Measuring Gene Expression

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Bioinformatics 301

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Notes at: http://wishartlab.com

Assignment Schedule

- **Gene finding - genome annotation**
  - **(Assigned Oct. 31, due Nov. 7)**
- **Microarray analysis**
  - **(Assigned Nov. 7, due Nov. 19)**
- **Protein structure analysis**
  - **(Assigned Nov. 21, due Nov. 28)**

Each assignment is worth 5% of total grade, 10% off for each day late

How To Do Your Assignment

- **Is this a eukaryote, prokaryote, virus, mix?**
- **Use several methods to find genes or predict proteins – BLASTX is best but it helps to run other gene prediction tools if the organism contains novel genes or gene fragments**
- **Once you have found your genes/proteins then start running some annotation tools – one protein at a time. *Don't use BASYS*. Try using PROSITE, PSI-BLAST, PPT-DB, Proteus2, PDB to figure out what these proteins might be. Use pictures & graphs**

How To Do Your Assignment

- **Gather as much information about each protein/ gene as possible and show its gene/protein sequence, where it is located in the genome (position), what it might look like, its functional sites, etc. (look at the data in CCDB or GeneCards to see how you should annotate each gene/protein)**
- **Some of the proteins may be familiar or similar to something else others will be totally weird. Use PubMed or other databases to figure out how some of the proteins you've identified may lead to disease. Explain how they may work**

How To Do Your Assignment

- **The assignment should be assembled using your computer (cut, paste, format and edit the output or data so it is compact, meaningful and readable – consider tables)**
- **No handwritten materials unless your computer/printer failed**
- **A good assignment should be 8-10 pages long and will take 5-6 hours to complete**
- **Hand-in hard copy of assignment on due date. Electronic versions are accepted only if you are on your death bed**

Objectives

- Review different methods to measure gene expression
- Understand the differences in methods and reliability
- Understand basic principles of DD, SAGE, RNA-Seq, RT-PCR, Microarrays
- Understand some of the limitations of Microarray measurements

Outline for Next 3 Weeks

- Genes and Gene Finding (Prokaryotes)
- Genes and Gene Finding (Eukaryotes)
- Genome and Proteome Annotation
- Measuring Gene Expression
- Introduction to Microarrays
- More details on Microarrays

Looking at Genes*

- **Where? (where are genes located?)**
  - **Genes are located using gene finding programs (Glimmer, Genscan)**
- **What? (what do these genes do?)**
  - **Genes are characterized using gene annotation tools (BaSys etc.)**
- **How Many? (how abundant are they?)**
  - **Gene expression is measured experimentally using SAGE or gene chips**

Different Kinds of "Omes"

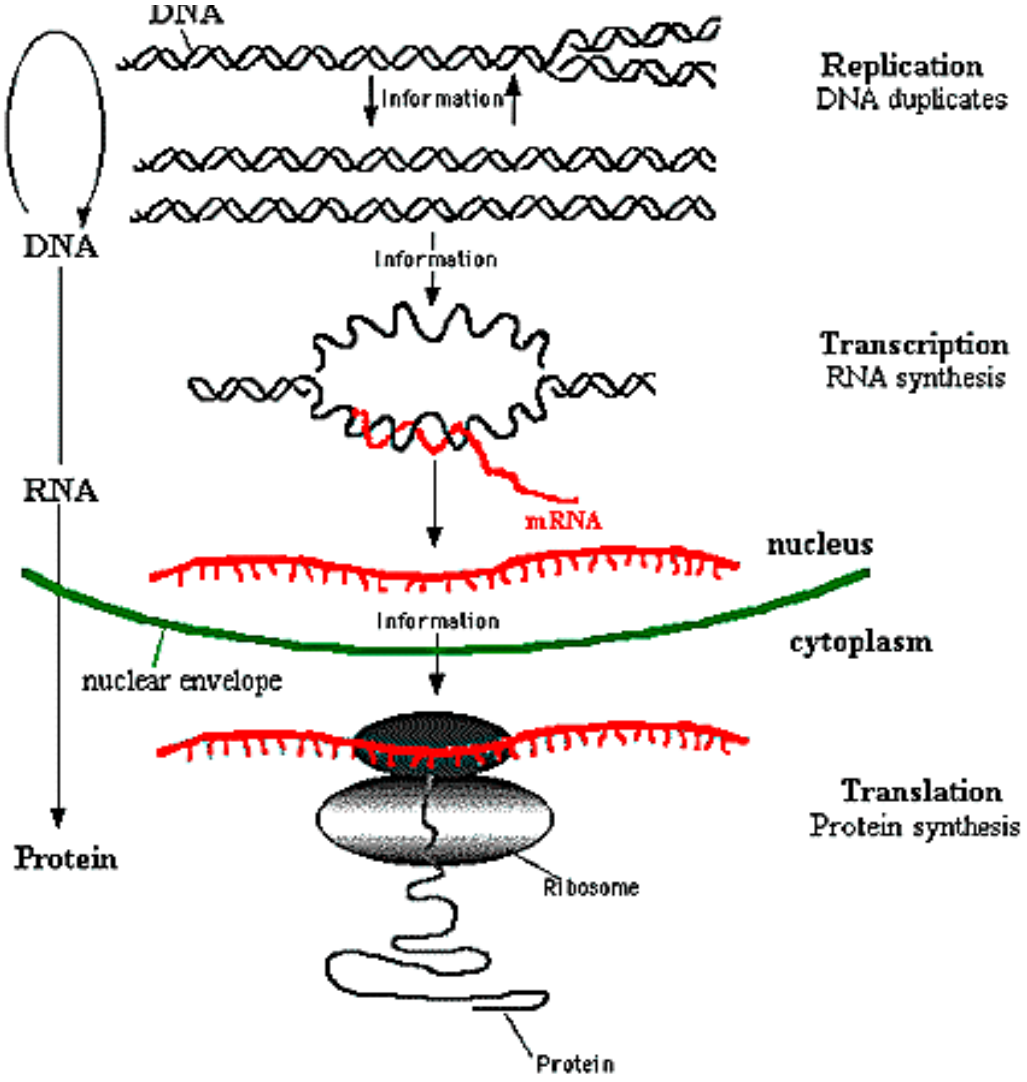
- **Genome**
  - **Complement of all genes in a cell, tissue, organ or organism**
- **Transcriptome**
  - **Complement of all mRNA transcripts in a cell, tissue, organ or organism**
- **Proteome**
  - **Complement of all proteins in a cell, tissue, organ or organism**

Different Kinds of "Omes"*

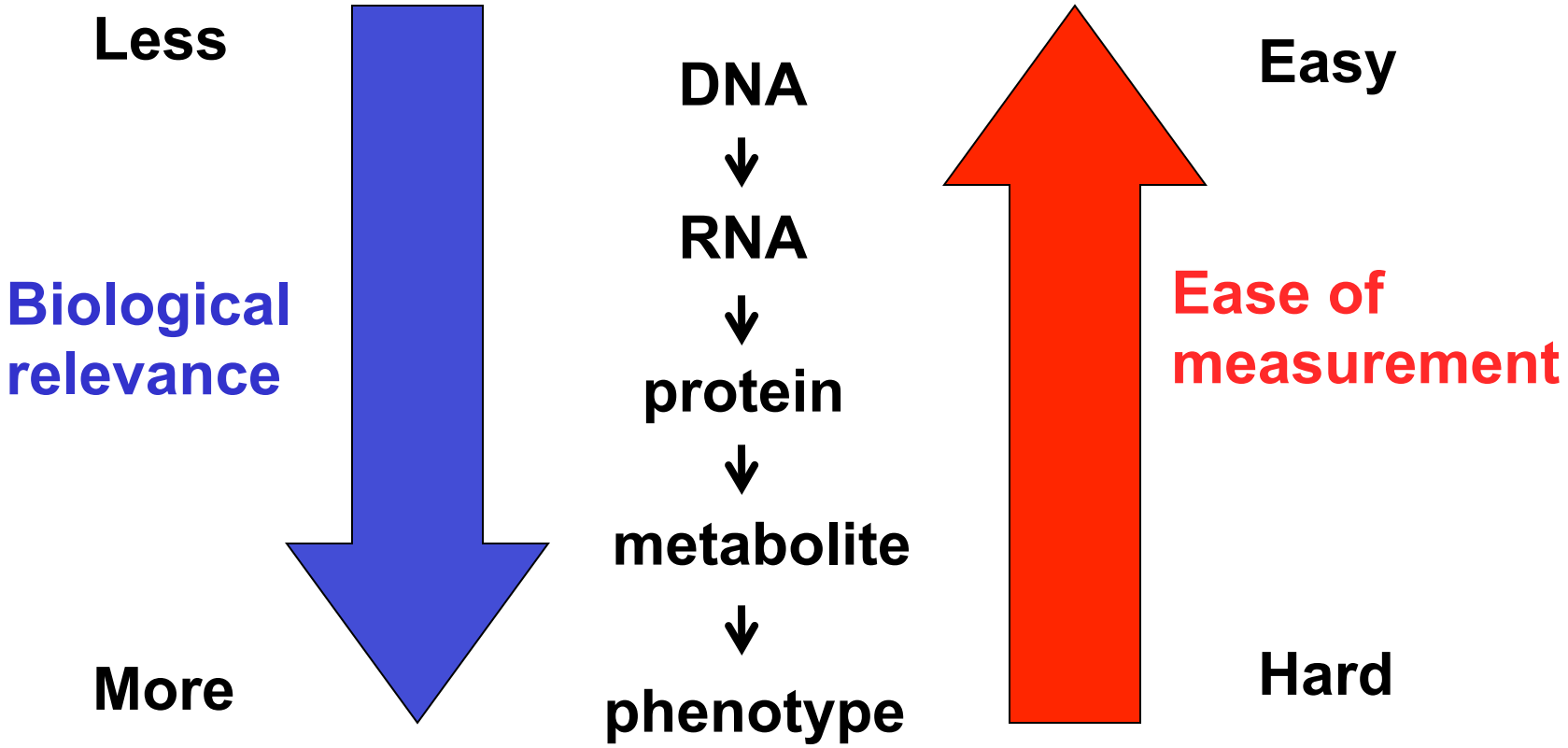
Genome

Transcriptome

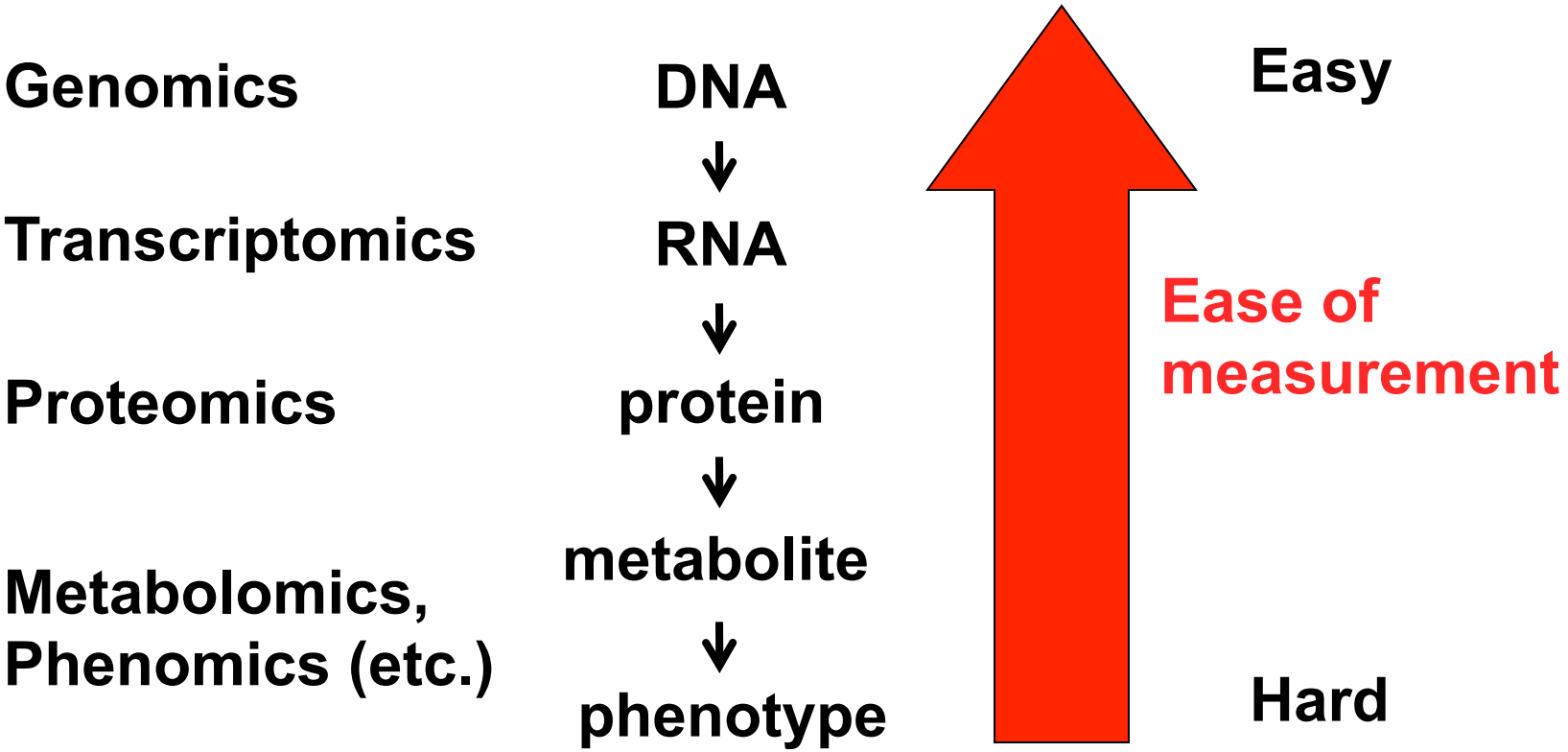
Proteome



The Measurement Dichotomy*



High Throughput Measurement



-Omics Mania

biome, CHOmics, cellome, cellomics, chronomics, clinomics, complexome, crystallomics, cytomics, cytoskeleton, degradomics, diagnomics™, enzymome, epigenome, expressome, fluxome, foldome, secretome, functome, functomics, genomics, glycomics, immunome, transcriptomics, integromics, interactome, kinome, ligandomics, lipoproteomics, localizome, phenomics, metabolome, pharmacometabonomics, methylome, microbiome, morphome, neurogenomics, nucleome, secretome, oncogenomics, operome, transcriptomics, ORFeome, parasitome, pathome, peptidome, pharmacogenome, pharmacomethylomics, phenomics, phylome, physiogenomics, postgenomics, predictome, promoterome, proteomics, pseudogenome, secretome, regulome, resistome, ribonome, ribonomics, riboproteomics, saccharomics, secretome, somatonome, systeome, toxicomics, transcriptome, translatome, secretome, unknome, vaccinome, variomics...

http://www.genomicglossaries.com/content/omes.asp

Why Measure Gene Expression?*

- Assumption that more abundant genes/transcripts are more important
- Assumption that gene expression levels correspond to protein levels
- Assumption that a normal cell has a standard expression profile/signature
- Changes to that expression profile indicate something is happening

