subject over time?
- Data analysis:
  - Cell type effects
  - Confounders

Human Studies: Which Tissue?

- Target tissues
  - Cancer tissues are easy to obtain
    - They do not necessarily reflect pre-disease status
    - FFPE are not easy to work with
  - For some disease we can get at least close
    - Bladder cancer → Cells in urine sediments
    - Leukemias → White blood cells
      (benzene effects in Bollati et al Cancer Res 2007)

Non-Target Tissues

- How about psychiatric diseases?
  - Embryo layer approach
    (Neuroectoderma→buccal; good only for in-utero exposure?)
  - Uniform effects approach: Do exposures modify epigenetic marks in the same way across all tissues?
    - Problem 1: Epigenetic marks are tissue specific
    - Problem 2: Distribution of pollutants: different doses in different tissues (e.g., particulate matter)
  - Highest dose/first target approach
    - Inhalable pollutants → nasal mucosa
    - Pollutants in food and water/smoking→ buccal cells

A Practical Starting Point

- Existing cohort/studies have collected:
  - Blood/buffy coat
  - Buccal cells
  - Urine
- Storage
  - Many studies extracted DNA and have no more cells available
- Questions:
  - Can we use them?
  - Which information can we get?

How we have been using WBCs

- **Requirement 1:** The gene is expressed in WBCs (http://www.ncbi.nlm.nih.gov/IEB/Research/Acembly/)
- **Requirement 2:** The gene has a function in WBCs that is related with:
  - Mechanisms of action of the exposure
  - Disease of interest (http://www.ncbi.nlm.nih.gov/omim/)
- E.g.: Particulate matter→ iNOS→Cardiovascular disease
  (Tarantini et al. Environ Health Perspectiv 2009)

WBC Methylation in Epidemiology

- When analyzing data, which are the confounders we need to control for?
- What do we know about determinants of DNA methylation in human populations?
- To address this question:
  - Pooled analysis of 1,465 subjects from five investigations
  - In all the investigations: DNA methylation analysis of LINE-1 and Alu

Pooled Analysis Data

	Study #1	**Study #2**	**Study #3**	**Study #4**	**Study #5**	**All studies**
	Boston, USA	Warsaw, Poland	Milan, Italy	Brescia, Italy	Trissino, Italy	*n*=1,465
Alu, 5%mC (SD)	26.3 (1.1)	25.1 (1.4)	27.1 (3.4)	25.8 (0.7)	24.9 (1.0)	26.0 (1.8)
LINE-1, 5%mC (SD)	76.9 (2.0)	80.2 (3.2)	71.7 (5.9)	78.8 (1.0)	78.7 (3.2)	76.2 (6.2)

Zhu et al., Int J Epidemiology 2012

Blood Count and Methylation

	Alu		LINE-1	
	Beta *	*P*-value *	Beta *	*P*-value *
White blood cells, 10^3cell/mm^3	0.002	0.938	0.078	0.168
Neutrophils, %	0.009	0.226	**0.036**	**0.005**
Lymphocytes, %	-0.009	0.246	**-0.039**	**0.004**
Monocytes, %	-0.001	0.981	-0.033	0.374
Eosinophils, %	-0.014	0.643	0.007	0.888
Basophils, %	0.005	0.968	-0.202	0.399

* Adjusted for age, gender and study.

Zhu et al., Int J Epidemiology 2012

Age, gender, alcohol

Variable	Alu					LINE-1				
	No.	%5mC[a]	95% CI[a]	Beta[a]	*P*-value[a]	No.	%5mC[a]	95% CI[a]	Beta[a]	*P*-value[a]
Age, years										
<48	285	26.2	25.9, 26.5			264	77.4	76.8, 77.9		
48-63	279	26.0	25.8, 26.2			271	77.5	77.1, 77.9		
64-69	314	26.0	25.8, 26.2			307	77.2	76.8, 77.6		
70-74	267	25.9	25.7, 26.1			263	77.1	76.7, 77.5		
≥75	320	25.9	25.7, 26.1	-0.012[b]	0.019[b]	313	77.1	76.7, 77.5	-0.003[b]	0.780[b]
Gender										
Female	206	26.3	26.0, 26.6			202	76.5	76.0, 77.0		
Male	1,238	25.9	25.8, 26.0	-0.361	0.012	1,216	77.4	77.2, 77.5	0.874	0.001
Alcohol drinking										
Nondrinker	609	26.1	26.0, 26.3			604	77.2	76.9, 77.4		
Ever-drinker	717	25.9	25.8, 26.0	-0.208	0.043	700	77.1	76.9, 77.3	-0.049	0.798

[a] Adjusted for age, gender and study.

