Quantification of microRNAs and DNA methylation status by qPCR

Dr. Nicholas Redshaw

LGC Molecular and Cell Biology

Science & Technology

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Science
for a safer world

Overview

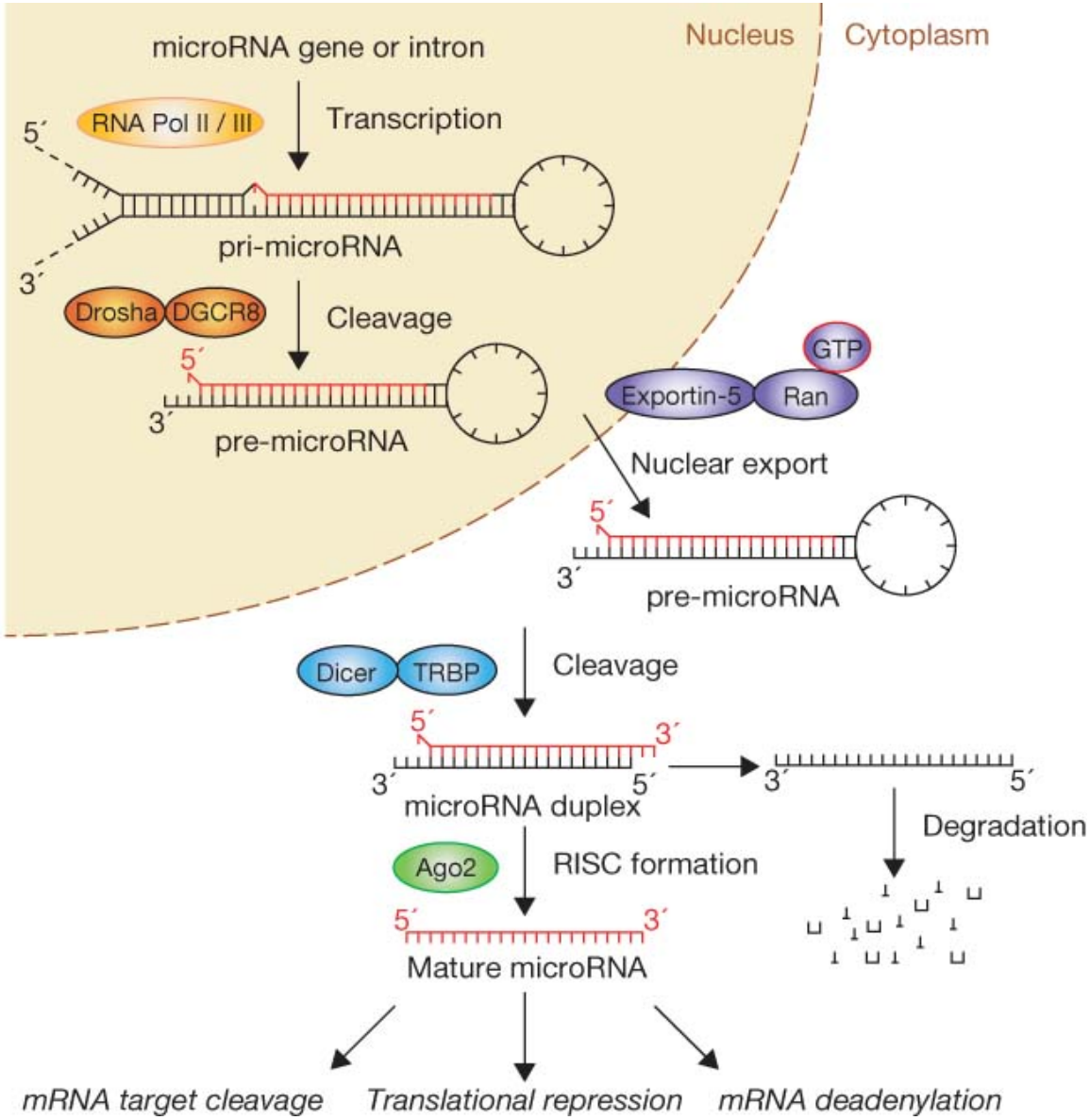
<u>microRNAs</u>

- Introduction to microRNAs (miRNAs)
- Methods for quantifying miRNAs by qPCR
- miRNA isolation
- Short RNA enrichment

<u>Methylation</u>

- Introduction to epigenetic regulation by DNA methylation
- Methods for quantifying DNA methylation status by qPCR

miRNA biogenesis

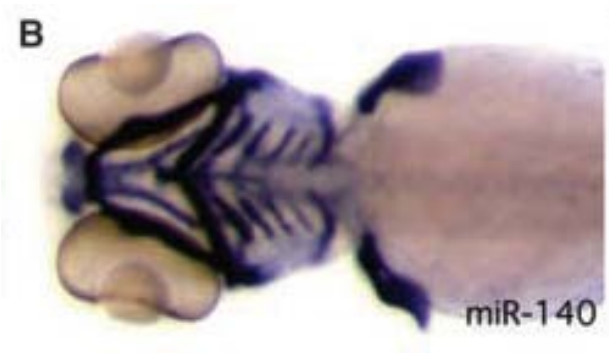


Winter *et al*. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol. Mar;11(3):228-34

miRNAs

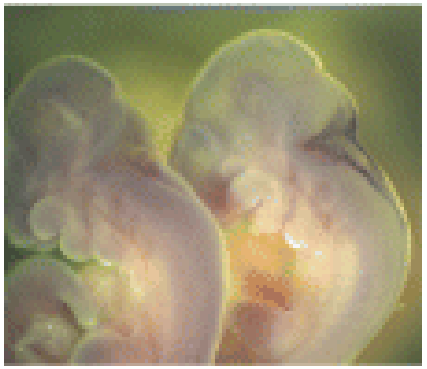
- First discovered in animals *(C.elegans)* in 1993
- Identified in humans in 2000
- > 2,500 miRNAs discovered in humans
- miRNAs predicted to regulate up to 2/3 of all human genes
- Highly cell/tissue-specific:

miR-140 in mouse zebrafish cartilage



Weinholds *et al.* 2005, Science

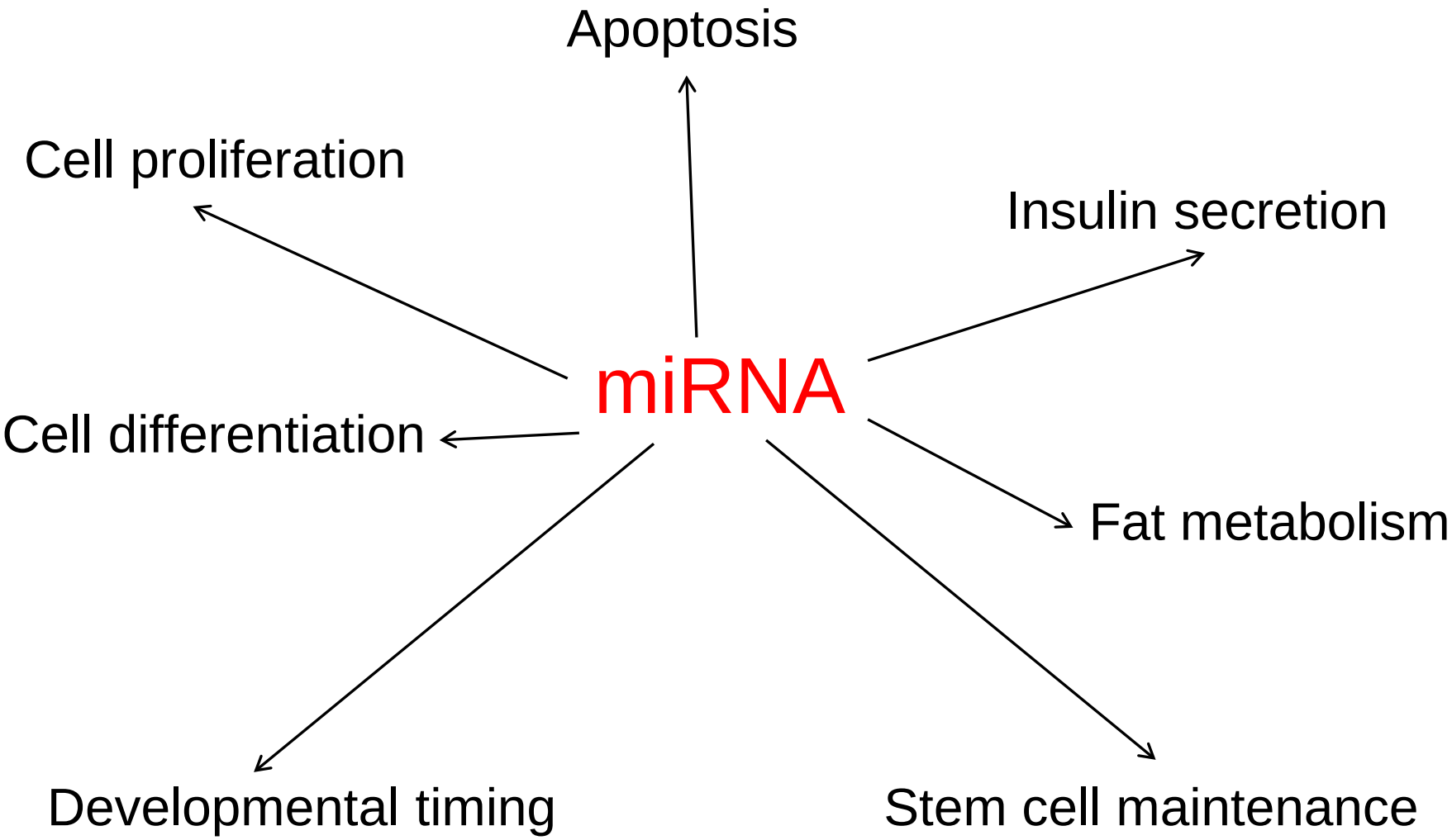
miR-449 expression in mouse hindbrain



Redshaw *et al*. 2009, *Brain Research*

References:
Weinholds *et al.* (2005) MicroRNA expression in zebrafish embryonic development. Science. 309:310–311
Redshaw *et al.* (2009) microRNA-449 is a putative regulator of choroid plexus development and function. Brain Res. 1250:20-6

Functions of miRNAs



miRNAs:
Small molecules with a big impact

- Biomarkers in diseases including cancer

  Mitchell *et al*. (2008): miR-141 expression in serum distinguishes prostate cancer patients from controls

- Cancer tissue profiling

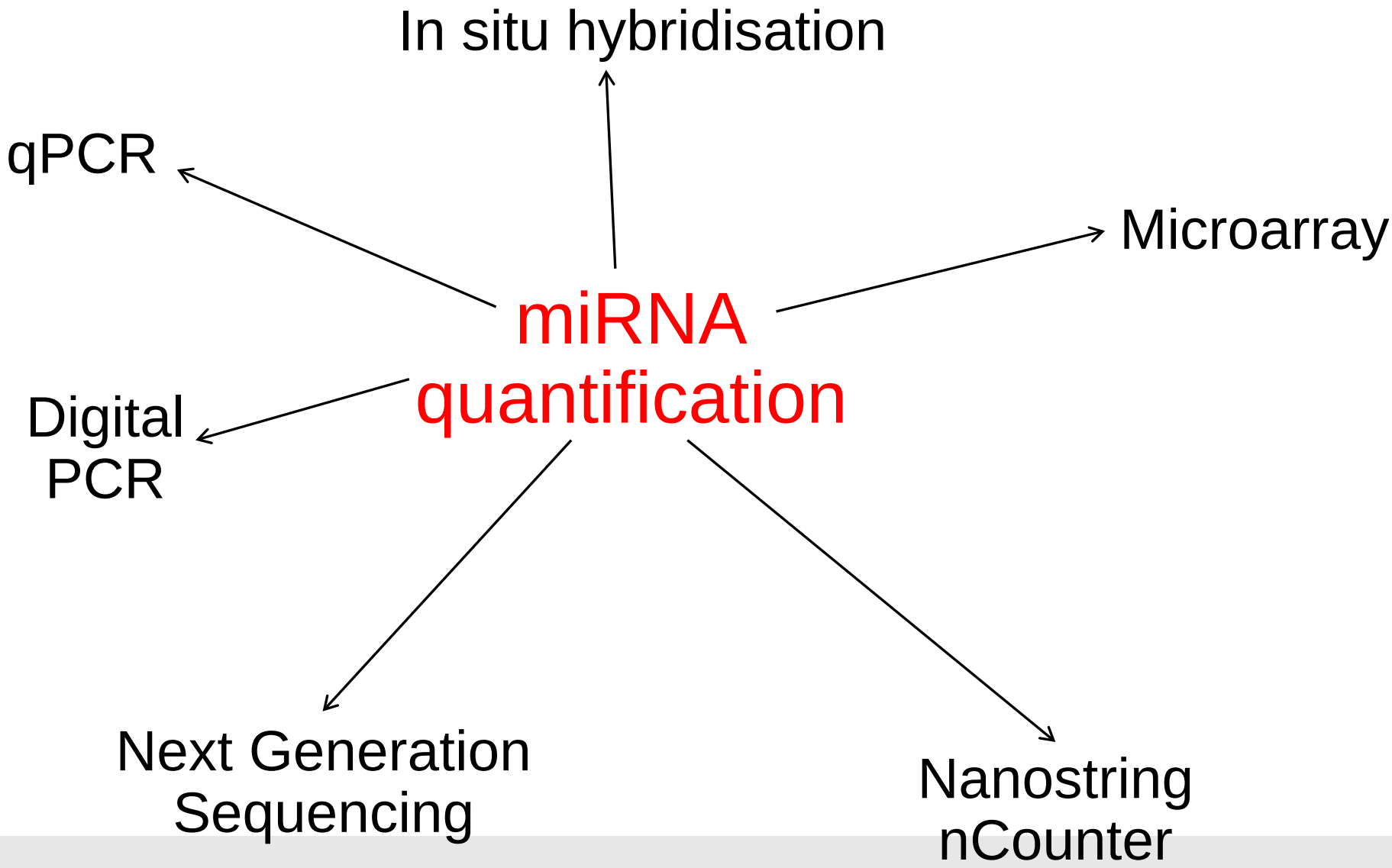
  Lu *et al*. (2005) were able to classify tumours based on their miRNA expression profiling