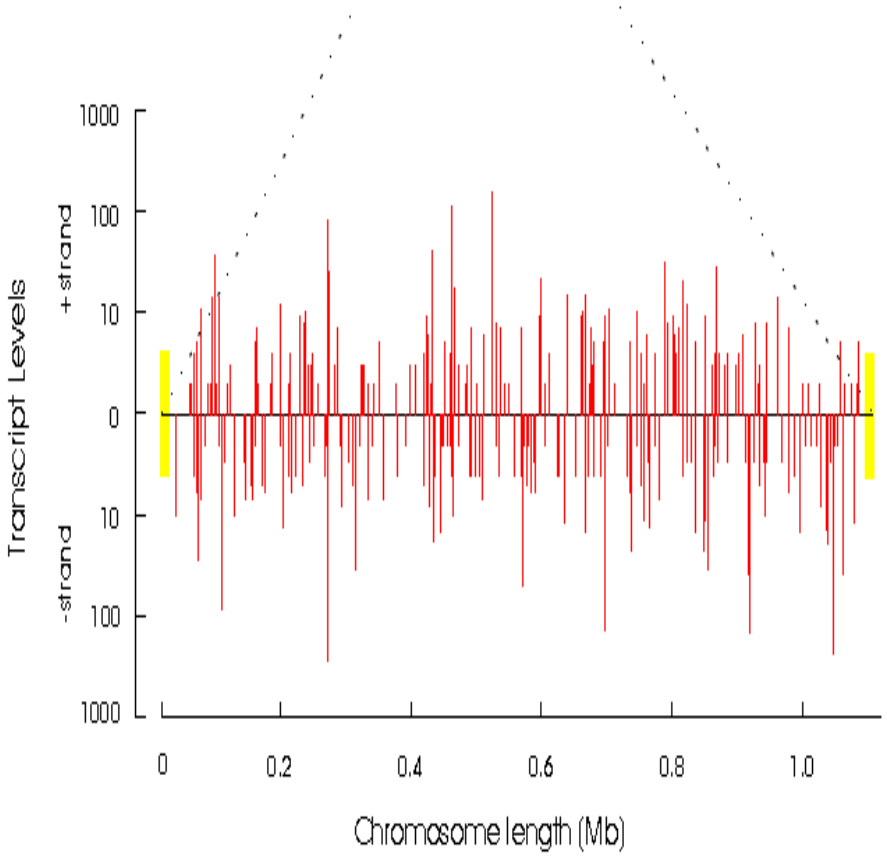
Why Measure Gene Expression?*

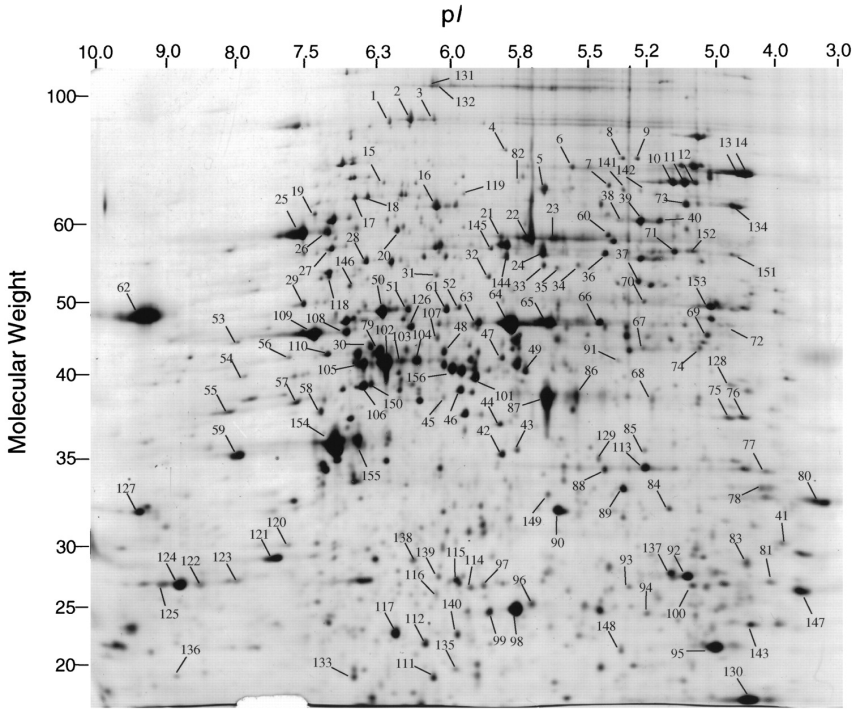
- Gene expression profiles represent a snapshot of cellular metabolism or activity at the molecular scale
- Gene expression profiles represent the cumulative interactions of many hard to detect events or phenomena
- Gene expression is a “proxy” measure for transcription/translation events

mRNA level = Protein level?*

- **Gygi et al. (1999) Mol. Cell. Biol. compared protein levels (MS, gels) and RNA levels (SAGE) for 156 genes in yeast**
- **In some genes, mRNA levels were essentially unchanged, but protein levels varied by up to 20X**
- **In other genes, protein levels were essentially unchanged, but mRNA levels varied by up to 30X**

SAGE vs. 2D Gel





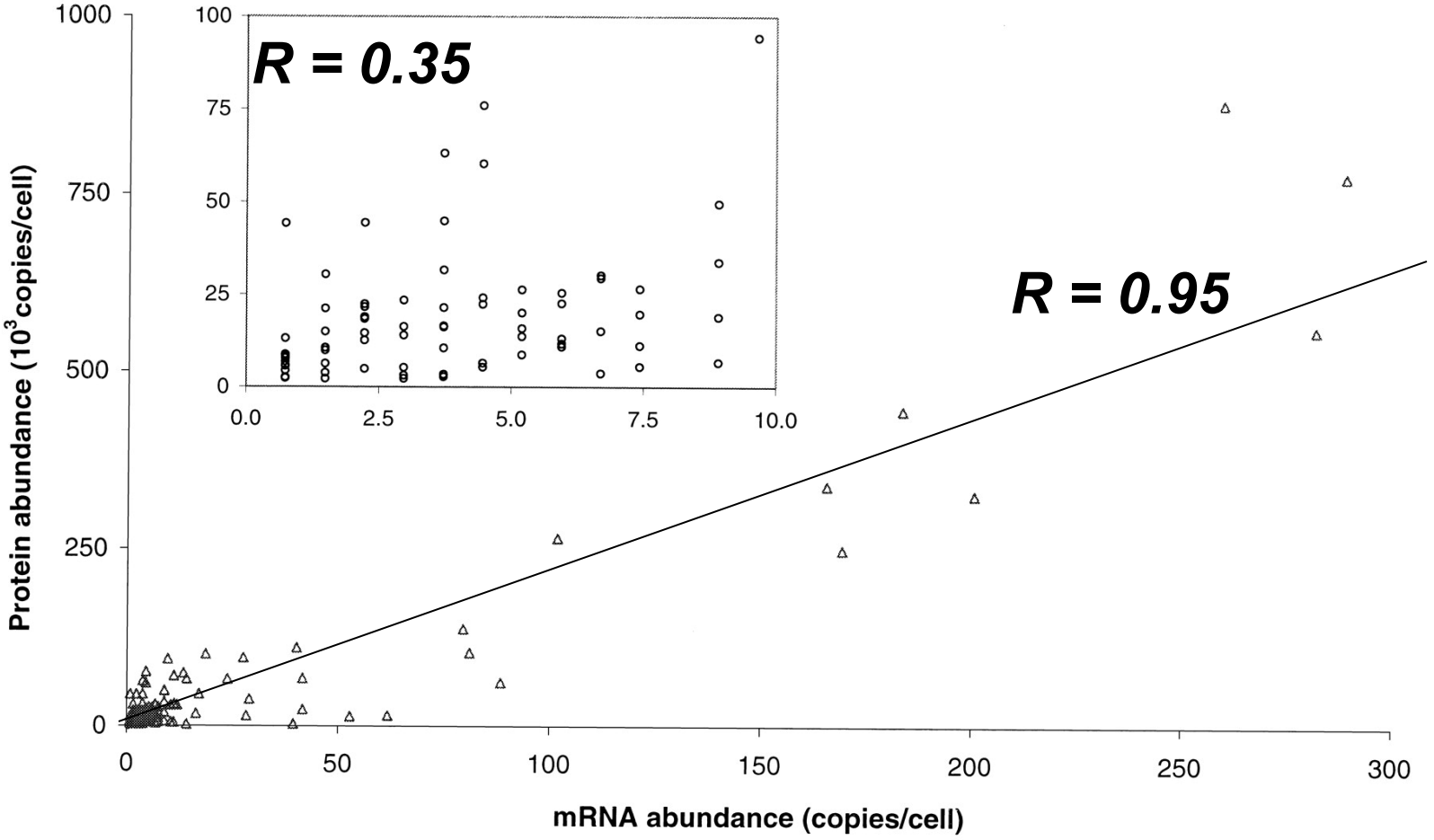
mRNA

Protein

mRNA level = Protein level?

*

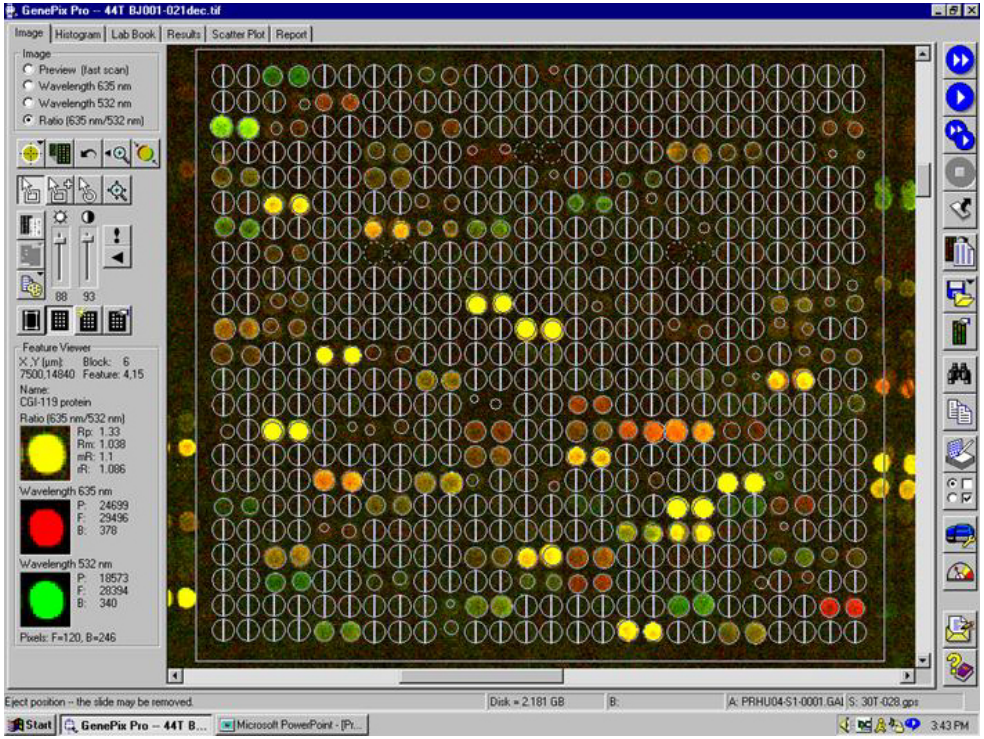
Gygi et al. (1999) Mol. Cell. Biol

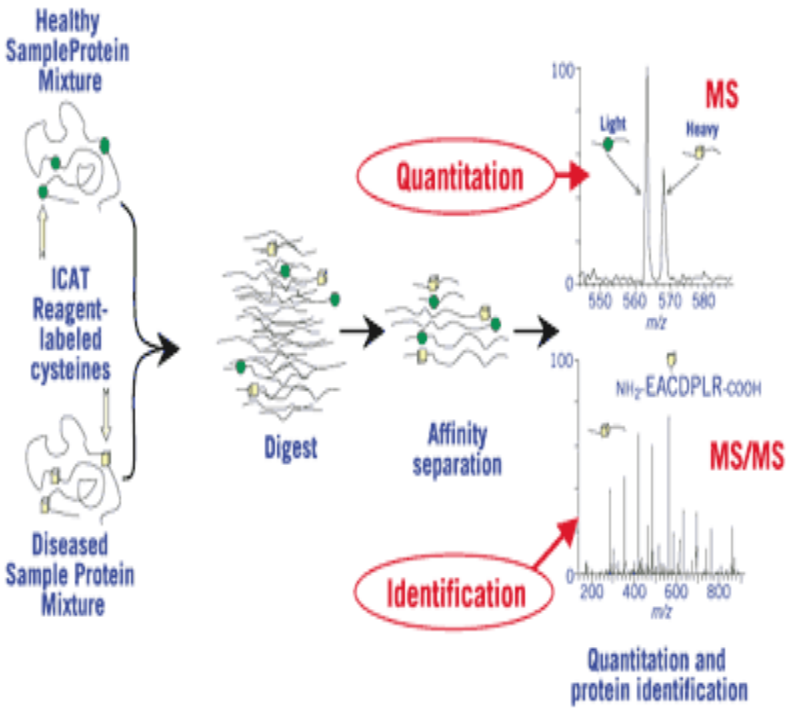


mRNA level = Protein level?

- **Griffen TJ et al. (2002) *Mol. Cell. Proteomics* 1:323-333**
- **Compared protein levels (MS, ICAT) and RNA levels (microarray) for 245 genes in yeast on galactose/ethanol medium**
- **“Significant number of genes show large discrepancies between abundance ratios when measured at the levels of mRNA and protein expression”**

