Welcome to our E-Seminar:

Using Lower Amounts of RNA for GE Microarray Experiments
Labeling RNA for Microarray Experiments
Outline

• Introduction
• Labeling, Amplification
  • Labeling and amplification techniques
  • Direct Labeling
  • Linear Amplification
• Kits selection for different Agilent microarrays
Important Issues for Gene Expression Labeling and Hybridization

- Sample limitation
- Correct representation of original mRNA population
- Simple, reproducible protocols
- Commercial kits
Many users are sample limited

Does the amount of cell/tissue/fluids sample that you can obtain limit the number or kinds of experiments you can conduct?
Agilent Market Research Study (4/02); N= 364
Two-Color Labeling and Competitive Hybridization onto Microarray

Probe Preparation
- Deposition cDNA
- Deposition: whole oligo or in situ oligo synthesis

Target Preparation
- Normal
- RNA isolation
- Deposition: whole oligo or in situ oligo synthesis
- Competitive hybridization
- Disease

Data Analysis

Chairperson: Rita Willis
Examples Labeling and Amplification Methods Used in Gene Expression

**Labeling**

- Single-step - direct incorporation of dyes into growing chain
- Two-step - incorporation of modified nucleotides followed by conjugation of dyes, e.g. aminoallyl or biotin/streptavidin method

**Amplification**

- Linear amplification – generates cRNA linearly, e.g. T7-based amplification
- Exponential amplification – generates samples exponentially, e.g. PCR-based amplification
Direct Labeling

- G2555A Fluorescent Direct Labeling kit
Agilent Fluorescent Direct Label Kit
(enzymatic, non-amplification method, Part Number G2557A, 20 reactions)

Total RNA or polyA+ RNA

Reverse transcription

Degradation of RNA Strand

RNase I “A”

Cleanup

Time: 3-4 hours

Non-Agilent Components Needed

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Agilent Fluorescent Direct Label Kit
Characteristics

- Generate fluorescent-labeled cDNA using MMLV RT
- Limit of detection 3 mRNA in $10^6$ cells using Agilent system
- Simple **direct label** procedure
  - No amplification - minimizes chance for amplification bias between different mRNAs
  - Fast - only 3-4 hour procedure
  - Uses minimal amount of Cy3, Cy5 dyes
  - Qualified for cDNA and oligonucleotide arrays
  - Recommend the use of 10 $\mu$g total RNA or 200 ng poly A+ RNA
  - Optimized target purification
  - Single column cleanup for both dyes (Cy3, Cy5)
Agilent Direct Label Kit

1) Using lower amounts of RNA
2) Incorporation efficiency
3) Using tiling array as a development tool
What is the effect of using less total RNA?

Hela-Cy3/Spleen-Cy5 differential expression data

Log ratio correlation between 20 µg and lower amounts of total RNA input
Differential Gene Expression Using Total RNA Input with Agilent Direct Label Kit

Count of Features

- Unchanged
- Over-expressed
- Under-expressed

Agilent Technologies

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What is the effect of using less poly A+ RNA?

Hela-Cy3/Spleen-Cy5 differential expression data

![Graph showing the correlation coefficient (R^2) as a function of PolyA+ RNA input (µg). The correlation coefficient decreases as the RNA input decreases.]
Differential Gene Expression Using PolyA+ RNA Input with Agilent Direct Label Kit

Feature Count

PolyA+ RNA (µg)

0.01 0.02 0.05 0.1 0.2 0.4 1

0 2000 4000 6000 8000 10000 12000

Unchanged
OverExpressed
UnderExpressed
Agilent Direct Label Kit

1) Using lower amounts of RNA
2) Incorporation efficiency
3) Using tiling array as a development tool
Cy3 and Cy5 Incorporation with Similar Efficiencies: Hybridization Data with Total RNA Input

Fluorescent Signal Distribution in a Hela Self/Self Hybridization
(input RNA: Total RNA)

Number of features in Bin

Fluorescent Signal Bin

Cy3 Signal

Cy5 Signal
Cy3 and Cy5 Incorporation with Similar Efficiencies: Hybridization Data Using Cy3 and Cy5 with poly + RNA Input

Fluorescent Signal Distribution in a Hela Self/Self Hybridization
input RNA: polyA+

- **Cy3 Signal**
- **Cy5 Signal**
Agilent Direct Label Kit

1) Using lower amounts of RNA
2) Incorporation efficiency
3) Using tiling array as a development tool
Characterizing Labeling Reactions Using Tiling Microarrays – Experimental Design

5’ to 3’ gene sequence
Similar Relative Signal Intensity for Cy3 and Cy5 Using MMLV RT Synthase: Results for Three Genes

