

# 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.

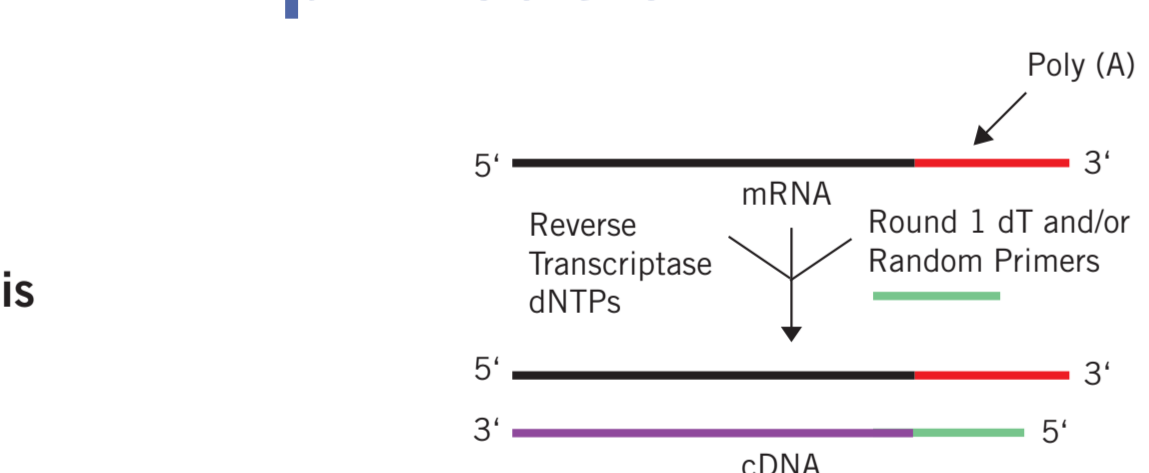
## RampUP Methodology

We developed a two-round RNA amplification protocol with an easy procedure and minimal purification and hands-on steps, which produces an accurate pool of senseRNA after 2 rounds of amplification. The process is designed around the use of tandem T7 and T3 RNA polymerase promoters, which allows for an easy transition of amplified RNA synthesized during round 1 into round 2. The procedure is also optimized to work with either intact or degraded RNA, by using either dT or random primers in the first reverse transcription step.