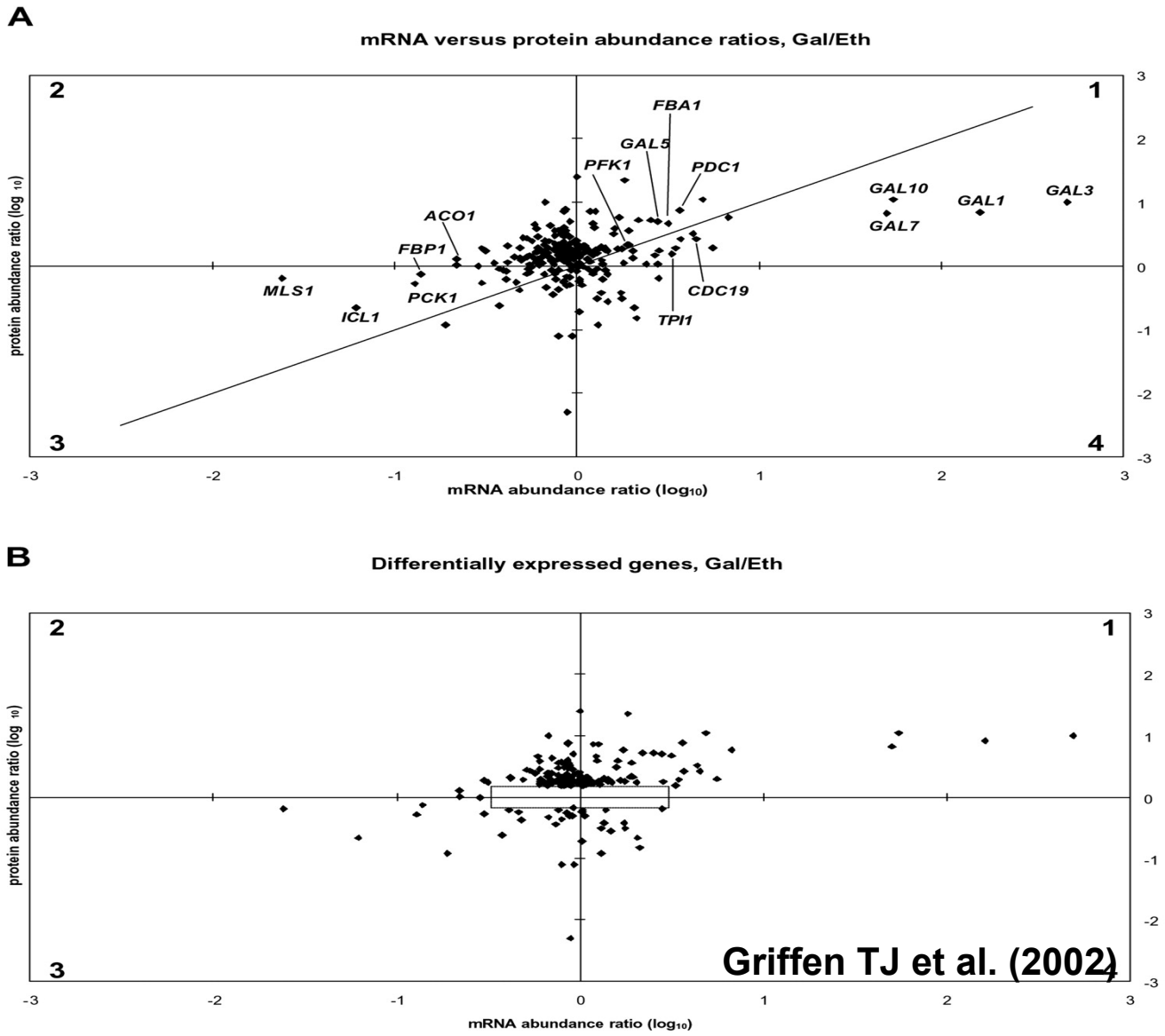
Microarray vs. ICAT*



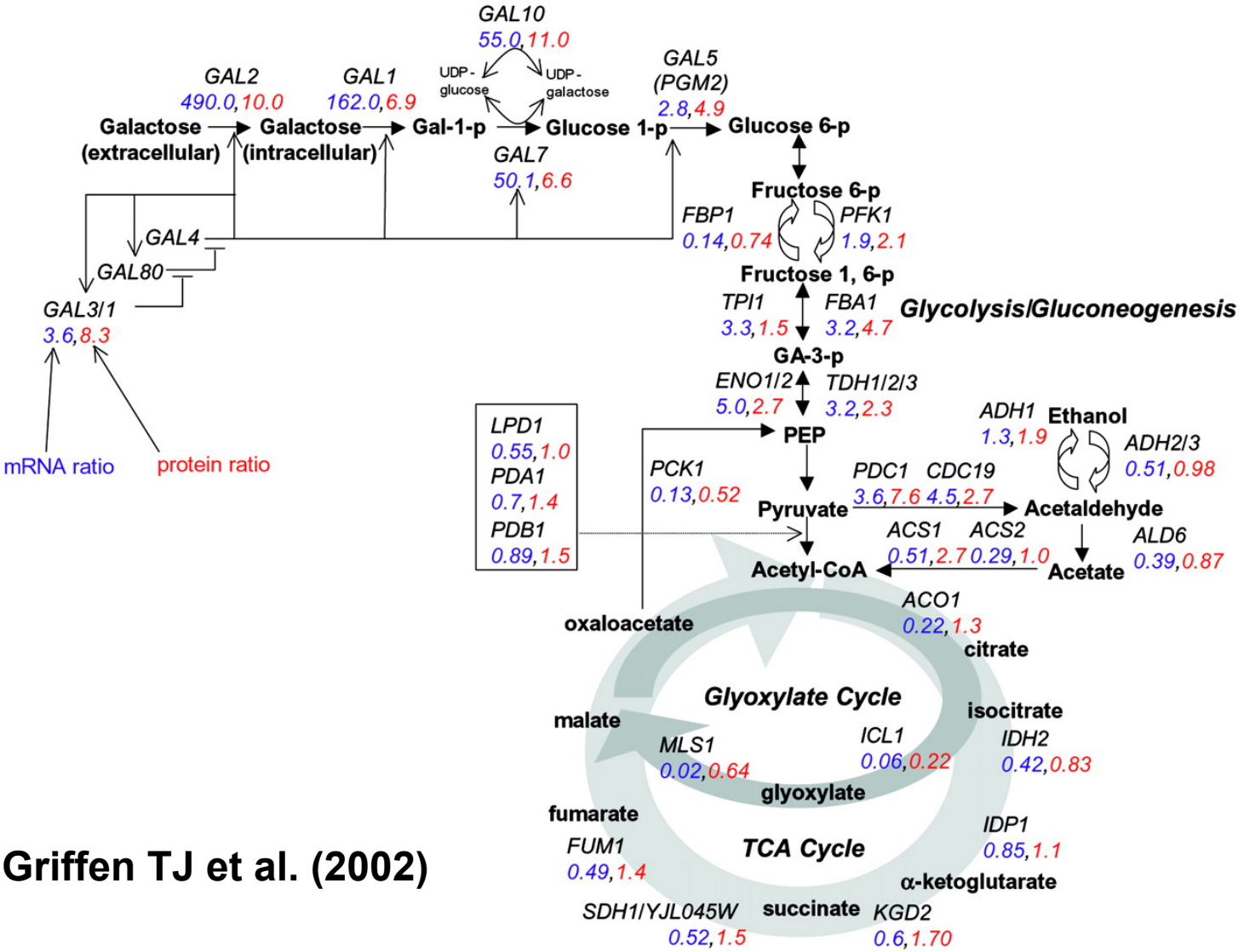


mRNA **Protein**

mRNA vs. Protein levels*

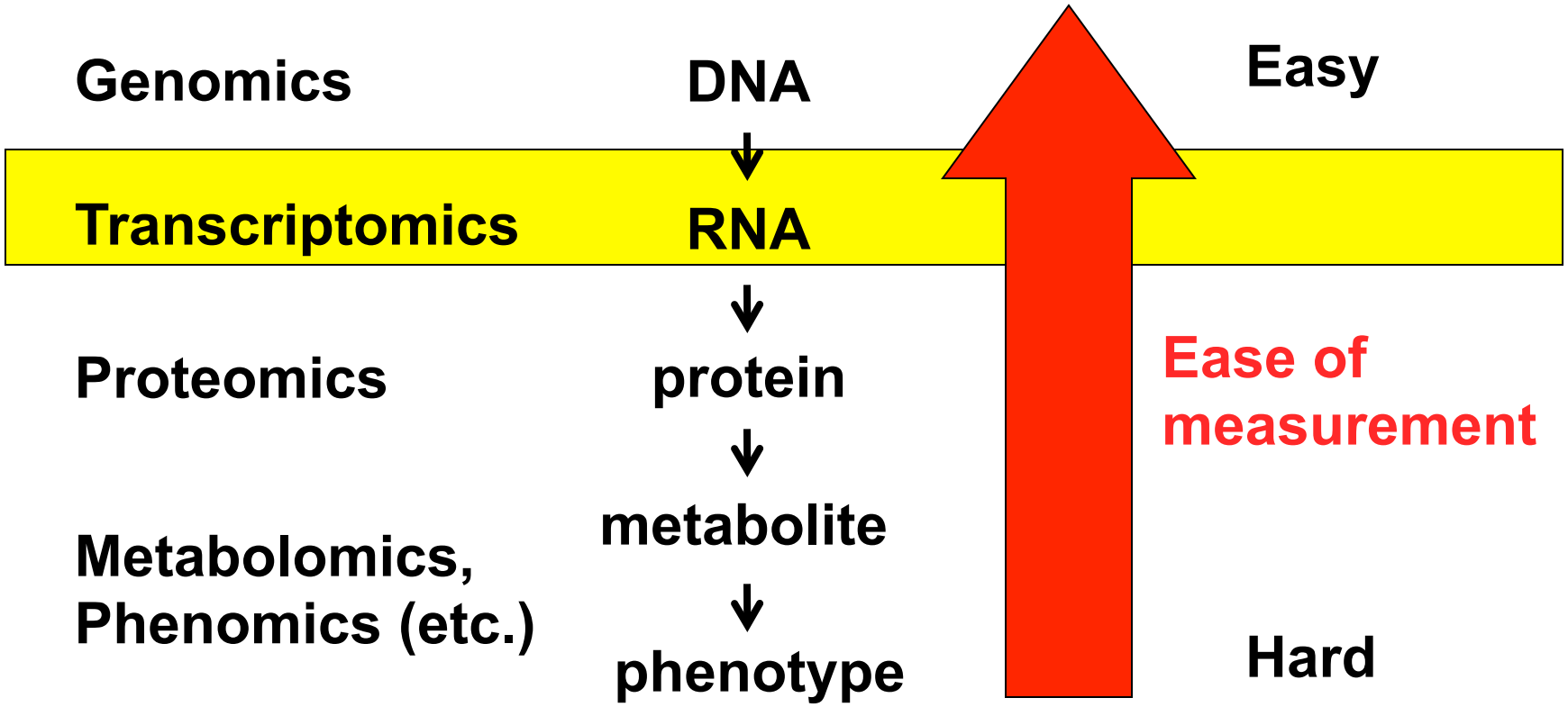


mRNA vs. Protein levels



Griffen TJ et al. (2002)

Why Do It?



It's easier to do than the other measurements

How Relevant are RNA Levels to Protein Levels?

“ [transcript abundance] doesn’t tell us everything, but it tells us a lot more than we knew before ”

--Pat Brown, Stanford
Microarray pioneer

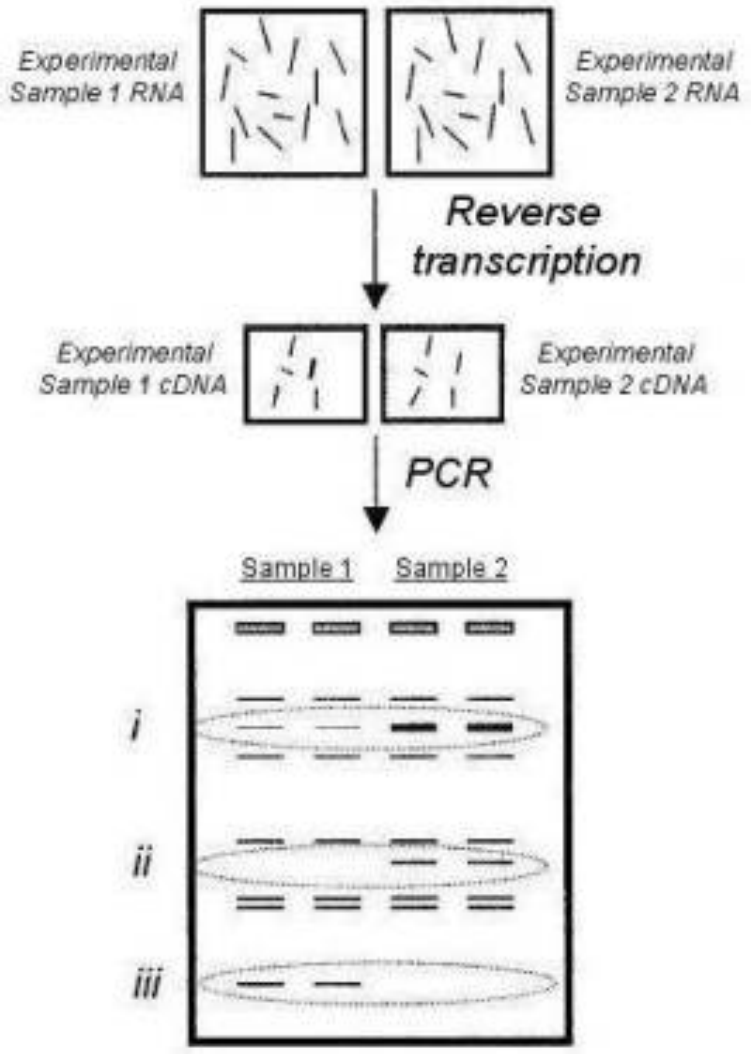
Measuring Gene Expression*

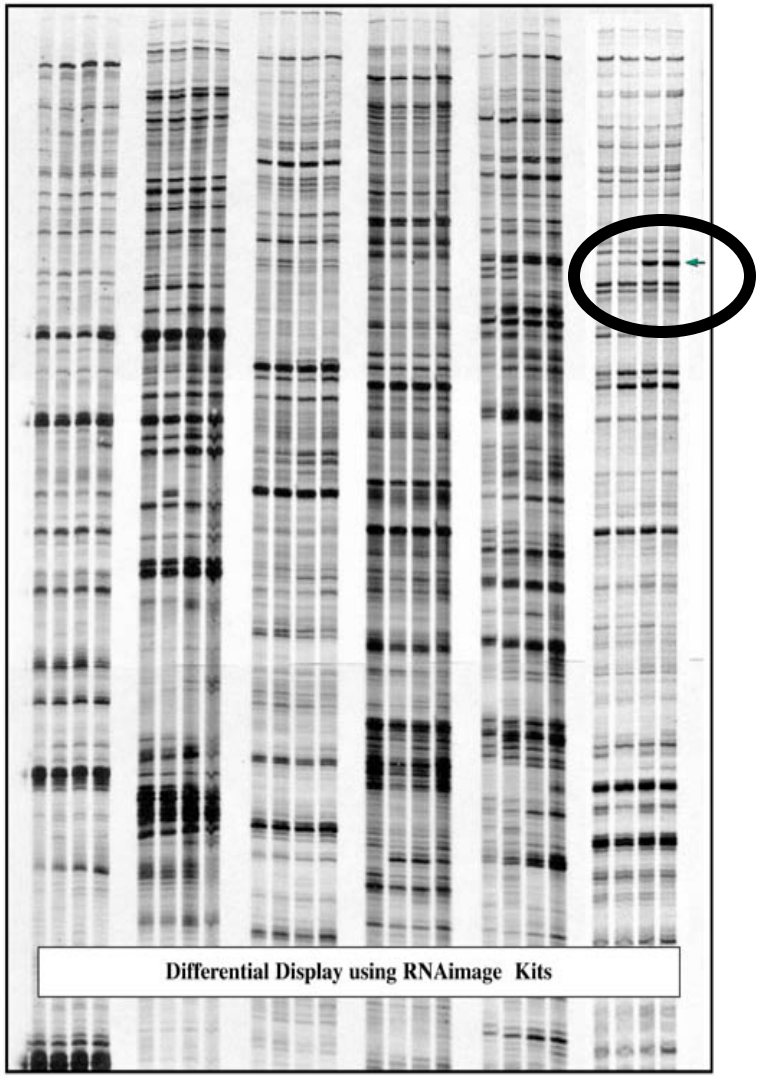
- Differential Display
- Serial Analysis of Gene Expression (SAGE)
- RNA-Seq
- RT-PCR (real-time PCR)
- Northern/Southern Blotting
- DNA Microarrays or Gene Chips

