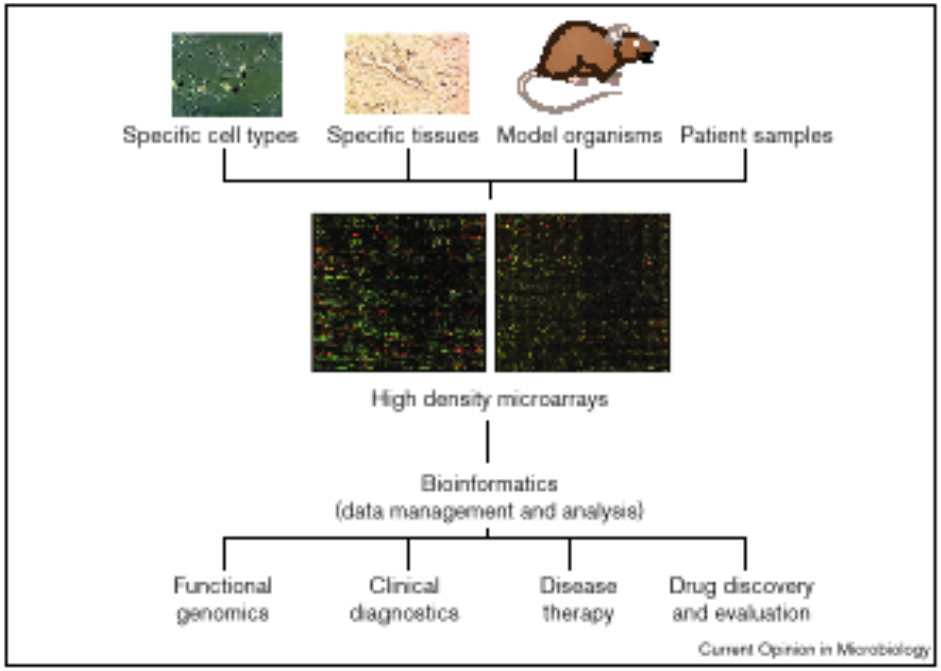
MICROARRAYS



PHASES: extraction and manipulation of nucleic acids to be analyzed, chip, production of microarrays throug probes transfer onto thechip or by in situ synthesis, hybridization to the target, scanner for chip reading, software for quantification and results analysis .
SITU SYNTHESIS of DNA on glass using combinatorial chemical approach (oligomers 10-25)
DEPOSITION: activated glass

Microarrays are used as a **screening tool** because of the high throughput capabilities

Quantitative real-time reverse-transcription PCR (qRT-PCR)

1. is able to detect low levels of individual mRNA and miRNAs
2. requires less highly specialized equipment
3. data analysis is less complex ➔less time consuming and less costly per sample

Basis of Microarrays

- ✓ Hybridization of labelled mRNA fragments to large number of specific probes attached to a solid support
- ✓ Probes: oligonucleotides (20-60 bases) or cDNA of the genes of interest
- ✓ Labelling of target mRNAs with fluorescent dyes
- ✓ Hybridization and scanning
- ✓ Data normalization to provide the relative abundance of each mRNA

DATA NORMALIZATION

Normalization is an essential procedure in the analysis of DNA microarrays to compare data from different arrays or colour channels. There are three types of internal controls that can be used for normalization.

1. Most commonly normalization is based on all genes on the array. The majority of genes do not change in terms of their expression level.
2. Previously known Housekeeping-genes.
3. Spiked-in control genes.