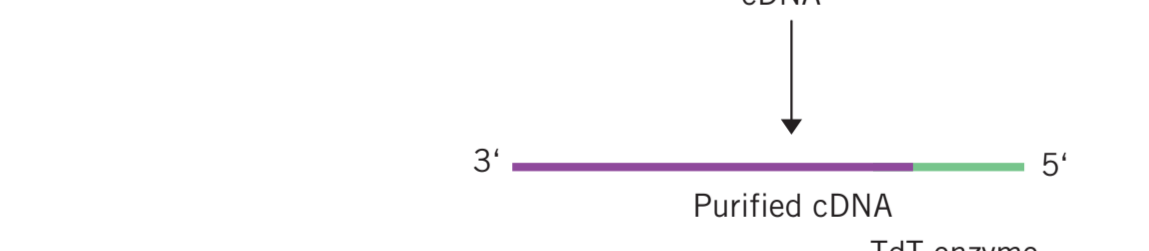
### RampUP RNA Amplification

#### ROUND 1

##### First strand cDNA synthesis



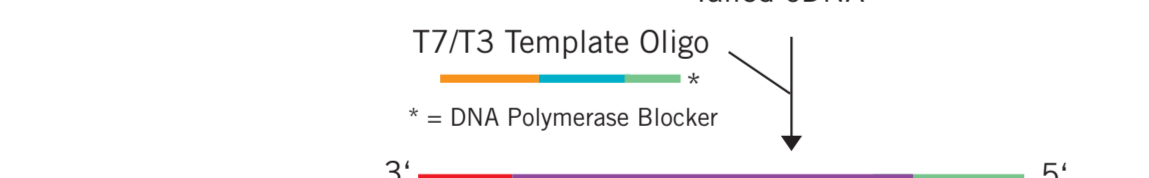
##### Purification of cDNA



##### Tailing of cDNA



##### Annealing of T7/T3 Template Oligo



##### T7/T3 Promoter Synthesis

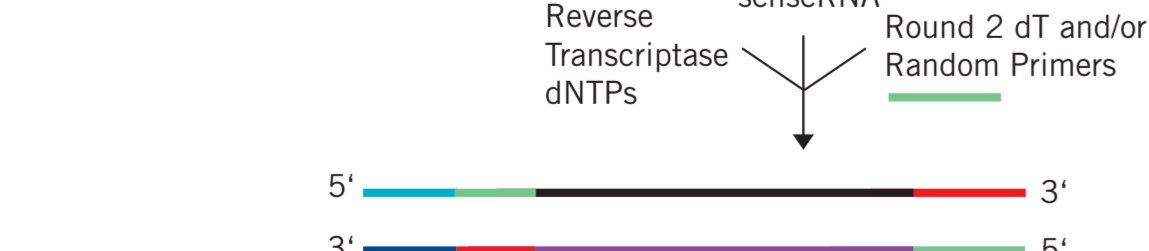


##### T7 In Vitro Transcription

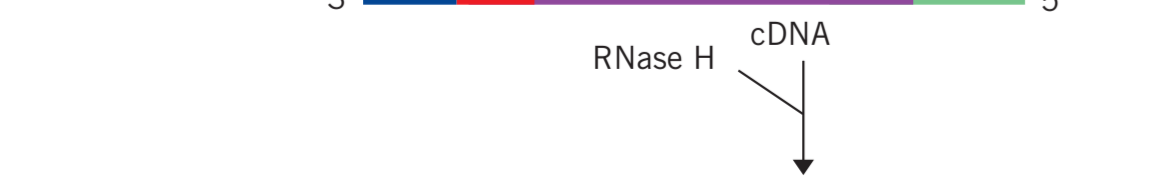


#### ROUND 2

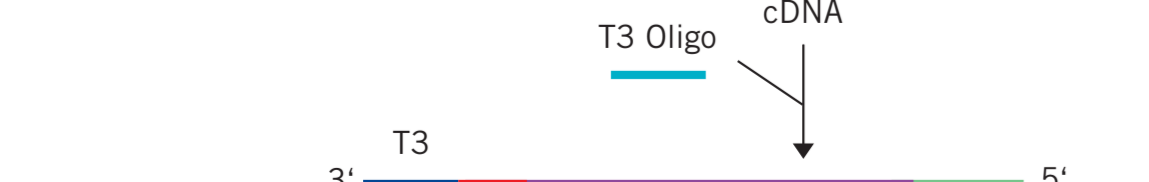
##### Reverse Transcription



##### RNAse H degradation of senseRNA



##### Annealing of T3 Oligo



##### T3 In Vitro Transcription

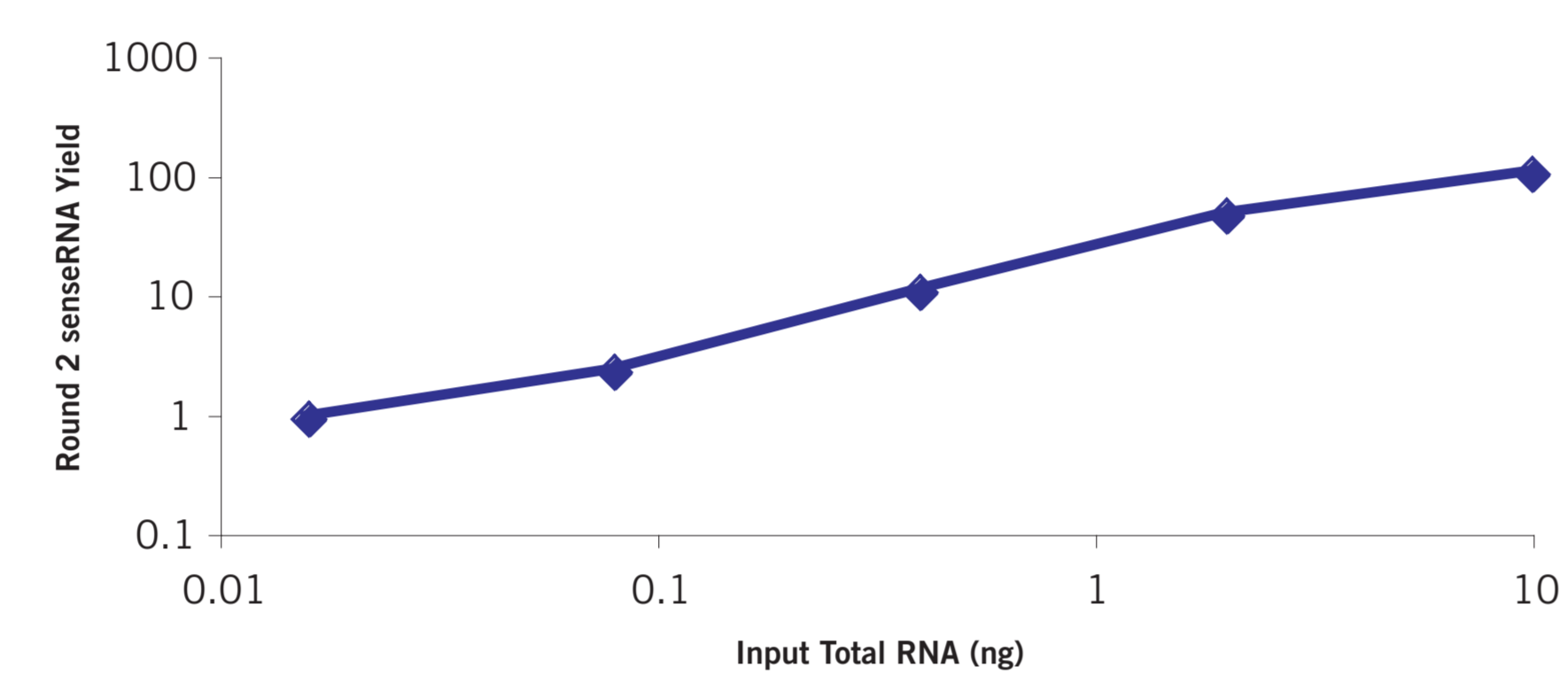