Differential Display (DD)*

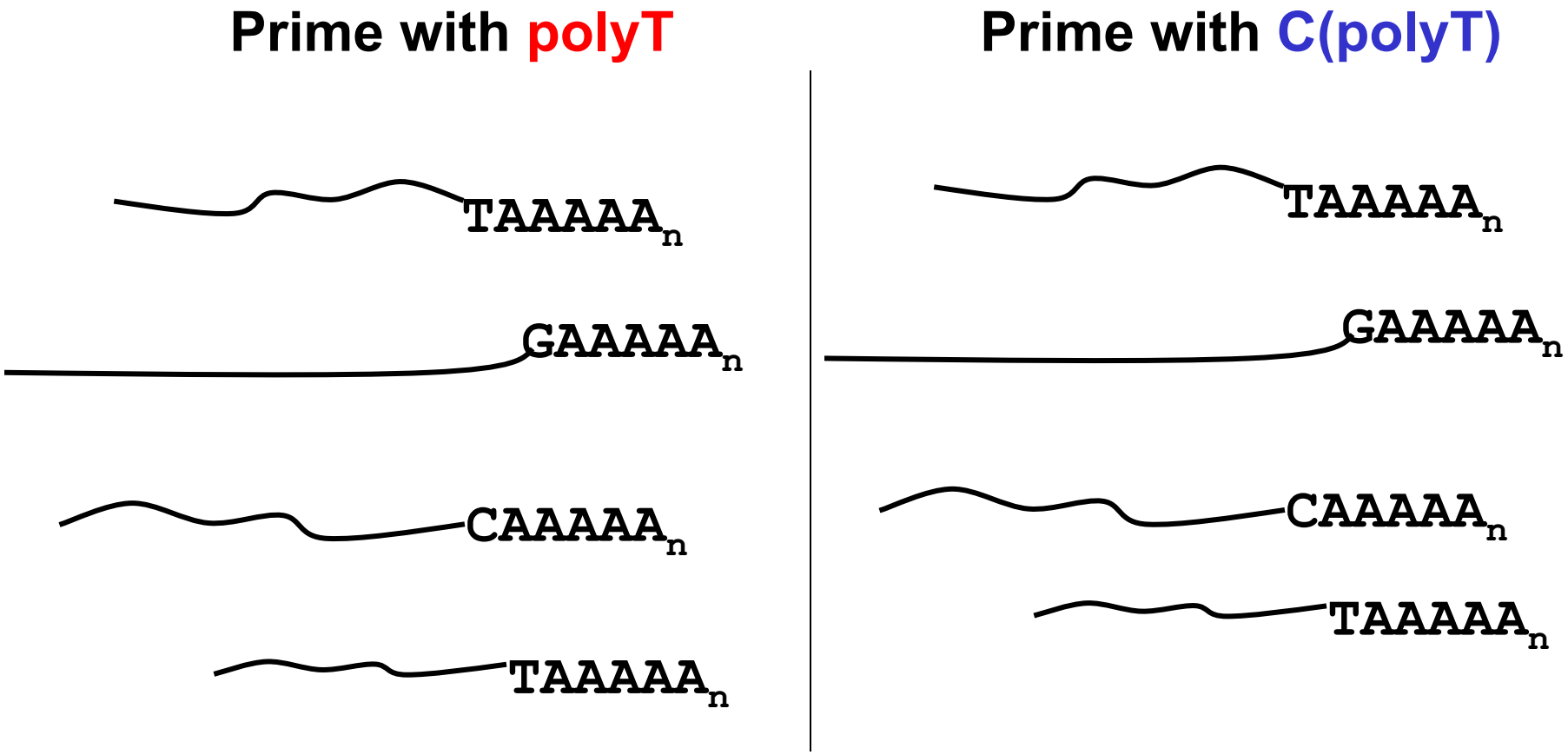
- **Basic idea:**
  - **Run two RNA (cDNA) samples side by side on a gel**
  - **Excise and sequence bands present in one lane, but not the other**
- **The clever trick:**
  - **Reduce the complexity of the samples by making the cDNA with primers that will prime only a subset of all transcripts**

Differential Display*

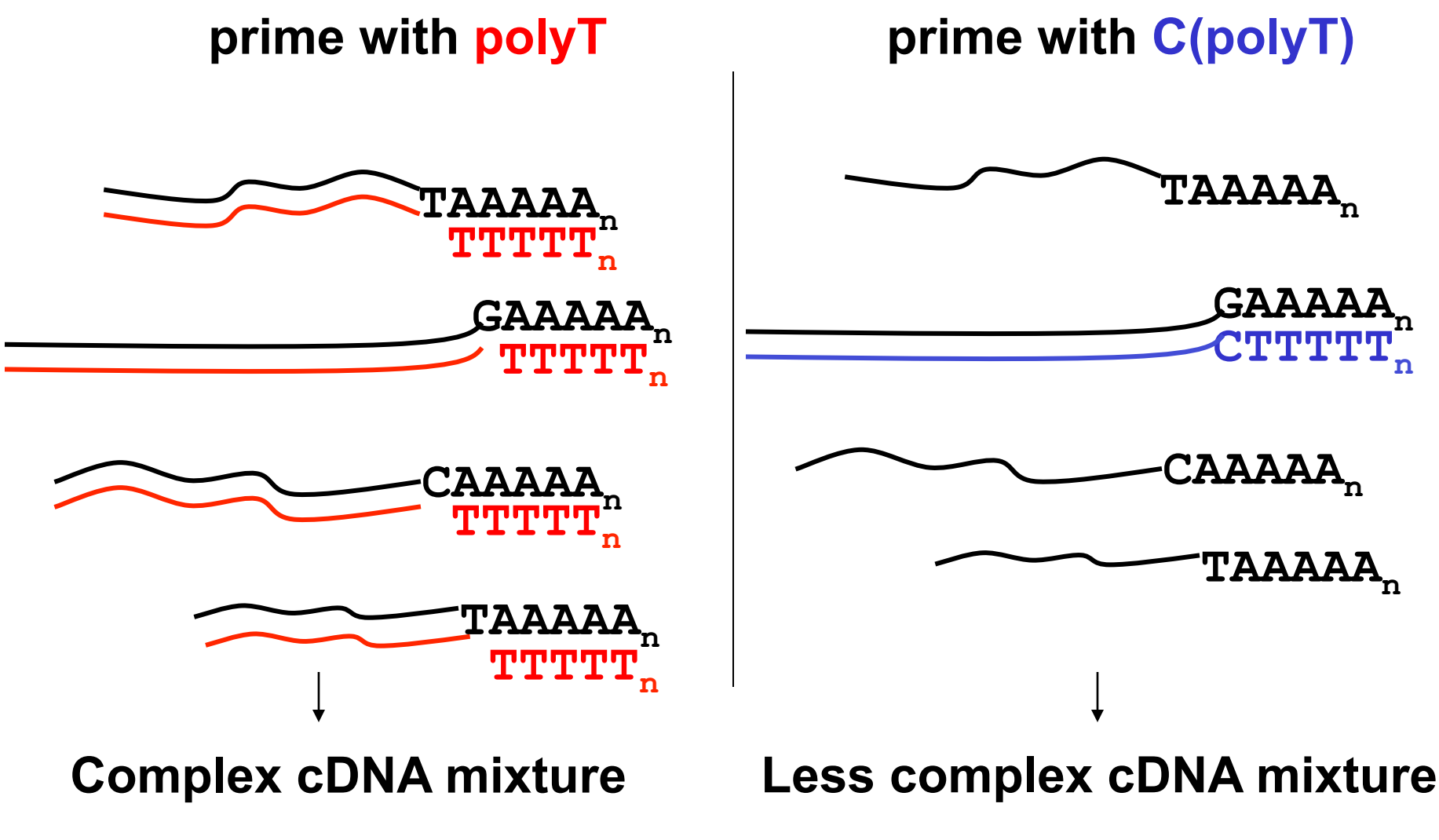




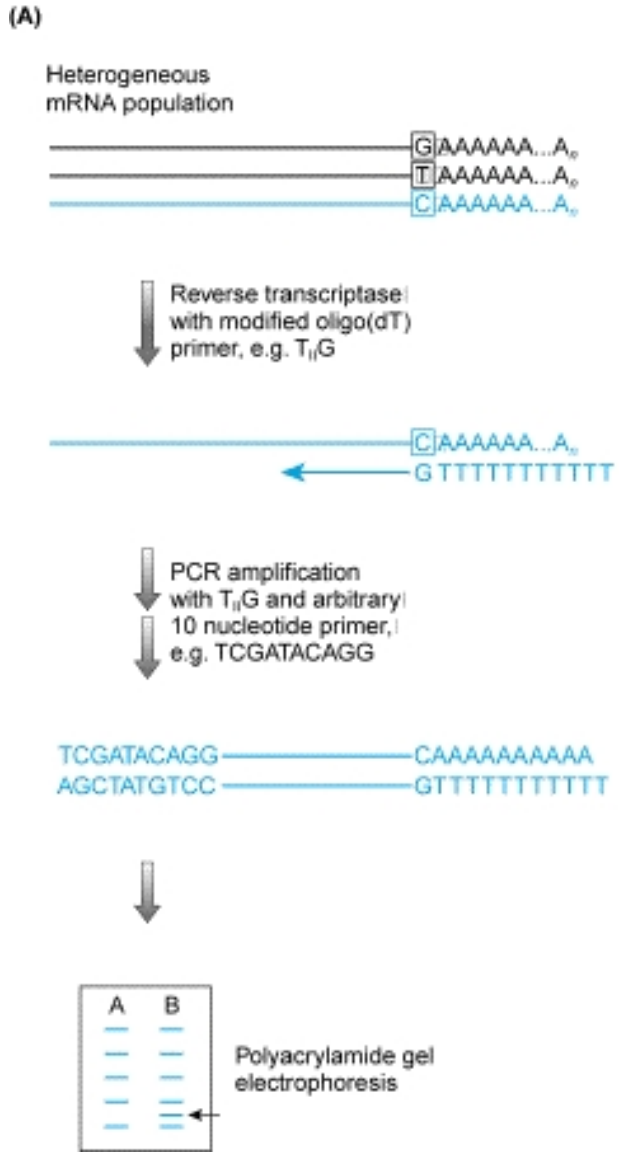
Differential Display (Detail)

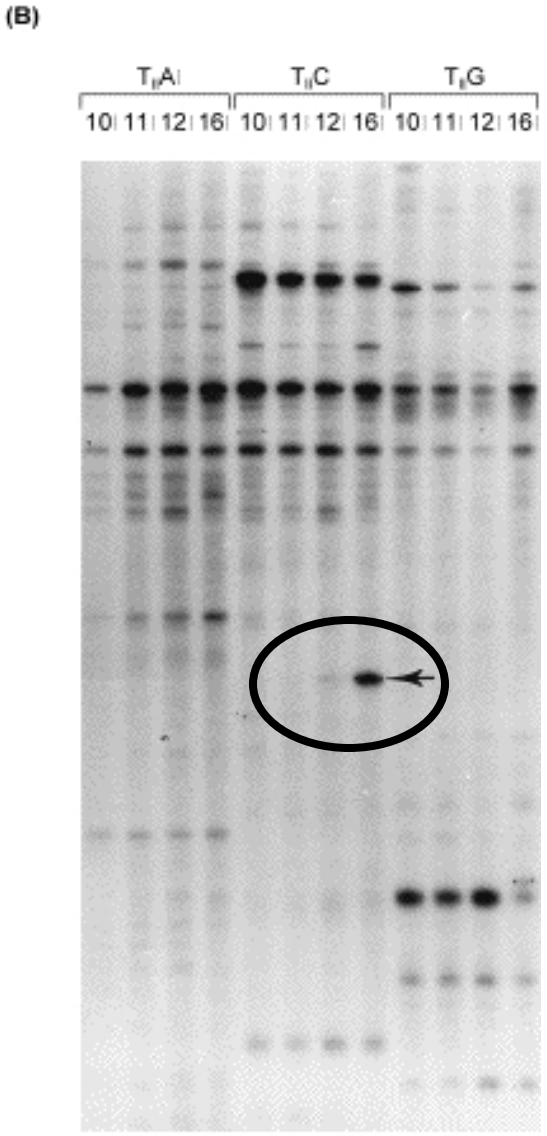


Differential Display (Detail)



Differential Display





10hr
11hr
12hr
16hr

Advantages of DD*

- Oldest of all transcript expression methods
- Technically and technologically simplest of all transcript methods
- Does not require ESTs, cDNA libraries, or any prior knowledge of the genome
- Open-ended technology

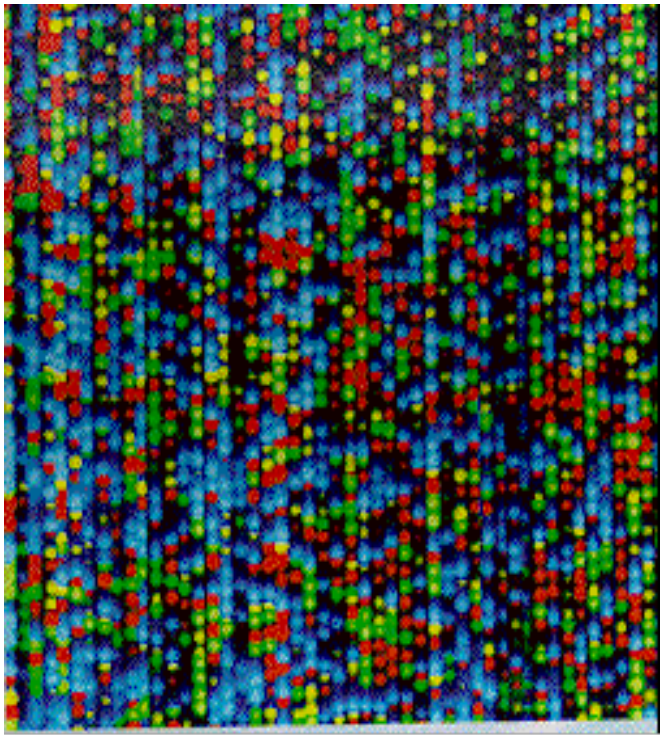
Disadvantages of DD*

- Not very quantitative
- Sensitivity can be an issue
- Only a fraction of the transcripts can be analyzed in any single reaction
- Prone to false positives
- Not easily automated or scaled-up

SAGE*

- Principle is to convert every mRNA molecule into a short (10-14 base), unique tag. Equivalent to reducing all the people in a city into a telephone book with surnames
- After creating the tags, these are assembled or concatenated into a long “list”
- The list can be read using a DNA sequencer and the list compared to a database to ID genes or proteins and their frequency

