RampUP RNA Amplification

Data Set

October 2006

RampUP RNA Amplification

ROUND 1

First strand cDNA synthesis
80 minutes

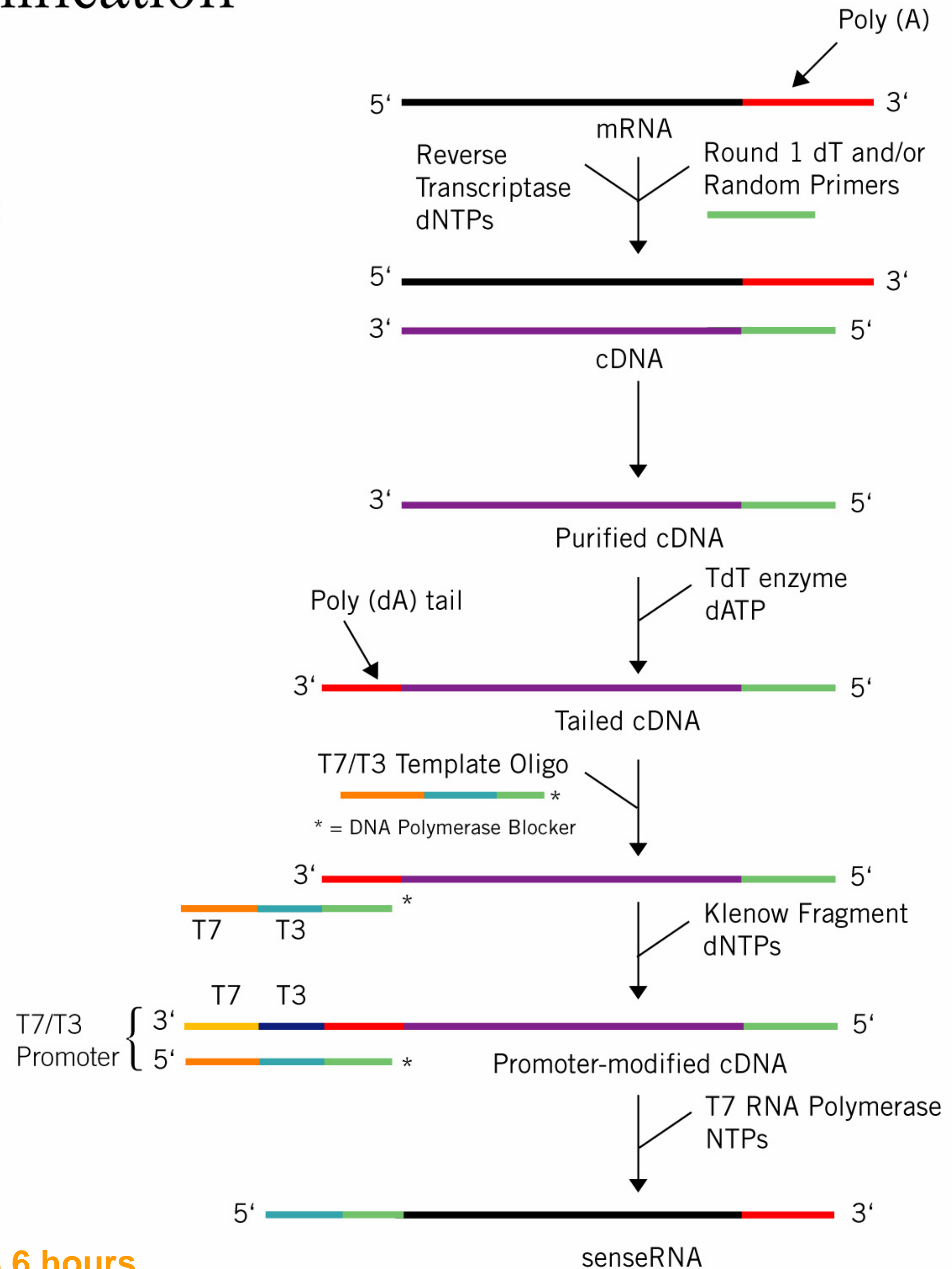
Purification of cDNA
40 minutes

Tailing of cDNA
30 minutes

Annealing of T7/T3 Template Oligo
15 minutes

T7/T3 Promoter Synthesis
50 minutes

T7 In Vitro Transcription