## RNA Input Titration

### Experiment 1:

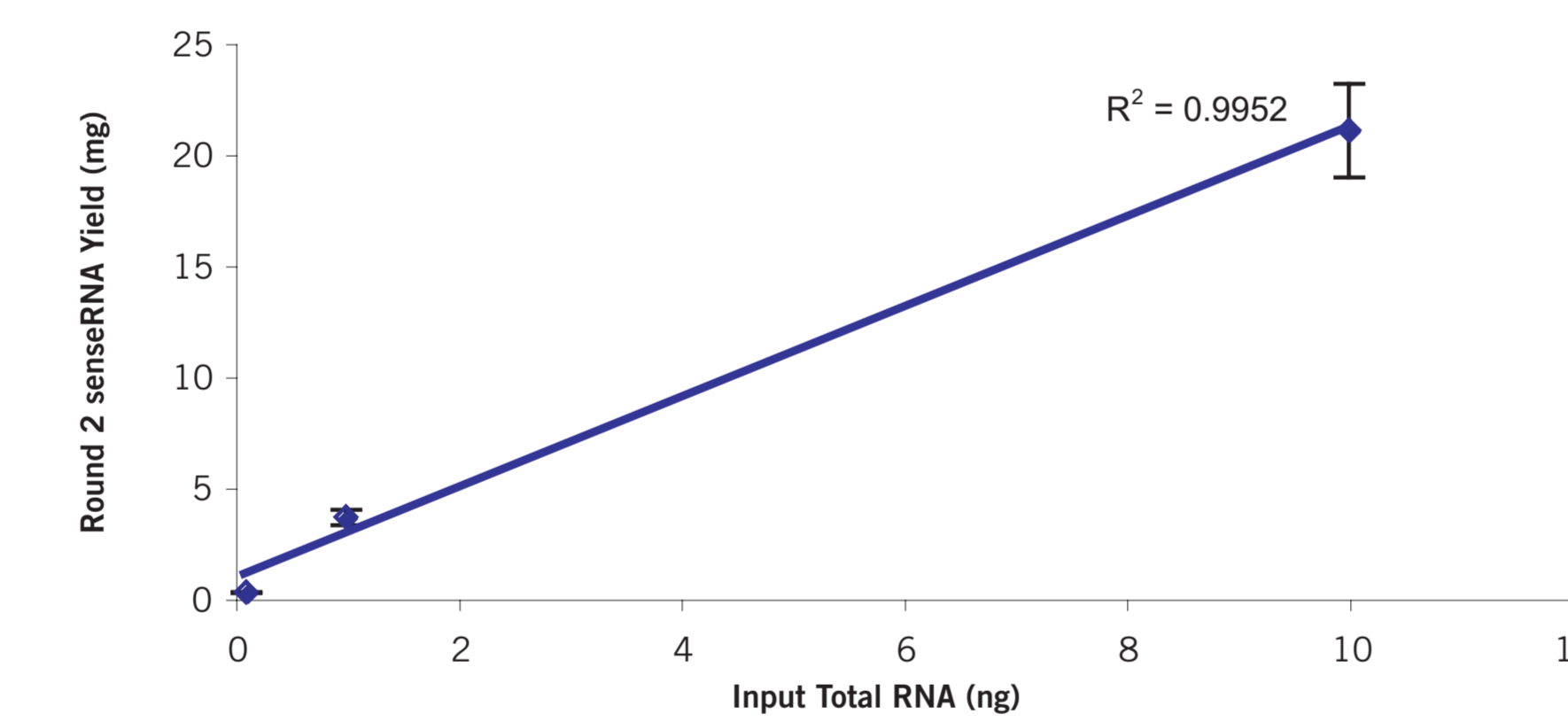
A total RNA input titration experiment was run to test the RampUP process. Using various input amounts of Mouse Brain Total RNA (Ambion cat. no. 7812), the following amounts of Round 2 senseRNA were recovered as determined by OD260:

Input Mouse Brain Total RNA (ng)	Round 2 senseRNA Yield (µg)
10	102
2	46
0.4	10.6
0.08	2.25
0.016	0.91
0 (Background)	0.61



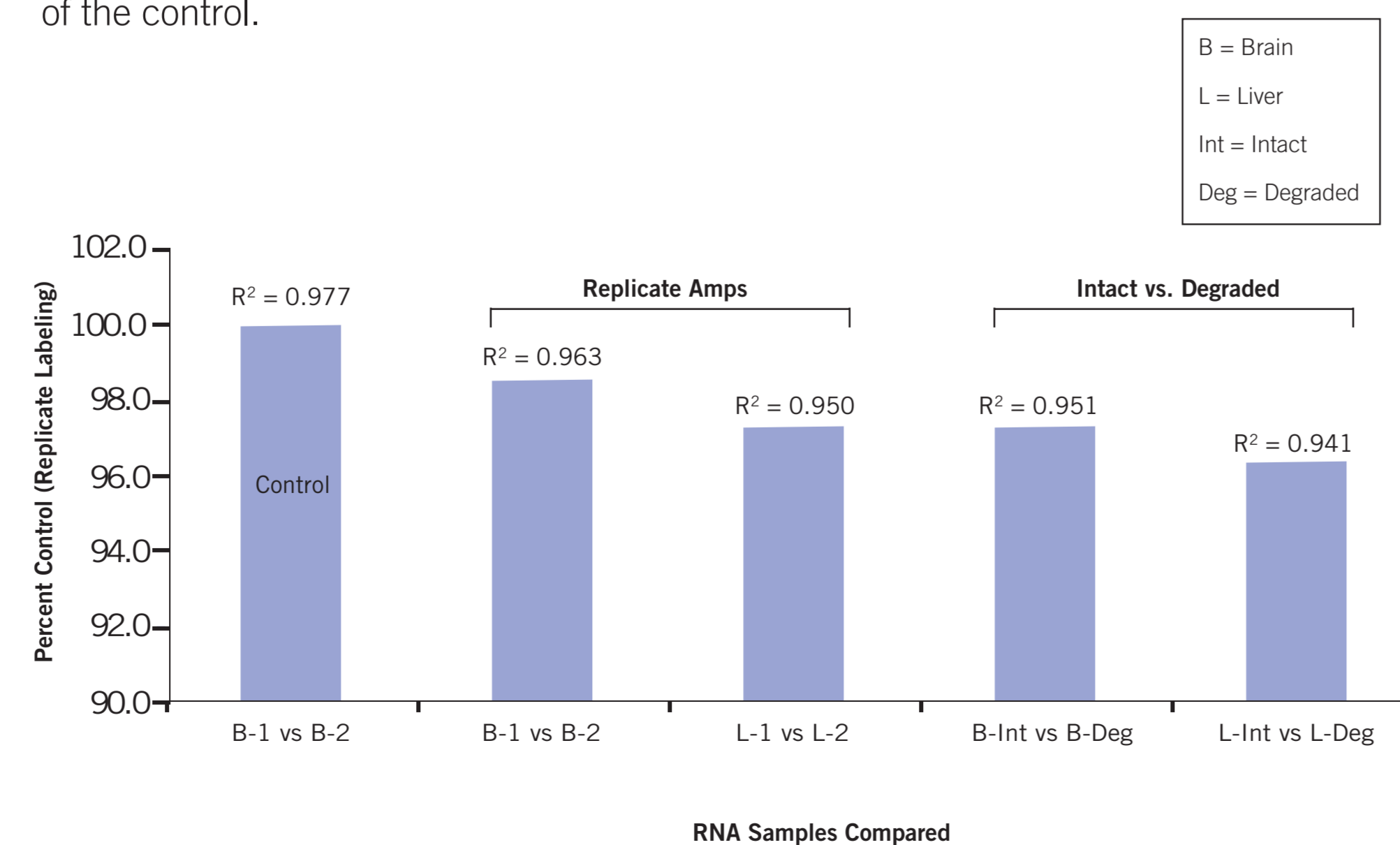
### Experiment 2:

Since most amplification processes produce non-sense or artifact-containing products, a second titration experiment was performed with Human RNA. For this experiment, the amplifications were quantitated using qRT-PCR to follow mRNA from the actin gene (rather than total RNA OD). Various input amounts of Total RNA were used in RampUP, and the resulting senseRNAs were measured for Actin with qRT-PCR. The results of this titration are illustrated below:



## Reproducibility of RampUP

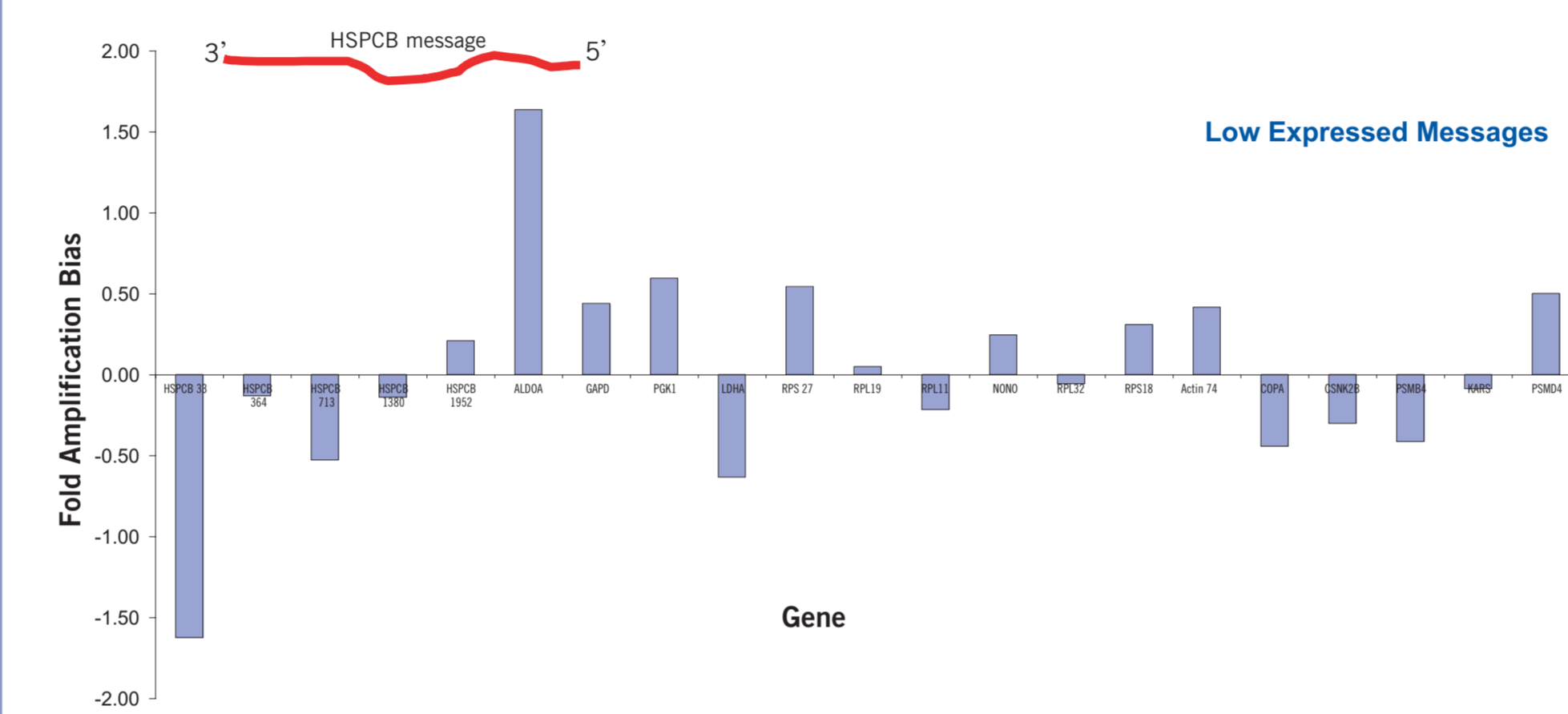
To determine the reproducibility of RampUP amplification, replicate analysis experiments were performed. Several intact and chemically degraded total RNA samples (5ng each) were amplified with RampUP, labeled by direct incorporation, and hybridized to 38.5K mouse oligo arrays (Microarrays Inc.). A replicate 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.