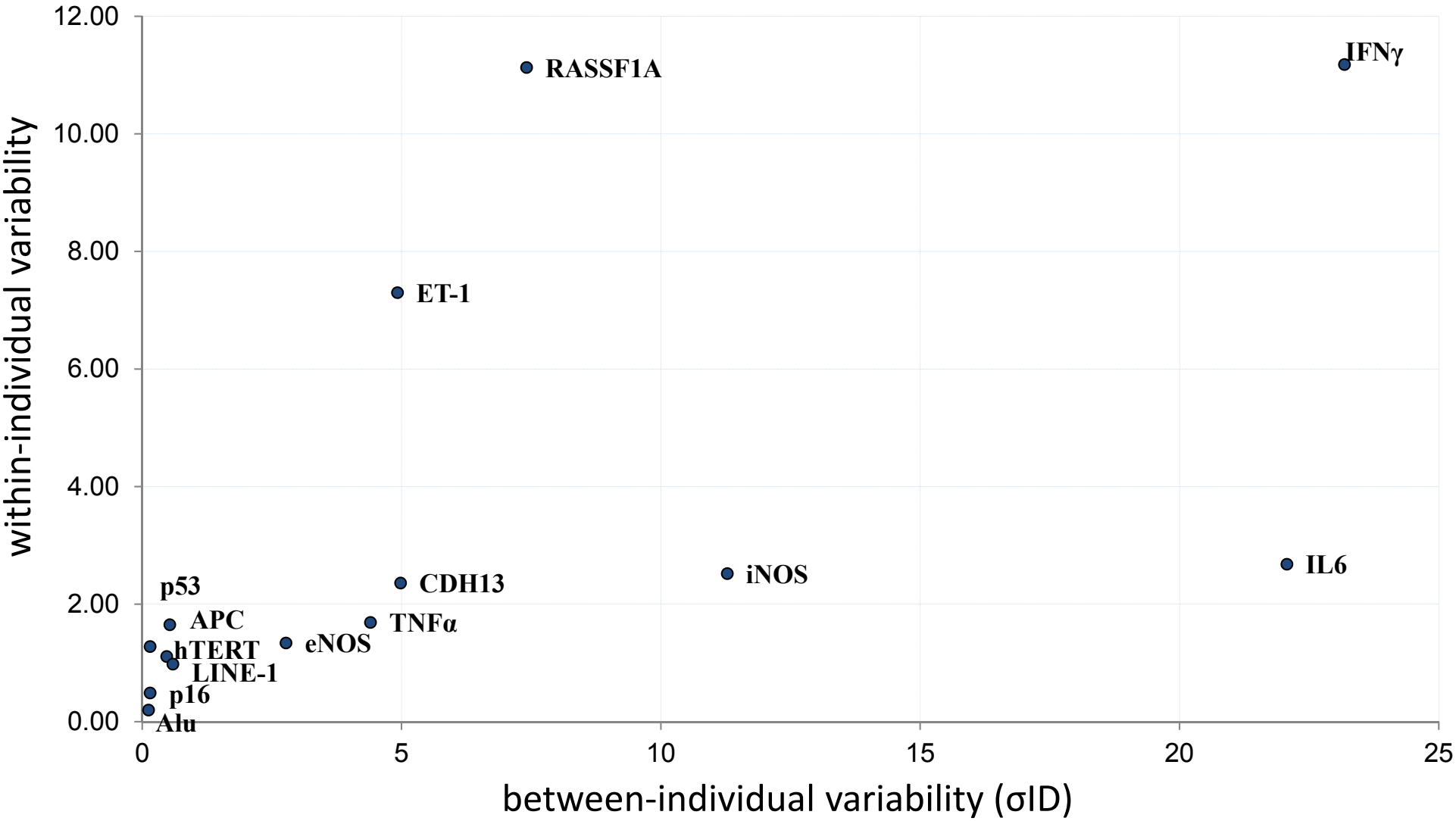
[b] Beta and *P*-value for age were obtained using continuous variables.