Overnight



Total Time to Round 1 IVT: ~3.6 hours

ROUND 2

Reverse Transcription

90 minutes

RNase H degradation of senseRNA

60 minutes

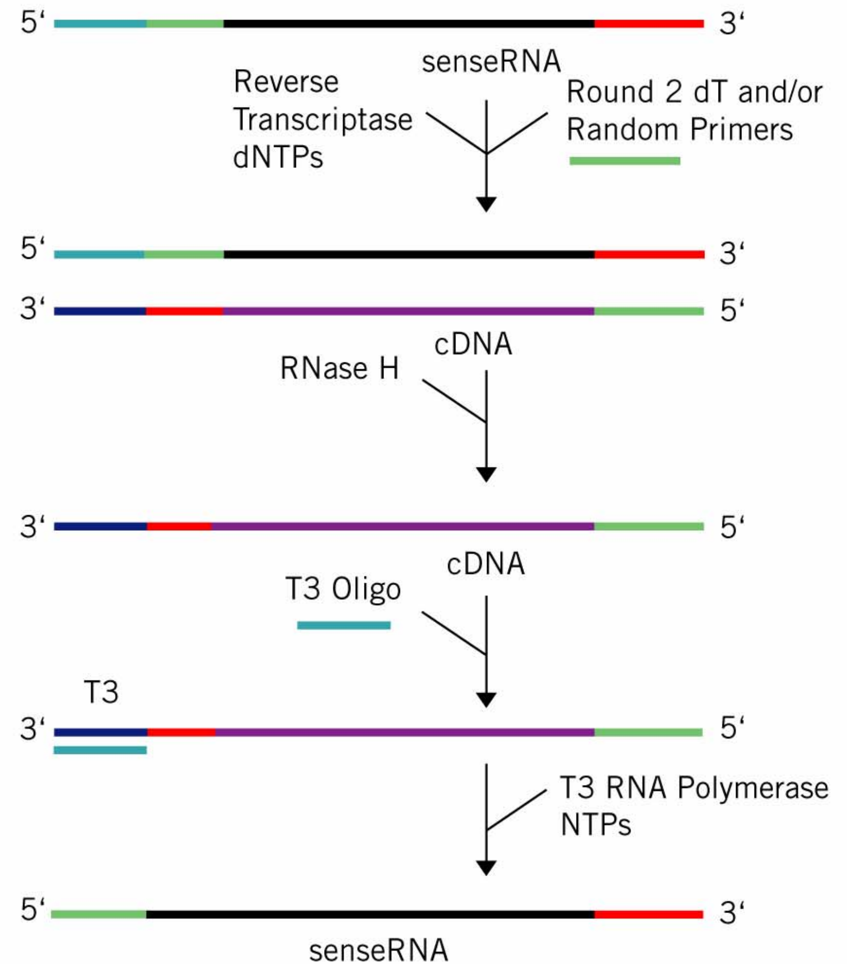
Annealing of T3 Oligo

10 minutes

T3 In Vitro Transcription

Overnight

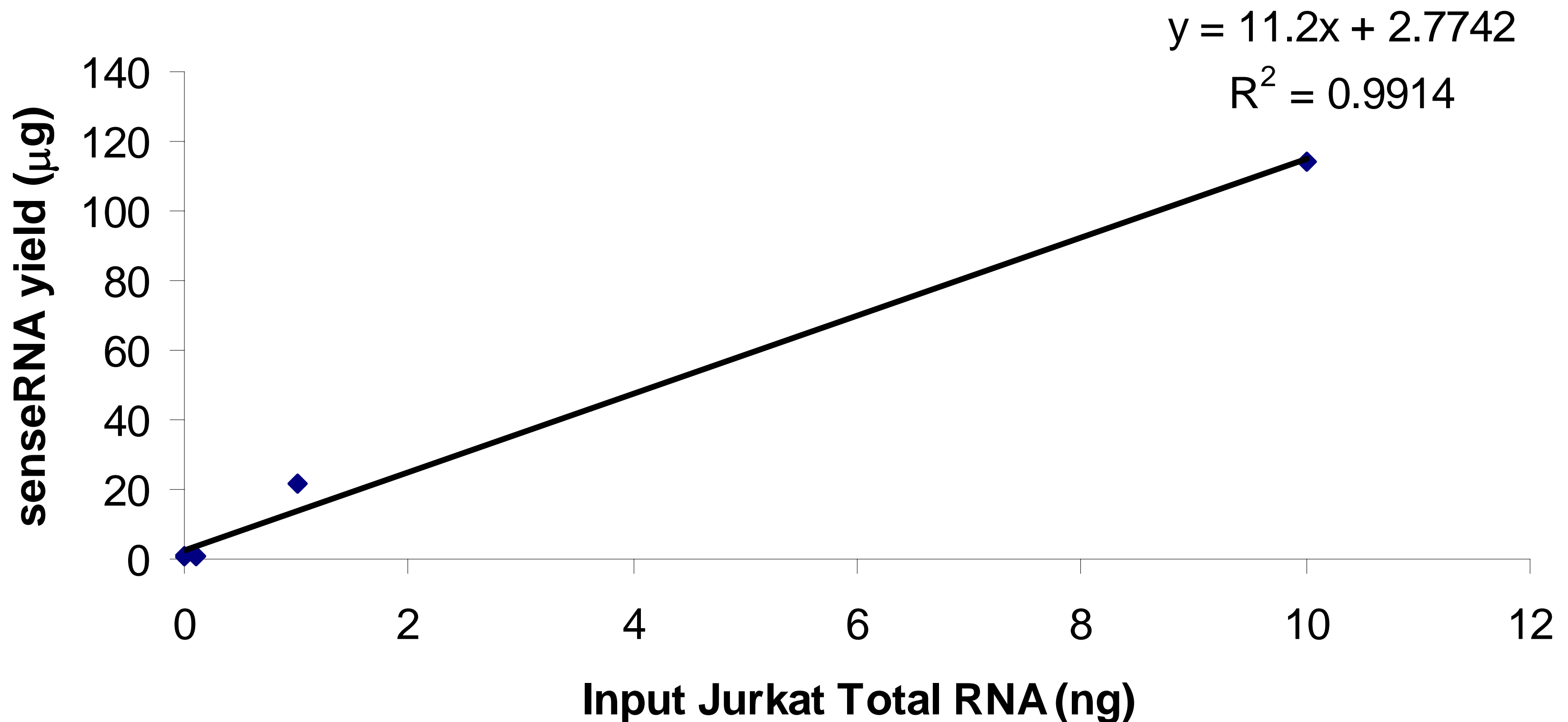
Total Time to Round 2 IVT: ~2.7 hours



