Agilent One Color RNA Spike-In Kit

Product Number 5188-5282

Introduction

Gene Expression microarray experiments are multi-step procedures where small differences among samples, procedures, or user-induced variations may confound the microarray data. The Agilent One Color RNA Spike-In Kit was developed to provide positive controls for monitoring the Agilent One Color Gene Expression microarray workflow from sample amplification and labeling to microarray processing. The Agilent One Color RNA Spike-In Kit consists of a set of 10 positive control transcripts optimized to anneal to complementary probes on the microarray with minimal self-hybridization or cross-hybridization. Many Agilent catalog and custom microarrays contain the complementary Spike-In probe sets. The Spike-In probe sets were designed to not have complementary sequences to the biological samples used on most microarrays.

The Agilent One Color RNA Spike-In Kit contains a mixture of 10 *in vitro* synthesized, polyadenylated transcripts derived from the Adenovirus E1A gene. The Agilent One Color RNA Spike-In Kit contains these transcripts premixed at concentrations that span six logs and differ by one log or half log increments. Agilent One Color microarray experiments with the Agilent One Color RNA Spike-In Kit can be analyzed with the Agilent Feature Extraction (version 8.5) software which will generate a QC Report. Tables and graphs from the Agilent QC Report detail the linear portion of the dynamic range of the microarray experiment, the high and low detection limits of the experiment, and the reproducibility of the controls with %CV calculations for each of the 10 Spike-In probes.

The Agilent One Color RNA Spike-In Kit is an invaluable tool for optimizing and troubleshooting experiments. The concentrated Agilent One Color RNA Spike-Mix stock must be diluted with the Dilution Buffer provided in the kit. The diluted RNA controls are spiked directly into the RNA samples prior to amplification and labeling to achieve the relative amounts shown in Table 1 below.

Table 1 Relative Spike-In amounts in the concentrated stock

RNA Spike-In name*	Log (relative concentration)	Kit stock concentration (pg/µL)	Mass ratio of E1A:total RNA (1:x)†
(+) E1A_r60_3	0.30	0.04	1:12,500,000,000
(+) E1A_r60_a104	1.30	0.4	1:1,250,000,000
(+) E1A_r60_a107	2.30	4	1:125,000,000
(+) E1A_r60_a135	3.30	40	1:12,500,000
(+) E1A_r60_a20	3.83	133	1:3,750,000
(+) E1A_r60_a22	4.30	400	1:1,250,000
(+) E1A_r60_a97	4.82	1333	1:375,000
(+) E1A_r60_n11	5.30	4000	1:125,000
(+) E1A_r60_n9	5.82	13333	1:37,500
(+) E1A_r60_1	6.30	40000	1:12,500

* The Spike-In probe names listed in Agilent's Feature Extraction Software v8.5 may or may not contain the prefix (+)E1A.

† The E1A mass to total RNA mass is based on the dilutions recommended in this protocol. The dilutions can be varied by the user to adjust for the mRNA content of the total RNA sample.

The Agilent One Color Spike-In controls are labeled and amplified together with the RNA samples of interest. After hybridization to the complementary probes on the microarray, the green signal intensities for each Spike-In transcript can be used to monitor the sample amplification and labeling and the 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 the Agilent DNA microarray kits for Gene Expression and are required for use with the Agilent One Color Gene Expression microarray workflow. The Agilent One Color microarray workflow can generate reliable and accurate results with high confidence in the microarray data.

Kit Contents

The Agilent One Color RNA Spike-In Kit contains:

- Agilent One Color RNA Spike-Mix, 10 µL
- Dilution Buffer, 1.2 mL

Related Agilent Reagents

Low RNA Input Linear Amplification Kit	5184-3523
Gene Expression Hybridization Kit	5188-5242
Gene Expression Wash Buffer 1	5188-5325
Gene Expression Wash Buffer 2	5188-5326
Gene Expression Wash Pack (1+2)	5185-5327

Storage

Store the Agilent One Color 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 Agilent One Color RNA Spike-In Kit positive controls can be stored up to 2 months in a nondefrosting freezer at –70°C to –80°C and freeze-thawed up to eight times.