Zhu et al., Int J Epidemiology 2012

Summary of pooled analysis

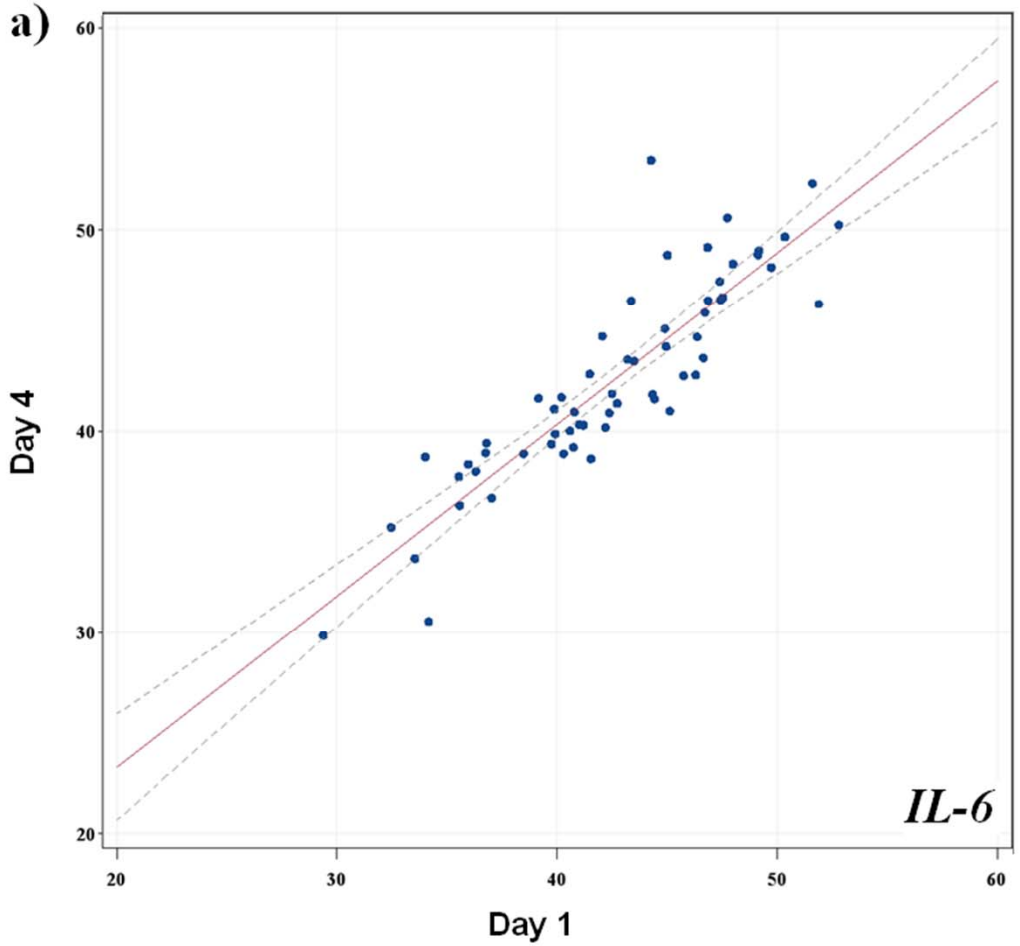
- Alu and/or LINE-1 methylation associated with:
  - WBC differential
  - Age
  - Gender
  - Alcohol
- It may not apply to gene specific methylation
- Need to adjust for WBC differential