Linearity of RampUP Amplification

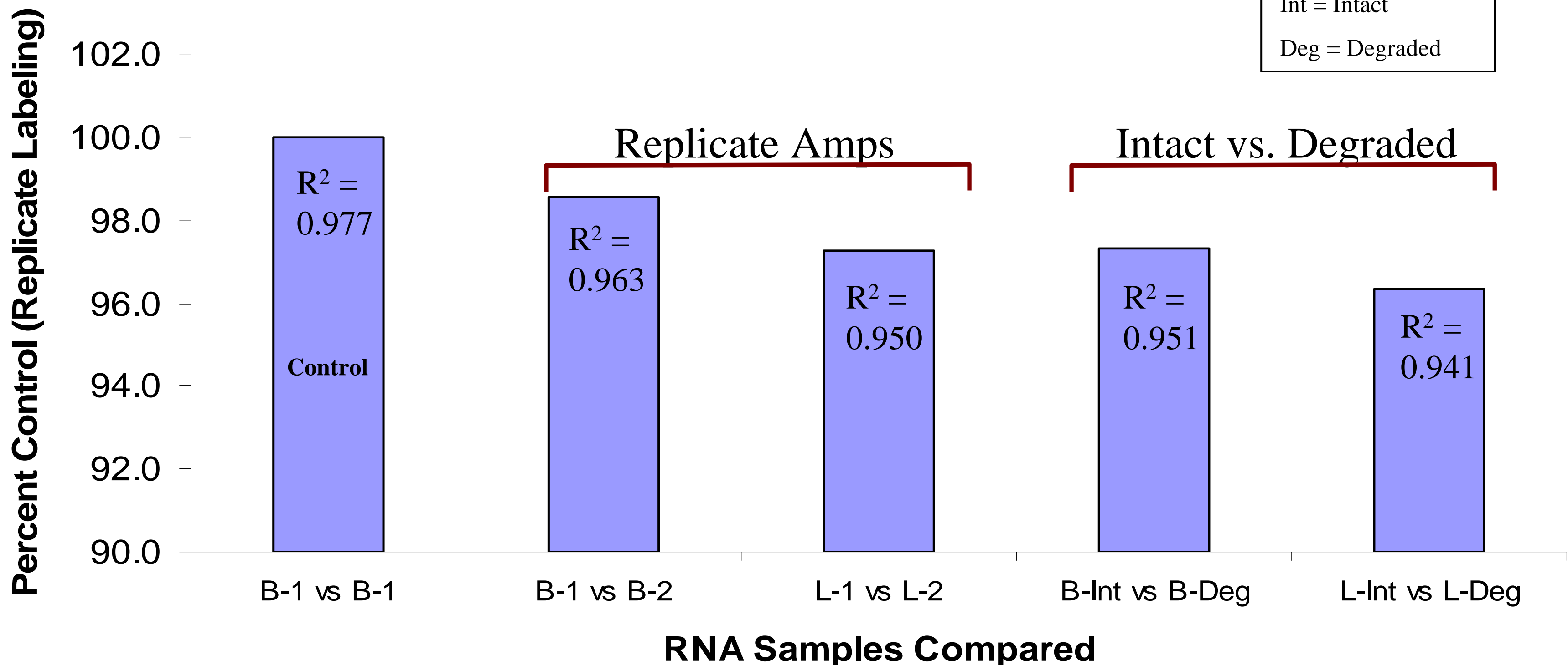
A total RNA input titration experiment was performed to test the linearity of RampUP amplification. Jurkat (Human Cell Line T-Cell Leukemia) Total RNA (Ambion cat. no. 7858) was titrated and the senseRNA yields obtained were determined by OD260 as reported below.