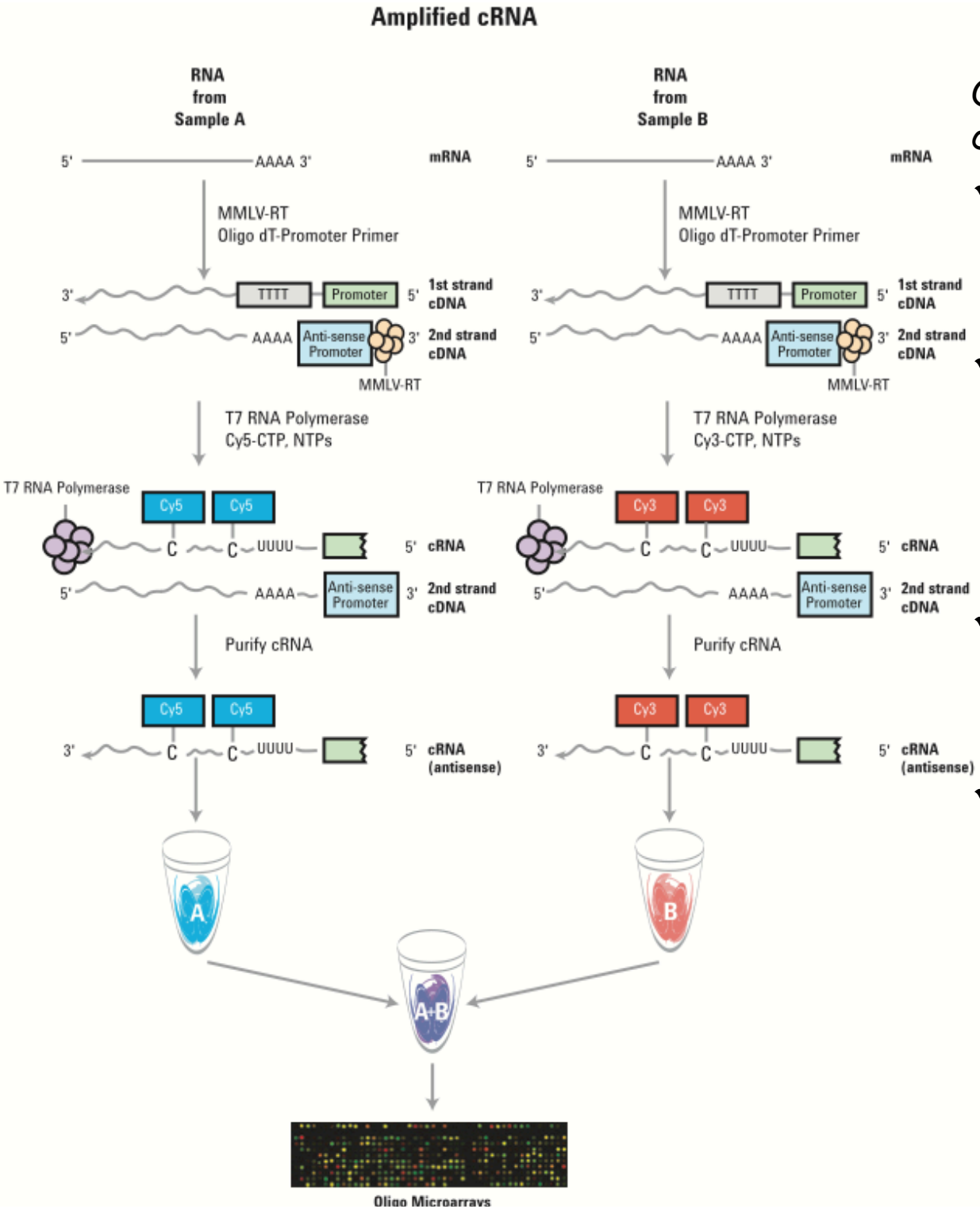
One method is used for data normalization, the others to valiidate results

Normalization consists of several steps:

a) Background Correction

b) Transformation: Microarray intensities should always be looked at using log2 scale.

c) Robust estimation of a “rescaling” factor (e.g. median of differences)

