Agilent RNA Spike-In Kit

Product Number 5188-5279

Introduction

The Agilent RNA Spike-In Kit was developed to provide positive controls for monitoring the microarray workflow from sample amplification and labeling to microarray processing. The Agilent RNA Spike-In Kit contains two spike-in mixtures. Each mixture contains 10 *in vitro* synthesized, polyadenylated transcripts derived from the Adenovirus E1A transcriptome that are premixed at various ratios. When the two mixtures are cohybridized onto a microarray, the user can use the expected versus observed log ratios to monitor the system for linearity, sensitivity, and accuracy.

Since a gene microarray experiment is a multistep procedure, small differences among samples, procedures, or user-induced variations confound the microarray data. Confidence in the experimental data is increased when it is compared to control transcripts of known concentrations and ratios. The RNA Spike-In Kit consists of two sets of positive control transcript mixtures optimized to anneal to their complementary probes on the microarray, with minimal self-hybridization and cross-hybridization. Most Agilent catalog microarrays, and some custom microarrays, contain these specific probe sets that are designed to not have complementary sequences in the biological samples being analyzed. This positive control kit is an invaluable tool for optimizing technique and for troubleshooting experiments. Concentrated RNA Spike-In Kit stocks must be diluted with the Dilution Buffer provided in the kit. These are spiked-in directly to the RNA samples prior to amplification and labeling to achieve the final relative amounts indicated in Table 1.

Table 1 Final relative sample amounts

RNA Spike-In name*	Spike A Mix relative copy number	Spike B Mix relative copy number	Expected ratio amounts (A/B)
(+) E1A_r60_1	10	10	1:1
(+) E1A_r60_n11	1.5	0.5	3:1
(+) E1A_r60_a20	100	100	1:1
(+) E1A_r60_3	3	9	1:3
(+) E1A_r60_a104	10	30	1:3
(+) E1A_r60_a107	30	10	3:1
(+) E1A_r60_a135	9	3	3:1
(+) E1A_r60_a22	10	100	1:10
(+) E1A_r60_a97	0.5	1.5	1:3
(+) E1A_r60_n9	100	10	10:1

* The Spike-In ProbeNames listed in Agilent's Feature Extraction Software v8.1 output table may or may not contain the prefix (+)E1A.

These controls are then labeled and amplified together with the RNA samples of interest being analyzed. After hybridization to the complementary probes on the microarray, the log ratios of red to green signal intensities for each Spike-In transcript can be used to monitor the sample amplification and labeling and microarray processing procedures used in the experiment, which are independent from the quality of the starting RNA sample. These controls were validated for use with Agilent DNA microarray kits for Gene Expression and are required for use with the Agilent Two-Color Microarray-Based Gene Expression Analysis Protocol, version 4.0.

Kit Contents

The Agilent RNA Spike-In Kit contains:

- Spike A Mix, 10 µL
- Spike B Mix, 10 µL
- Dilution buffer, 1.2 mL

Related Agilent Reagents

Low RNA Input Linear Amplification Kit	5184-3523
cRNA Cleanup Module	5188-2770
Gene Expression Hybridization Kit	5188-5242
Gene Expression Wash Pack	5188-5327
Stabilization and Drying Solution	5185-5979

Storage

Store the Agilent RNA Spike-In Kit at –70°C to –80°C in a nondefrosting freezer for up to 1 year from the date of the receipt.

The first dilution of the RNA Spike-In Kit PolyA RNA Positive Controls can be stored up to 3 months in a nondefrosting freezer at –20°C and freeze-thawed up to eight times.

Preparation of RNA Spike A Mix and Spike B Mix for Target Labeling

Vortex stock solutions vigorously, heat at 37°C for 5 minutes, and vortex once more. Briefly centrifuge to spin contents to bottom of tube prior to opening. Settlement of the solution on the sides or lid of the tubes may occur during shipment and storage.

Table 2 provides the amount of Spike A Mix and Spike B Mix for three different starting sample ranges of total RNA or 0.2 µg of polyA mRNA. We recommend that Spike A Mix always be labeled with cyanine 3 and Spike B Mix be labeled with cyanine 5. The output of the Agilent SpikeIns LogRatio Statistics table in the eQC report, generated when using Agilent Feature Extraction Software v8.1, uses this orientation. It is built into the software code and cannot be set by the user. If the polarity is flipped, the user will have to manually adjust the output.

Detailed protocols for the Low Input RNA Linear Amplification Kit and Agilent Hybridization and Wash Kits are described in their respective manuals. The latest updates are available at www.agilent.com.