SAGE Tools



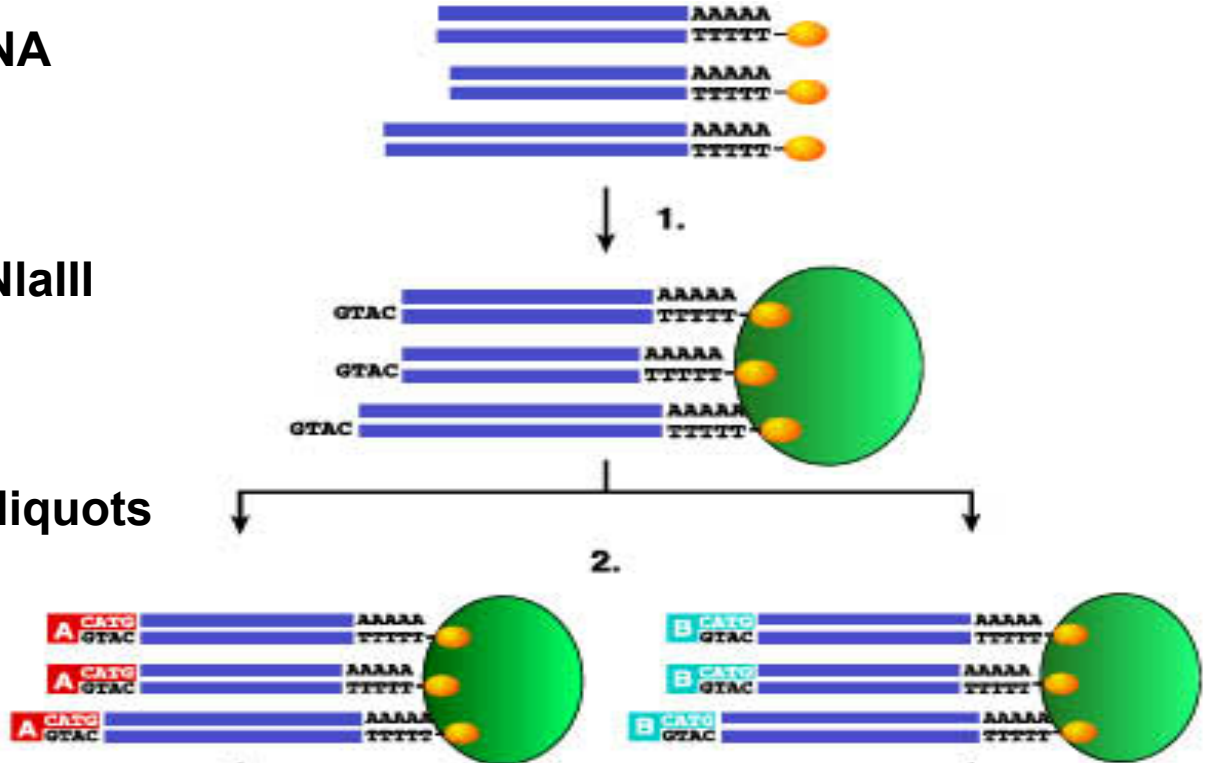
SAGE*

Convert mRNA to dsDNA

Digest with NlaIII

Split into 2 aliquots

Attach Linkers



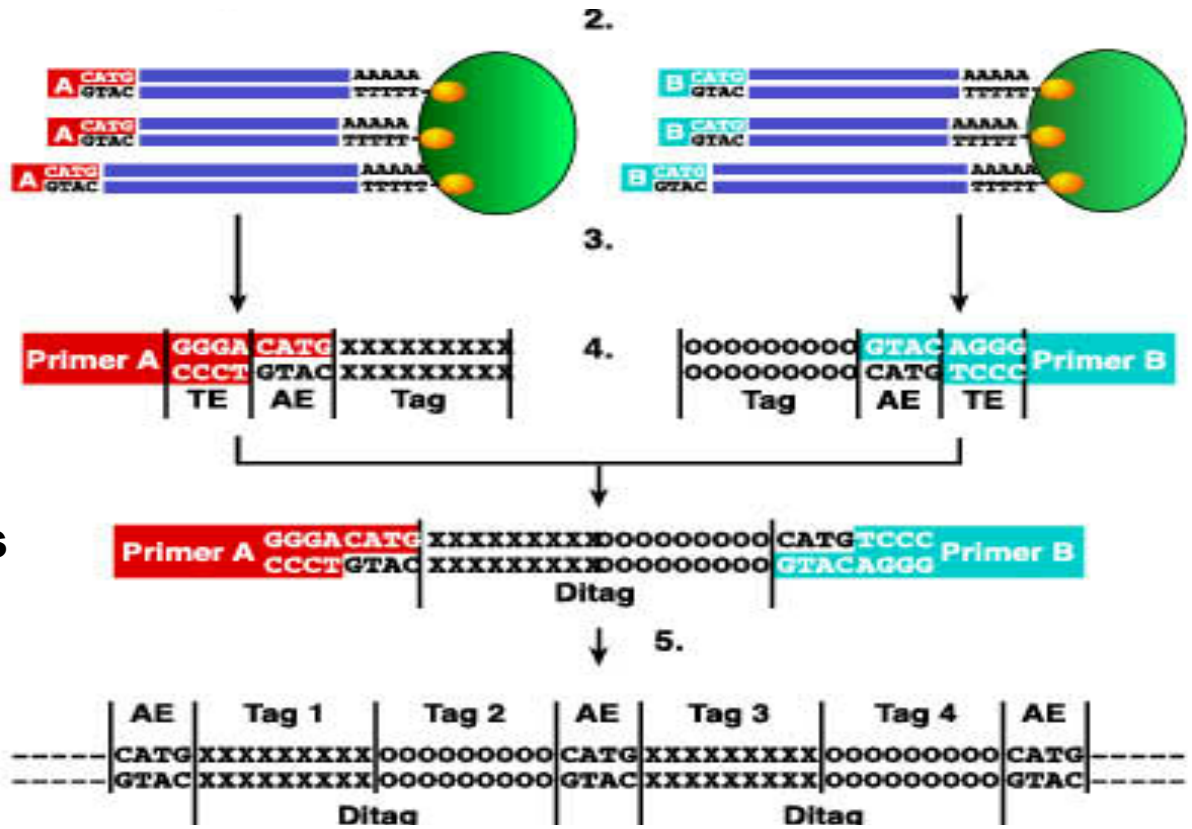
SAGE*

Linkers have PCR & Tagging Endonuclease

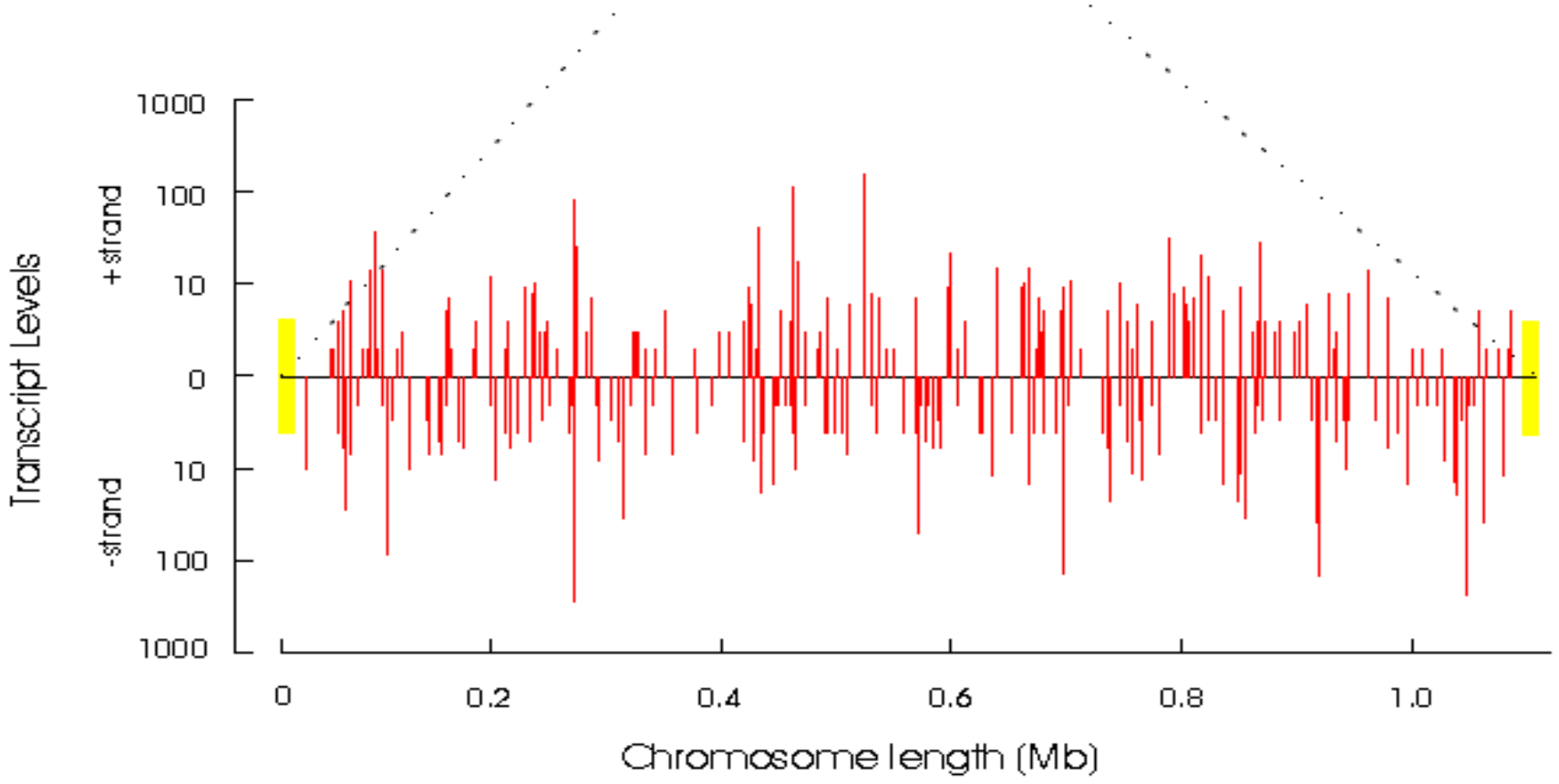
Cut with TE BsmF1

Mix both aliquots Blunt-end ligate to make “Ditag”

Concatenate & Sequence



SAGE of Yeast Chromosome



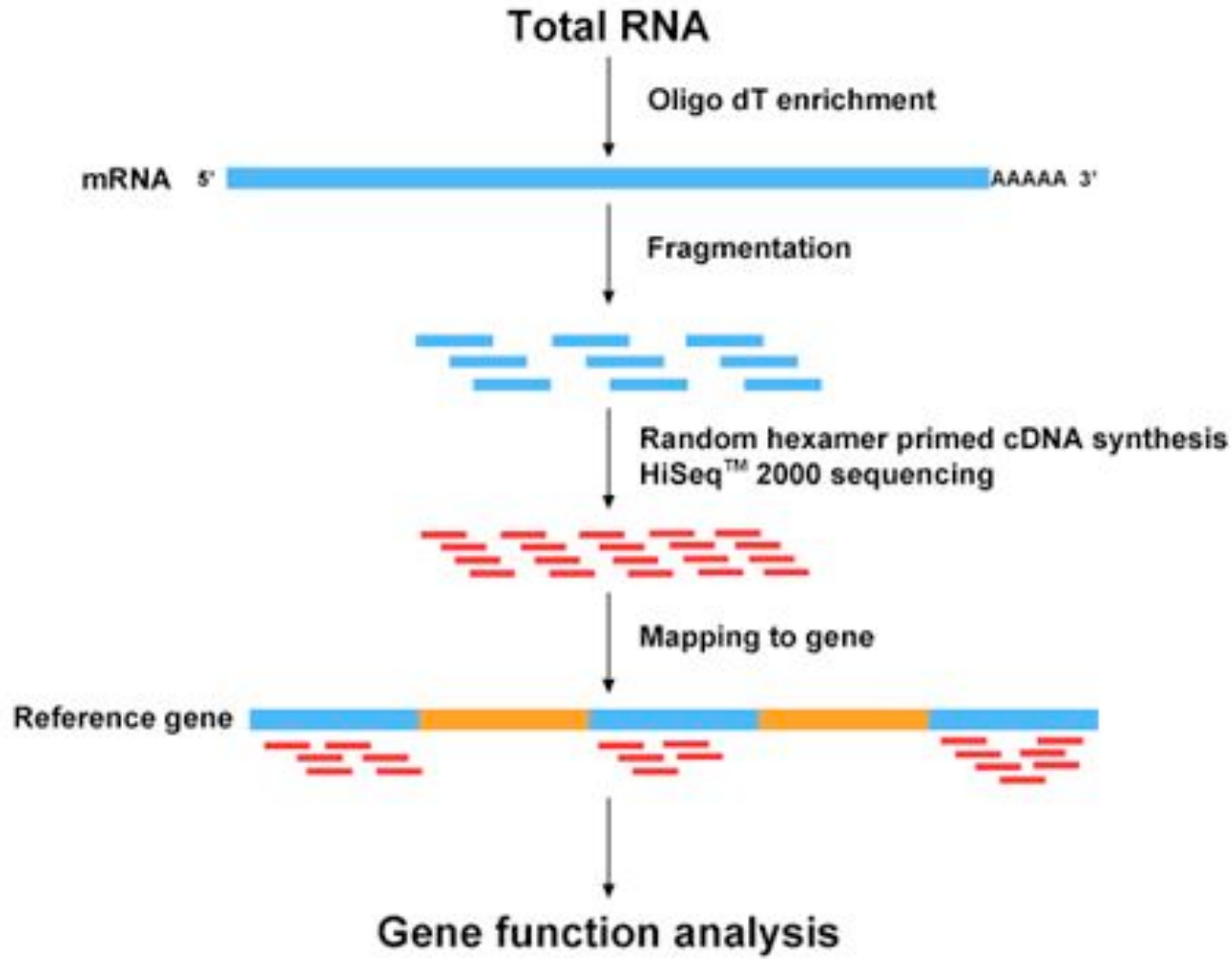
Advantages of SAGE*

- Very direct and quantitative method of measuring transcript abundance
- Open-ended technology
- Near infinite dynamic range
- Built-in quality control:
  - e.g. spacing of tags & 4-cutter restriction sites

Disadvantages of SAGE*

- Expensive, time consuming technology - must sequence >50,000 tags per sample (>$5,000 per sample)
- Most useful with fully sequenced genomes (otherwise difficult to associate 15 bp tags with their genes)
- 3’ ends of some genes can be very polymorphic

RNA-Seq


Total RNA
Oligo dT enrichment
mRNA 5'
AAAAA 3'
Fragmentation
Random hexamer primed cDNA synthesis
HiSeq™ 2000 sequencing
Mapping to gene
Reference gene
Gene function analysis

Advantages of RNA-Seq*

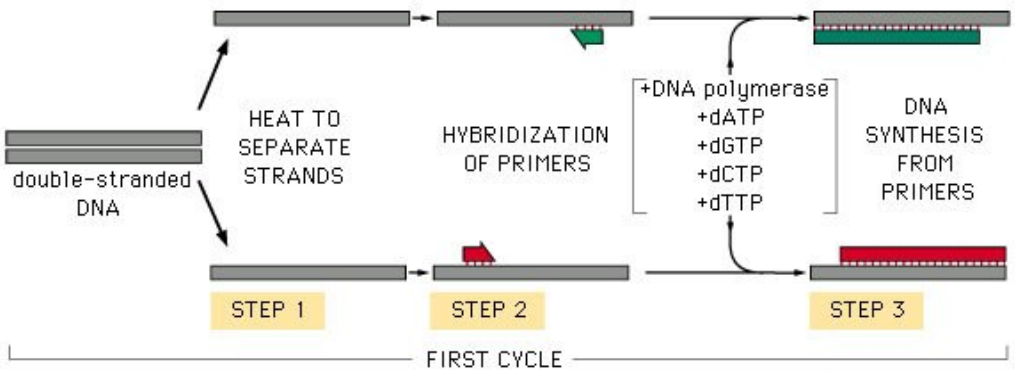
- Very direct and quantitative method of measuring transcript abundance
- Open-ended technology
- Near infinite dynamic range
- No prior knowledge of genome required
- Discriminates among regions with high sequence identity

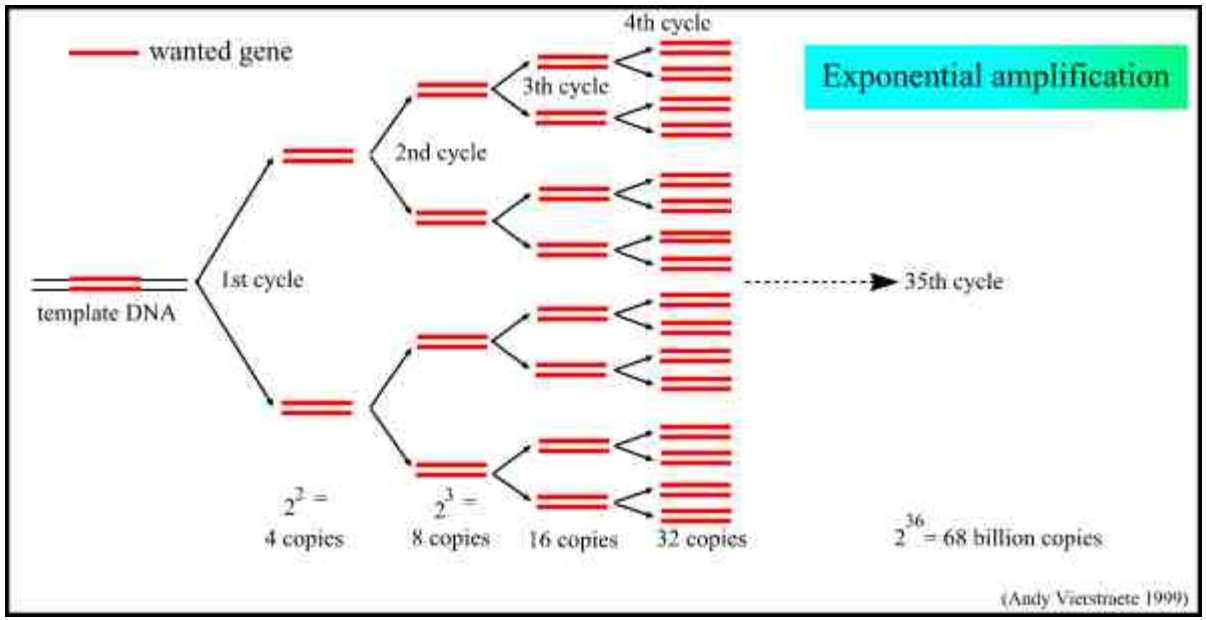
Disadvantages of RNA-Seq*

- Expensive equipment (instruments are >$500,000)
- Expensive to run (at least for now)
- Amplification steps can distort the balance between abundant and rare RNA species
- Selection and hybridization methods may introduce artifacts
- Software is still evolving/improving