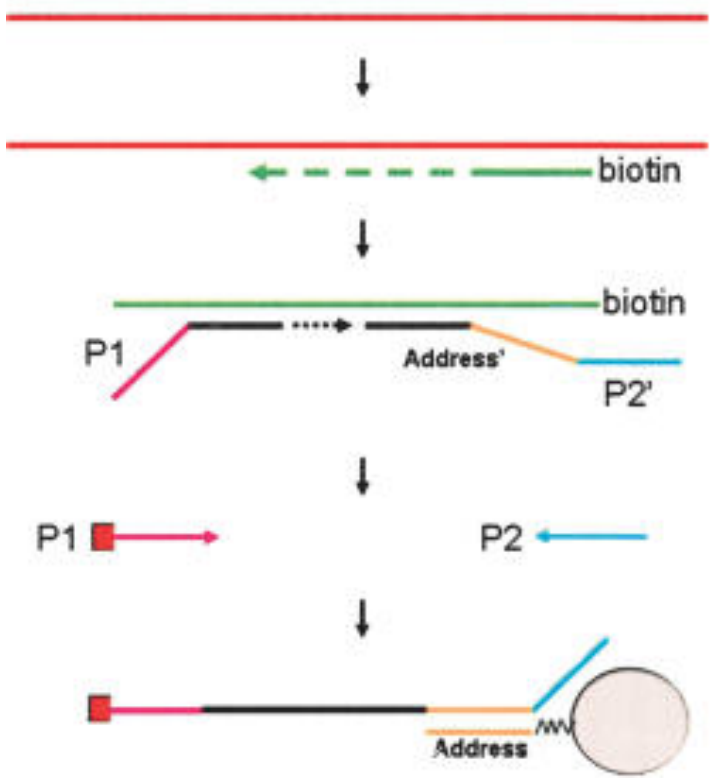


One sample is labeled with cyanine 3 and one with cyanine 5.

- ✓ A primer, which contains poly dT and a T7 polymerase promoter, is annealed to the poly A+ RNA.
- ✓ Reverse transcriptase is added to synthesize the first and second strands of cDNA. At this point, double-stranded cDNA has been synthesized.
- ✓ Next, cRNA is synthesized using T7 RNA polymerase, which incorporates cyanine 3- or cyanine 5-labeled CTP.
- ✓ Once labeling is complete, both samples are combined and hybridized to the microarray. Genes whose expresssion differs between the samples are easily identifiable by scanning the microarray with a laser-based detection system.