Table 2 Sample dilutions for RNA Spike A Mix for cyanine 3 and Spike B Mix for cyanine 5 labeling

Starting amount of RNA		Serial dilutions			Spike A Mix or Spike B Mix volume to be used (µL)
Total RNA (ng)	PolyA RNA (ng)	First	Second	Third	
50–200		1:20	1:40	1:16	2
201–2000		1:20	1:40	1:4	2
>2000	200	1:20	1:40	0	2

NOTE It is important to use RNAse-free microfuge tubes and tips. Avoid pipetting less than 2 µL in volume to ensure accuracy.

For example, to prepare the Spike A Mix dilution appropriate for 50 ng of total RNA starting sample:

1. Vortex thawed RNA Spike A Mix concentrate vigorously. Heat at 37°C in a circulating waterbath for 5 minutes. Vortex RNA Spike Mix A stock tube vigorously again. Briefly centrifuge to spin contents to bottom of tube.
2. Add 2 µL of RNA Spike A Mix stock to 38 µL of Dilution Buffer provided in the kit. (1:20)
3. Mix thoroughly by vortexing and spin down quickly to collect all liquid at the bottom of the tube. This tube contains the First Dilution.
4. Add 2 µL of First Dilution Solution to 78 µL of Dilution Buffer for Second Dilution. (1:40)
5. Mix thoroughly by vortexing and spin down contents to the bottom of the tube. This tube contains the Second Dilution.
6. Add 2 µL of the Second Dilution to 30 µL of Dilution Buffer for the Third Dilution. (1:16)
7. Mix thoroughly by vortexing and briefly spin down the contents. This tube contains the Third Dilution.
8. Add 2 µL of Third Dilution to 50 ng of sample total RNA to be labeled with cyanine 3 and proceed with Low Input Fluorescent RNA Linear Amplification Kit method, as specified in manual.

Sample Data

Sample data shown in Figure 1 were obtained for RNA Spike-In Kit following the dilutions specified in the above tables.

RNA Spike-In Mix with Total RNA

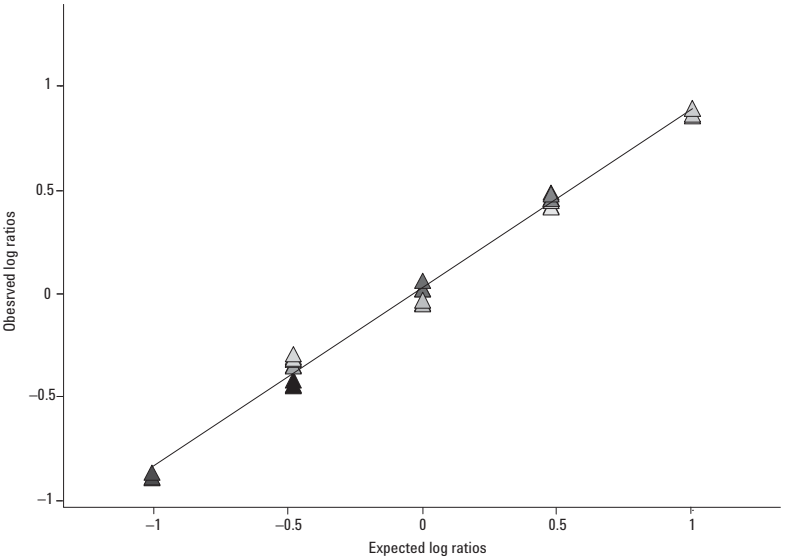

Figure 1 Performance of Spike-In controls in three different starting total RNA amounts (50 ng, 500 ng, 5000 ng)

Data in Figure 1 were generated by amplifying and labeling RNA Spike A Mix with Hela total RNA (Clontech) in the presence of cyanine 3-CTP, and RNA Spike B Mix with human spleen total RNA (Clontech) for each of three different starting total RNA amounts (50 ng, 500 ng, and 5000 ng). Cyanine 3- and cyanine 5-labeled targets were cohybridized to Agilent Human 1A (v.2, part number G4110B) oligo microarrays and log ratios for cyanine 5 versus cyanine 3 signal intensities were calculated. Data representing the observed versus expected log ratio for each spike-in transcript is plotted, with different data point shadings indicating different total RNA input amounts. The correlation coefficient is 0.9, demonstrating the high correlation of ratios for each transcript across the ten-transcript mixture.

Guidelines for the analysis of RNA Spike-Ins are addressed in the current Feature Extraction User Guide (part number G2566-90012) and Reference Guide (part number G2566-90014). These are available at www.agilent.com.

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