## Validation of qRT-PCR Methods

Universal Human Reference RNA (Stratagene) samples were chemically degraded with sodium hydroxide, to a fragment size between 50 and 200 bases. These degraded RNAs were compared to intact Reference RNAs with qRT-PCR. 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 sufficient (in quality) for amplification experiments. These PCR primers were used in further studies to determine the accuracy of the amplification process.

### Comparison of Intact versus Degraded RNA (No RNA Amplification)



## Accuracy of Amplification Comparison of RNA Amplification Kits

Several products are available for RNA amplification, but only some products are recommended for use with partially degraded RNA samples, like those obtained from FFPE samples. We compared RNA amplification kits that were designed for use with degraded RNA in order to assess the fidelity of the RampUP amplification process. Intact and degraded RNA samples were used in four RNA amplification kits, according to the protocol of the manufacturer. The amplified RNAs and unamplified RNAs were analyzed with qRT-PCR. 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 results for each amplification kit to further illustrate the results.

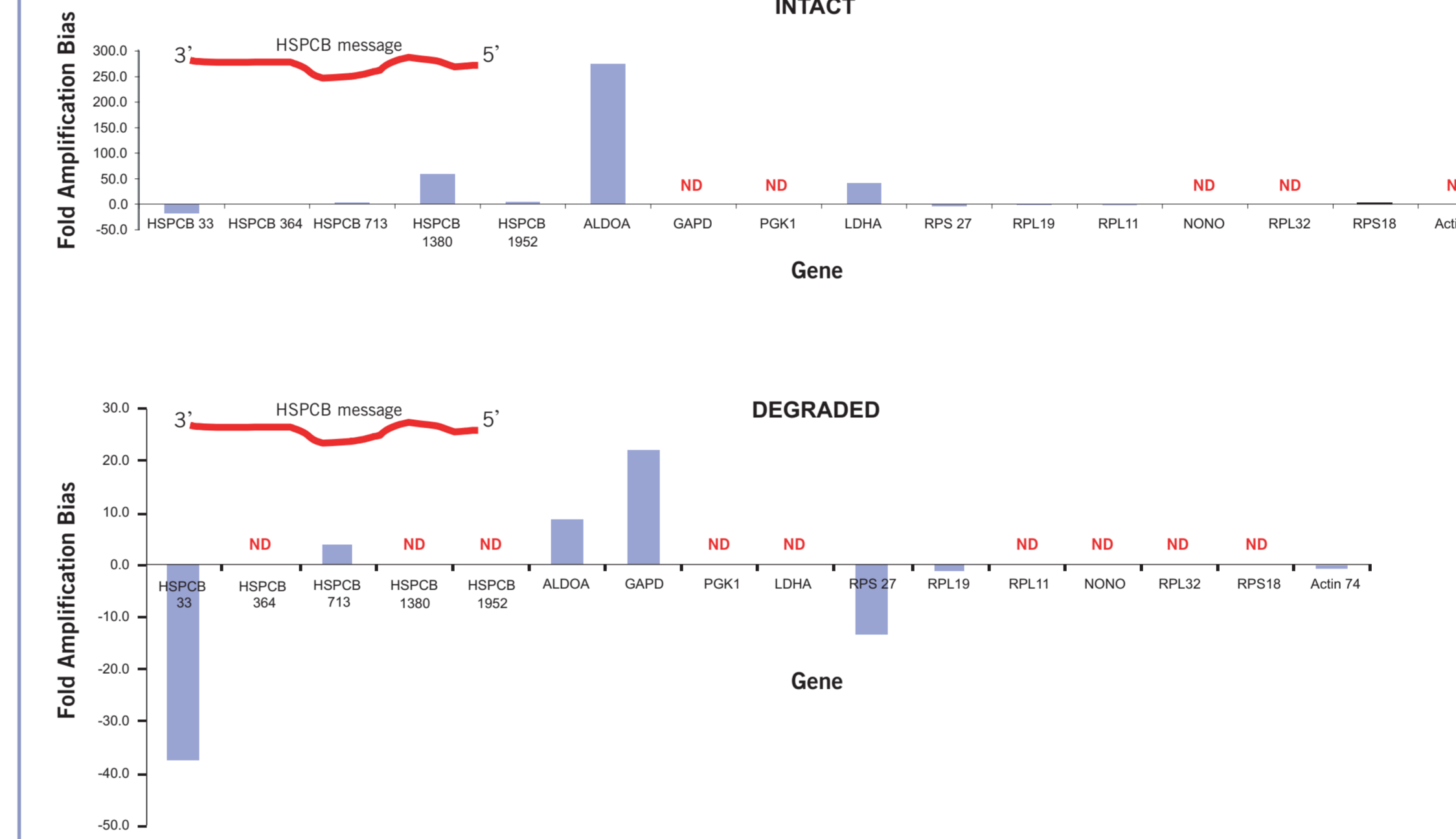
RNA Amplification Kit	Input RNA	Range of Fold Amplification Bias	Number of Lost Transcripts
Full Spectrum RNA Amplification (Systems Biosciences)	100ng Intact	-192 to 43.7	0 out of 16
Full Spectrum RNA Amplification (Systems Biosciences)	100ng Degraded	-8.8 to 25	4 out of 16
Paradise (Arcturus)	100ng 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

Note: The ideal Fold Amplification Bias is 0.