- Therapeutic targets

  Lanford *et al*. (2010): anti-miR-122 inhibitor used to reduce cholesterol levels

References:
Mitchell *et al.* (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 105(30):10513-8
Lu *et al.* (2005) MicroRNA expression profiles classify human cancers. Nature 435, 834-838
Lanford *et al.* (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. 327(5962):198-201.

miRNA quantification platforms



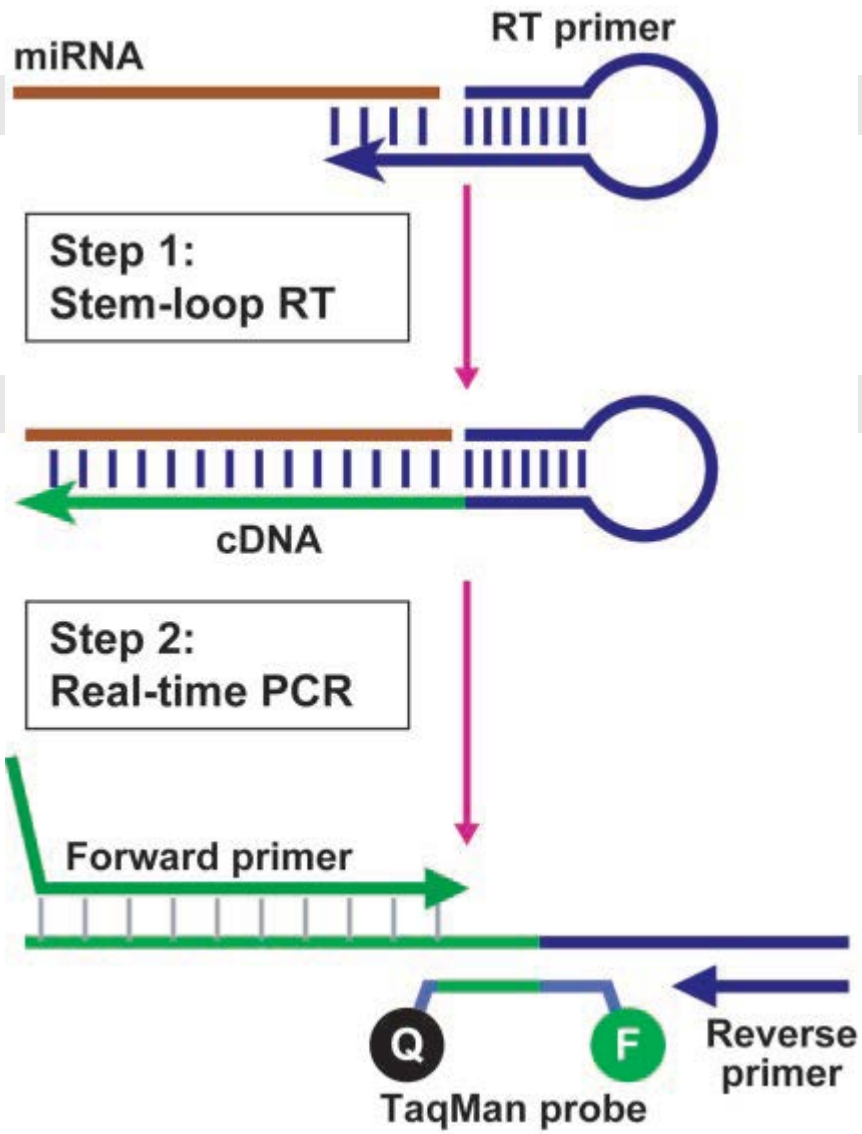
Challenges for miRNA qPCR

- mature miRNAs are short (~22 nts)
- miRNAs are heterogeneous in their GC content, resulting in a wide variation in melting temperatures for a population of miRNAs
- mature miRNAs lack a common sequence feature that would facilitate their selective purification, e.g. a poly(A) tail
- The target sequence is also present in the primary transcript (pri-miRNA) and the precursor (pre-miRNA), in addition to the mature miRNA
- miRNAs within the same family may differ by a single nucleotide (e.g., Let-7 family).

miRNA RT-qPCR technologies

- Taqman miRNA assay (Life Technologies)
- miRCURY LNA™ Universal RT microRNA PCR assays (Exiqon)
- miScript PCR System (Qiagen)

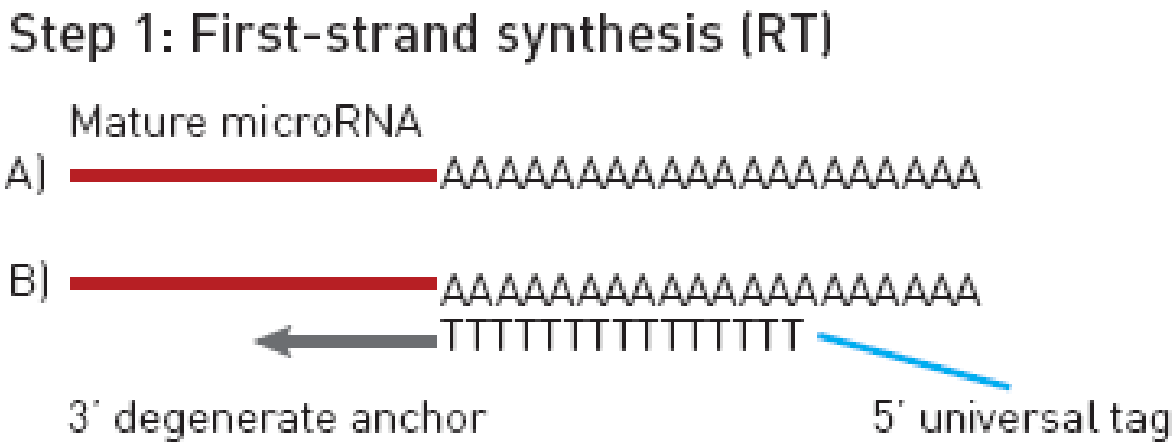
Stem-loop RT-qPCR: Life Technologies



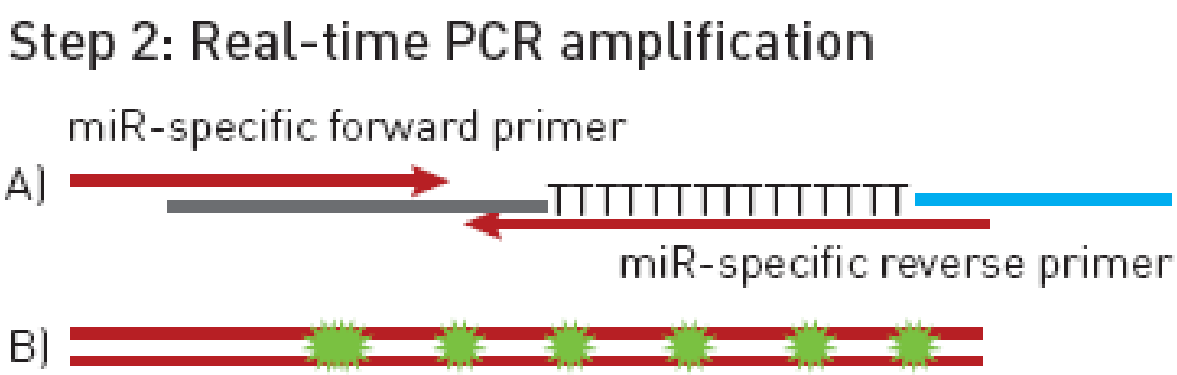
- Leading miRNA qPCR technology
- miRNA-specific stem-loop RT primer (not universal)
- Taqman qPCR amplification

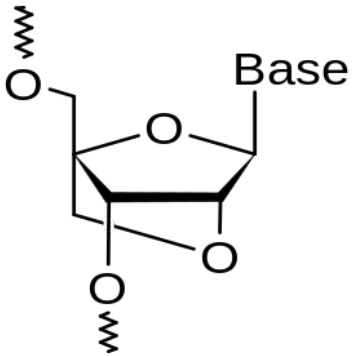
Chen *et al.* (2005) Nucleic Acids Research,33 No. 20

Universal RT priming: Exiqon



- Exiqon technology uses Locked Nucleic Acid (LNA) containing probes:

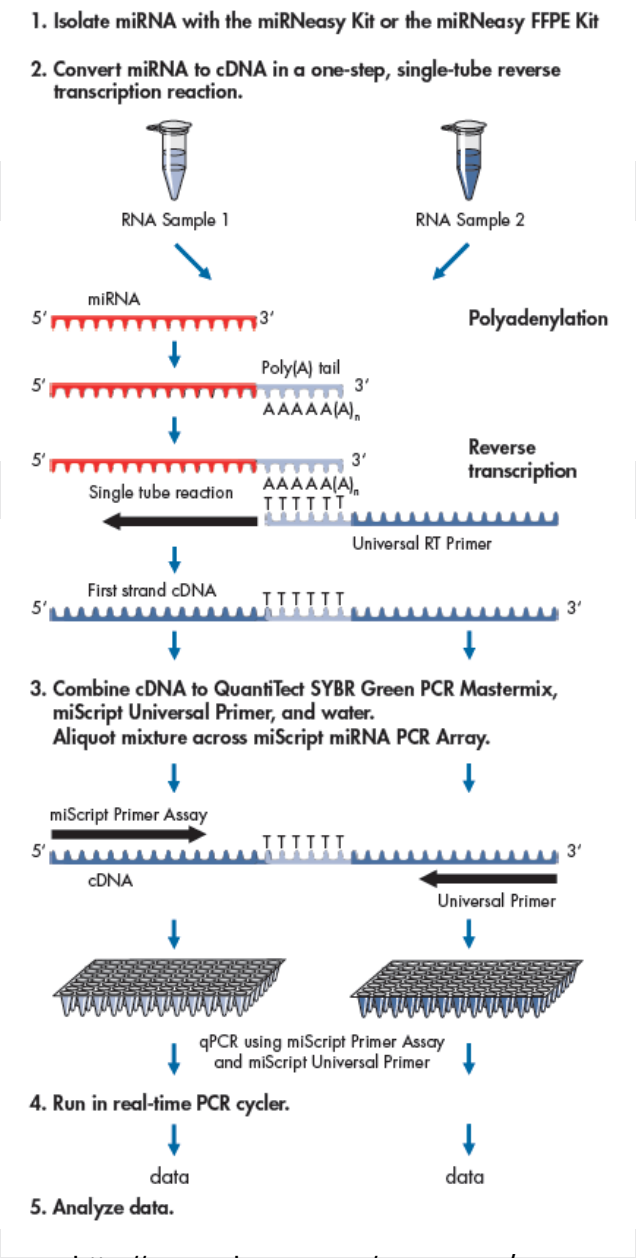




LNA monomer

http://www.exiqon.com/

miScript PCR System (Qiagen)