RT-PCR

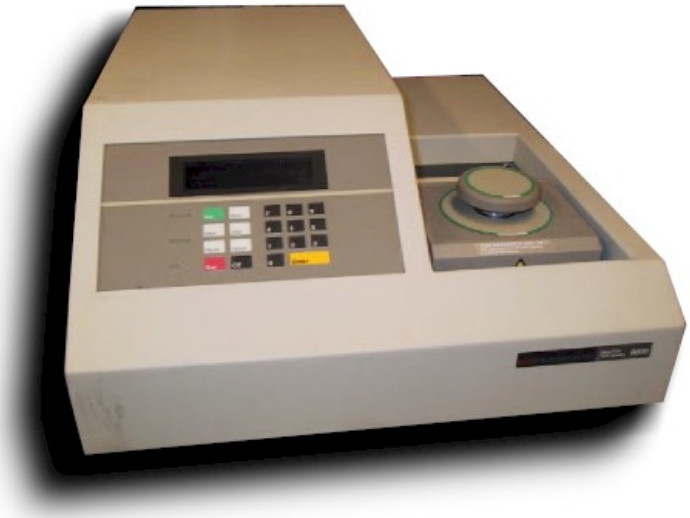
Principles of PCR*





Polymerase Chain Reaction

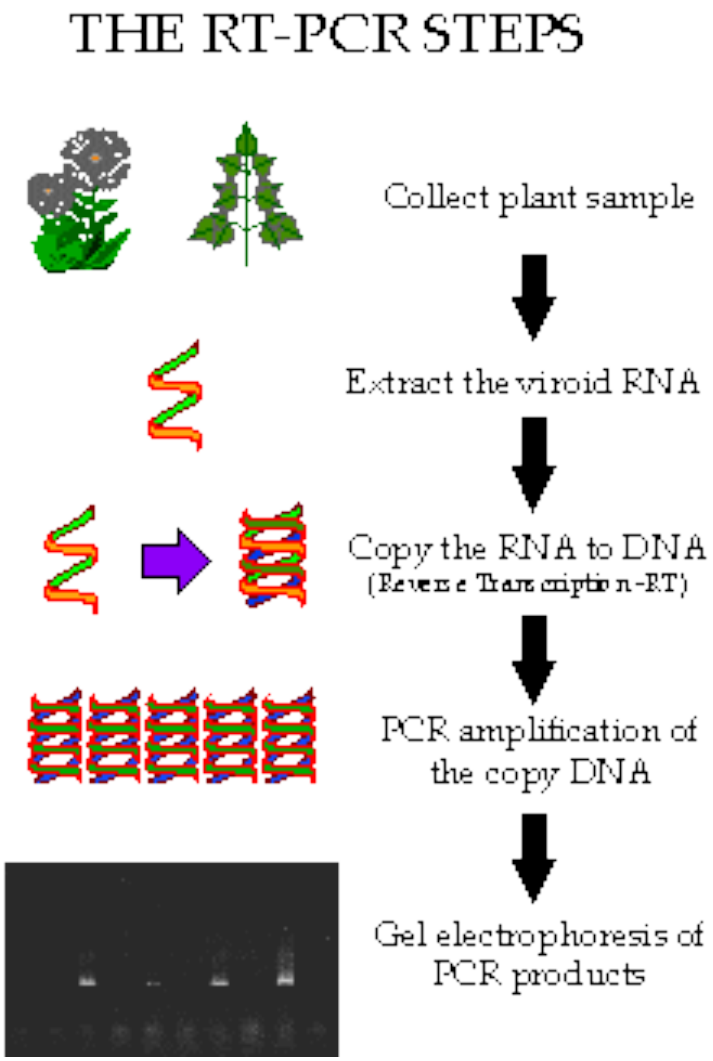
PCR Tools



Thermocycler

Oligo Synthesizer

Reverse Transcriptase PCR*

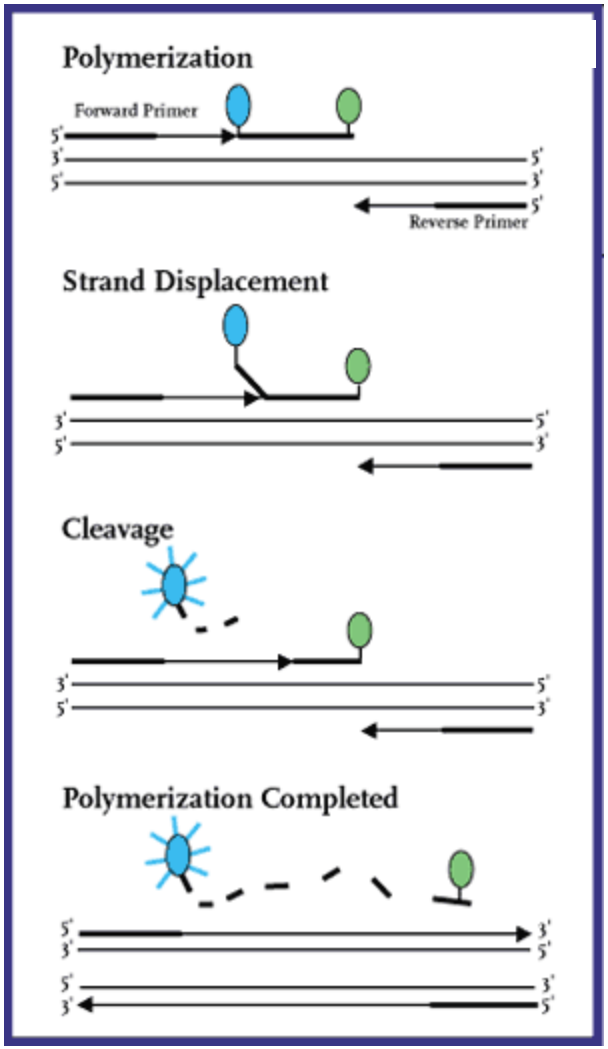


- Two kinds of "RT-PCR" - confusing
- One uses reverse transcriptase (RT) to help produce cDNA from mRNA
- Other uses real time (RT) methods to monitor PCR amplification

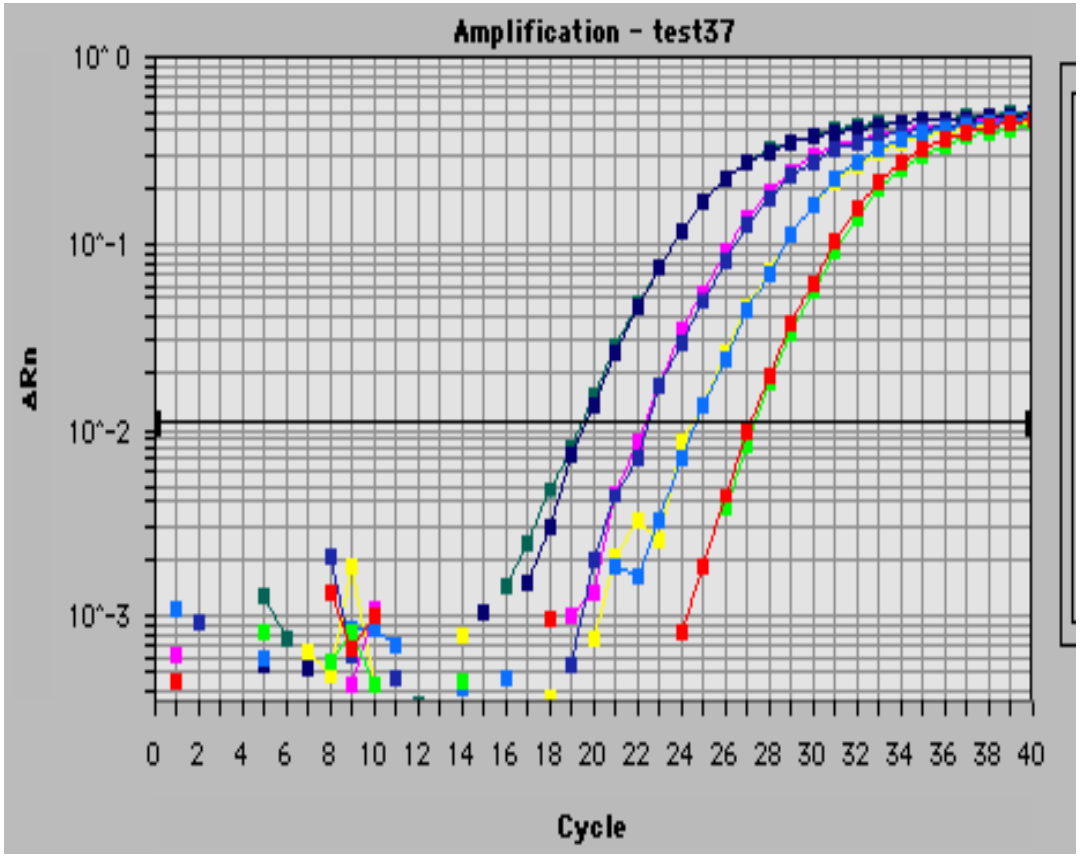
RT-PCR*

- RT (Real Time) PCR is a method to quantify mRNA and cDNA in real time
- A *quantitative PCR method*
- Measures the build up of fluorescence with each PCR cycle
- Generates quantitative fluorescence data at earliest phases of PCR cycle when replication fidelity is highest

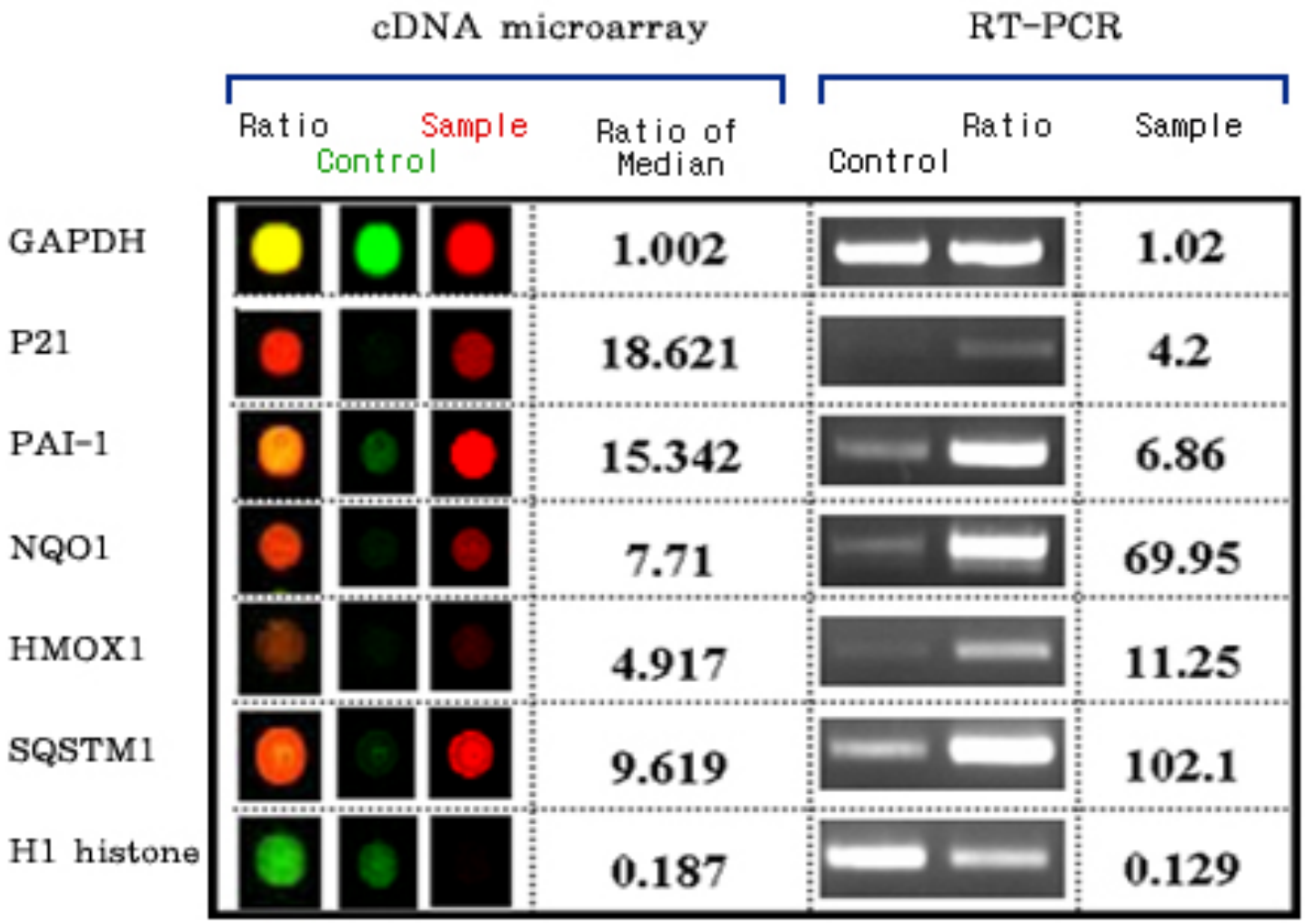
RT-PCR (Taqman)*



An oligo probe with 2 flurophores is used (a quencher & reporter)



RT-PCR vs. Microarray**



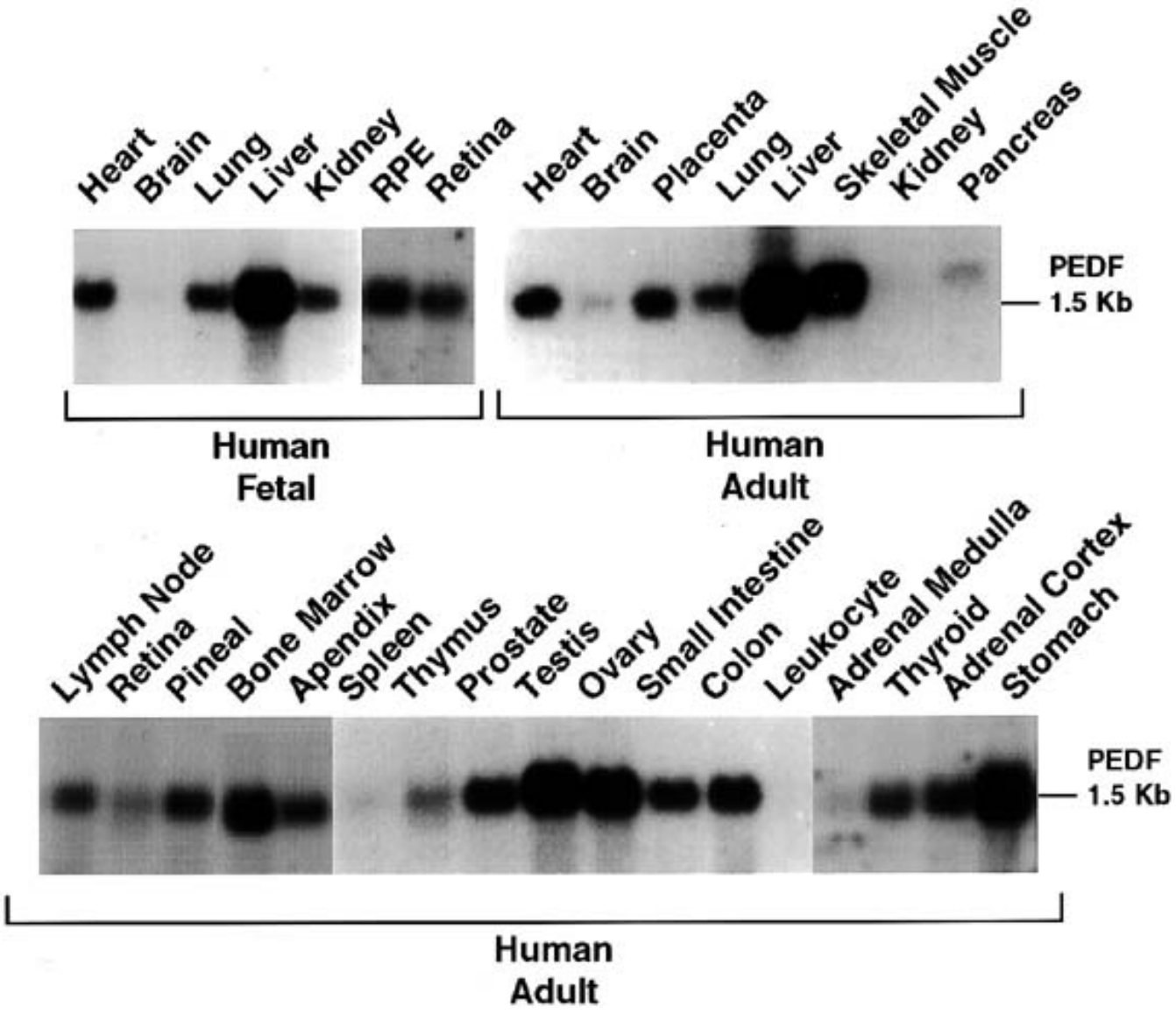
Advantages of RT-PCR*

- **Sensitive assay, highly quantitative, highly reproducible**
- **Considered "gold standard" for mRNA quantitation**
- **Can detect as few as 5 molecules**
- **Excellent dynamic range, linear over several orders of magnitude**