From:
Genome Res. May 2004; 14(5): 878–885.
doi: 10.1101/gr.2167504

DASL ASSAY



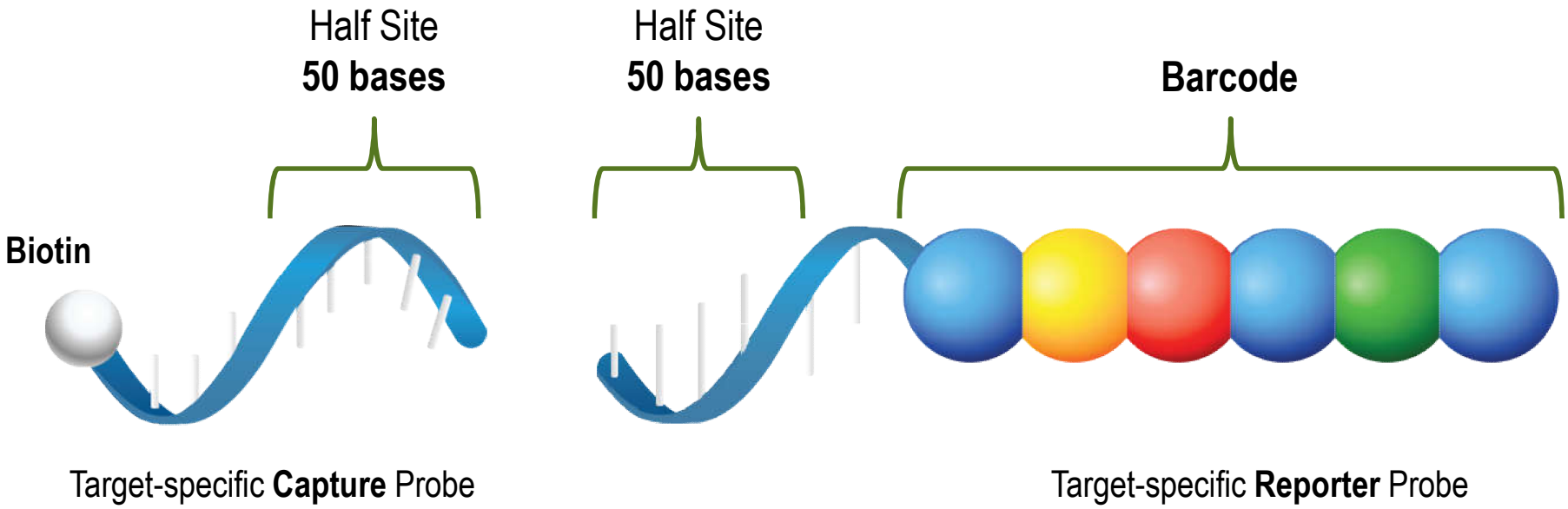
DASL assay

- small size of the targeted gene sequence (~50 nucleotides),
- the use of random primers in the cDNA synthesis
- three independent probe sets per gene

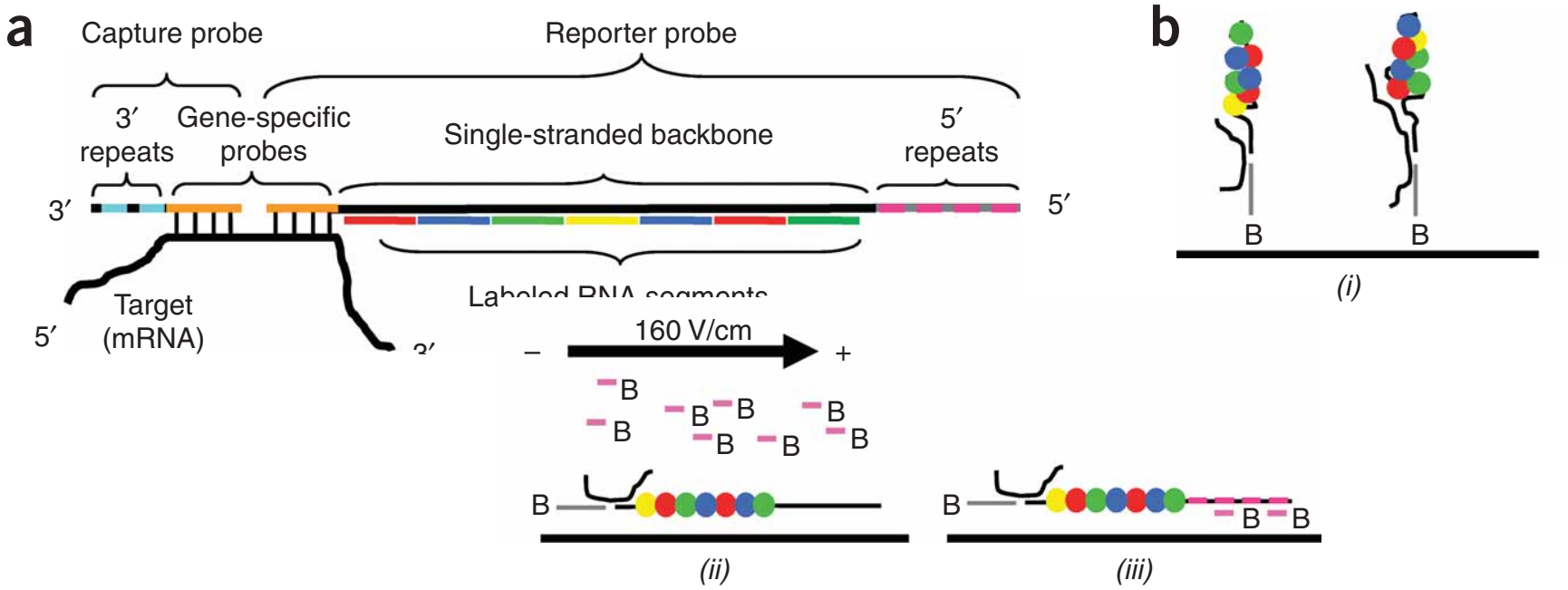
Those factors are important for expression profiling of degraded RNAs on bead array-based platform using a minimal amount of total RNA

Two oligonucleotides for each target site on the cDNA.
The upstream oligonucleotide consists of two parts, the gene-specific sequence and a universal PCR primer sequence (P1) at the 5′-end.
The downstream oligonucleotide consists of three parts, the gene-specific sequence, a unique address sequence which is complementary to a capture sequence immobilized on the array, and a universal PCR primer sequence (P2′) at the 3′-end. A single address sequence is uniquely associated with a single target site.
PCR products, which are fluorescently labelled by incorporation of the 5′-labeled primer P1, are hybridized to capture sequences on the beads in the array.