LogRatio vs start

Spiked in synth transcript (red)
- HPVE7 (green)
- ADP/ATP carrier protein (yellow)
- TRAF4 (blue)

5’ to 3’ sequence
BREAK NUMBER 1

For questions, at break please dial 1 on your phone or type onto the chat screen at any time during the presentation.
Linear Amplification and Labeling

- G2554A Fluorescent
  Linear Amplification Kit

Agilent Technologies
Description: Fluorescent Linear Amp Kit G2554A*

**Total RNA or polyA+ RNA**

- mRNA
  - Add TTTTT-T7 Promoter 5’, Reverse transcriptase (MMLV-RT),

**Reverse transcription**

- mRNA
  - 1st strand cDNA
  - mRNA/DNA
    - 1st strand cDNA
    - TTTTT T7 Promoter 5’,

**cRNA synthesis and fluorescent labeling**

- cRNA
  - T7 RNA Polymerase
  - UUUUU 5’

**Final Product**

- cRNA
  - UUUUU 5’
  - Typically 200-500x amplification

Time: 5-8 hours

*US Patent Number US6132997 A1
Benefits: Agilent Fluorescent Linear Amp Kit (G2554A)

- Single tube
- Single purification step
- Reduced time
Question: Are mRNA Ratios Maintained After Amplification?

mRNA Pool
(400 ng mRNA)
(1000s of mRNAs, all at different levels)

T7 amplification
300-fold

Amplified cRNA
Is every mRNA amplified 300-fold?
Experiment:

Spike in 3 different plant mRNAs at different concentrations into mammalian mRNA pool

Agilent Linear Amplification Kit

Measure the resulting concentrations of the plant mRNAs, using radiolabeled hybridization with plant mRNA probes, and compare with total RNA amplification levels
Spiked Transcript in mRNA Pool is Amplified to Same Extent as Total mRNA Pool and Shows Linearity

\[ y = 241.67 \times x^{1.0945} \quad R = 0.99931 \]

242x Amplification
Experimental Result Using Agilent Linear Amplification Kit

• 250-fold amplification of total mRNA in sample

• 3 different spiked in transcripts were all amplified ~250x, confirming that amplification is sequence independent

• Transcripts spiked in at different concentrations were amplified to the same extent as the total mRNA population showing that amplification is linear
Consistent Dye Incorporation With the Agilent Fluorescent Linear Amp Kit

Consistent Dye incorporation by Fluorescent Linear Amplification kit

![Graph showing consistent dye incorporation with Agilent Fluorescent Linear Amp Kit. The x-axis represents Total RNA input (ng), and the y-axis represents pmol Cy3/μg RNA. Two lines represent HeLa and Spleen samples. The graph shows a general trend of consistent dye incorporation across different RNA input levels.](image-url)
Log Ratio Correlation Coefficient Between 5000 ng and Lower Amounts of total RNA Inputs

Log ratio correlation coefficient between 5000 ng and lower amounts of total RNA inputs

R²

Total RNA input (ng)
BREAK NUMBER 2

For questions, at break please dial 1 on your phone or type onto the chat screen at any time during the presentation.
Agilent Low RNA Input Linear Amplification Kit
(Part Number 5184-3523)

Total RNA or mRNA

MMLV-RT

ds cDNA

Kit Protocol 1
T7 RNA polymerase, Cy3- or Cy5- CTP and NTPs

T7 RNA polymerase and NTPs

fl-cRNA

cRNA

Target for use with \textit{in situ} oligo array

Kit Protocol 2
MMLV RT, Cy3- or Cy5- dCTP and dNTPs

fl-cDNA (label sense strand)

Target for use with deposition cDNA
Characteristics:
Low Input RNA Linear Amplification Kit (5184-3523)

• For *in situ* (oligonucleotide) and deposition (cDNA and oligonucleotide) arrays
• Requires 50 ng or less Total RNA
• Uses only one amplification step
• Makes either cRNA or cDNA
• Simple procedure – amenable to automation
• User friendly protocol (no cleanup after major steps)
Generating micrograms of labeled cRNA from nanograms of total RNA after a one-round of amplification.