- Universal RT primer
- qPCR contains miRNA specific forward primer and universal reverse primer
- Potentially lower specificity compared with Life Tech and Exiqon methods

http://www.qiagen.com/resources/

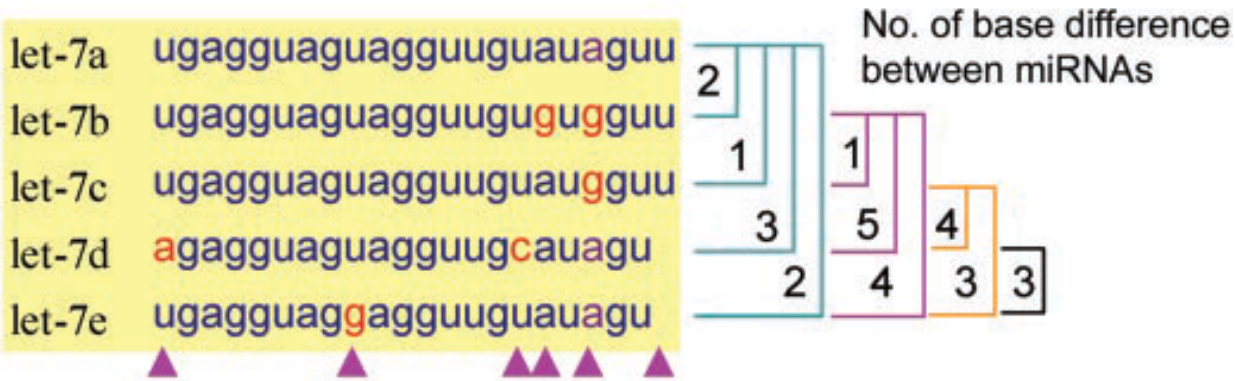
Summary of miRNA qPCR methods

- The stem-loop Taqman assay provides the most specific amplification of target miRNAs
- Exiqon and Qiagen assays utilise a universal RT step
- LNA containing primers (Exiqon) may possess a reduced amplification efficiency (Levin *et al.* 2006)

Reference:
Levin *et al.* (2006) Position-dependent effects of locked nucleic acid (LNA) on DNA sequencing and PCR primers. Nucleic Acids Res. 34:e142.

miRNA qPCR specificity

		Synthetic miRNA target					
		let-7a	let-7b	let-7c	let-7d	let-7e	
miRNA assay	let-7a	100.0	0.3	3.7	0.0	0.0	Relative detection (%)
	let-7b	0.0	100.0	0.3	0.0	0.0	
	let-7c	0.0	2.5	100.0	0.1	0.0	
	let-7d	0.1	0.0	0.0	100.0	0.0	
	let-7e	0.0	0.0	0.0	0.0	100.0	



Chen *et al. Nucleic Acids Research*, 2005, Vol. 33, No. 20

- miRNAs can be found in families where sometimes only one nucleotide differs between species
- Cross-reactivity between species?

miRNA qPCR normalisation

- Many studies have used snoRNA (e.g. U6) or 5s rRNA as reference gene normalisers
- More recent data has shown that using other stable miRNAs are more effective as reference genes
- As with any RT-qPCR experiment, reference genes must be validated for every experiment

miRNA RT-qPCR arrays

- TaqMan MicroRNA Arrays (Life Technologies)
- miRCURY Ready-to-Use PCR (Exiqon)
- miScript miRNA PCR Arrays (Qiagen)

miRNA RT-qPCR arrays

- TaqMan MicroRNA Arrays (Life Technologies)

e.g. Taqman Open Array – run 3 samples with up to 758 unique assays/sample

- miRCURY Ready-to-Use PCR (Exiqon)

e.g. Serum/Plasma Focus microRNA PCR Panel: 2 x 384-well plates containing panel of 179 LNA microRNA primer sets for 4 samples

- miScript miRNA PCR Arrays (Qiagen)

Profiling miRNA expression using up to 84 assays

miRNA RT-qPCR arrays

- Multiplex RT-qPCR arrays based on the same basic technology as the singleplex assays, however they posses many different properties, e.g. The Life Tech Taqman array uses a pre-amp step

- Can require specialised equipment, e.g. Exiqon arrays only compatible with Roche LightCycler 480 instruments.

- Favourable performance vs. miRNA microarrays (Chen *et al.* 2009)

References:
Jensen *et al.* (2011) Evaluation of two commercial global miRNA expression profiling platforms for detection of less abundant miRNAs. BMC Genomics.12:435.
Chen *et al.* (2009) Reproducibility of quantitative RT-PCR array in miRNA expression profiling and comparison with microarray analysis. BMC Genomics. 10:407.

Obtaining accurate and consistent miRNA data

- Sample collection
- miRNA isolation
- miRNA quantification
- Data analysis
- Standardisation of miRNA data

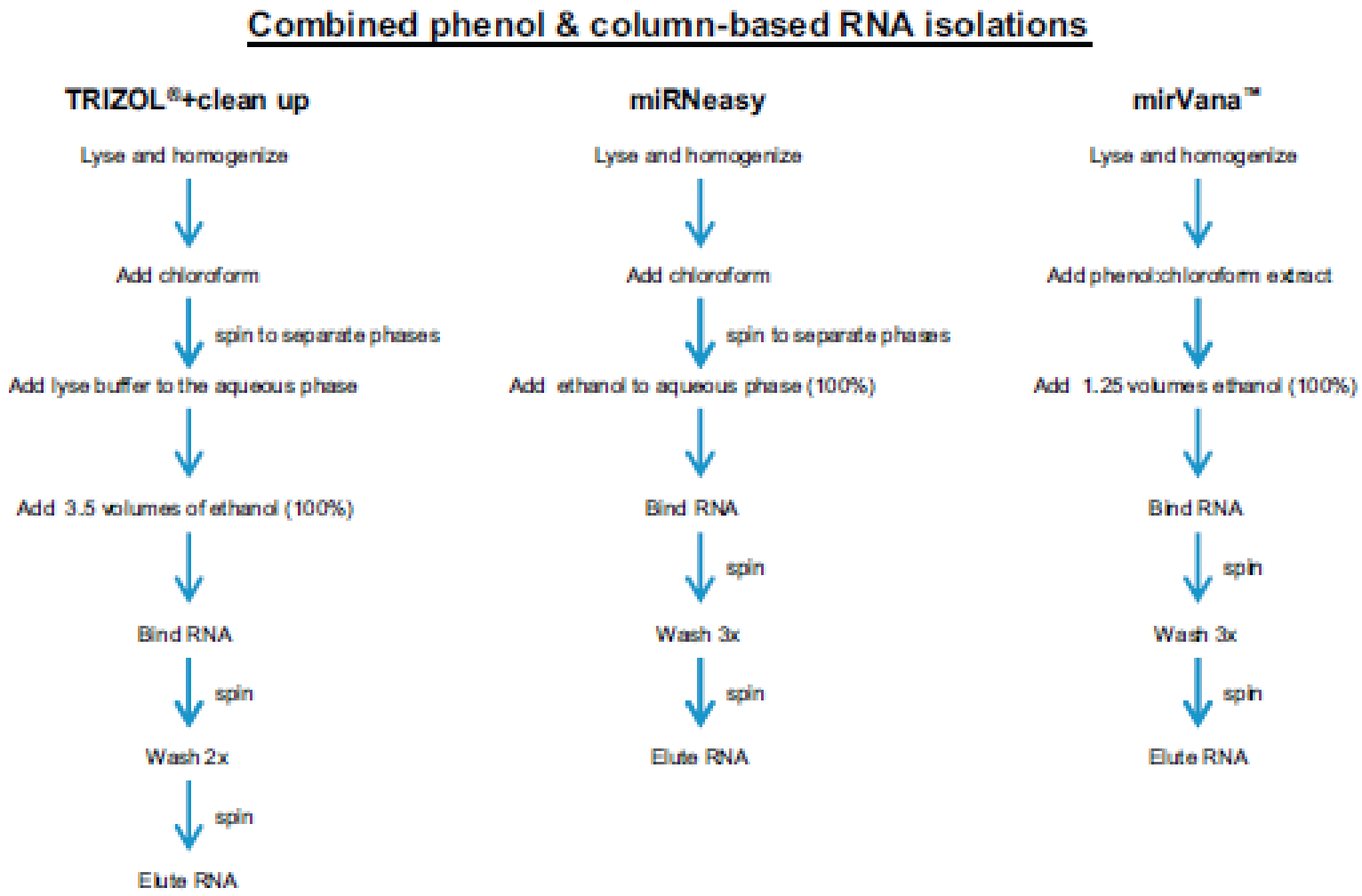
miRNA extraction methods

Trizol (Life Technologies)

miRVana miRNA isolation kit (Life Technologies)

miRNeasy (Qiagen)

miRNA extraction methods



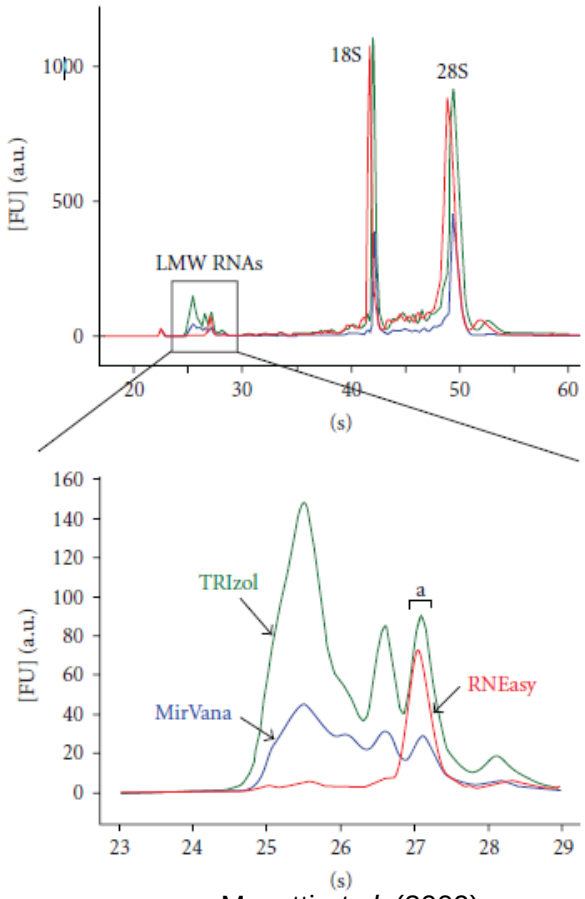
Eldh *et al.* (2012) Molecular Immunology 50: 278–286

Extraction yield

Cell Line	Prep Type	No. of Preps	RNA Yield Mean (SD)	260:280 Mean (SD)	260:230 Mean (SD)	RIN Mean (SD)
HeLa	TRIzol	10	45.59 (7.17)	1.89 (0.0406)	1.28 (0.208)	9.9 (0.14)
HeLa	*mir*Vana	8	43.16 (12.01)	1.96 (0.0364)	1.36 (0.323)	9.9 (0.11)
HeLa	miRNeasy	11	34.07 (5.40)	2.08 (0.00874)	1.97 (0.389)	9.9 (0.11)
ZR-75-1	TRIzol	4	23.52 (1.39)	1.84 (0.0432)	0.815 (0.0759)	9.8 (0.075)
ZR-75-1	*mir*Vana	4	16.92 (5.84)	2.01 (0.0356)	1.28 (0.361)	9.1 (0.17)
ZR-75-1	miRNeasy	4	18.37 (1.93)	2.05 (0.0311)	1.87 (0.337)	9.8 (0.050)

Ach *et al.* (2008)

- Trizol generally gives higher yields than mirVana and miRNeasy
- Quality and purity is comparable between methods



Masotti *et al.* (2009)