Digital Counting: How it Works



a) A schematic representation of the hybridized complex (not to scale).

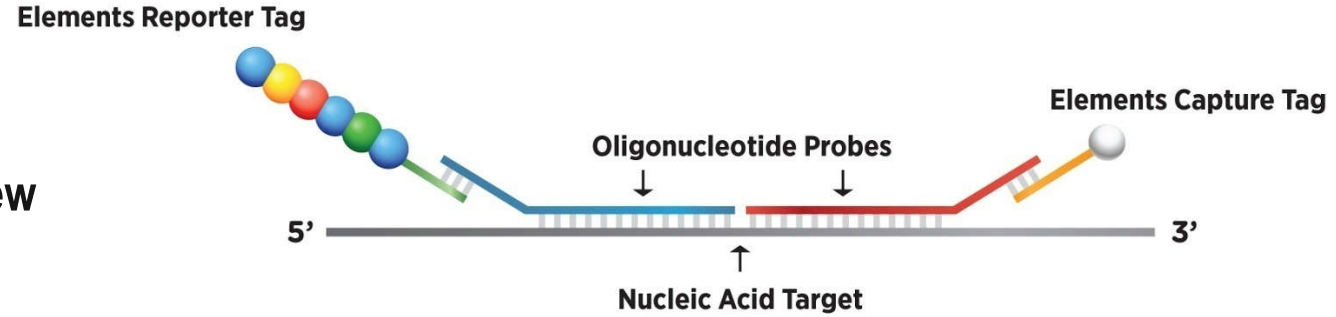


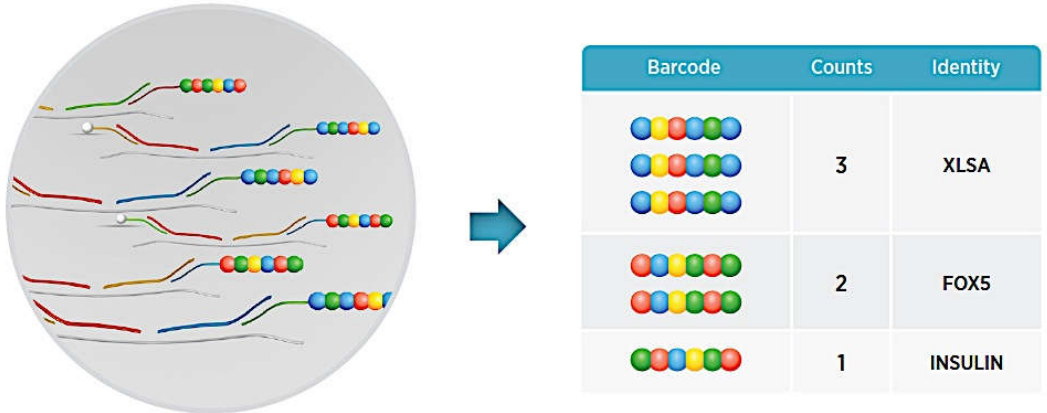
(The capture probe and reporter probe hybridize to a complementary target mRNA in solution via the gene-specific sequences .