Jurkat Total RNA Input (ng)	senseRNA yield (μg)
10	114
1	22
0.1	1
0.01	0.7
0	0.6



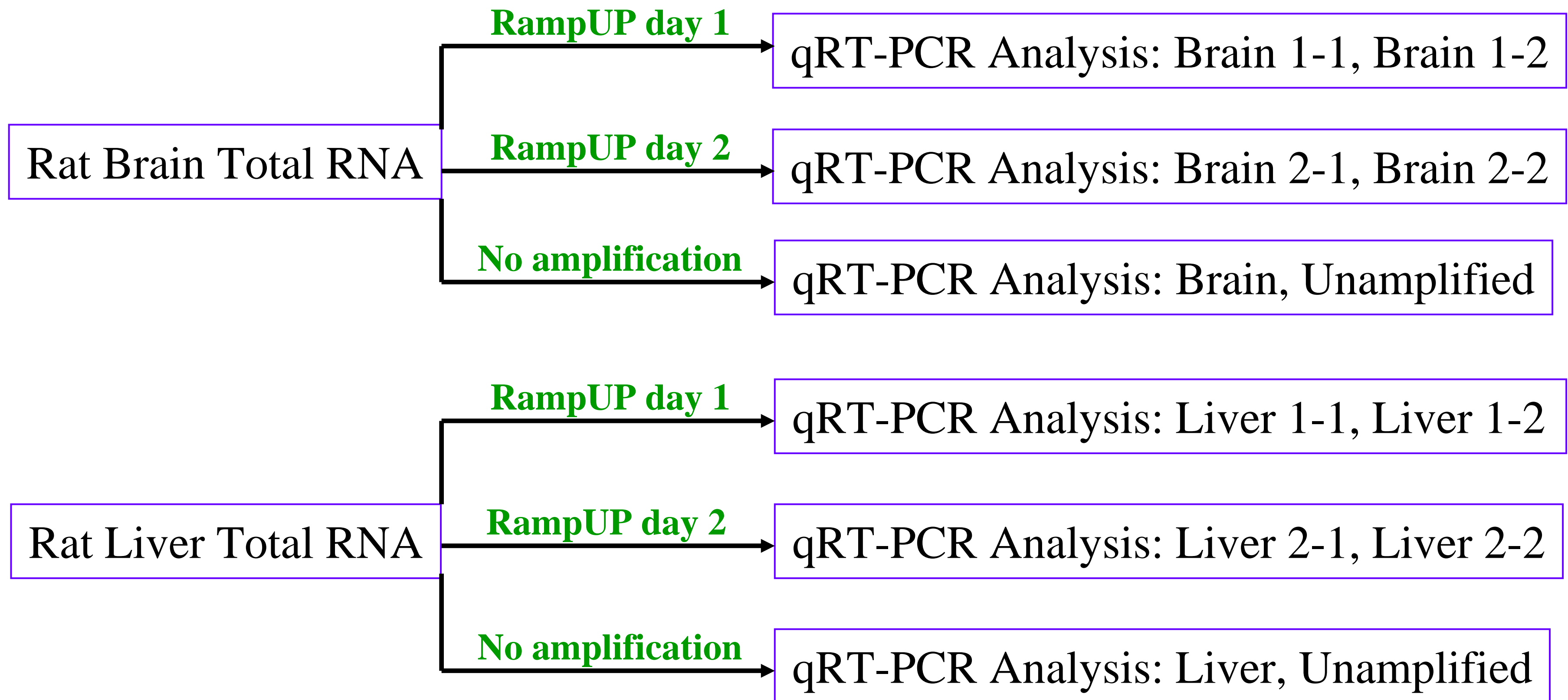
Reproducibility of RampUP

Experiment 1: Glass microarray validation: To determine the reproducibility of RampUP amplification, replicate analysis experiments were performed. Several intact and chemically degraded total RNA samples (5ng each) were amplified with RampUP, labeled by direct incorporation, and hybridized to 38.5K mouse oligo arrays (Microarrays Inc.). A replicate labeling array was set up as a control. Replicate amplifications of mouse brain and mouse liver resulted in 98.6% and 97.2% correlation values as compared to the control, respectively. SenseRNA generated from intact mouse brain was compared to partially degraded mouse brain senseRNA, resulting in a correlation value that was 97.3% of the control. Likewise, senseRNAs prepared from intact and degraded mouse liver RNA resulted in a correlation value that was 96.3% of the control.



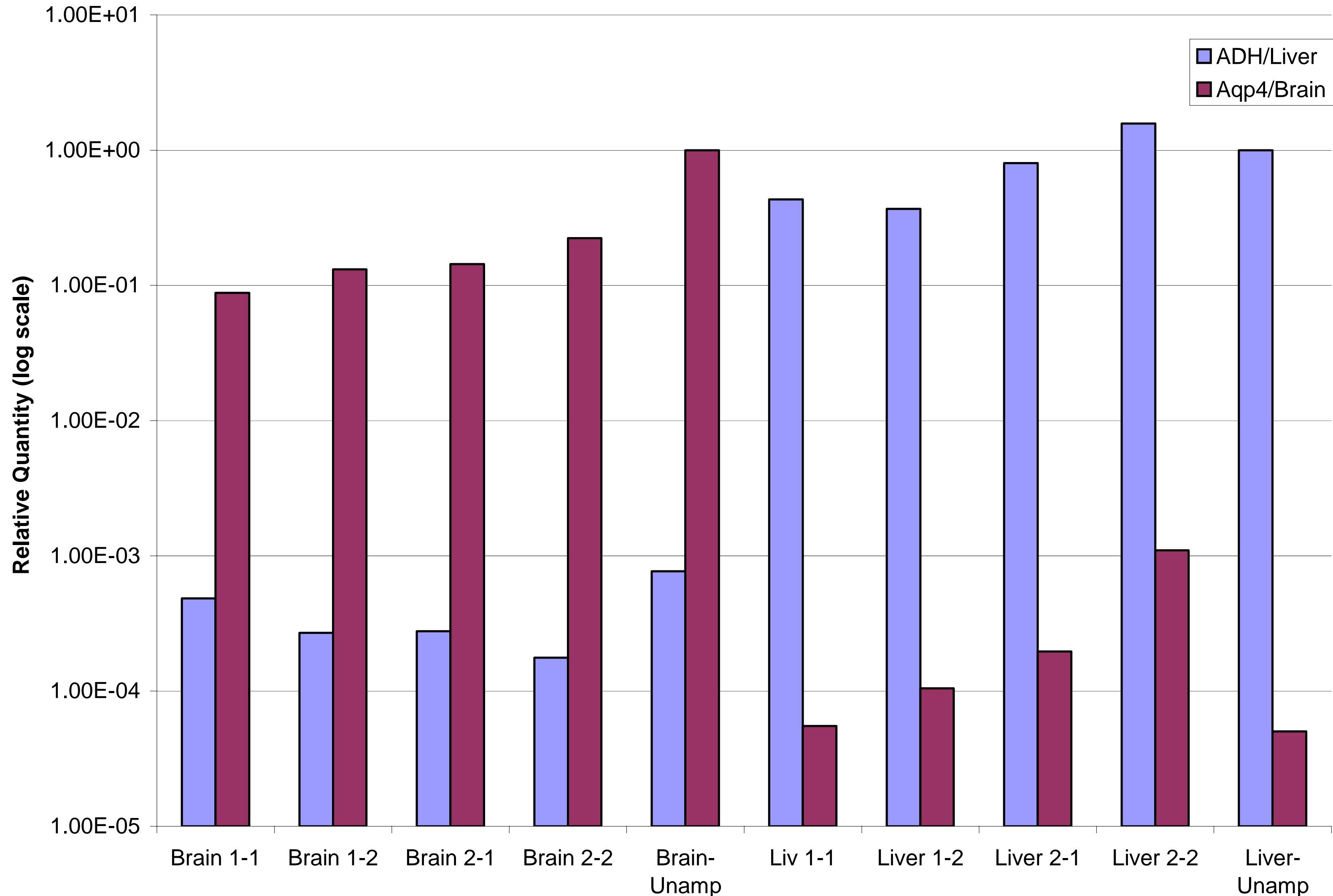
Reproducibility of RampUP

Experiment 2, Design: qRT-PCR validation: To study the reproducibility of RampUP, replicate amplification experiments were completed, one on day 1 and one on day 2. In each experiment replicate amplification reactions were prepared starting from rat brain total RNA and rat liver total RNA. The resulting senseRNA was analyzed using qRT-PCR. Unamplified total RNA samples were also analyzed using qRT-PCR to provide the standard for comparison. Results are shown on the next slide.