Preparation of RNA Spike Mix for Target Labeling

Vortex stock solutions vigorously, heat at 37°C for 5 minutes, and vortex once more. Briefly centrifuge to drive 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 Agilent One Color RNA Spike-In Kit for a range of total RNA input amounts. These are diluted such that 1 µL of Spike-Mix RNA is added for every 100 ng of total RNA in the labeling reaction up to 500 ng, and 0.5 µL added for every 100 ng total RNA for input amounts greater than 500 ng.

Table 2 Sample dilution for Agilent One Color RNA Spike Mix for cyanine 3 labeling

Starting amount of RNA		Serial dilutions			Spike-In Mix volume to be used in each labeling reaction(µL)
Total RNA (ng)	Maximum volume of RNA (µL)	First	Second	Third	
200	8.3	1:20	1:25	1:10	2
300	7.3	1:20	1:25	1:10	3
400	6.3	1:20	1:25	1:10	4
500	5.3	1:20	1:25	1:10	5
600	7.3	1:20	1:25	1:5	3
700	6.8	1:20	1:25	1:5	3.5
800	6.3	1:20	1:25	1:5	4
900	5.8	1:20	1:25	1:5	4.5
1000	5.3	1:20	1:25	1:5	5

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

For example, to prepare the Agilent One Color RNA Spike-In Kit dilution appropriate for 200 ng of total RNA starting sample:

1. Vortex thawed Agilent One Color RNA Spike-Mix concentrate vigorously. Heat at 37°C in a circulating water bath for 5 minutes. Vortex Agilent One Color RNA Spike-Mix tube vigorously again. Briefly centrifuge to spin contents to the bottom of the tube.
2. Add 2 µL of Agilent One Color RNA Spike-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 of the liquid at the bottom of the tube. This tube contains the First Dilution.
4. Add 2 µL of the First Dilution to 48 µL of Dilution Buffer for the Second Dilution (1:25).
5. Mix the Second Dilution thoroughly by vortexing and spin down quickly to collect all of the liquid at the bottom of the tube.

6 Add 4 µL of the Second Dilution to 36 µL of Dilution Buffer for the Third Dilution (1:10).

7 Mix the Third Dilution thoroughly by vortexing and spin down quickly to collect all of the liquid at the bottom of the tube.

8 Add 2 µL of Third Dilution to 200 ng of sample total RNA and proceed with cyanine 3 labeling using the Agilent Low RNA Input Linear Amplification Kit protocol as specified in the user manual.

NOTE

Discard the tubes containing the Second Dilution and the Third Dilution after use. The First Dilution (1:20) of the Agilent One Color RNA Spike-Mix controls can be stored up to 2 months in a nondefrosting freezer at –70°C to –80°C and freeze-thawed up to eight times.

Sample Data

Agilent Feature Extraction v8.5 software was used to generate the Agilent QC Report, a subset of which is presented in the following figures and tables:

Agilent One Color RNA Spike-Mix with MG63 Total RNA

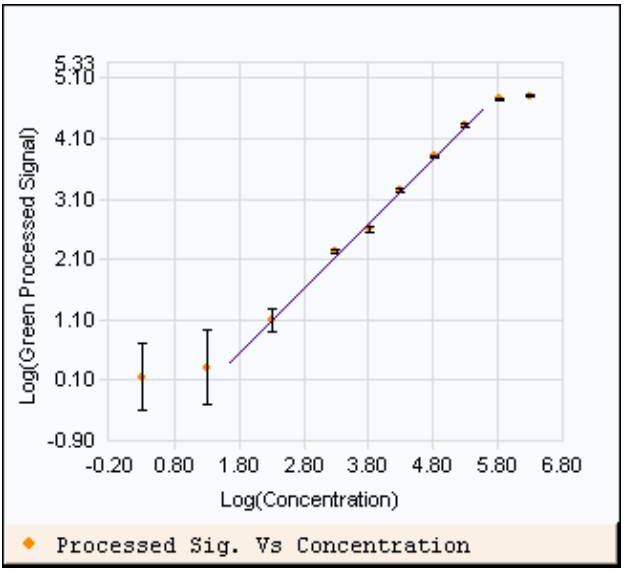

Figure 1 Agilent Spike-In One Color QC Report: Log (Signal) versus Log (Relative Concentration) Plot

Data in Figure 1 were generated by amplifying and labeling a 1:5000 final dilution of the Agilent One Color RNA Spike-Mix with Osteosarcoma cell line (MG63) total RNA (Ambion) in the presence of cyanine 3-CTP starting with 200 ng total RNA. Cyanine 3-labeled targets were hybridized to Agilent Human 1A v2 oligo microarrays (Agilent Product Number G4110B), and green signals were measured and processed. Data representing the log of the green processed signal for each Spike-In transcript is plotted against the log of the relative concentration.