After hybridization, the tripartite molecule is affinity-purified first by the 3'-repeat sequence and then by the 5'-repeat sequence to remove excess reporter and capture probes, respectively. (b) Schematic representation of binding, electrophoresis, and immobilization. (i) The purified complexes are attached to a streptavidin-coated slide via biotinylated capture probes. (ii) Voltage is applied to elongate and align the molecules. Biotinylated anti-5' oligonucleotides that hybridize to the 5'-repeat sequence are added. (iii) The stretched reporters are immobilized by the binding of the anti-5¢ oligonucleotides to the slide surface via the biotin. Voltage is turned off and the immobilized reporters are prepared for imaging and counting. (c) False-color image of immobilized reporter probes.

nCounter Elements - Digital Molecular Barcoding Chemistry

- **General Purpose Reagents** for Laboratory Use
- Custom designs **for as few as 12 samples**
- Ideal for complex **projects requiring iterative design**
- Target-specific **oligonucleotide probes can be re-used in multiple studies**





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Hybridise

~50 base probes per mRNA that hybridize in solution. The Reporter Probe carries the signal; the Capture Probe allows the complex to be immobilized for data collection

Purify

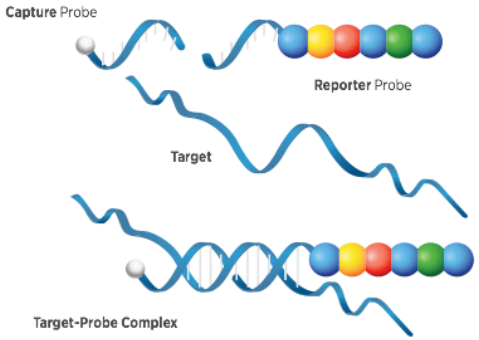
After hybridization, the excess probes are removed and the probe/target complexes aligned and immobilized in the nCounter Cartridge.

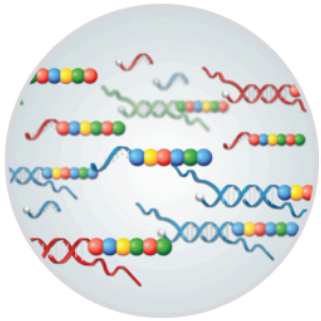
Count

Sample Cartridges are placed in the Digital Analyzer for data collection. Color codes on the surface of the cartridge are counted and tabulated for each target molecule. **1COUNT=1 MOLECULE**

1

HYBRIDIZE





solution phase hybridization

2

PURIFY

and

IMMOBILIZE

3

COUNT

Barcode	Counts	Identity
	3	XLSA
	2	FOX5
	1	INSULIN

nCounter

NanoString does not require amplification, and up to **800 genes** can be detected in a single sample, which reduces sample-handling errors and removes enzymatic reaction errors, although reference gene stability can be a challenge as with qPCR, because of differences in reference gene expression in different tissues

This technique is feasible in RNA extracts from FFPE

Application to diagnostics: The Prosigna assay analyzes the activity of 58 genes and calculates a risk of recurrence score (low, intermediate, or high). Research suggests the Prosigna assay eventually may be used more to make treatment decisions based on the risk of distant recurrence within 10 years of diagnosis of early-stage hormone-receptor positive disease with up to three positive lymph nodes after 5 years of hormonal therapy treatment in postmenopausal women. The Prosigna test is approved by the FDA and it is a CE-IVD product.