Disadvantages of RT-PCR*

- Expensive (instruments are >$150K, materials are also expensive)
- Not a high throughput system (10's to 100's of genes – not 1000's)
- Can pick up RNA carryover or contaminating RNA leading to false positives

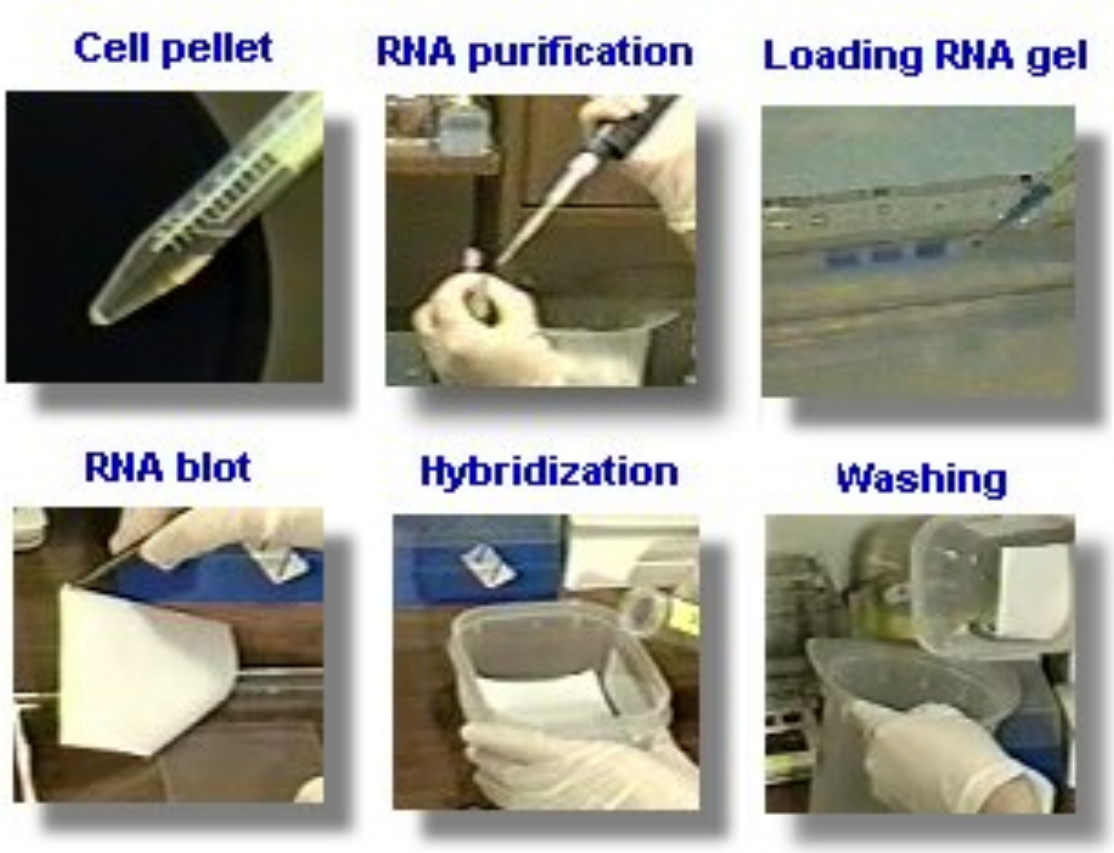
Northern Blots

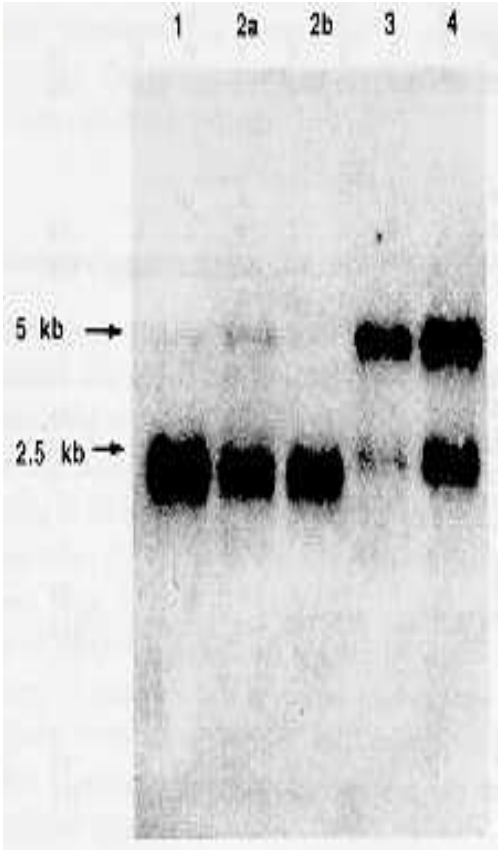


Northern Blots*

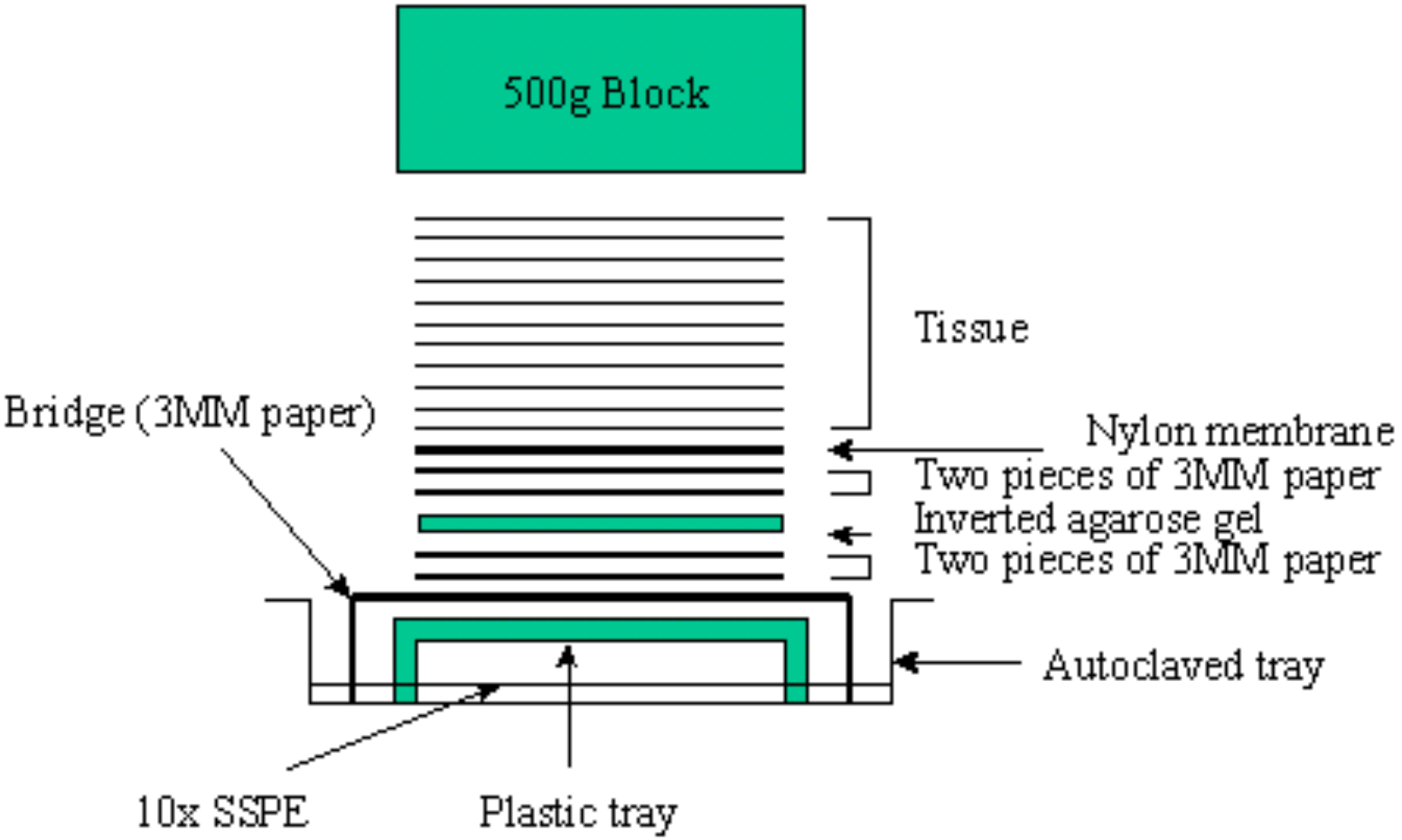
- Method of measuring RNA abundance
- Name makes "fun" of Southern blots (which measure DNA abundance)
- mRNA is first separated on an agarose gel, then transferred to a nitrocellulose filter, then denatured and finally hybridized with ^{32}P labelled complementary DNA
- Intensity of band indicates abundance

Northern Blotting*





The "Blot" Block



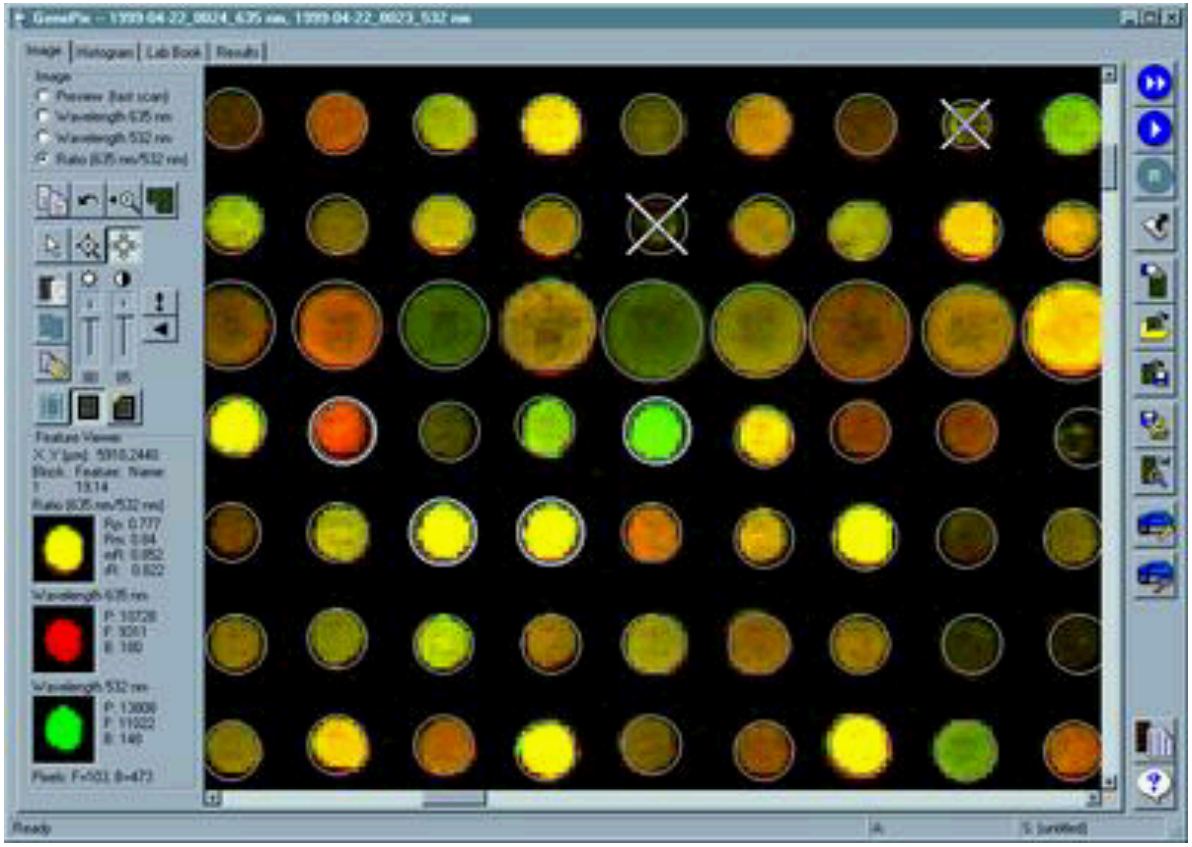
Advantages of Northerns*

- Inexpensive, quantitative method of measuring transcript abundance
- Well used and well understood technology
- Use of radioactive probes makes it very sensitive
- Near infinite dynamic range

Disadvantages of Northerns*

- Relies on radioactive labelling – "dirty" technology
- Quality control issues
- "Old fashioned" technology, now largely replaced by microarrays and other technologies

Microarrays



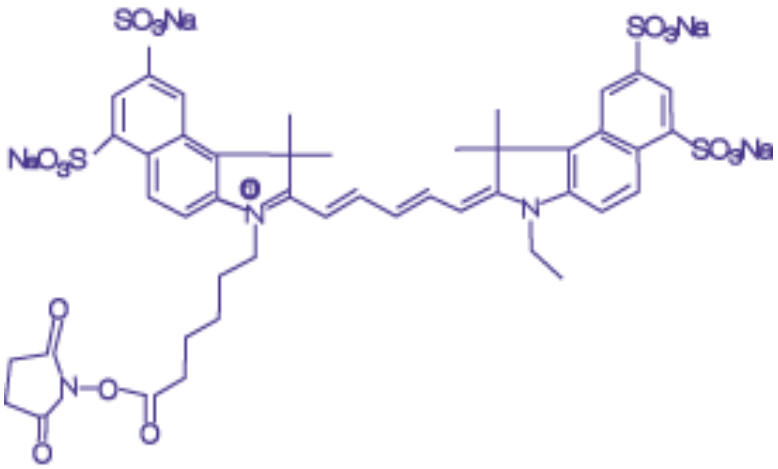
Microarrays*

- **Basic idea:**
  - **Reverse Northern blot on a huge scale**
- **The clever trick:**
  - **Miniaturize the technique, so that many assay can be carried out in parallel**
  - **Hybridize control and experimental samples simultaneously; use distinct fluorescent dyes to distinguish them**

