MARG Research Group Study:
Evaluation of Small Sample Nucleic Acid Amplification Technologies for Gene Expression Profiling

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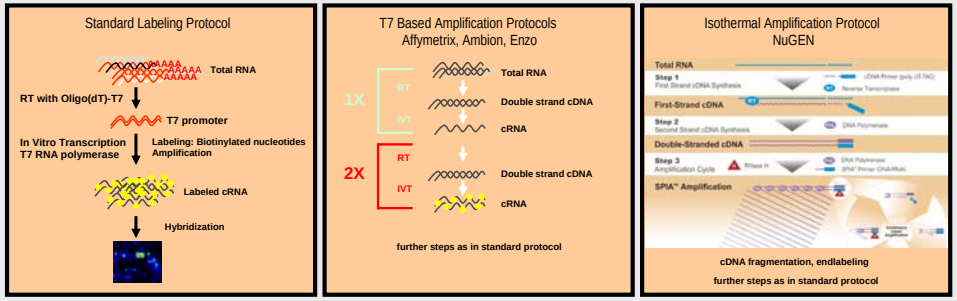
Microarrays have a significant impact on many areas of biology. However, there are still many areas of research that can not benefit from microarray technology due to a limiting amount of biological material that can be obtained (e.g. samples obtained by small biopsy, fine needle aspiration, and Laser Capture Microdissection). In response to this demand, a number of technologies have been developed which boast the capability of generating microarray targets from very small amounts of RNA (<10ng). To evaluate the relative merits of each of these technologies for the microarray community, the ABRF-MARG has completed a study which evaluates commercial small sample nucleic acid amplification protocols when compared to a single round T7 based amplification approach using the Affymetrix GeneChip platform. The results of this experiment are described herein. In addition to data quality and comparison of the small sample protocols , the results are validated by quantitative PCR in addition to the evaluation of the technical merits of each procedure.

Experimental Design

Four amplification chemistries have been tested:
- Affymetrix Two Cycle Labeling
- Ambion MessageAmp aRNA Amplification
- Enzo BioArray High Yield RNA Transcript Labeling
- NuGen Ribo-SPIA RNA amplification

- 20 ng total RNA starting material, 2 RNA samples have been processed in triplicates
- Microarray platform – Affymetrix U133A 2.0
- Probe level analysis (RMA) prior to measuring differential expression
- Results of differential expression were compared to results from 1x T7 amplification and quantitative PCR

The Principles of Amplification Chemistries



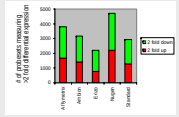
Questions

- Reproducibility:
  How similar are the results of replicates within one chemistry?
- Sensitivity:
  How many transcripts are detected as over two fold differentially expressed?
- Correlation to 1x T7 Amplification:
  How do amplification results comparable to one round of T7 amplification
- Accuracy:
  How far do measurements of differential expression represent true quantitative differences between samples?
- Biological Interpretation:
  Can the same conclusions be reached irrespective of amplification technology?

Reproducibility & Sensitivity

Reproducibility: Pairwise linear relationship between replicates
- Standard protocol $r^2 > 0.99$
- Affymetrix $r^2 > 0.99$
- Ambion $r^2 > 0.99$
- Enzo $r^2 > 0.99$
- NuGEN $r^2 > 0.99$

Sensitivity: Number of probesets detecting >2 fold differential expression
- Standard protocol: 2918
- Affymetrix: 3792
- Ambion: 3157
- Enzo: 2211
- NuGEN: 4718



Correlation to Standard Protocol

Pairwise linear relationship between differential expression measured by 1x T7 vs. four amplification chemistries
- Affymetrix $r^2 > 0.96$
- Ambion $r^2 > 0.89$
- Enzo $r^2 > 0.94$
- NuGEN $r^2 > 0.84$*

*NuGEN is a cDNA hybridization and not a cRNA hybridization

Qualitative Conflicts: Up (down) according to 1x T7 and down (up) according to amplification results

	>2 fold in the other direction
- Affymetrix:	1
- Ambion:	3
- Enzo:	1
- NuGEN:	90

Total number of differences out of over 22,000 probe sets

Correlation to Quantitative PCR

9 transcripts were validated, where either T7 based chemistries or isothermal amplification measured differential expression on at least one probeset while the other technology measured either no expression, no differential expression or (minimal) differential expression in the other direction.

Differential expression measured by T7 based chemistries		Differential expression measured by isothermal amplification only
ApoE	Not all 2x T7 amplification technologies identified these genes as differentially expressed	HOXD3
CDKN1c		Trim10
NUP210		CLTC
SRRM2		TRPC3
		RBP4

In all cases, differential expression was confirmed by quantitative PCR. If one chemistry detected strong differential expression, quantitative PCR measured differential expression in the same direction.

Practical Aspects

	Labeling time	Costs per sample (list price)	cRNA yield	Present Calls
- Affymetrix	2.5 days	$ 153	149 µg	60 %
- Ambion	3 days	$ 143	102 µg	54 %
- Enzo	2.5 days	$ 231	80 µg	49 %
- NuGEN	1.5 days	$ 194	5 µg*	65 %
			* cDNA	

Summary

All amplification chemistries show excellent reproducibility, but there are significant differences in sensitivity. Multiple round T7 based amplification chemistries show better correlation to the single round T7 based protocol making comparative analysis more straightforward. Isothermal amplification showed the highest sensitivity for measurement of differential expression. Currently, no evidence for false positive measurements by any of the chemistries could be clearly observed. Cursory functional analysis demonstrates that biological conclusions are similar with some technologies providing more “detailed” pathway information.