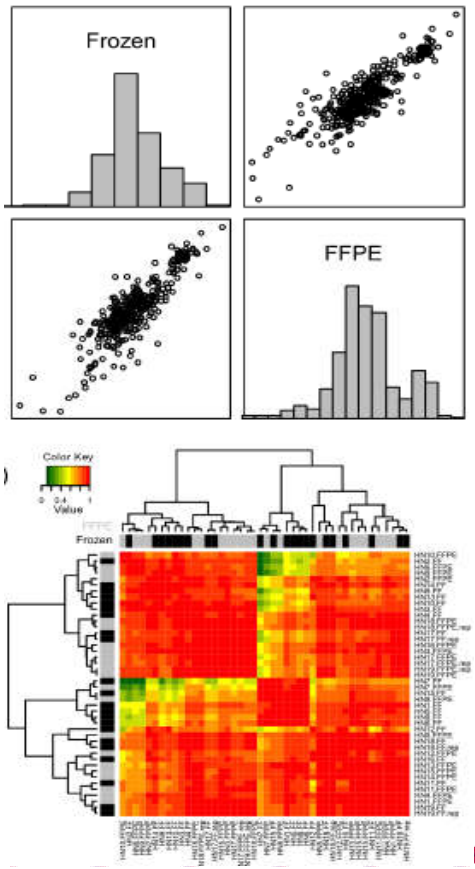
diatech labline

Outstanding Performance on FFPE Samples

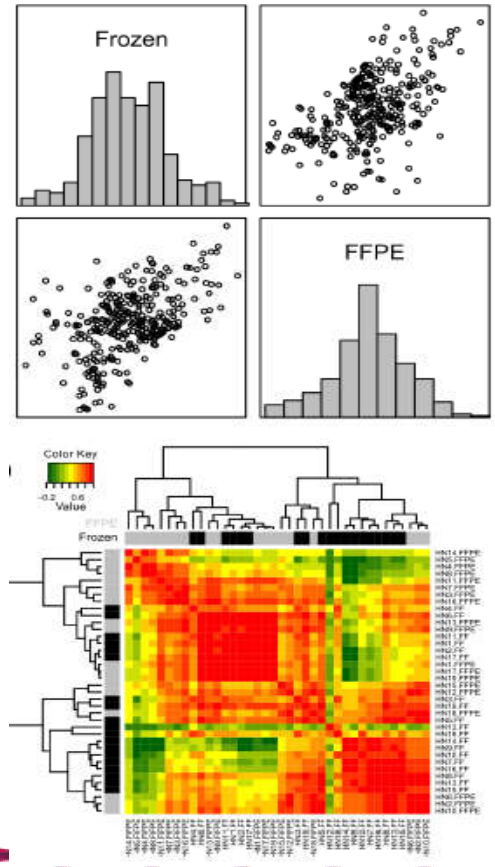
mRNA transcript quantification in archival samples using multiplexed, color-coded probes

Reis, P.P. *et al.*, *BMC Biotechnology;* May 9, 2011

nCounter® (r = 0.90)



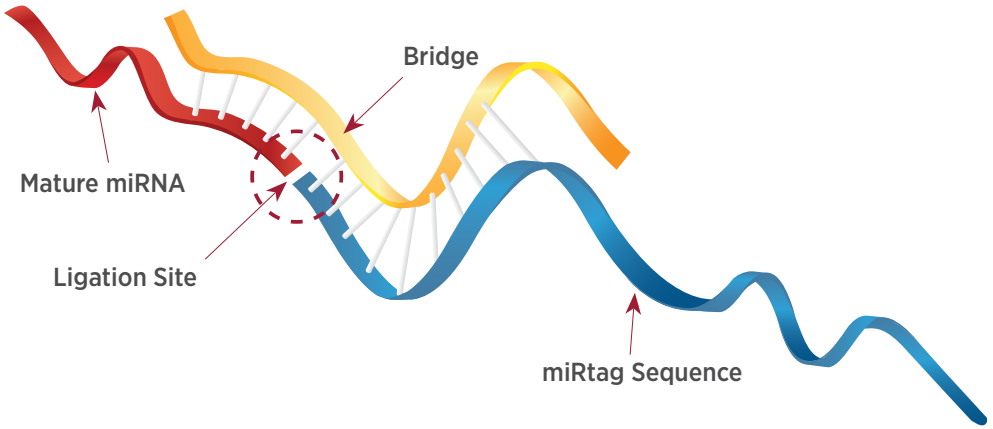
qPCR (r = 0.50)



"… the probe-based **NanoString method achieved superior gene expression quantification results** when compared to RQ-PCR in archived FFPE samples. We believe that this newly developed technique is optimal for large-scale validation studies using total RNA isolated from archived, FFPE samples."

nCounter-miRNA

miRNA Sample Preparation



DNA sequences called miRtags are ligated to the mature miRNAs through complementarity with sequence-specific bridging oligonucleotides (bridges). Excess tags and bridges are removed via a simple enzymatic step in the same tube. No further purification is required.

Sample Hybridization

The miR tagged mature miRNA is then hybridized to a probe pair in the standard nCounter gene expression assay workflow.