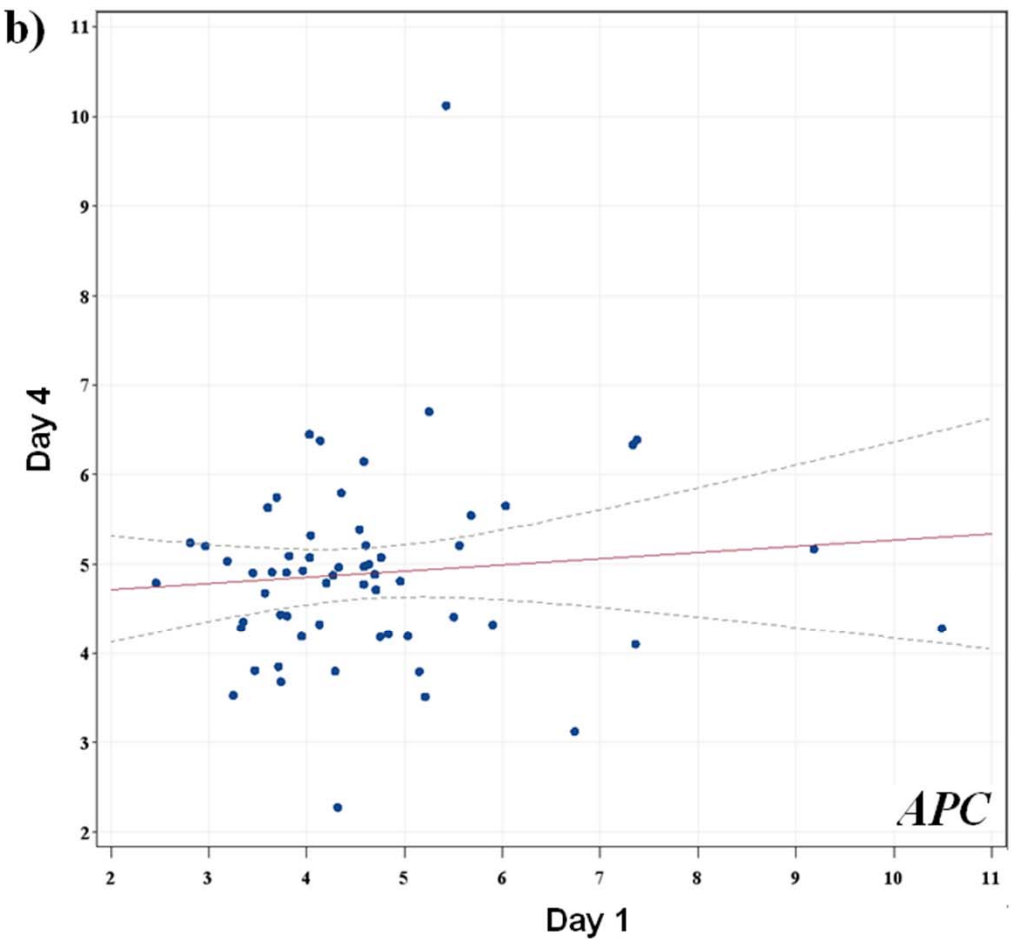
How stable is blood methylation? Variability between Day 1 and 4