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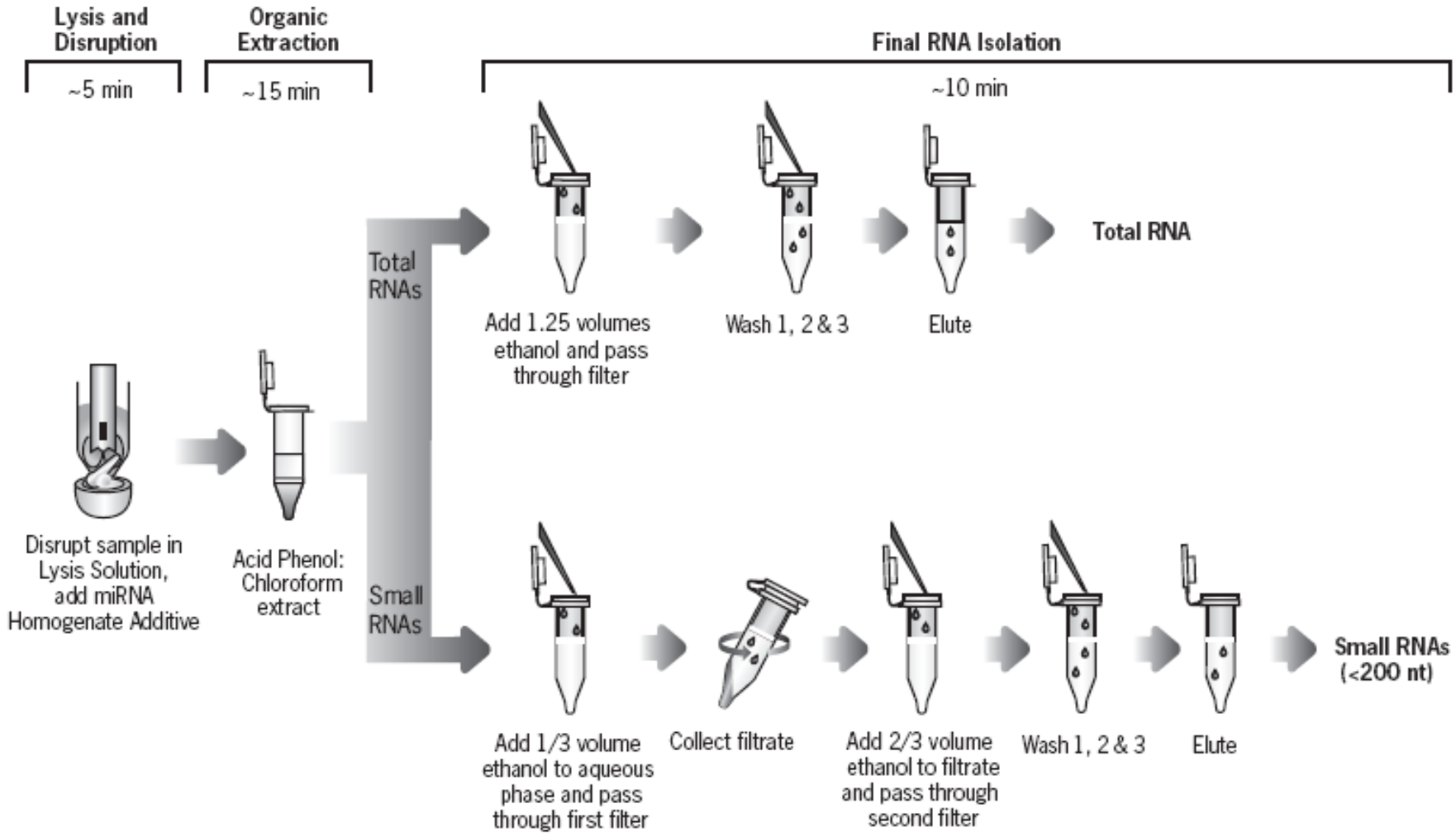
References:

Ach *et al.* (2008) Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. BMC Biotechnol. 8:69.

Masotti *et al.* (2009) Quantification of small non-coding RNAs allows an accurate comparison of miRNA expression profiles. J Biomed Biotechnol. 2009:659028

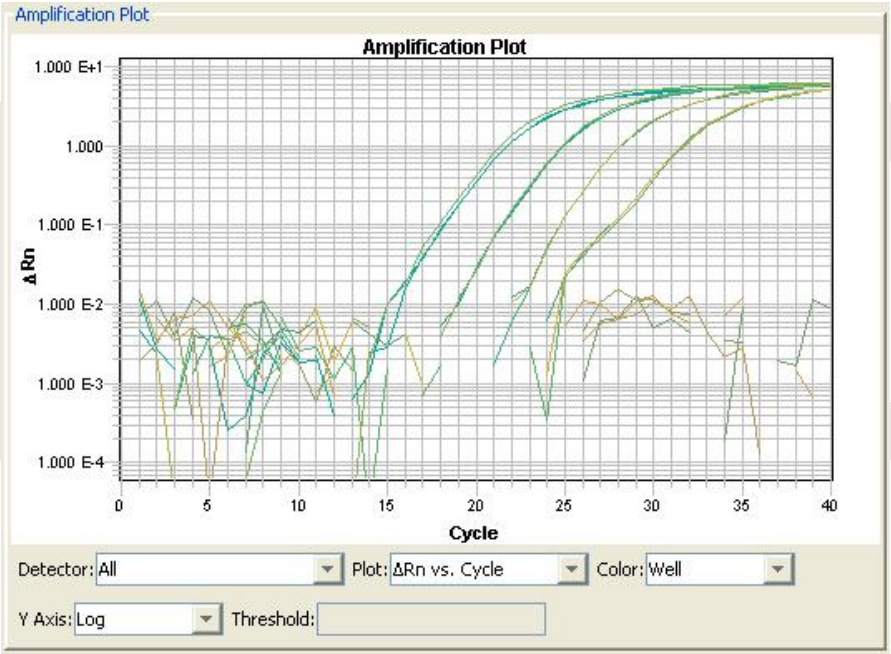
Total RNA vs. short RNA enrichment?

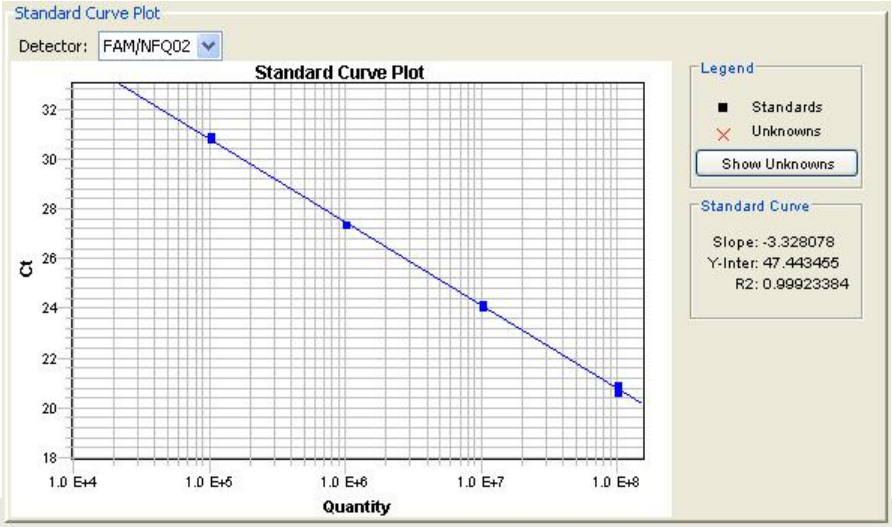


miRVana miRNA isolation kit

miRNA qPCR assay performance

Let-7a (Life Technologies)



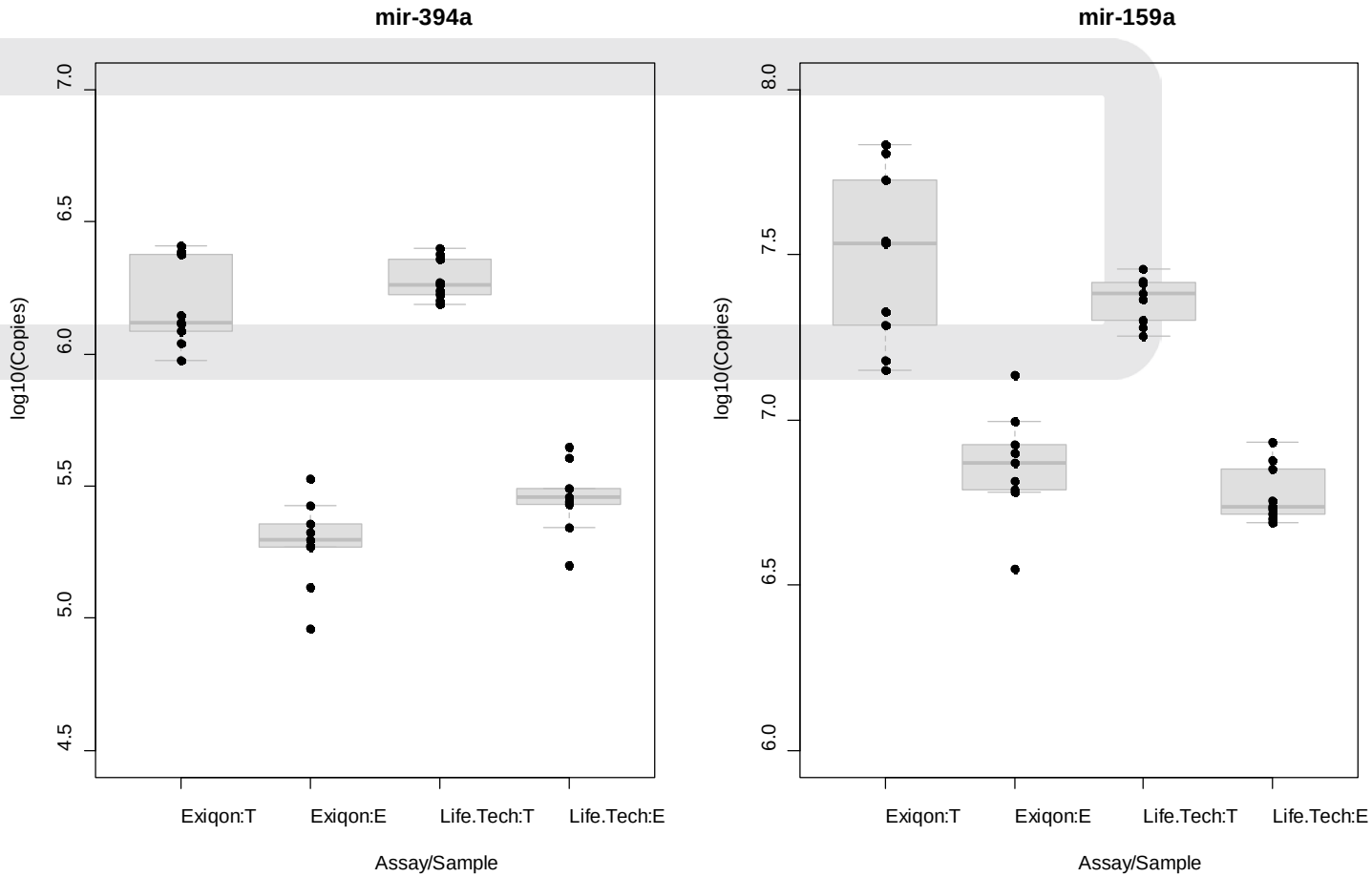


miRNA	Life Technologies		Exiqon	
	Slope	R^2	Slope	R^2
Let-7a	-3.33	0.999	-3.36	0.99
Let-7c	-3.46	0.999	-3.78	0.99
miR-21	-3.36	0.999	-3.54	0.98
miR-16	-3.31	0.999	-3.39	0.99
miR-26b	-3.38	0.997	-3.46	0.98
miR-159a	-3.07	0.996	-3.65	0.99
miR-172a	-3.46	0.997	-3.50	0.99
miR-394a	-3.11	0.996	-3.29	0.99

Summary

- All of the assays for both technologies showed a good linearity
- Some of the miRNAs cross reacted with carrier RNA but at a very low level (>7 Cqs higher than lowest standard dilution).