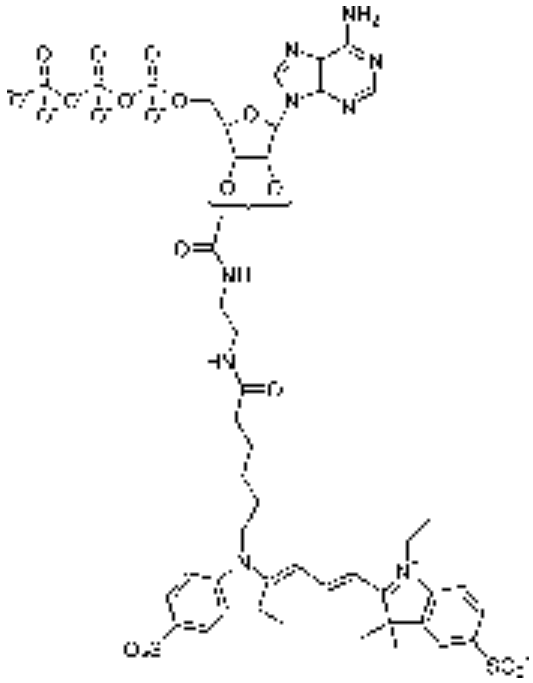
DNA Microarrays*

- Principle is to analyze gene (mRNA) or protein expression through large scale non-radioactive Northern (RNA) hybridization analysis
- Essentially high throughput Northern Blotting method that uses Cy3 and Cy5 fluorescence for detection
- Allows expressional analysis of up to 20,000 genes simultaneously

Cy3 and Cy5 Dyes

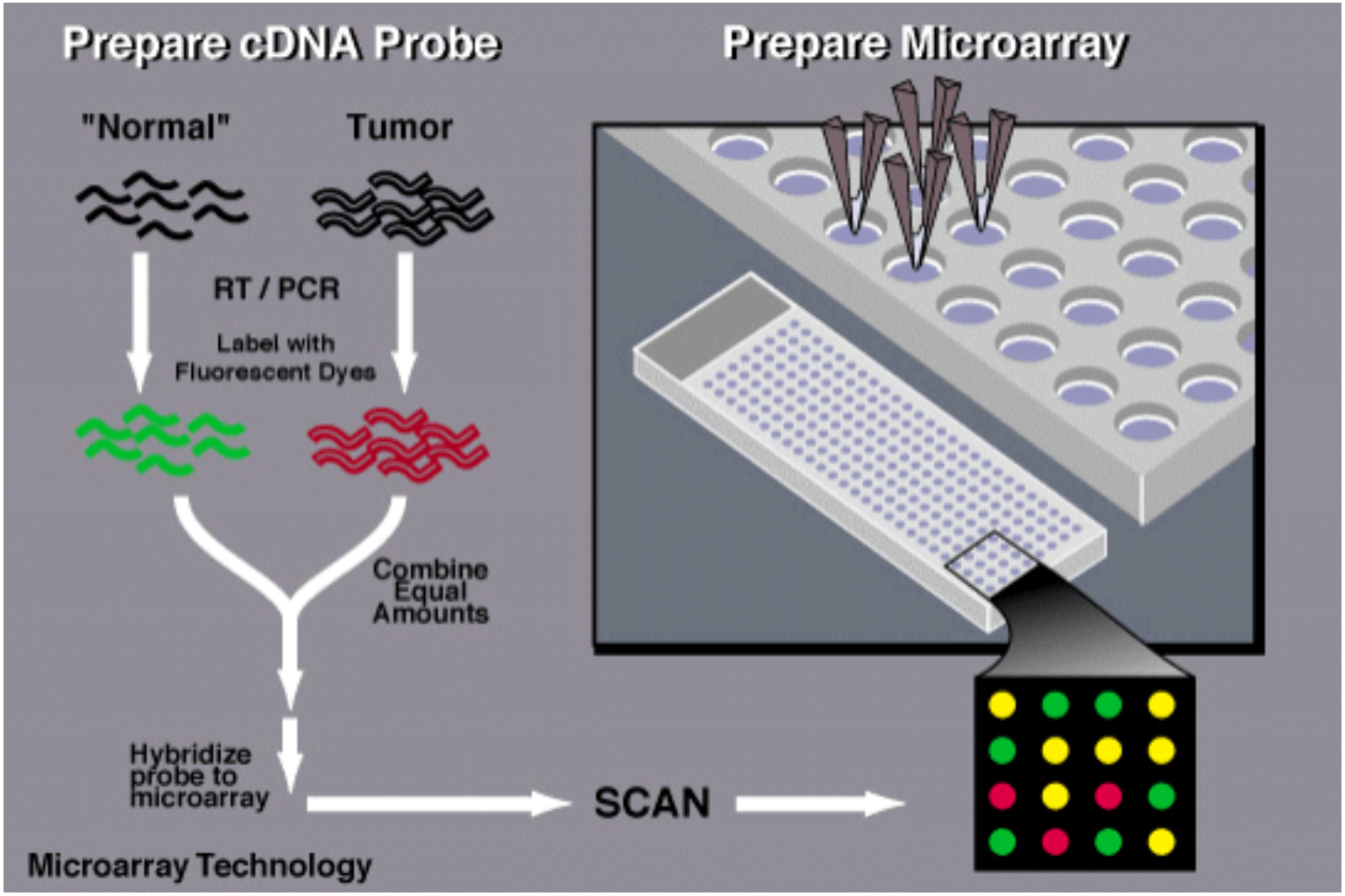


Cy5

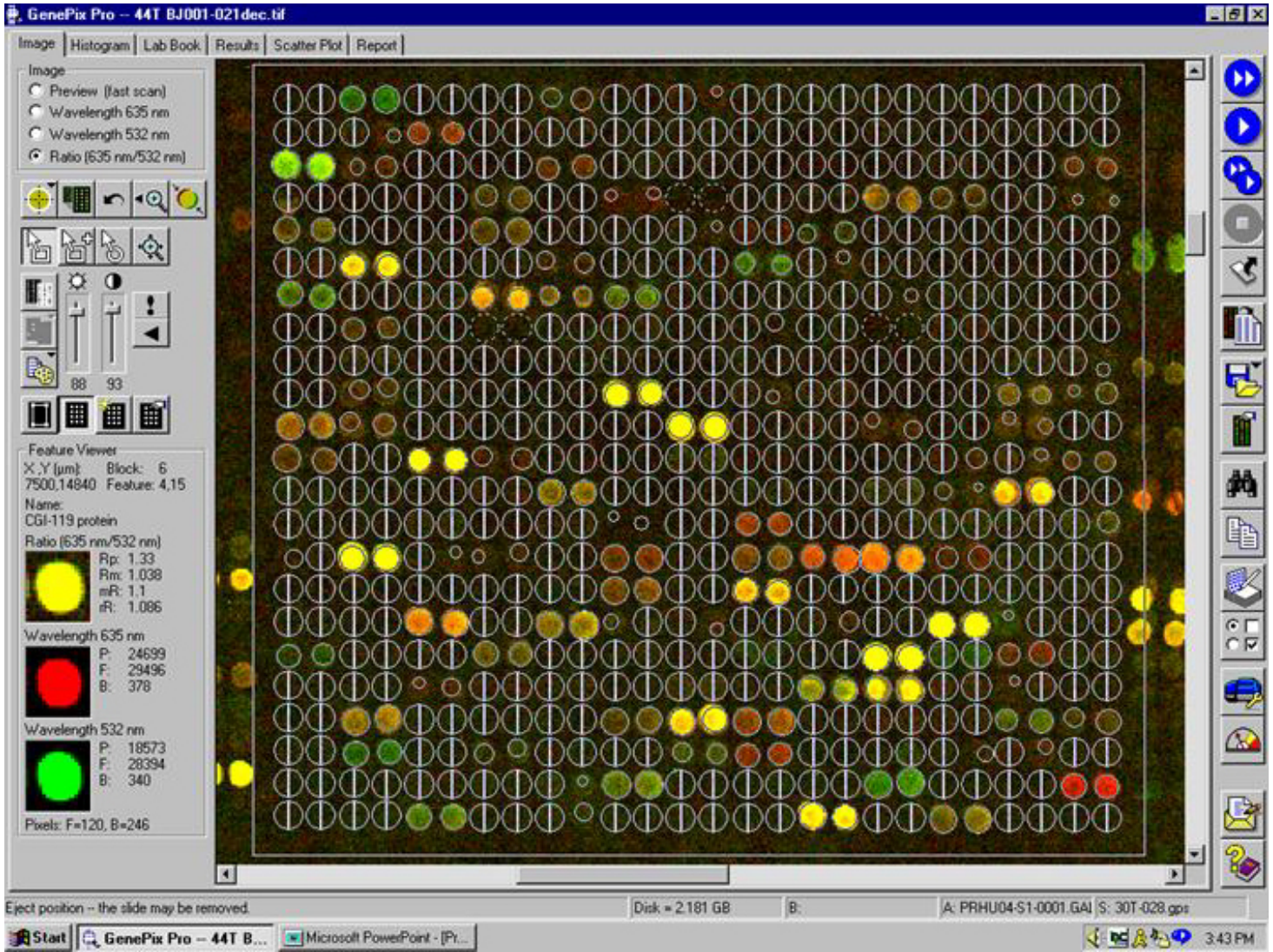


Cy3-ATP

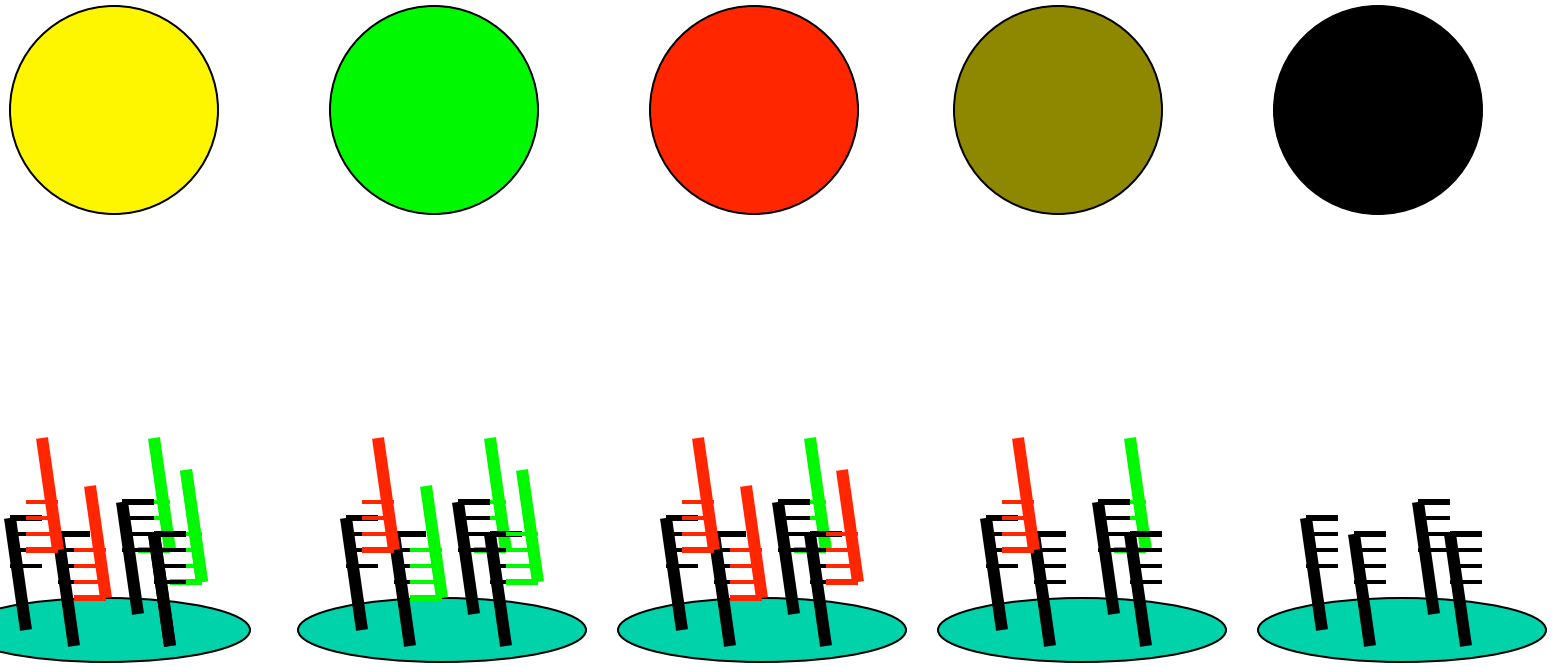
Principles of Microarrays**



Typical Microarray Data



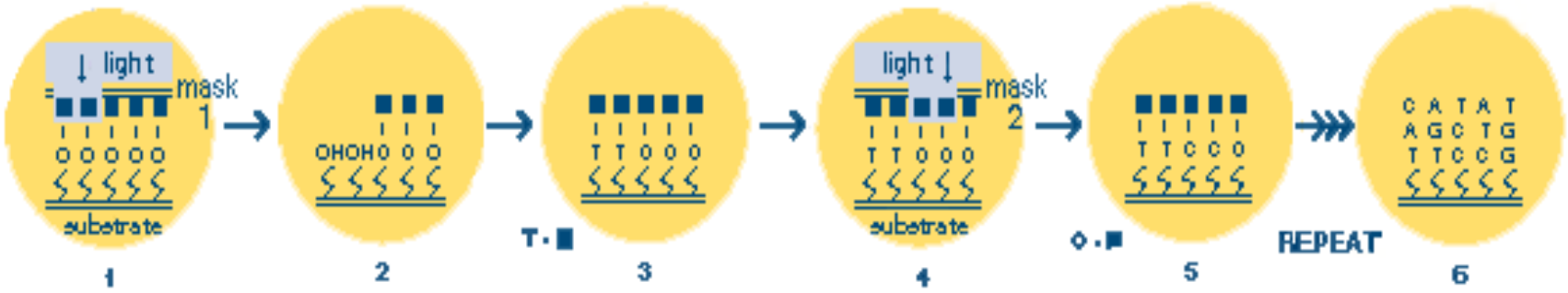
Microarrays & Spot Colour*

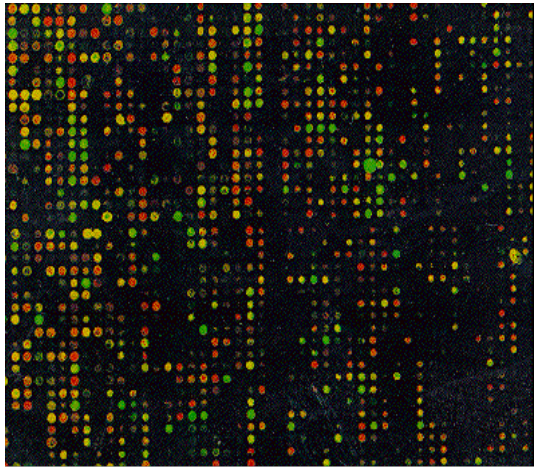


Four Types of Microarrays*

- **Photolithographically prepared short oligo (20-25 bp) arrays**
- **Spotted glass slide cDNA (500-1000 bp) arrays**
- **Spotted nylon cDNA (500-1000 bp) arrays**
- **Spotted glass slide oligo (70 bp) arrays**

Affymetrix GeneChips





Glass Slide Microarrays

Advantages to Microarrays*

- High throughput, quantitative method of measuring transcript abundance
- Avoids radioactivity (fluorescence)
- Kit systems and commercial suppliers make microarrays very easy to use
- Uses many “high-tech” techniques and devices – cutting edge
- Good dynamic range

Disadvantages to Microarrays*

- Relatively expensive (>$1000 per array for Affy chips, $300 per array for "home made" systems)
- Quality and quality-control is highly variable
- Quantity of data often overwhelms most users
- Analysis and interpretation is difficult

Conclusions

- **Multiple methods for measuring RNA or transcript abundance**
  - **Differential Display**
  - **Serial Analysis of Gene Expression (SAGE)**
  - **RNA-Seq**
  - **RT-PCR (real-time PCR)**
  - **Northern Blotting**
  - **DNA Microarrays or Gene Chips**

Conclusions

- Some methods are better or, at least, more reliable than others
- Agreement between mRNA levels and protein levels is generally very poor – calls into question the utility of these measurements
- All mRNA measurement methods require a “second opinion”