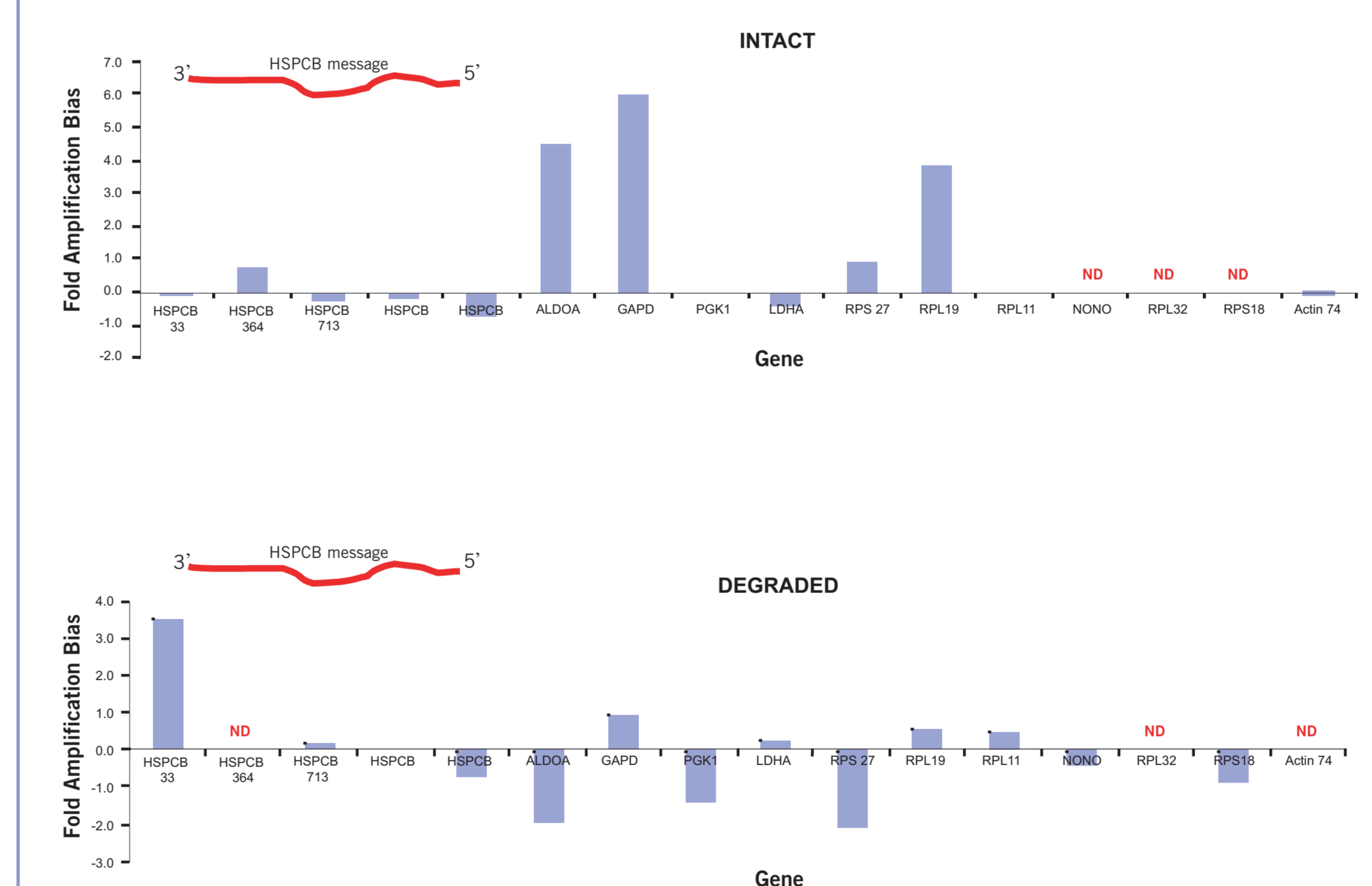
### Systems Biosciences Full Spectrum RNA Amplification Kit 100 ng Input RNA (1 Round)