Comparison of extraction and qPCR technologies for miRNA quantification

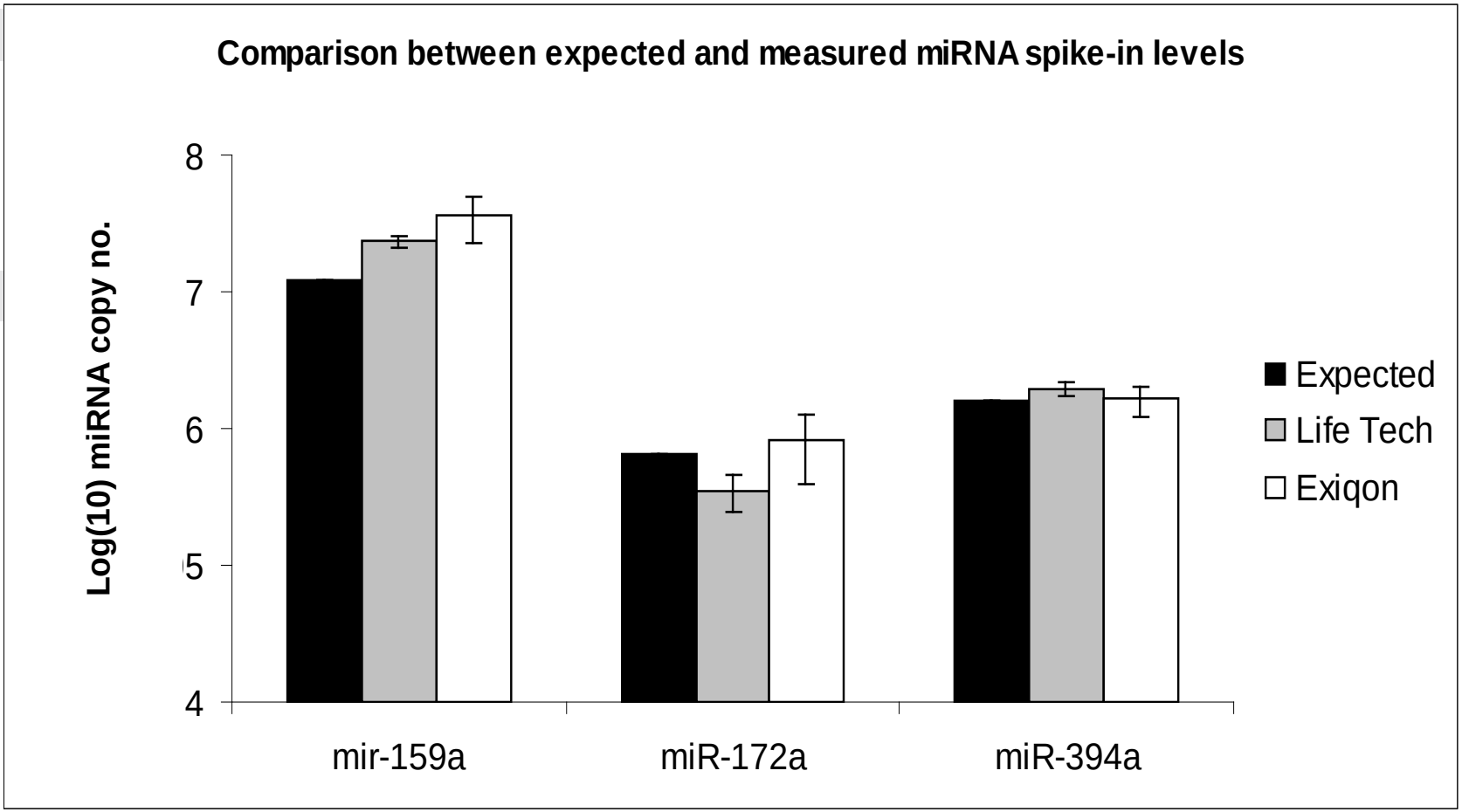


Redshaw *et al.* (2013) RT-qPCR was performed using the Life Technologies and Exiqon assays on total (T) and enriched (E) RNA as indicated. Log(10) copy numbers of miRNA levels are shown for miRs-394a and -159a as boxplots, with the constituent data points overlaid in order to illustrate distribution.

Reference:

Redshaw *et al.* (2013) A comparison of miRNA isolation and RT-qPCR technologies and their effects on quantification accuracy and repeatability. Biotechniques. 54(3):155-64.

Measurement of miRNA spike-ins



Redshaw *et al*. (2013)

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Reference:
Redshaw *et al.* (2013) A comparison of miRNA isolation and RT-qPCR technologies and their effects on quantification accuracy and repeatability. Biotechniques. 54(3):155-64.

Part 2:

Quantification of DNA methylation status by qPCR

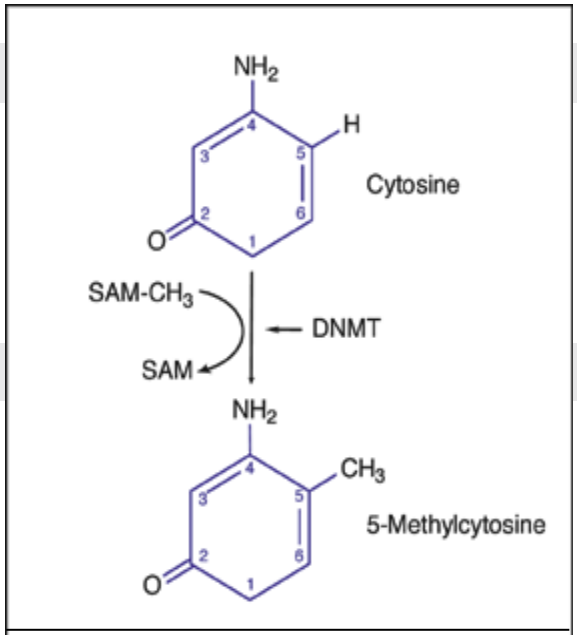
Epigenetics

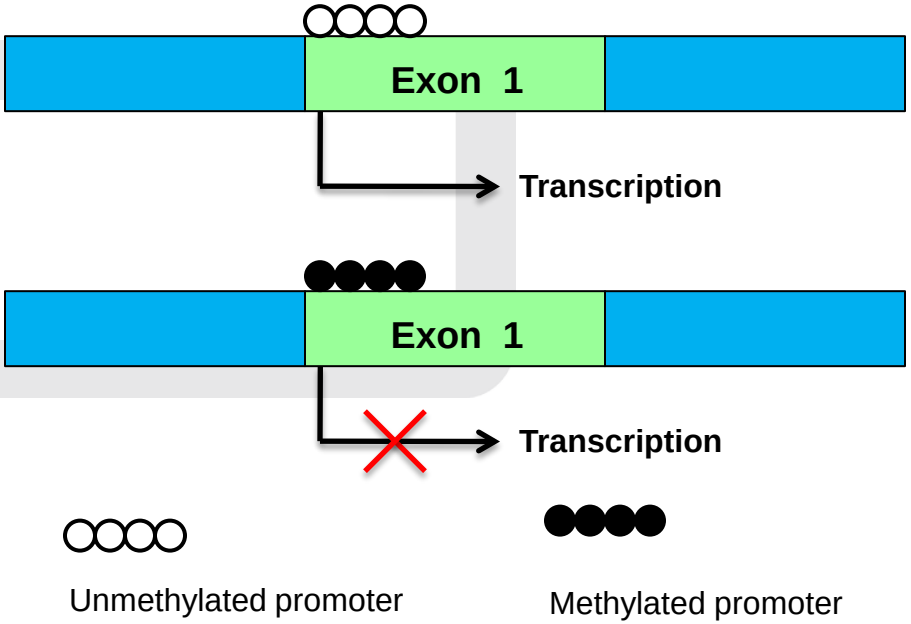
Epigenetics: Study of heritable changes in gene expression that occur without changes in the primary DNA sequence.

Types of epigenetic regulation include:

- **Methylation of Cytosines**
- Histone modifications
- Non-coding RNA (miRNAs)

DNA methylation





Methyl groups are added to cytosine in the DNA by DNA methyltransferases (DNMT).

Genes in which Cytosines in the promoter are heavily methylated are generally silent whereas unmethylated genes are expressed.

- In mammals, methylation typically occurs in CpG dinucleotides

Gene Regulation: DNA methylation in diagnostics

- Changes in methylation status of several genes identified as biomarkers in a number of cancers
  - e.g. p14(ARF) and p16 (CDKN2A)
- Methylated targets are <u>stable</u> biomarkers (unlike mRNA)
  - Allow retrospective analysis in FFPE samples
- Offer possibilities for non-invasive diagnosis
  - Cell free DNA present in body fluids such as blood and urine

Barekati *et al. BMC Cancer* 2012, **12**:244
http://www.biomedcentral.com/1471-2407/12/244

BMC Cancer

RESEARCH ARTICLE Open Access

Methylation signature of lymph node metastases in breast cancer patients

Zeinab Barekati[1], Ramin Radpour[1], Qing Lu[2], Johannes Bitzer[3], Hong Zheng[4,5], Paolo Toniolo[6], Per Lenner[7] and Xiao Yan Zhong[1*]

Current Molecular Medicine 2010, *10*, 123-132 123

DNA Methylation Based Biomarkers in Non-Invasive Cancer Screening

N. Shivapurkar*[,1,2] and A.F. Gazdar[1,2]

Letelier *et al. Clinical Epigenetics* 2012, **4**:11
http://www.clinicalepigeneticsjournal.com/content/4/1/11

CLINICAL EPIGENETICS

REVIEW Open Access

DNA promoter methylation as a diagnostic and therapeutic biomarker in gallbladder cancer

Pablo Letelier[1], Priscilla Brebi[1], Oscar Tapia[1,3] and Juan Carlos Roa[1,2,4*]

Methods and platforms

- A multitude of methods are available for both genome-wide and single locus analysis of DNA methylation

Digital PCR

Methylation sensitive restriction enzyme (MSRE) digestion

MethyLight

Next generation sequencing (NGS)

Single locus methods

Table 1. Methods discussed in this review compared for several parameters important for their clinical applicability.[a]

Method	Analytical sensitivity	Throughput	Closed tube	Quantitative accuracy	Advantage	Disadvantage	References
Direct bisulfite sequencing	Low	Medium	No	Low	The methylation status of individual CpG sites can be resolved	Cloning procedures or digital PCR is necessary if single molecules are to be sequenced	Frommer et al. *(23)*
Pyrosequencing	Medium	Medium	No	High	Quantitative data on individual CpG sites can be obtained	Cloning procedures or digital PCR is necessary if single molecules are to be sequenced	Collela et al. *(35)*, Uhlmann et al. *(36)*, Tost et al. *(37)*
COBRA	Medium	Medium	No	High	Cost-effective	Restriction enzymes recognizing a given sequence context may not be available	Xiong and Laird *(42)*
MS-SnuPE	Medium	Medium	No	High	Multiplexing is possible	Radioactive labeling required	Gonzalgo and Jones *(44)*
MS-MCA	Medium	High	Yes	Medium	Not much labor required	Interpretation can be difficult if too many CpG sites occur between the primers	Worm et al. *(49)*
MS-HRM	High	High	Yes	Medium	Reversal of PCR bias	Interpretation can be difficult if too many CpG sites occur between the primers	Wojdacz and Dobrovic *(31)*
MALDI-TOF	Medium	Ultrahigh	No	High	Quantitative data on individual CpG sites can be obtained	Expensive equipment required	Ehrich et al. *(58)*
HeavyMethyl	High	High	Yes	High	Low false-positive rate	Many oligonucleotides are used	Cottrell et al. *(32)*
MSP	High	Medium	No	Low	Cost-effective	False positives	Herman et al. *(63)*
MethyLight	High	High	Yes	High	Not much labor required	A control assay is needed	Eads et al. *(68)*
McMSP	High	High	Yes	Low	Cost-effective	False positives	Akey et al. *(73)*
SMART-MSP	High	High	Yes	High	Low false-positive rate	A control assay is needed	Kristensen et al. *(61)*
MS-FLAG	High	Medium	No	High	Multiplexing is possible	Gel electrophoresis is used for post-PCR analysis	Bonanno et al. *(75)*
Next-generation sequencing	High	Ultrahigh	No	High	The methylation status of individual CpG sites can be resolved	Expensive equipment required	Taylor et al. *(24)*, Cokus et al. *(79)*, Korshunova et al. *(80)*

[a] Analytical sensitivity and quantitative accuracy are dependent on the specific assay and parameters such as the concentration and quality of input DNA and PCR conditions. For this reason, we have not defined absolute values for these parameters but have evaluated the methods relative to each other.