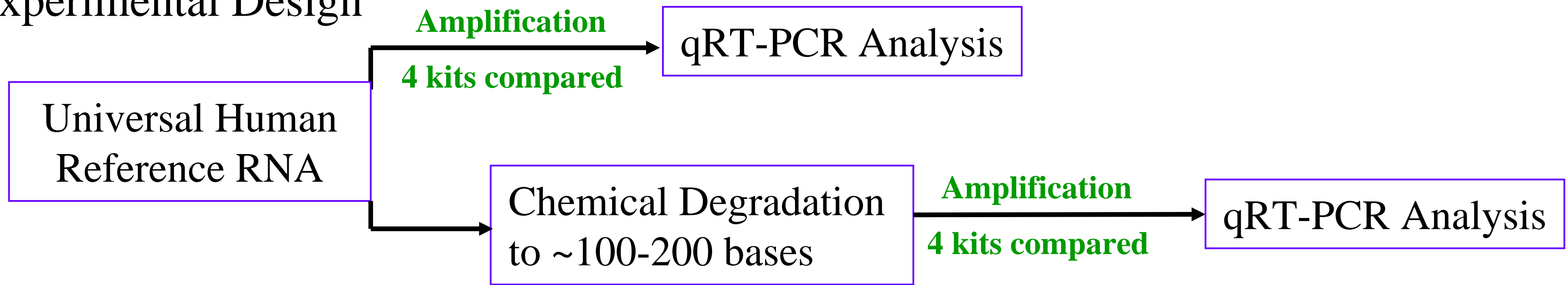
Reproducibility of RampUP

Experiment 2, Results: qRT-PCR data for Aquaporin 4 (Aqp4), a high abundance brain mRNA, and Alcohol Dehydrogenase (ADH), a high abundance liver mRNA, are shown below. Note that both genes are also weakly expressed in the other tissue. Data is shown as the relative quantity compared to GAPDH (an endogenous control). Among the four replicate RampUP amplification reactions, the expression profiles of Aqp4 and ADH are conserved in both brain and liver, as compared to the unamplified controls.



Accuracy of RampUP

Experimental Design



Validation of qRT-PCR Methods

Intact and chemically degraded Universal Human Reference RNA samples (Stratagene, cat. no. 740000) were compared in qRT-PCR prior to amplification. PCR primers were designed for 21 genes, each with ~75bp amplicons. The Ct values of the degraded RNAs were compared to the Ct values of the intact RNAs. The observed Fold Amplification Bias was very low, less than 2 fold, for all 21 genes, confirming that the selected primer pairs (amplicons) were valid for both intact and degraded samples and that the degraded RNA was of sufficient quality for amplification experiments. These PCR primers were used in further studies to determine the accuracy of the RampUP amplification process in comparison to three other kits.

Comparison of Intact versus Degraded RNA
(No RNA Amplification)

Low Expressed Messages

HSPCB message
3'
5'

Fold Amplification Bias
2.00
1.50
1.00
0.50
0.00
-0.50
-1.00
-1.50
-2.00

HSPCB 33
HSPCB 364
HSPCB 713
HSPCB 1380
HSPCB 1952
ALDOA
GAPD
PGK1
LDHA
RPS 27
RPL19
RPL11
NONO
RPL32
RPS18
Actin 74
COPA
CSNK2B
PSM B4
KARS
PSM D4

Gene

Accuracy of RampUP

Comparison of RNA Amplification Kits: Intact and degraded universal human reference RNA samples were used in four RNA amplification kits designed for use with degraded RNA, according to the protocol of the manufacturer, in order to assess the relative fidelity of the RampUP amplification process. The amplified RNAs and unamplified RNAs were then analyzed with qRT-PCR as validated. The Fold Amplification Bias was calculated for each gene by comparing the Ct values between amplified and unamplified samples. A negative Fold Amplification Bias means that the amplified RNA sample has underrepresented amounts of the gene. A positive Fold Amplification Bias means that the amplified RNA sample has over-represented amounts of the gene. Ideally, the smaller the Fold Amplification Bias, the more accurate the amplification. An overall grouped summary of these experiments is presented in the table below, followed by individual qRT-PCR data for the RampUP titration to further illustrate the results.

RNA Amplification Kit	Input RNA	Range of Fold Amplification Bias	Number of Lost Transcripts
Full Spectrum RNA Amplification (Systems Biosciences)	100ng Intact	-192 to 43.7	0 out of 16
Full Spectrum RNA Amplification (Systems Biosciences)	100ng Degraded	-8.8 to 25	4 out of 16
Paradise (Arcturus)	100ng Intact	-18.3 to 274.9	5 out of 16
Paradise (Arcturus)	100ng Degraded	-37.2 to 22.2	9 out of 16
SenseAmp (Genisphere)	100ng Intact	-0.8 to 6.0	3 out of 16
SenseAmp (Genisphere)	100ng Degraded	-2.1 to 3.6	3 out of 16
RampUP (Genisphere)	10ng Intact	-3.53 to 2.69	0 out of 21
RampUP (Genisphere)	10ng Degraded	-1.49 to 0.93	0 out of 21
RampUP (Genisphere)	1ng Intact	-3.14 to 4.51	0 out of 21
RampUP (Genisphere)	1ng Degraded	-1.01 to 1.91	1 out of 21
RampUP (Genisphere)	0.1ng Intact	-3.61 to 9.08	0 out of 21
RampUP (Genisphere)	0.1ng Degraded	-0.88 to 4.19	3 out of 21

Accuracy of RampUP

10ng Input RNA for RampUP

Intact

Low Expressed Messages

3' HSPCB message 5'

Fold Amplification Bias

5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00

HSPCB 33, HSPCB 364, HSPCB 713, HSPCB 1380, HSPCB 1952, ALDOA, GAPD, PGK1, LDHA, RPS 27, RPL19, RPL11, NONO, RPL32, RPS18, Actin 74, COPA, CSNK2B, PSM B4, KARS, PSM D4

Gene

Degraded

Low Expressed Messages

3' HSPCB message 5'