### Arcturus Paradise Kit 100 ng Input RNA (1 Round)



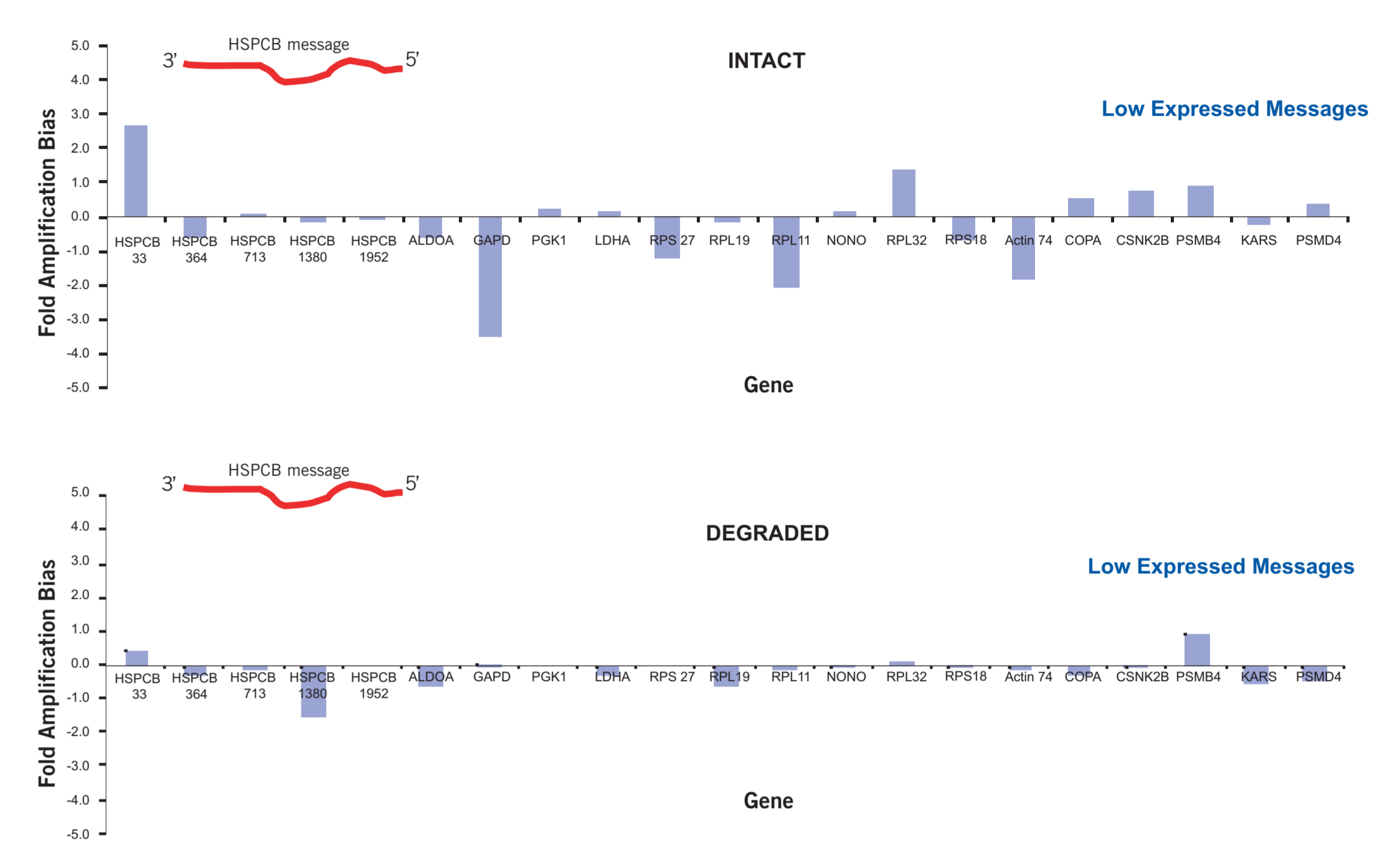
### Genisphere SenseAmp Kit 100 ng Input RNA (1 Round)