A

B
Correlation coefficient ($R^2$) comparison of log ratios using a range of total RNA quantities (50ng to 5000ng)

A. 50ng vs 500ng, $R^2=0.98$
B. 50ng vs 5000ng, $R^2=0.95$
C. 500ng vs 5000ng, $R^2=0.97$
Log Ratio Comparison Result

![Graph showing the correlation coefficient (log ratios) for different total RNA inputs. The x-axis represents total RNA input (5000ng vs 1ng, 5000ng vs 10ng, 5000ng vs 25ng, etc.), and the y-axis represents the correlation coefficient (log ratios) ranging from 0.2 to 1.1. The graph shows an increasing trend as the total RNA input increases.]
Differential Gene Expression

- **Gene Number**
  - 1 ng
  - 50 ng
  - 500 ng
  - 2000 ng

- **Total RNA Input**
  - 1 ng: 1,000 units
  - 50 ng: 2,000 units
  - 500 ng: 4,000 units
  - 2,000 ng: 8,000 units

- **Categories**
  - unchanged
  - Down-regulated
  - Up-regulated
Correlation of signature genes using 50 ng or less total RNA

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<th>Input Total RNA</th>
<th>$R^2$ (all signature genes)</th>
<th>Anticorrelated genes</th>
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<tr>
<td>50 ng vs 1 ng</td>
<td>0.59</td>
<td>297 (1.7%)</td>
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<tr>
<td>50 ng vs 10 ng</td>
<td>0.62</td>
<td>349 (1.9%)</td>
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<td>50 ng vs 25 ng</td>
<td>0.94</td>
<td>24 (0.1%)</td>
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<tr>
<td>50 ng vs 50 ng</td>
<td>0.97</td>
<td>1 (0.006%)</td>
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Minimum dye-bias of Low RNA Linear Amp Kit
(~50 ng total RNA, dye-swap experiment)

>97% of Genes Correlated Demonstrating Consistency of the Procedure
(<1% anticorrelated)
Performance of Amplification of Low Input Total RNA Kit: Log Ratio of Spike-in's cDNA/cRNA

![Bar graph showing the average log ratio across 4 arrays for different spike-ins generated from different amounts of input. The x-axis represents the types of spike-ins generated from different amounts of input, and the y-axis represents the average log ratio across 4 arrays. The bars are color-coded to indicate the expected log ratio of 1 to 5 and the input ratios for cDNA and cRNA.]

- Expected Log Ratio of 1 to 5
- cDNA: Input Ratio of 500 fg to 2500 fg
- cDNA: Input Ratio of 50 fg to 250 fg
- cDNA: Input Ratio of 20 fg to 100 fg
- cRNA: Input Ratio of 500 fg to 2500 fg
- cRNA: Input Ratio of 50 fg to 250 fg
- cRNA: Input Ratio of 20 fg to 100 fg
A

HeLa poly (A)+ RNA Input

B

Spleen poly (A)+ RNA Input

Amplified Cyanine-3 cRNA (µg)

Amplified Cyanine-5 cRNA (µg)
Log Ratio Comparison Result

Correlation Coefficient (Log Ratios)

Poly (A)+ RNA Input

200ng vs 0.1ng
200ng vs 1ng
200ng vs 10ng
200ng vs 50ng
200ng vs 200ng
Differential Gene Expression Analysis

![Graph showing gene expression levels with different input amounts of Poly (A)+ RNA. The x-axis represents different input levels: 0.1 ng, 1 ng, 10 ng, 50 ng, and 200 ng. The y-axis represents gene number, ranging from 0 to 10,000. The graph uses color coding: blue for unchanged, green for down-regulated, and red for up-regulated genes.]

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Slide 45

Chairperson: Rita Willis

- 2000 ng Total RNA
- 50 ng Poly (A)+ RNA

Correlation Coefficients:
- Visible: Non-Weighted = 0.914, Weighted = 0.823
- Selected: N/A, N/A
- Common Signature: Non-Weighted = 0.938, Weighted = 0.851
- All Signature: Non-Weighted = 0.918, Weighted = 0.825
- All (no failure/controls): Non-Weighted = 0.914, Weighted = 0.823

Legend:
- Unchanged (4,549/4,549)
- Query Signature (9,080/9,080)
- Target Signature (10,768/10,768)
- Common Signature (8,013/8,013)
- Anticorrelated (371/371)
- Selection (0/0)
# Agilent Labeling/Microarray/Hybridization Kits

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*For updated information please also check [http://www.chem.agilent.com/](http://www.chem.agilent.com/)

Chairperson: Rita Willis
Summary

- All labeling/amp, hybridization kits verified for use with Agilent microarrays
  - **Direct Label Kit**
    - Less RNA (10 µg total RNA, 200 ng poly A+ RNA)
    - Poly A and Total RNA protocols
    - Tiling arrays show uniform labeling
    - Uses significantly less dye
  - **Linear Amp Kit**
    - New protocol uses less RNA (5 µg total RNA, 200 ng poly A+ RNA)
    - Poly A and Total RNA protocols
    - Single tube protocol
  - **Hybridization Kits**
    - additional components for easy ordering
    - Combined with Agilent scanner, provides excellent S/N
  - **New Low RNA Linear Amp Kit**
    - uses only 50 ng total RNA
Acknowledgements

R&D
• Diane Ilsley
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