Fold Amplification Bias

5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00

HSPCB 33, HSPCB 364, HSPCB 713, HSPCB 1380, HSPCB 1952, ALDOA, GAPD, PGK1, LDHA, RPS 27, RPL19, RPL11, NONO, RPL32, RPS18, Actin 74, COPA, CSNK2B, PSM B4, KARS, PSM D4

Gene

Accuracy of RampUP

1ng Input RNA for RampUP

Intact

Low Expressed Messages

HSPCB message
3’
5’

Fold Amplification Bias
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00

HSPCB 33, HSPCB 364, HSPCB 713, HSPCB 1380, HSPCB 1952, ALDOA, GAPD, PGK1, LDHA, RPS27, RPL19, RPL11, NONO, RPL32, RPS18, Actin 74, COPA, CSNK2B, PSMB4, KARS, PSMD4

Gene

Degraded

Low Expressed Messages

HSPCB message
3’
5’

Fold AMplification Bias
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00

ND

HSPCB 33, HSPCB 364, HSPCB 713, HSPCB 1380, HSPCB 1952, ALDOA, GAPD, PGK1, LDHA, RPS27, RPL19, RPL11, NONO, RPL32, RPS18, Actin 74, COPA, CSNK2B, PSMB4, KARS, PSMD4

Gene

ND: no template detected or data out of range

Accuracy of RampUP

0.1 ng Input RNA for RampUP

Intact

HSPCB message

3’

5’

Low Expressed Messages

Fold Amplification Bias

9.00
7.00
5.00
3.00
1.00
-1.00
-3.00
-5.00

HSPCB 33
HSPCB 364
HSPCB 713
HSPCB 1380
HSPCB 1952
ALDOA
GAPD
PGK1
LDHA
RPS 27
RPL19
RPL11
NONO
RPL32
RPS18
Actin 74
COPA
CSNK2B
PSM B4
KARS
PSM D4

Gene

Degraded

HSPCB message

3’

5’

Low Expressed Messages

Fold Amplification Bias

5.00
4.00
3.00
2.00
1.00
0.00
-1.00
-2.00
-3.00
-4.00
-5.00

HSPCB 33
HSPCB 364
HSPCB 713
HSPCB 1380
HSPCB 1952
ALDOA
GAPD
PGK1
LDHA
RPS 27
RPL19
RPL11
NONO
RPL32
RPS18
Actin 74
COPA
CSNK2B
PSM B4
KARS
PSM D4

ND
ND
ND

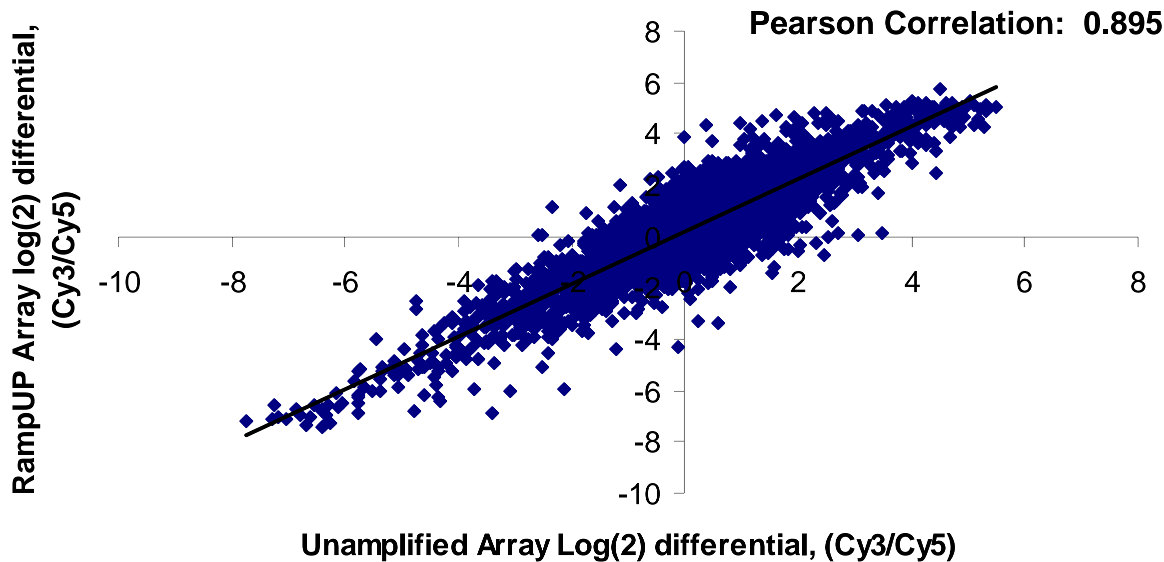
Gene

ND: no template detected or data out of range

Accuracy of RampUP

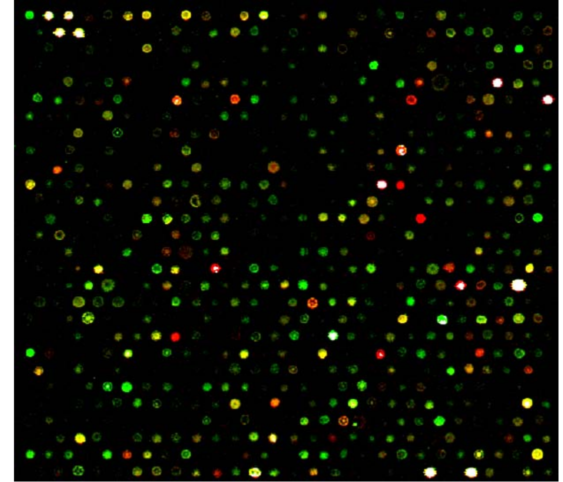
Differential Analysis: Glass Microarray Experiment

To determine the accuracy of the RampUP amplification process, unamplified total RNA was compared to RampUP senseRNA in a differential analysis experiment on 38.5K mouse oligo arrays (Microarrays Inc.). Unamplified mouse brain and mouse liver total RNA samples were differentially labeled (1.5µg per channel) using Genisphere's Array 900MPX kit. Likewise, mouse brain and mouse liver senseRNA samples generated using RampUP were differentially labeled (3 µg per channel) with Genishpere's Array 900MPX kit. The comparison of the differential arrays gave a 0.895 Pearson correlation.