Byun HM et al, under review

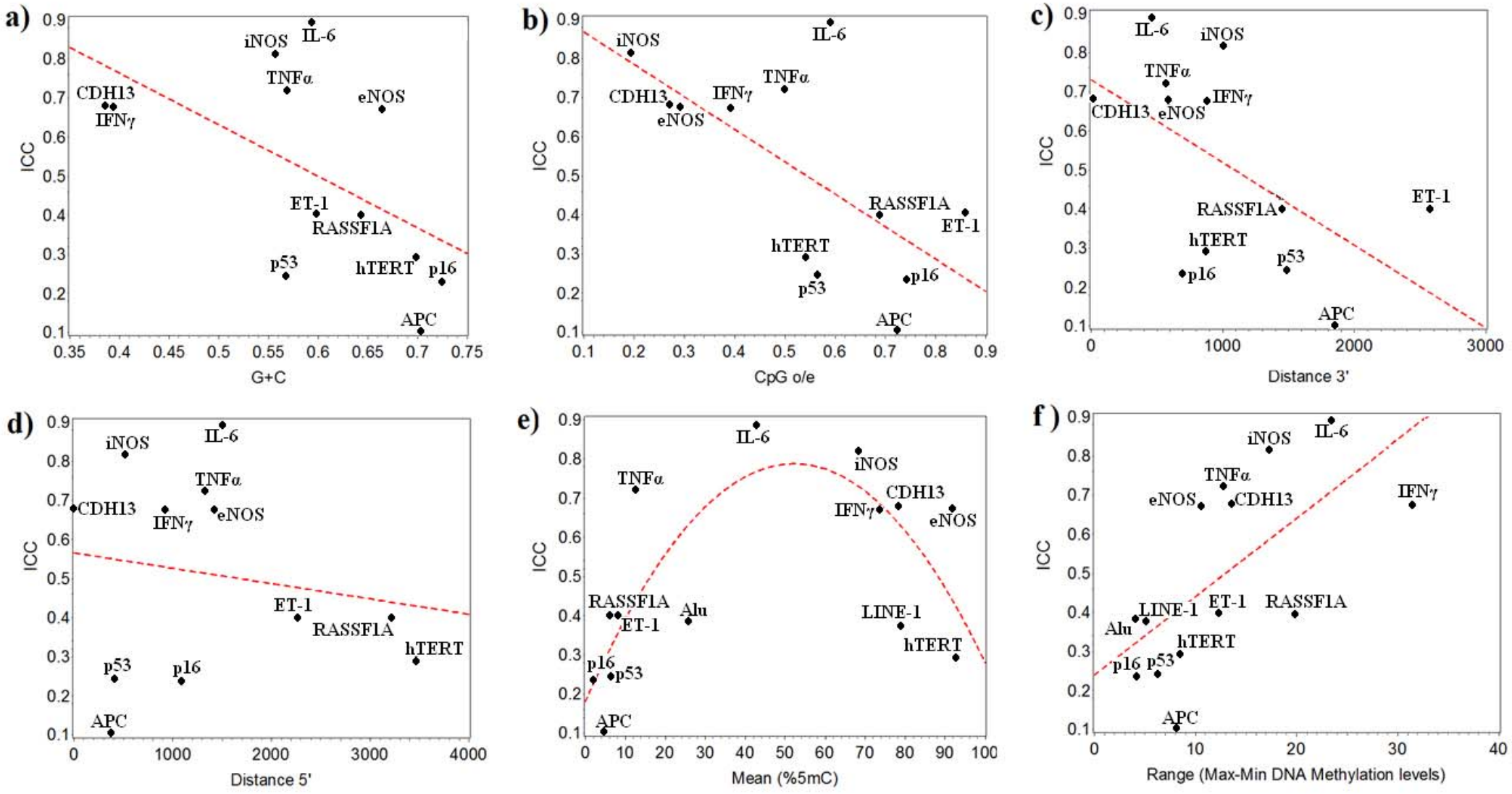
Examples of variability





Byun HM et al, under review

Determinants of variability



Byun HM et al, under review

Mela Valley Asthma Study (Sicily, Italy)

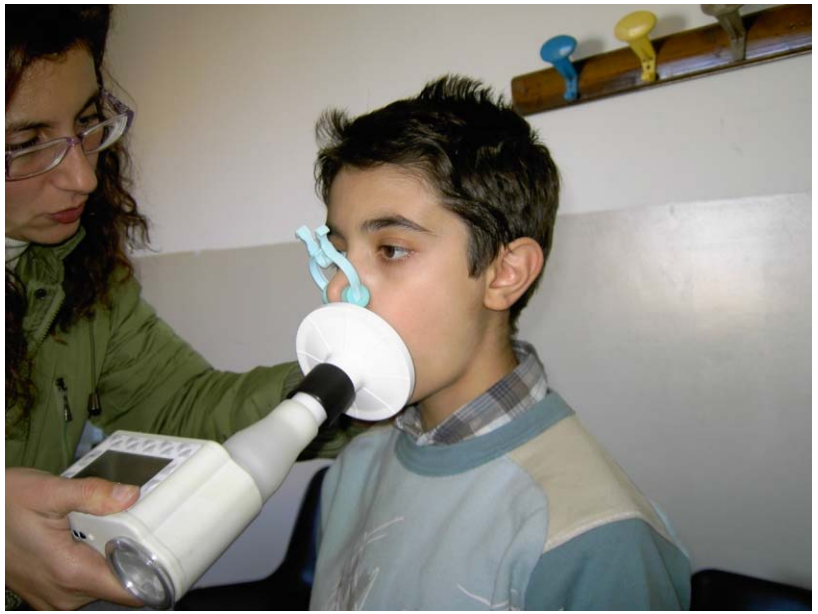
- DNA from nasal swab in 38 third graders with asthma symptoms
- Lung function testing and exhaled nitric oxide measures