Kristensen and Hansen (2009) *Clin. Chem.*

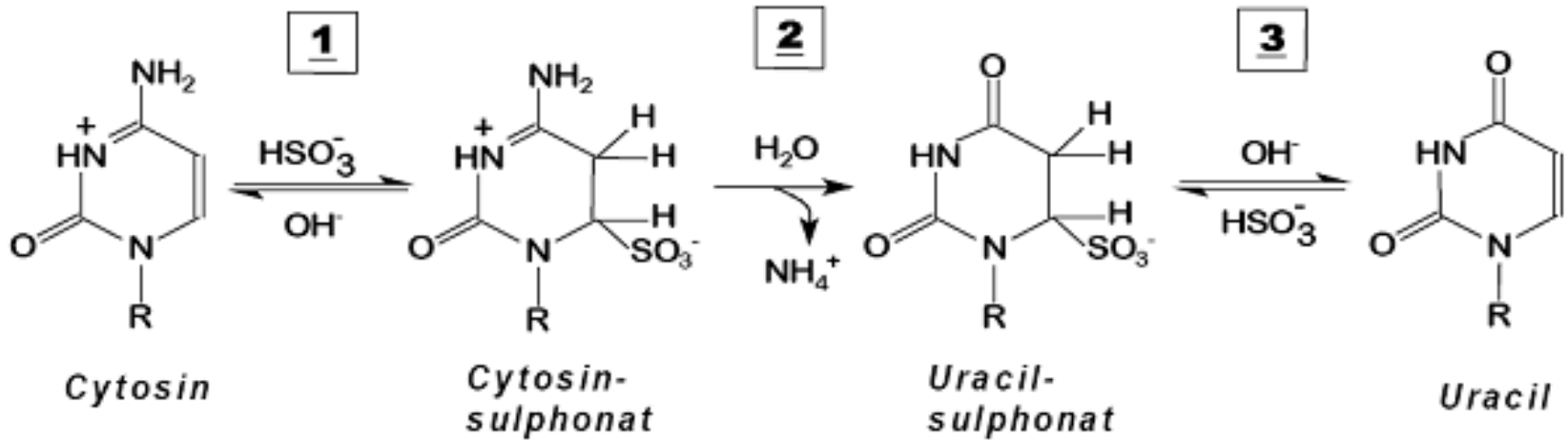
Methylation quantification by PCR

- **Methylight qPCR** – Bisulfite conversion of unmethylated cytosines followed by specific amplification of methylated or unmethylated DNA template

- **Restriction enzyme qPCR** – Digestion of unmethylated or methylated DNA with methylation sensitive or methylation resistant restriction enzymes followed by qPCR amplification of digested template

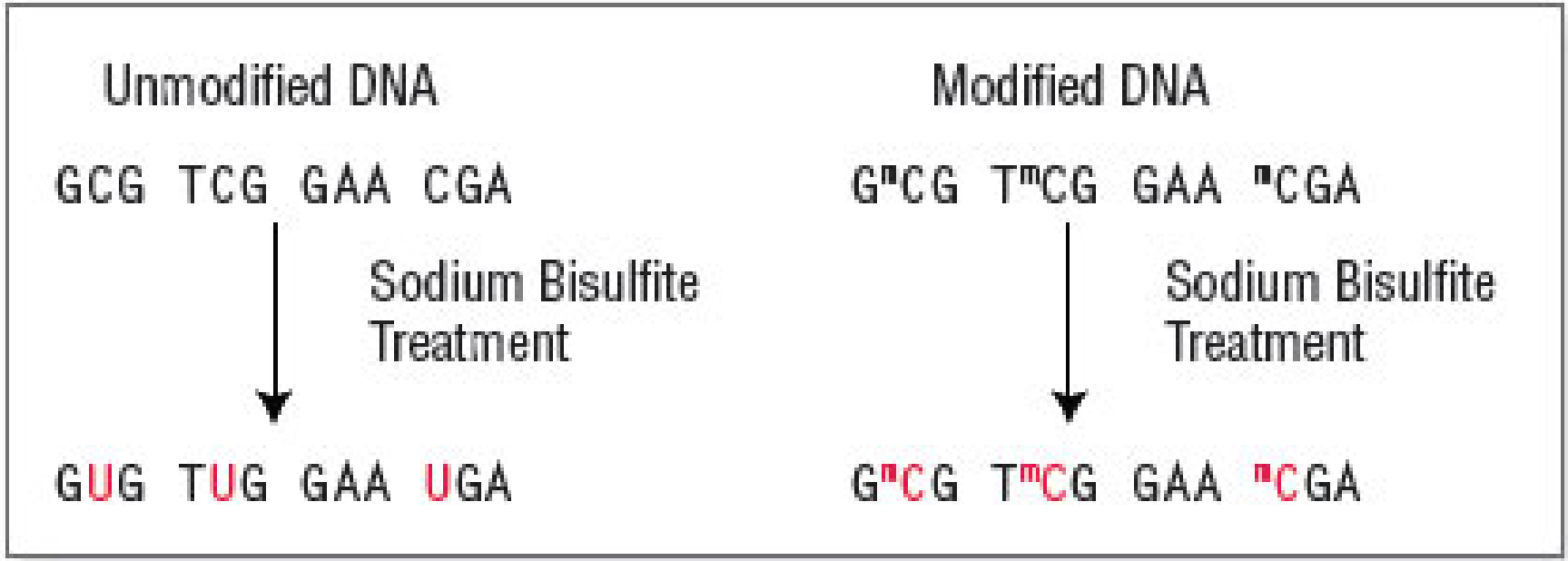
- **Combined Bisulfite and Restriction Analysis (COBRA)** – Combined bisulfite conversion and restriction enzyme digestion of DNA

Bisulfite conversion of unmethylated Cytosine residues



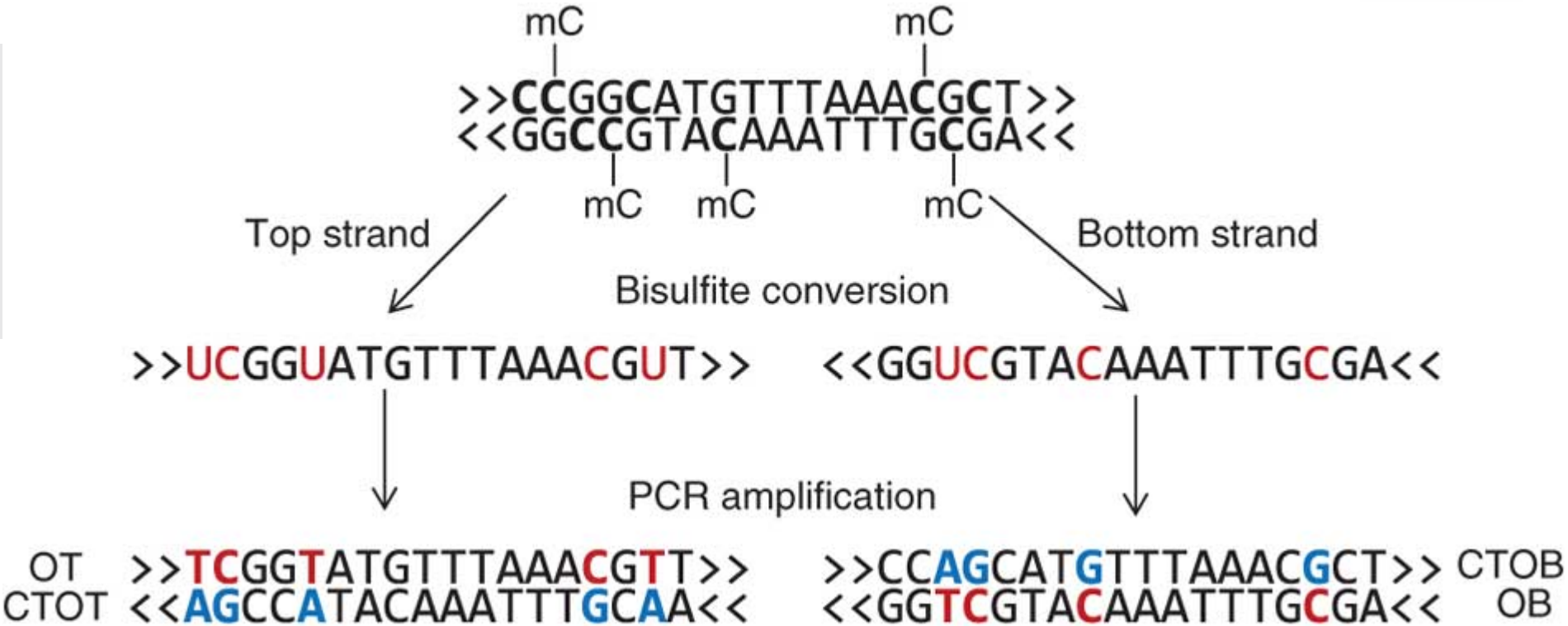
- Bisulfite conversion can be performed by commercially available kits (e.g. Epitect Bisulfite Kits, Qiagen; EZ Methylation kits, Zymo Research)
- Cytosine residues are deaminated to Uracil in the presence of NaOH and sodium bisulfite

Bisulfite conversion



- Only unmethylated Cytosines are converted to Uracil allowing unmethylated and methylated bases to be distinguished

Bisulfite PCR



Primer/probes must be specific to either the top or bottom strands of the bisulfite converted template as they are not complementary

OT = Original Top strand; CTOT = complementary strand to original top strand; OB = original bottom strand; CTOB = complementary strand to original bottom strand

Methylight qPCR

- Methylight qPCR – amplification of bisulfite converted DNA using primers and probes designed to specifically bind methylated or unmethylated template DNA

- Primer/probes must be specific to one strand of the bisulfite converted template as they are not 100% complementary

- To calculate % methylation, data must be normalised to a 100% methylated control sample

References:
Eads *et al.* (2000) MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28: E32.
Trinh BN, Long TI, Laird PW (2001) DNA methylation analysis by MethyLight technology. Methods 25: 456-462.

Bisulfite PCR primer design

- Methylation-specific primers and probes must contain CpGs in the target region
- CpGs should ideally be located at the 3’ end of methylation-specific primers in order to maximally discriminate between meth-/unmethylated target
- Methylation independent primers must not contain any CpGs in the target region
- Both methylation-specific and -independent primers must contain an adequate number of non-CpG Cytosines in their target sequence in order to be specific to the bisulfite converted target sequence and not amplify the unconverted target DNA
- Several methylation primer design tools are freely available online:

  http://www.zymoresearch.com/tools/bisulfite-primer-seeker

Reference:
Wojdacz *et al.* (2008) A new approach to primer design for the control of PCR bias in methylation studies. BMC Res Notes 1: 54.

Bisulfite PCR – warning!

- Do not use Uracil DNA glycosylase in bisulfite PCR reactions (Taqman) – Bisulfite converted DNA contains Uracil!

- Uracil DNA glycosylase is included in some Taqman reagents to reduce cross-contamination between reactions

Methylight qPCR performance

Table 3. PMR Values of Case 3 in MethyLight for *MGMT* Using *COL2A1* as a Reference

		Different MethyLight runs							
		M1	M2	M3	M4	M5	Mean of PMR	SD	CV(M)
Different bisulfite treatments	B1	36.75	28.71	33.57	43.92	22.69	33.13	8.04	0.24
	B2	39.52	35.63	40.11	42.61	31.90	37.95	4.21	0.11
	B3	35.53	36.22	30.21	44.78	29.74	35.30	6.07	0.17
	B4	36.16	40.01	33.42	42.43	41.46	38.70	3.80	0.10
	B5	39.08	37.83	33.56	61.04	37.44	41.79	10.96	0.26
	B6	31.62	36.00	34.49	46.19	32.56	36.17	5.85	0.16
	B7	30.26	34.54	40.80	51.03	36.73	38.67	7.89	0.20
Mean of PMR		35.56	35.56	35.16	47.43	33.22			
SD		3.50	3.50	3.86	6.67	6.10			
CV(B)		0.10	0.10	0.11	0.14	0.18			

Abbreviations: CV(B), bisulfite-to-bisulfite coefficient of variation (CV) within each run; CV(M), run-to-run CV within each bisulfite-treated sample.

Ogino et al. analysed Methylight qPCR by performing replicate bisulfite conversions and qPCR runs

- Standard deviations for Run-to-Run variability were slightly higher than between independent bisulfite treatments
- Correlation between expected and observed values was good (r > 0.9).

Reference:

Ogino *et al.* (2006) Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 8: 209-217.