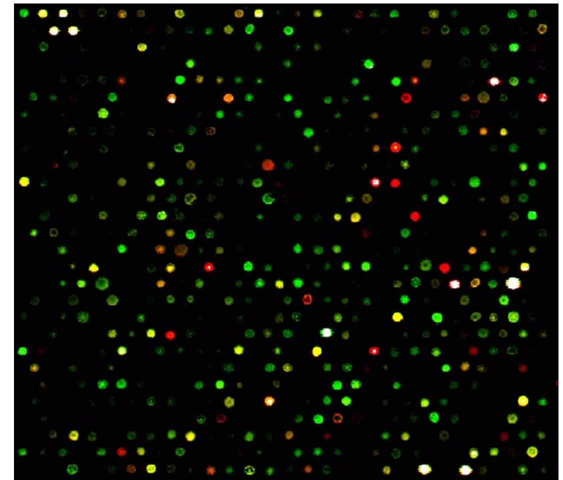
Unamplified RNA

Mouse Brain Cy3 Vs. Mouse Liver Cy5



RampUP senseRNA

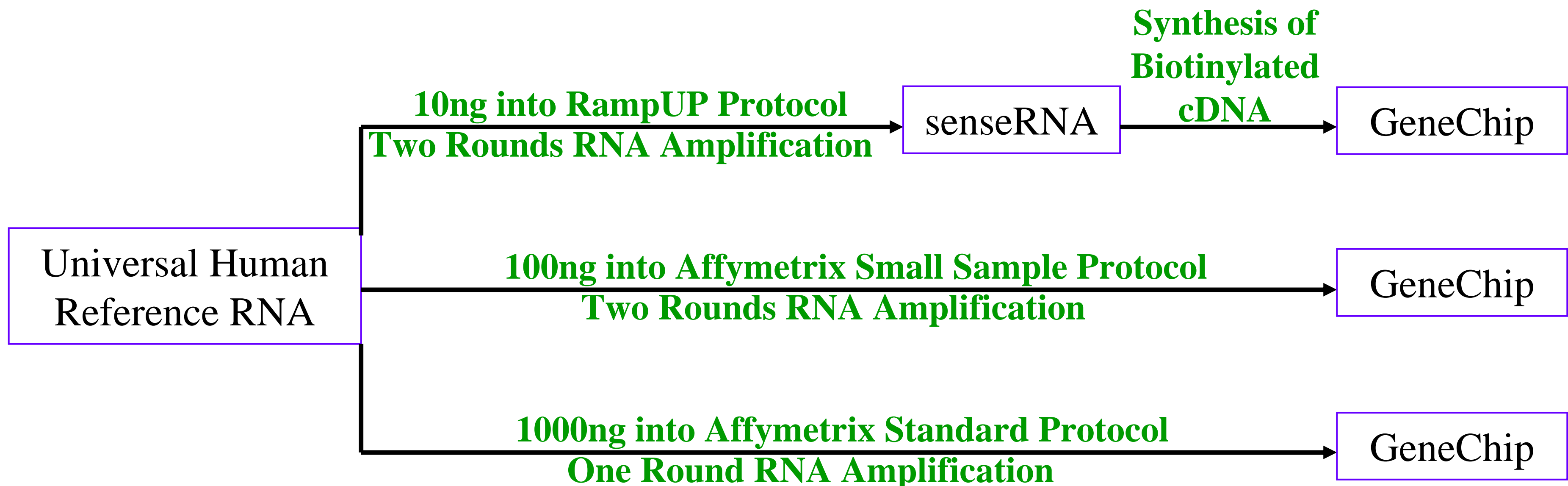
Mouse Brain Cy3 Vs. Mouse Liver Cy5



Compatibility with Affymetrix Platform

Experimental Design

To demonstrate the compatibility of RampUP with the Affymetrix platform as well as the data quality achieved, an experiment was performed using Universal Human Reference RNA (Stratagene, cat. no. 740000) and Human Genome U133A arrays (Affymetrix, cat. no. 900366). RampUP senseRNA was reverse transcribed using a random 12mer primer and biotin-16-dUTP (Roche, cat no. 11093070910) to generate labeled cDNA that was hybridized to the Affymetrix GeneChip. The results were compared to the Affymetrix standard protocol and Affymetrix small sample protocol. The data comparison is shown on the next slide.



Compatibility with Affymetrix Platform

Amplification Strategy	Input Total RNA	Rounds of Amplification	1st Round RT Primer	Correlation to Standard Labeling (Pearson)	Genes Present (% Affy Std)	Scale Factor	3'/5' Ratio GAPDH	3'/5' Ratio Actin
Genisphere Experimental:								
Affymetrix Standard Labeling	1000ng	1.0	T7/dT primer	N/A	55.0%	1.53	2.20	2.80
Affymetrix Small Sample Version II	100ng	2.0	T7/dT primer	0.90	52.0%	1.10	2.50	22.40
RampUP + Biotin RT	10ng	2.0	RP/dT	0.89	64.5%	0.72	1.40	2.70
Literature Values:								
Affymetrix Small Sample Version II (1)	10-100ng	2.0	T7/dT primer	0.90	(93-95%)	1-2.5	2.0-3.0	5.6-13.1
1 Round T7 Amp. + Standard Labeling (2)	10-100ng	2.0	T7/dT primer	0.87-0.88	~(92-93%)	not reported	4.0-19.0	4.0-19.0