Airway inflammation and DNA methylation

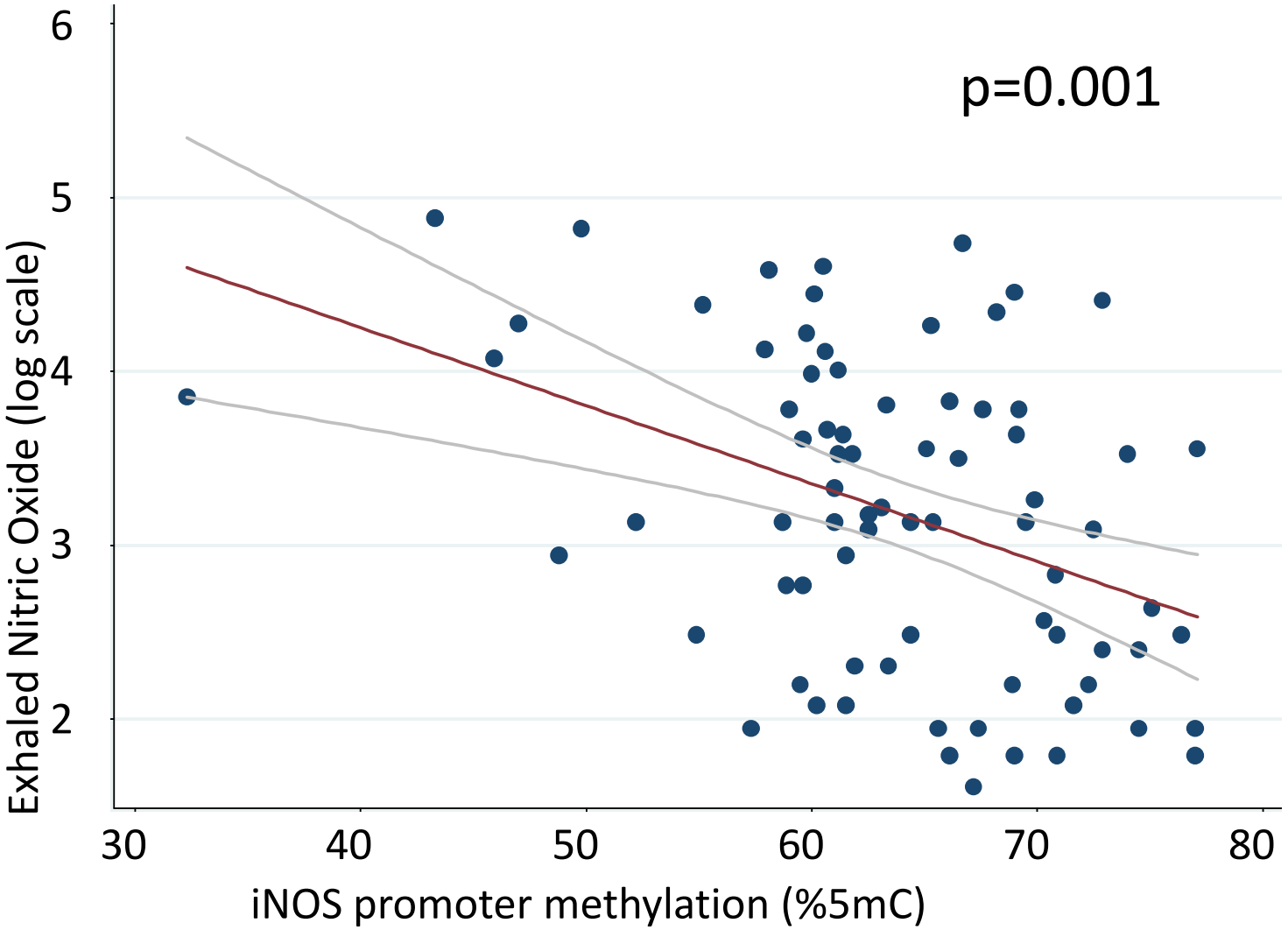
- Interleukine-6 (IL-6) expression:
  - Associated with reduced DNA methylation of the gene promoter
  - Associated with reduced forced expiratory volume in 1 second (FEV1) in asthma patients
- Inducible Nitric Oxide Synthase (iNOS) expression:
  - Associated with reduced DNA methylation of the gene promoter
  - increased NO production in the airway epithelium
  - NO production in asthma can be non-invasively measured as Fractional exhaled Nitric Oxide (FeNO).