At high signal levels the error bars are small due to scanner saturation. At low signal levels the error bars are visible because the signal is dropping into the background noise. Since the graph in Figure 1 is plotted on a log scale, the error bars can be evaluated as CVs.

Additional data is also presented in table format in the Agilent One Color QC Report. Table 3 below details experimental Spike-In Signal Statistics including the %CV and standard deviation (StdDev) for each of the 10 Spike-In transcripts. Data describing the lower end of the linear range as well as the low signal and the noise around the low signals are calculated in Table 4 below.

Table 3 Agilent One Color QC Report - Spike-In Signal Statistics

Agilent SpikeIns Signal Statistics				
Probe Name	Log(Relative Conc.)	Log(Median Proc. Sig.)	% CV	StdDev
(+)E1A_r60_3	0.30	0.15	105.74	0.55
(+)E1A_r60_a104	1.30	0.30	89.52	0.61
(+)E1A_r60_a107	2.30	1.08	36.28	0.19
(+)E1A_r60_a135	3.30	2.24	7.76	0.03
(+)E1A_r60_a20	3.83	2.59	7.40	0.03
(+)E1A_r60_a22	4.30	3.24	5.99	0.03
(+)E1A_r60_a97	4.82	3.80	5.94	0.03
(+)E1A_r60_n11	5.30	4.32	6.91	0.03
(+)E1A_r60_n9	5.82	4.75	4.86	0.02
(+)E1A_r60_1	6.30	4.81	5.82	0.02

This table shows the Spike-In Probe Name, the median Processed Signal (median of Log[ProcessedSignal]), %CV (StdDev of Processed-Signal /Average of ProcessedSignal) and StdDev (Log[ProcessedSignals]) for each of the 10 Spike-In sequences. There was a median %CV of 5.99 for the Spike-Ins in this experiment. The Agilent Feature Extraction v8.5 software also generates Spike-In concentration response statistics as presented in Table 4.

Table 4 Agilent One Color QC Report - Spike-In Concentration-Response Statistics

Agilent Spike-In Concentration-Response Statistics

Linear Range Statistics:

Low Signal	0.40
High Signal	4.60
Low Relative Concentration	1.67
High Relative Concentration	5.58
Slope	1.07
R^2 Value	1.00

Detection Limit Statistics

Saturation Point	4.82
Low Threshold	0.07
Low Threshold Error	0.85

Table 4 details the values calculated from the log signal versus log concentration plot shown in Figure 1. All of the statistics in this table are calculated using a parameterized sigmoidal curve fit to the data. For more details on the curve fitting or the calculation of these statistics, please refer to the Agilent Feature Extraction Software (v8.5) Reference Guide (pp. 60–65).

Troubleshooting

Ideally the curve for the Agilent One Color Spike-In Kit should contain two transcripts with a low signal in the noise and two transcripts in the range of scanner saturation to give a sigmoidal-shaped curve with 2 asymptotes. If a given total RNA sample has a relatively high mRNA content, the following data may be obtained:

Agilent RNA Spike-Mix in High mRNA Content Total RNA

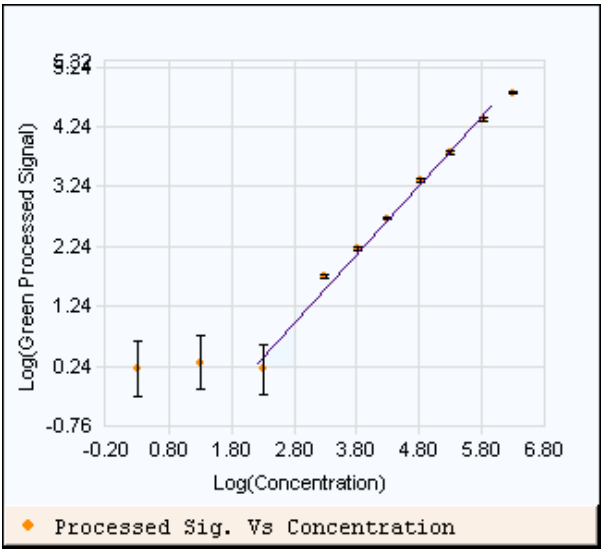

Figure 2 Agilent Spike-In One Color QC Report: Example of a Log (Processed Signal) versus Log (Relative Concentration) Plot with only one target in the Signal Saturation Range

Data in Figure 2 were generated by amplifying and labeling a 1:5000 final dilution of the Agilent One Color RNA Spike-Mix with Cervical Adenocarcinoma cell line (HeLa-S3) total RNA (Ambion) in the presence of cyanine 3-CTP starting with 200 ng total RNA.
Cyanine 3-labeled targets were hybridized to Agilent Human 1A v2 oligo microarrays (Agilent Product Number G4110B) and green signals were measured and processed. Data representing the log of the green processed signal for each Spike-In transcript is plotted against the log of the relative concentration.

For the data presented in Figure 2, only one of the E1A targets fell into the range of scanner saturation, which results in an increased slope of 1.16 for the linear fit of the line. In a case like this we recommend

using a lower dilution of the Agilent One Color RNA Spike-In Mix to obtain the desired sigmoidal-shaped curve. The statistics reported for the linear range will be more robust if a sigmoid shape with 2 asymptotes is obtained.

When the Agilent One Color Spike-In control targets are spiked in at too low of a dilution (too concentrated) the following effects may be observed:

Low Dilution of Agilent RNA Spike-Mix in Total RNA

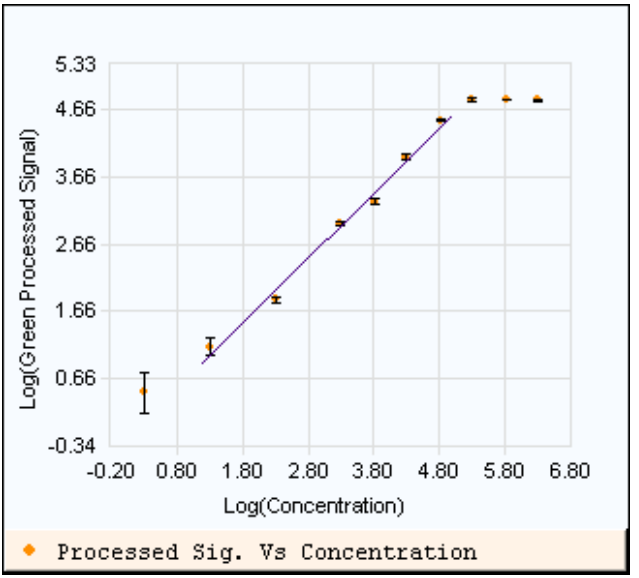

Figure 3 Agilent Spike-In One Color QC Report: Example of a Log (Processed Signal) versus Log (Relative Concentration) Plot with only one target in the Low Signal Range

Data in Figure 3 were generated by amplifying and labeling a 1:1000 final dilution of the Agilent One Color RNA Spike-Mix with Osteosarcoma cell line (MG63) total RNA (Ambion) in the presence of cyanine 3-CTP starting with 200 ng total RNA. Cyanine 3-labeled targets were hybridized to Agilent Human 1A v2 oligo microarrays (Agilent Product Number G4110B) and green signals were measured and processed. Data representing the log of the green processed signal for each Spike-In transcript is plotted against the log of the relative concentration.

The data presented in Figure 3 represents a five-fold lower dilution (reduced from 1:5000 final to 1:1000 final) from the same experiment presented in Figure 1. Only one of the E1A targets fell into the low range of the scanner, which results in a decreased slope of 0.96 for the linear fit of the line. In this case, we recommend using a higher dilution of the Agilent One Color RNA Spike-In Mix to obtain the desired sigmoidal-shaped curve similar to the curve obtained in Figure 1.

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