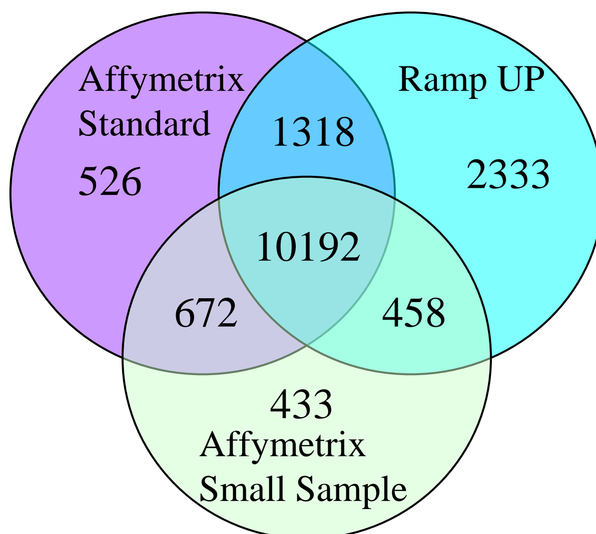
(1) Affymetrix Small Sample Sample Target Labeling Assay Version II, Technical Note, Affymetrix, INC (2002).

(2) Kenzelmann, M, et.al. High-accuracy Amplification of Nanogram Total RNA Amounts for Gene Profiling, Genomics 83 (2003), 550-558

Data Comparison

91% genes common between RampUP and Affymetrix Standard verses

85% common between Affymetrix Small Sample and Affymetrix Standard



RampUP Reproducibility

Biotinylated cDNA was synthesized from replicate RampUP senseRNA samples and compared on the Affymetrix platform.

Data Concordance : $R^2 = 0.985$

P-call Concordance: $R^2 = >96\%$

Development of a streamlined, high fidelity, two round sense RNA amplification method

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Abstract

As advances are made in genetic screening, clinical samples of vanishing quantities become of increasing interest to research. Amplification is required to study such minuscule amounts of RNA generally obtained from archived formalin fixed paraffin embedded (FFPE) tissue or through laser capture microdissection (LCM) technology. Under these circumstances, one round of standard amplification is not sufficient, and two rounds is cumbersome and plagued with artifacts, loss of data, and poor data quality. We have developed a novel two round sense strand amplification process that produces little to no artifact, while maintaining high fidelity in a streamlined methodology. The two round process is simplified due to the use of a tandem T7/T3 RNA polymerase promoter which is built onto the 3' end of the first strand cDNA and serves as the amplification template for round one. Furthermore, by avoiding the conventional Eberwine method, nearly full-length transcripts are produced allowing for the elimination of 3' bias to improve data quality. Each round typically gives approximately 500-1000 fold amplification. Moreover, we have designed a third round antisense amplification-labeling strategy that results in an additional 100-500 fold amplification. In this process a biotinylated or fluorophore labeled NTP may be incorporated for use in expression analysis or any other downstream applications which require labeled antisense RNA. This method will prove to be an important research tool for the study of the most critical clinical samples.

RampUP Methodology

RampUP RNA Amplification

RNA Input Titration

Experiment 1:

Experiment 2:

Reproducibility of RampUP

Validation of qRT-PCR Methods

Comparison of Intact versus Degraded RNA (No RNA Amplification)

Accuracy of Amplification

Comparison of RNA Amplification Kits

RNA Amplification Kit	Input RNA	Range of Fold Amplification Bias	Number of Lost Transcripts
Full Spectrum RNA Amplification (Systems Biosciences)	100ng Intact	-19.2 to 43.7	0 out of 16
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RampUP (Genisphere)	1ng Degraded	-1.01 to 1.91	1 out of 21
RampUP (Genisphere)	0.1ng Intact	-2.61 to 9.08	0 out of 21
RampUP (Genisphere)	0.1ng Degraded	-0.88 to 4.19	2 out of 21

Note: The ideal Fold Amplification Bias is 0.

Systems Biosciences Full Spectrum RNA Amplification Kit 100 ng Input RNA (1 Round)

Arcturus Paradise Kit 100 ng Input RNA (1 Round)

Genisphere SenseAmp Kit 100 ng Input RNA (1 Round)

Genisphere Ramp UP Kit 10 ng Input RNA (2 Rounds)

Genisphere Ramp UP Kit 1 ng Input RNA (2 Rounds)

Genisphere Ramp UP Kit 0.1 ng Input RNA (2 Rounds)

Conclusions

- RampUP is a two round linear amplification process that generates predictable amounts of product.
- RampUP is reproducible in amplifying both intact and partially degraded RNA samples in two rounds.
- RampUP amplifies RNA samples accurately, as confirmed by qRT-PCR.

References

RampUP poster presented at the 2006 AACC meeting in Chicago.

A link to the full size pdf document can be found on the Genisphere web site.

RampUP RNA Amplification

- **Single Cell Expression Profiling**

  Based on our observations, the reverse transcription of RNA from a single cell is not proficient, because of sample quality, sample contamination, and/or enzyme efficiency. For best results, RampUP should be run with at least 100pg of input Total RNA.

- **Amplification of any RNA type**

  RampUP is compatible with any RNA type including mRNA, partially degraded RNA, and prokaryotic RNA.

- **Higher Fidelity Amplification**

  RampUP senseRNA is a more accurate copy of unamplified RNA than amplified RNA generated by competitive techniques.

- **Artifact Free**

  Little non-specific amplification product is generated with RampUP.

- **Simple Two-Round Procedure**

  The protocol requires minimal hands-on time, which increases the throughput efficiency.

Acknowledgments

Many thanks the following collaborators:

Mat Moore and Mike Opel - Combimatrix Corp.

Accuracy and Kit Comparison qRT-PCR Data

Ron Hart Lab - Rutgers University

Reproducibility qRT-PCR Data

Don Baldwin Lab - University of Pennsylvania

Affymetrix Platform Compatibility Data