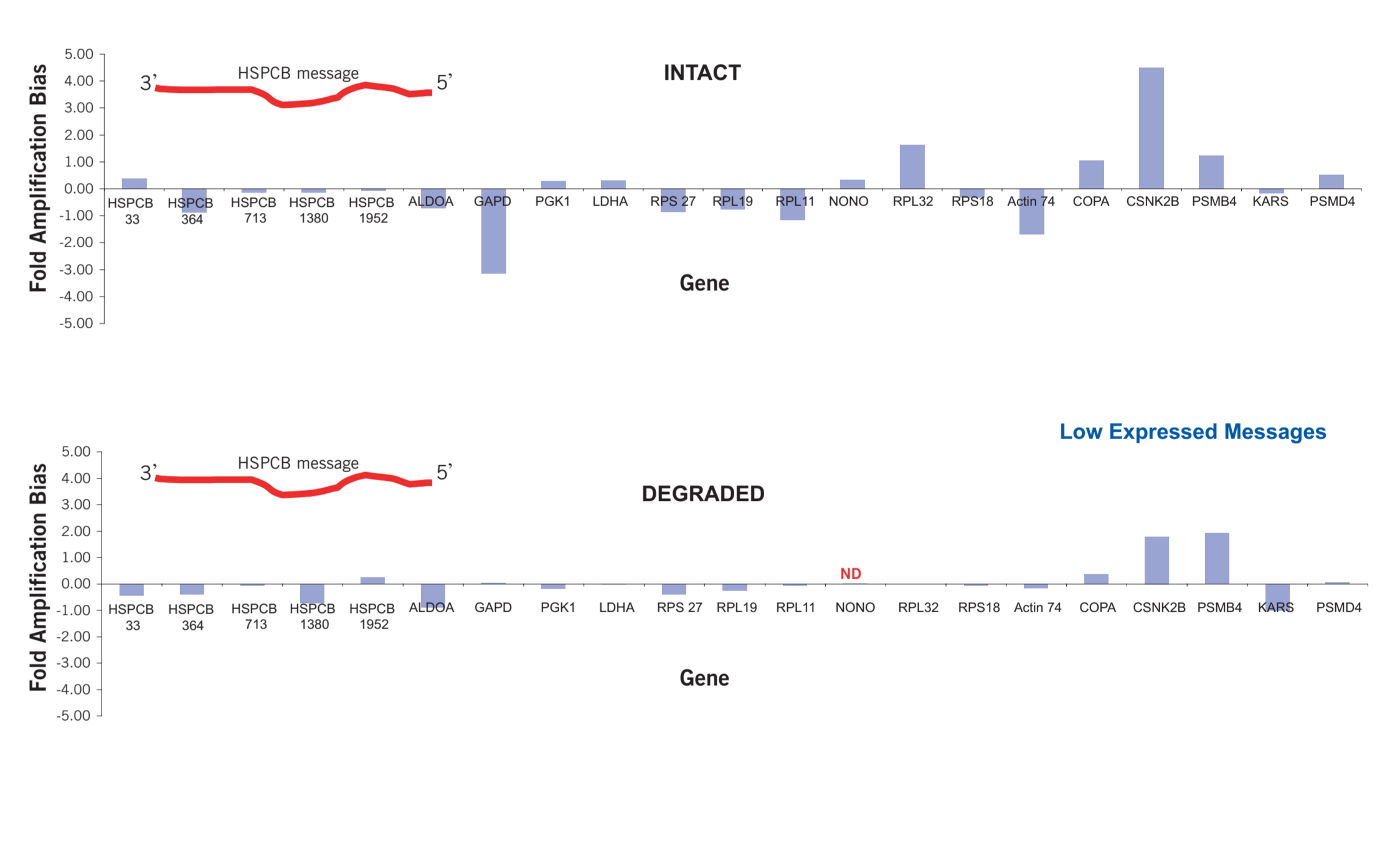
## Conclusions

- 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.

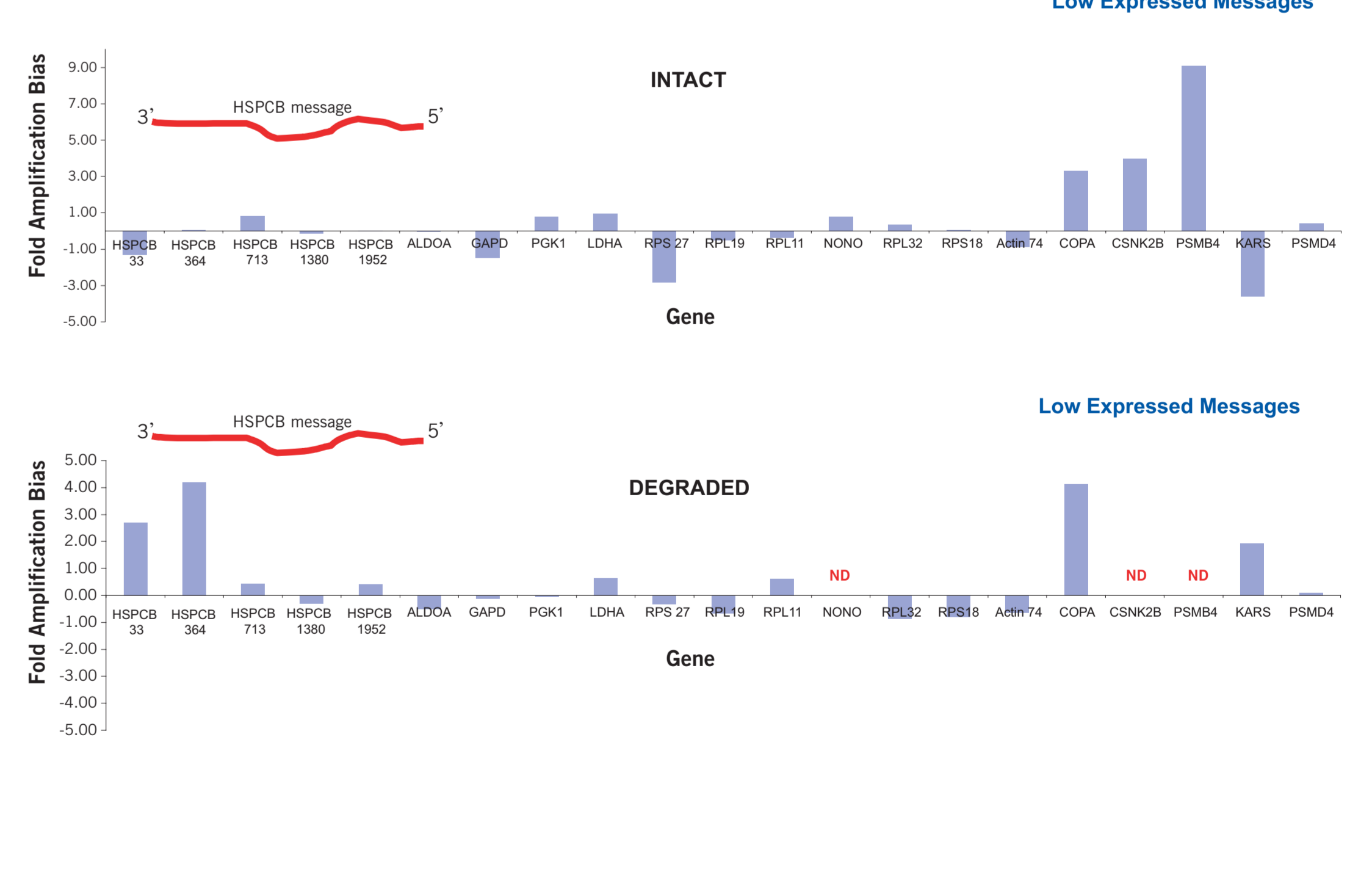
### Genisphere Ramp UP Kit 10 ng Input RNA (2 Rounds)



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



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



## References

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- Sambrook J, Fritsch EF, and Maniatis T. Molecular Cloning, A Laboratory Manual (Second Edition) Cold Spring Harbor Laboratory Press (1989).
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- Baugh LR, Hill AA, Brown EL, and Hunter CP. Quantitative analysis of mRNA amplification by in vitro transcription. Nucleic Acids Research 29:5e29 (1991).