Piko FEV-1
(obstruction)

FeNO
(inflammation)

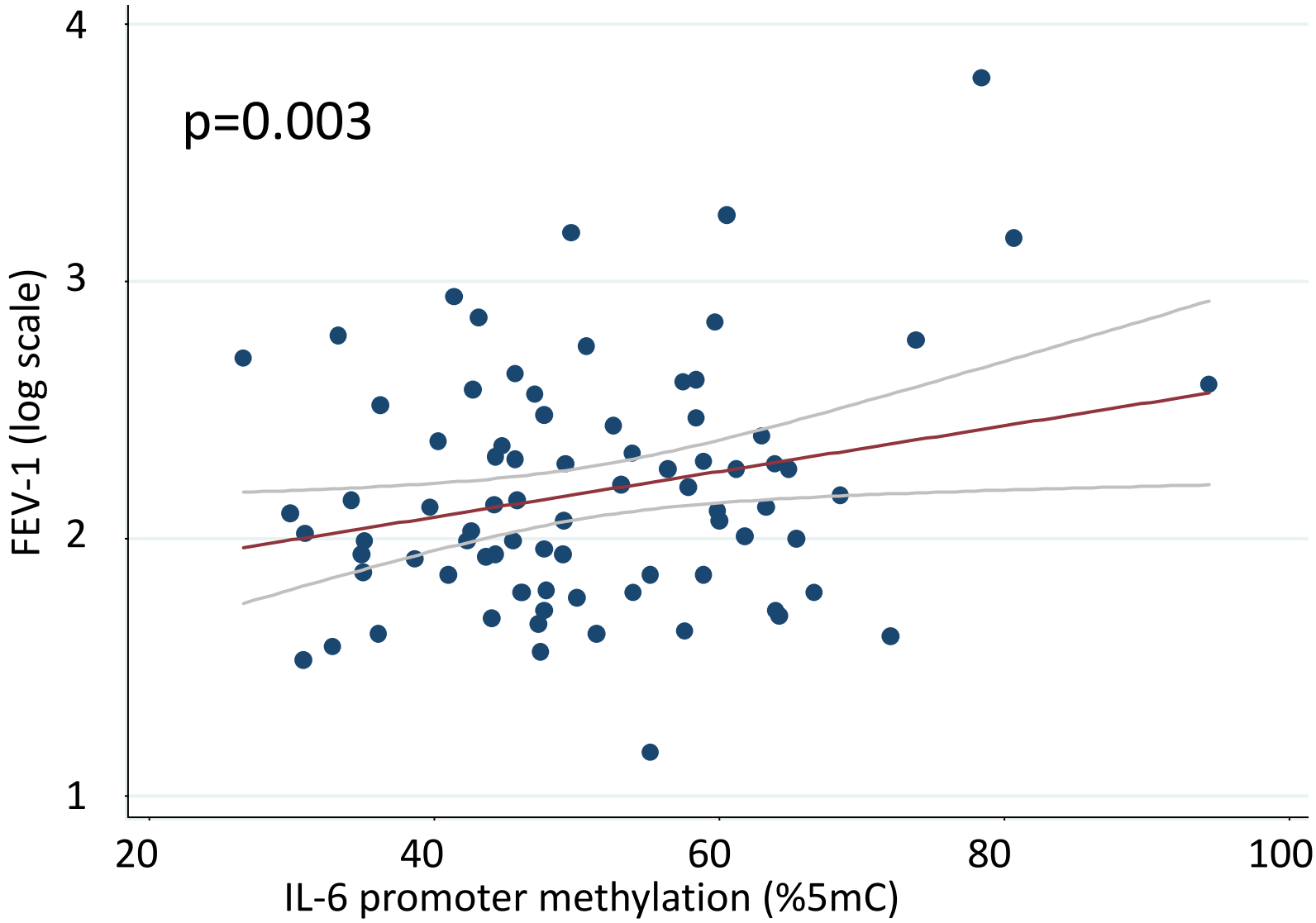


iNOS Methylation in Nasal Epithelial Cells & Exhaled Nitric Oxide



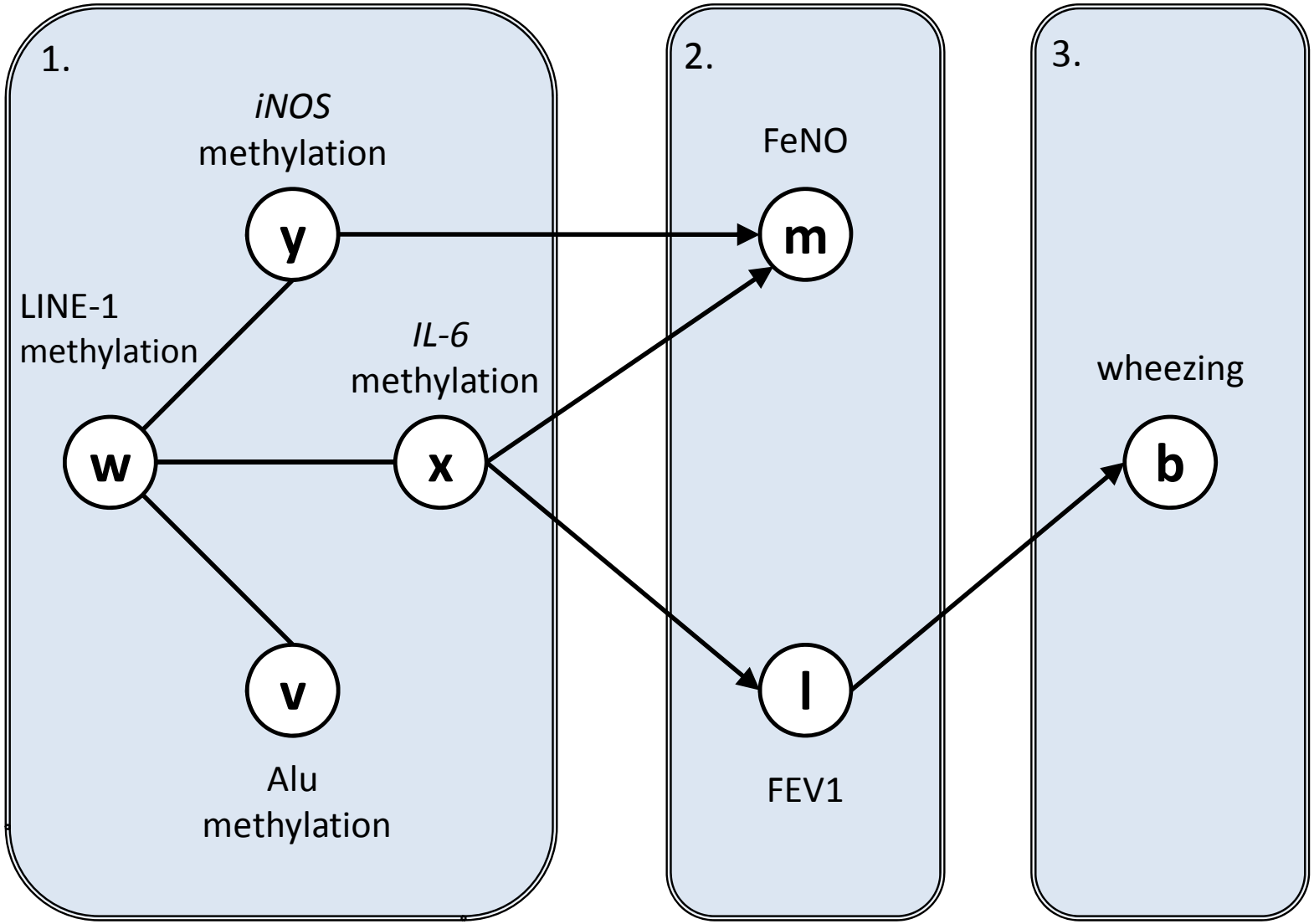
Baccarelli et al. under review

IL-6 Methylation in Nasal Epithelial Cells vs. Lung Function



Baccarelli et al. under review

Graphical Chain Modeling



an IQR decrease in *IL-6* methylation:
OR= 2.5 (90% CI 1.1-5.9, p=0.076) for wheezing

Conclusions

- Different methods for different questions
  - Gene-specific, global, genome-wide
  - Need to balance number of samples vs. coverage
    - Pyrosequencing and Illumina chips are a good fit for large human studies (Bisulfite treatment is easier and cheaper)
- Progress is fast
  - First methylation microarray chip released in 2006
  - Need for additional head-to-head comparisons between different methods
  - Bioinformatic/biostat methods to integrate data between different platforms
- Tissue specificity
  - Target tissues
  - Pure cell populations