LGC Methylight qPCR analysis

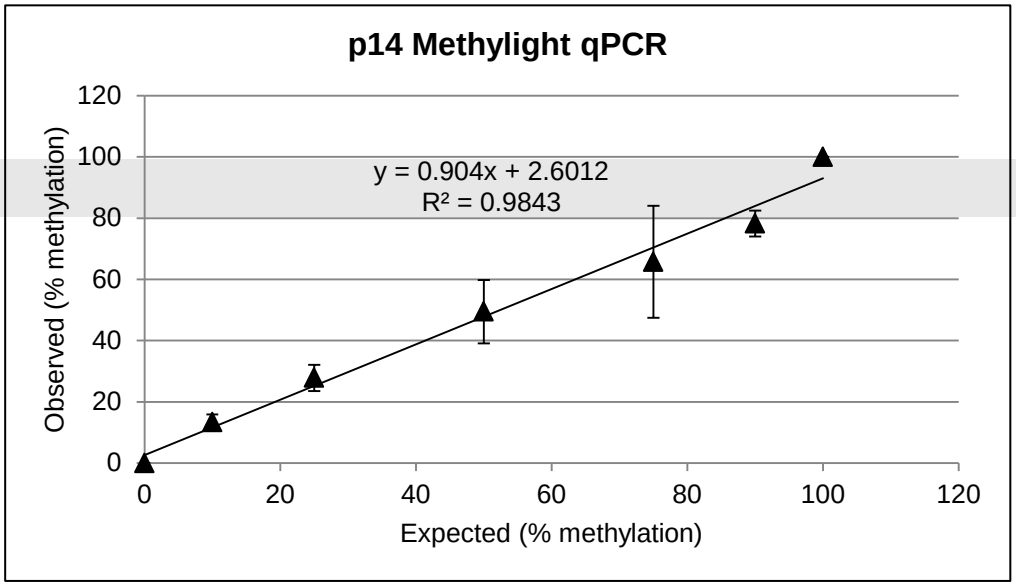
- Panel composed of 7 samples prepared from Zymo methylated and unmethylated gDNA standards

Sample #	1	2	3	4	5	6	7
% methylation	100	90	75	50	25	10	0

- Gene targets:
  - P14 (INK4A)
  - COL2A1 (control for MethyLight)

LGC Methylight qPCR analysis

- Methylation-specific P14 gene normalised to methylation independent COL2A1 gene:



- Average run-to-run Coefficient of Variation = 18%
- Differences of >25% methylation could be discriminated

- 3 independent bisulfite conversions and Methylight assays
- Gene targets:
  - P14 (INK4A)
  - COL2A1 (control for MethyLight)

Methylight qPCR: Normalisation

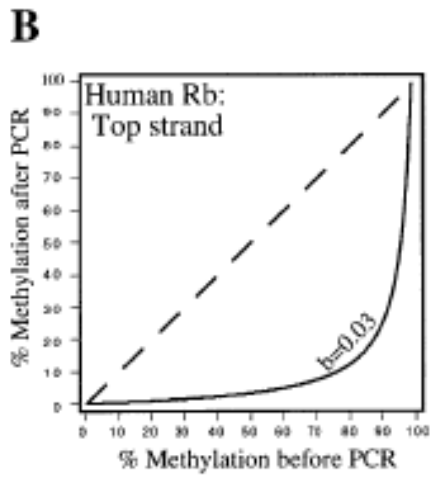
- Normalisation to a fully methylated control is required to calculate % methylation

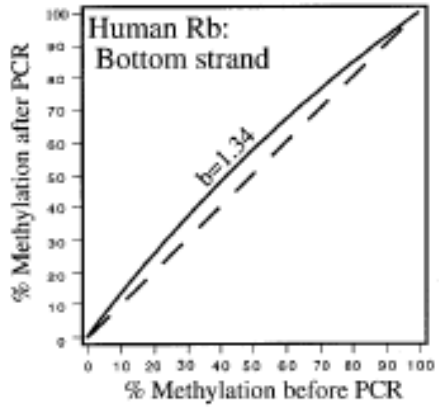
- Typically, CpG Methyltransferase (M.SssI) treated DNA is used to obtain ‘100% methylated’ DNA – how efficient is the rate of methylation?

- Several studies have used sequencing validated clones of bisulfite target DNA as a control (Munson et al. 2007) – however plasmid DNA may not be a suitable control for genomic DNA analysis and the control has not been subjected to bisulfite treatment

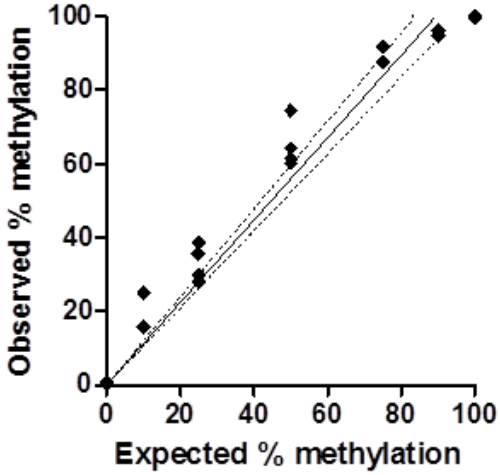
Reference:
Munson *et al.* 2007; Recovery of bisulfite-converted genomic sequences in the methylation-sensitive QPCR. Nucleic Acids Res. 2007;35(9):2893-903.

Methylight qPCR: Bias





Warnecke *et al*. (1997)



LGC methylation analysis

- It has been shown that bisulfite PCR can exhibit preferential amplification for the methylated or unmethylated template leading to over/under-estimation of % methylation (e.g. Warnecke et al. 1997)
- This can be improved by using appropriate controls (Wojdacz & Hansen, 2006)

References:

Warnecke *et al.* (1997) Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. Nucleic Acids Res 25: 4422-4426.

Wojdacz & Hansen (2006) Reversal of PCR bias for improved sensitivity of the DNA methylation melting curve assay. Biotechniques 41: 274, 276, 278.

Wojdacz *et al.* (2008) A new approach to primer design for the control of PCR bias in methylation studies. BMC Res Notes 1: 54.

Methylight qPCR

Pros:

- Good correlation with expected % methylation across full range (0-100% methylation)
- Bisulfite conversion of DNA is relatively simple and inexpensive (commerically available kits are efficient)

Cons:

- Bisulfite conversion can be incomplete and lead to sample loss
- Methylight qPCR assay design is complex
- Bisulfite PCR can exhibit amplification bias
- Difficulties associated with developing reliable methylation controls

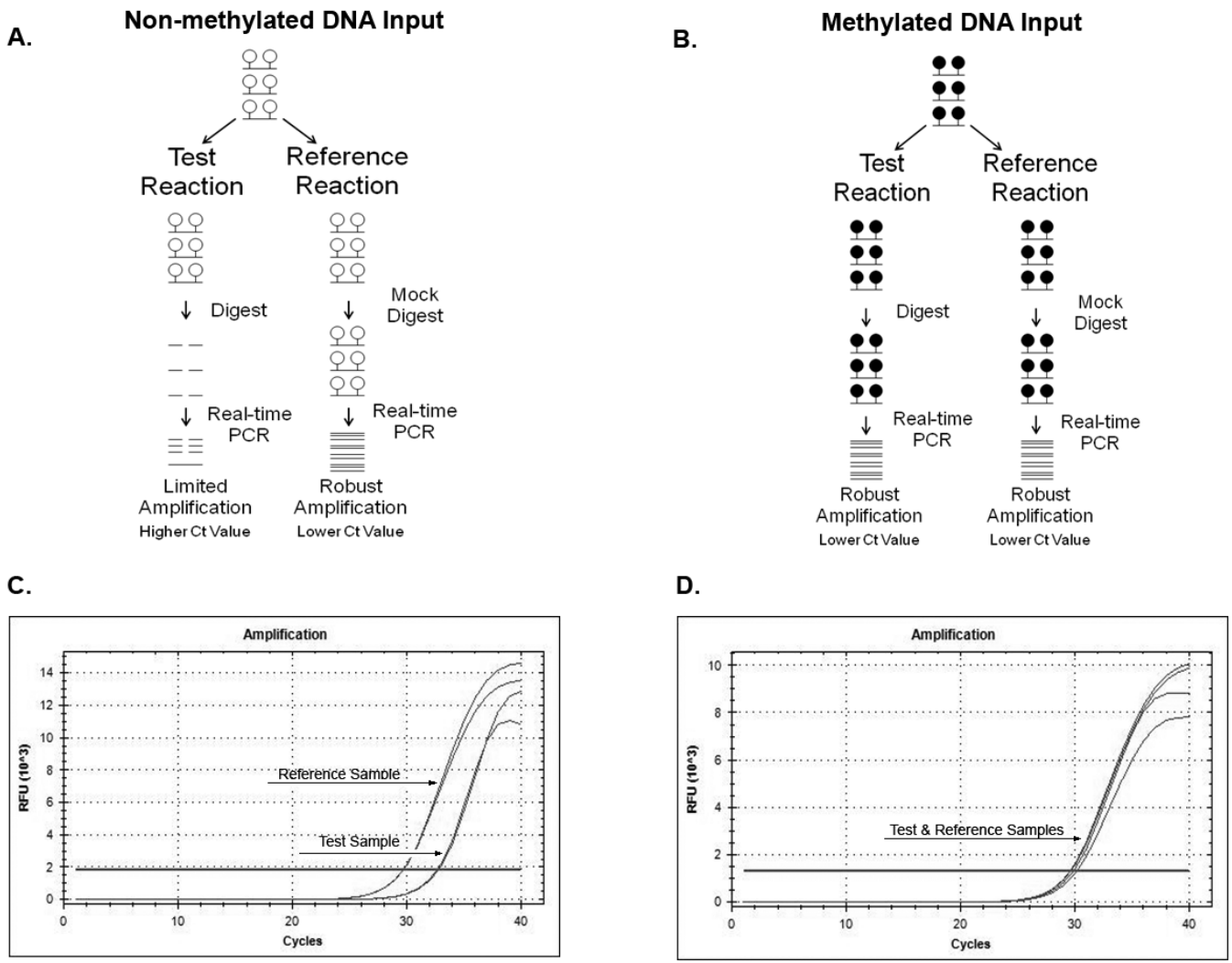
Quantification of methylation using Restriction Enzyme qPCR

- Methylated and unmethylated DNA can be distinguished by pre-treating DNA with methylation sensitive/resistant restriction enzymes
- Restriction enzymes that cleave restriction sites only when they are methylated are termed **M**ethylation **D**ependent **R**estriction **E**nzymes (**MDRE**s)
- Restriction enzymes that are resistant to methylation and only digest DNA when unmethylated are termed **M**ethylation **S**ensitive **R**estriction **E**nzymes (**MSRE**s)
- Digestion products quantified by qPCR
- % methylation quantified by normalising to a mock-treated control

References:
Oakes *et al.* (2006) Evaluation of a quantitative DNA methylation analysis technique using methylation-sensitive/dependent restriction enzymes and real-time PCR. Epigenetics 1: 146-152.
Hashimoto *et al.* (2007) Improved quantification of DNA methylation using methylation-sensitive restriction enzymes and real-time PCR. Epigenetics 2: 86-91.
Holemon *et al.* (2007) MethylScreen: DNA methylation density monitoring using quantitative PCR. Biotechniques 43: 683-693.

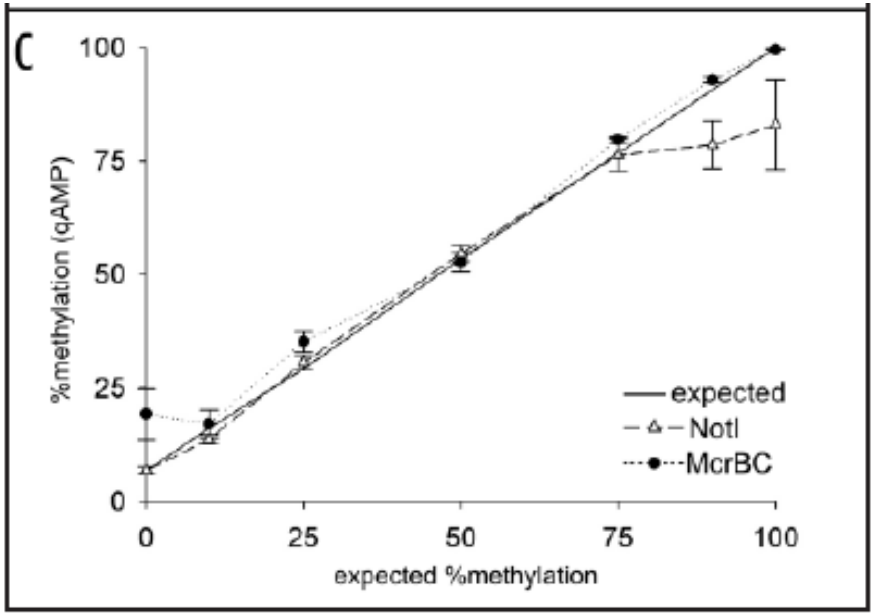
Quantification of methylation using Restriction Enzmye qPCR

- Digestion using methylation sensitive restriction enzymes



Quantification of methylation using Restriction Enzmye qPCR

- Methylation Sensitive Restriction Enzyme-qPCR analysis:



Modified from Oakes *et al*. (2006)

- Several studies have shown a good correlation with expected % methylation but only within a limited range (e.g. Oakes *et al.* 2006)
- Accuracy of % methylation decreases as the difference in Cq between digested and mock digested template becomes very small

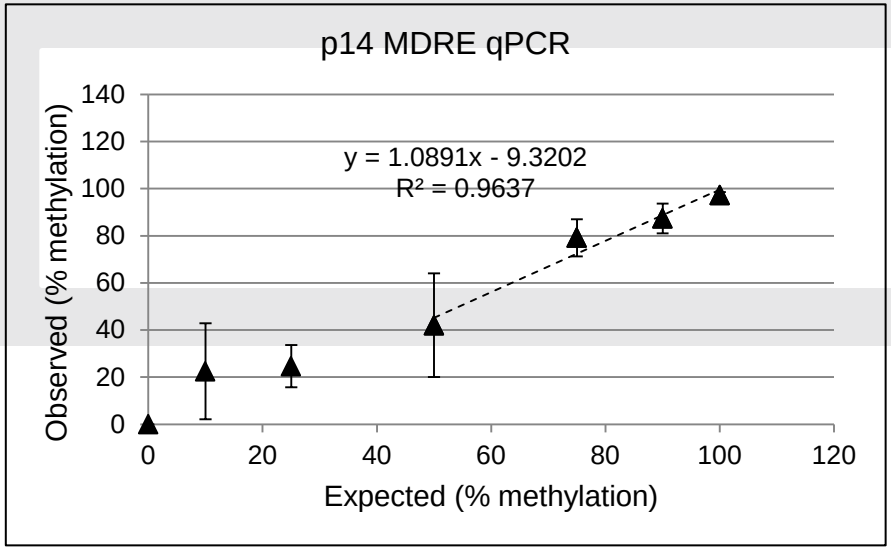
References:

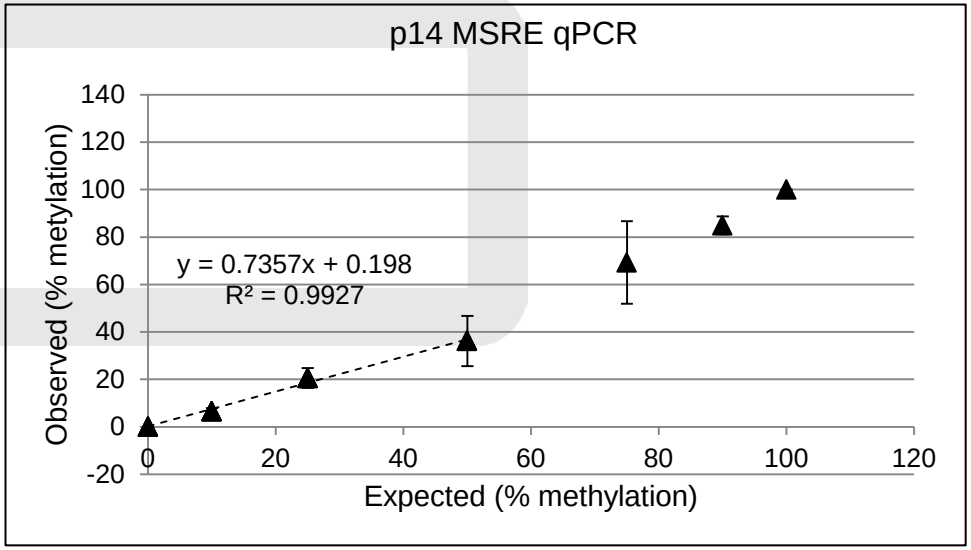
Oakes *et al.* (2006) Evaluation of a quantitative DNA methylation analysis technique using methylation-sensitive/dependent restriction enzymes and real-time PCR. Epigenetics 1: 146-152.

Hashimoto *et al.* (2007) Improved quantification of DNA methylation using methylation-sensitive restriction enzymes and real-time PCR. Epigenetics 2: 86-91.

Holemon *et al.* (2007) MethylScreen: DNA methylation density monitoring using quantitative PCR. Biotechniques 43: 683-693.

LGC Restriction Enzyme qPCR analysis





- Data obtained from 3 independent restriction enzyme digestions and qPCR assays
- Correlations between expected vs. observed % methylation were good within a limited range: 0-50% methylation with MSRE, 50-100% methylation with MDRE (dotted lines)
- Repeatability of measurement was comparable to Methylight qPCR analysis (average CV = 20%)
- Precision of measurement comparable to Methylight approach (differences in % methylation >25% were statistically significant)

Restriction enzyme qPCR

Pros:

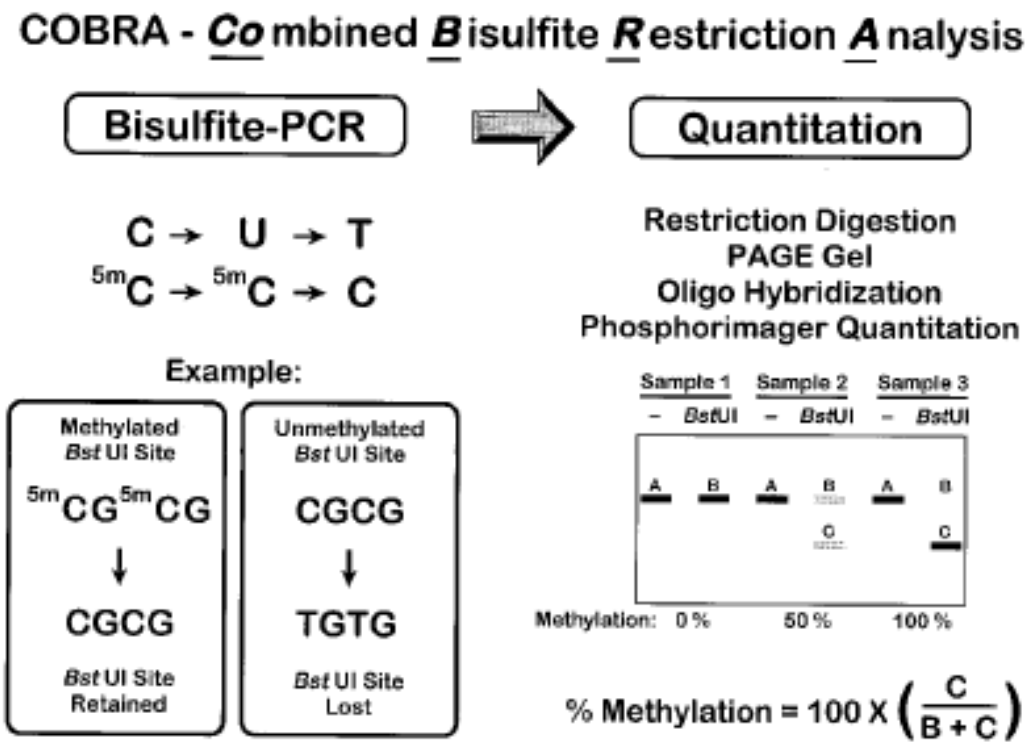
- Good correlation with expected % methylation *within a limited range*
- Restriction enzymes can be used that will cleave a large number of restriction sites within a target region
- Does not require bisulfite treatment

Cons:

- Method relies on the presence of suitable restriction enzymes within the qPCR assay target region which may not be present in some cases
- As assay performance is only optimal within a limited range of % methylation, initial preliminary experiments and/or digestion with both enzyme classes is required

Combined Bisulfite Restriction Analysis (COBRA)

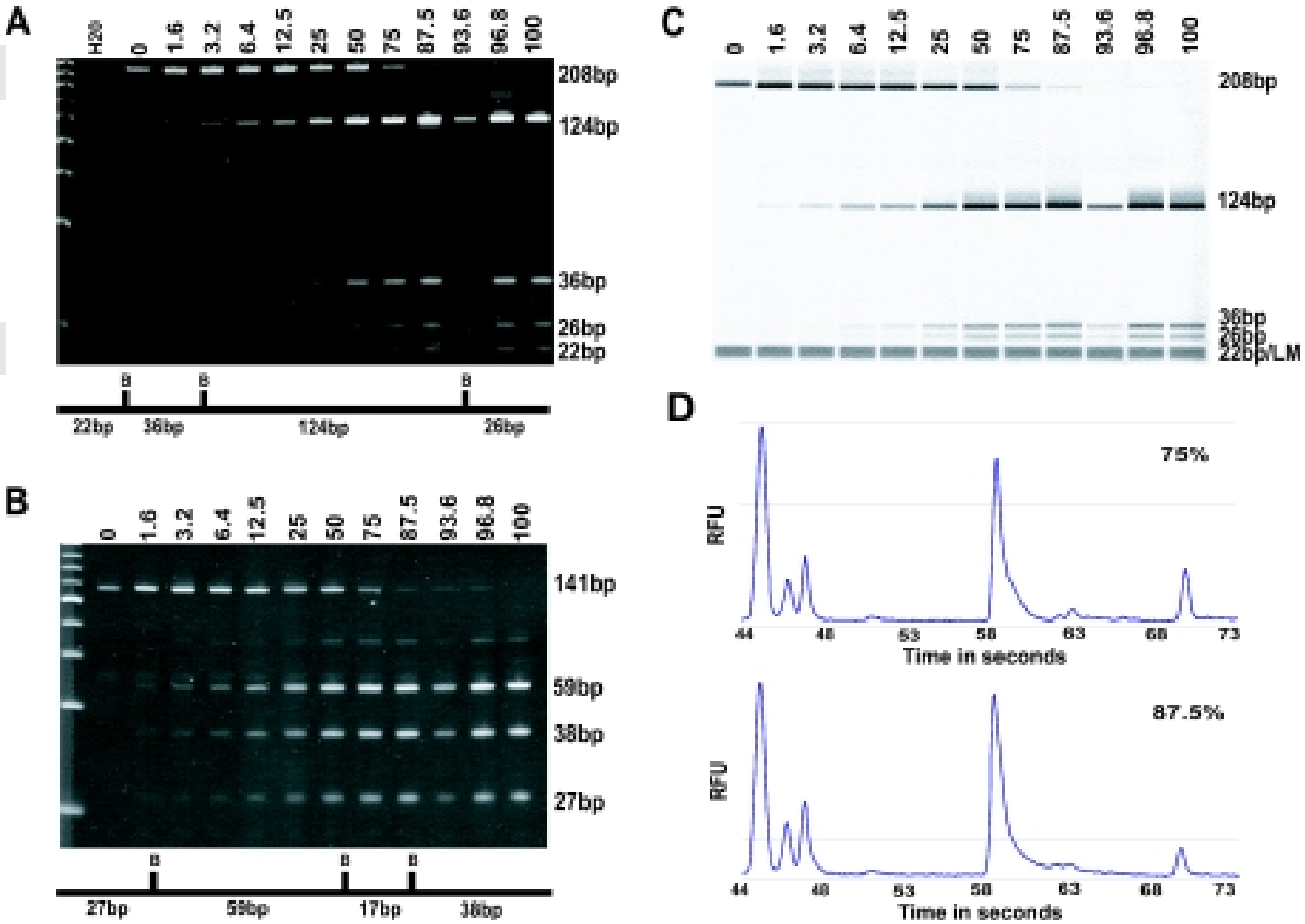
Bisulfite treated DNA is PCR amplified using methylation independent primers and subsequently digested with restriction enzymes that are specific to either the original methylated or unmethylated sites:



Xiong & Laird (1997)

Reference:
Xiong & Laird (1997) COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 25(12):2532-4.

COBRA analysis



Brena et al. (2006)

Reference:
Brena *et al*. (2006) Accurate quantification of DNA methylation using combined bisulfite restriction analysis coupled with the Agilent 2100 Bioanalyzer platform. Nucleic Acids Res. 34(3):e17.

COBRA

Pros:

- Restriction enzymes can be used that will cleave a large number of restriction sites within a target region
- Technique also allows for the analysis of specific loci

Cons:

- Both incomplete bisulfite conversion and incomplete/non-specific restriction enzyme digestion can impact the results
- More labour-intensive and costly than other methods
- Method requires semi-quantitative analysis of gel electrophoresis products

Summary

- Methylight and restriction enzyme qPCR yield comparable performance characteristics in terms of repeatability, accuarcy and precision
- All three of the described methods have been used successfully in multiple publications
- The pros and cons of each technique need to be assessed before deciding which approach to use for a particular experiment

Thank you for listening!