

## Frequently Asked Questions

# FlashTag Biotin HSR

## RNA Labeling Kit for Affymetrix GeneChip miRNA Arrays

### How should I isolate/purify my RNA?

Any kit for purification of total RNA or LMW RNA will be compatible with FlashTag Biotin HSR. Elute or resuspend the RNA in nuclease-free water. Ensure that the purification method retains low molecular weight species. Some commercial products that have been tested successfully with FlashTag Biotin HSR include:

- Applied Biosystems: *mirVana*<sup>™</sup> miRNA Isolation Kit
- Applied Biosystems: RecoverAll<sup>™</sup> Total Nucleic Acid Isolation Kit for FFPE
- Qiagen: miRNeasy Mini Kit
- Invitrogen: PureLink<sup>™</sup> miRNA Isolation Kit
- Invitrogen: TRIzol<sup>®</sup> reagent (total RNA only) with additional overnight -20°C precipitation step during isopropanol precipitation:  
<http://www.jneurosci.org/cgi/content/full/28/5/1213?maxtoshow=&HITS=&hits=&RESULTFORMAT=1&andorexacttitle=and&fulltext=lna+array&andorexactfulltext=and&searchid=1&FIRSTINDEX=10&sortspec=relevance&fdate=//&resourcetype=HWCIT#SEC2>

### Should I enrich my total RNA for miRNA?

Either Total RNA or LMW (Low Molecular Weight) RNA can be labeled with FlashTag Biotin HSR. Using total RNA can save time and money, and prevent sample loss.

### How much RNA is required for labeling?

The table below describes general recommendations for RNA input for FlashTag Biotin HSR labeling. The amount of microRNA relative to total RNA is less in cultured cells than in tissues. Therefore, Genisphere recommends using more RNA isolated from cultured cells.

RNA Sample	Input for FlashTag Biotin HSR Labeling
Total RNA containing LMW RNA	100 - 1000ng total RNA from tissues 500 - 1000ng total RNA from cultured cells
Enriched LMW RNA, quantitated	100 - 400ng LMW RNA from tissues 200 - 400ng LMW RNA from cultured cells
Enriched LMW RNA, not quantitated	Enriched from 100 - 1000ng total RNA

### Should I treat my RNA sample with DNase?

DNase treatment is optional. It is not necessary for RNA samples that have trace amounts of genomic DNA contamination, but it may be beneficial for RNA samples that are highly contaminated with genomic DNA, to more accurately quantitate the RNA. After treating your RNA with DNase, it is essential that the DNase be inactivated completely before proceeding with the FlashTag procedure, to prevent degradation of the FlashTag reagent (Vial 5). A variety of RNA purification columns/kits may be used to inactivate the DNase. Inactivation of the DNase by high temperature may not completely inactivate the enzyme.

**My RNA sample contains EDTA. Can I use it?**

The maximum amount of EDTA in a sample, prior to FlashTag labeling, can be 0.1-1mM. If more EDTA is present, the sample should be desalted/precipitated or purified prior to FlashTag labeling.

**In my sample, which RNA species are labeled with FlashTag?**

Any piece of RNA with a 3'OH will be labeled with FlashTag.

**Will total RNA labeling affect the miRNA profile on the array?**

No, specificity is defined by the array itself, using the recommended hybridization conditions. Single nucleotide discrimination is achieved when hybridizing the miRNA Arrays according to the FlashTag product insert.

**What are the recommended 260:280 ratios for input RNA?**

Genisphere doesn't have a specific recommendation. A high-quality RNA sample will have a ratio of at least 1.95. However, if microRNAs are present in samples with lower 260:280 ratios, these samples can still be used.

**Can I use FFPE total RNA?**

Yes, as long as the FFPE sample contains microRNA. Regardless of the degradation of the FFPE total RNA, use an ATP dilution of 1:500.

**What are the specifications for microRNA (within total RNA) on the Agilent Bioanalyzer?**

Currently Genisphere does not have any specifications for microRNA on the Agilent Bioanalyzer.

**What does Genisphere use as reference RNA?**

Genisphere uses Ambion FirstChoice® Total RNA samples as reference RNA.

**How is PAP tailing of plant miRNA achieved?**

The FlashTag kit contains a unique buffer (Vial 1) which enables PAP tailing of plant miRNA (and all RNA molecules).

**The FlashTag HSR Range of Sensitivity is described in amoles; how does that translate to copies of microRNA?**

The dynamic range of detection on a per molecule basis for any given miRNA is:

0.5 amoles = 300,000 copies  
7400 amoles = 4,440,000,000 copies

**Does FlashTag label DNA?**

If the DNA has a 3' polyA, it will ligate to the Vial 5 ligation mix. If the DNA oligo does not have a 3' polyA, it will not be polyA tailed by PAP enzyme, and will not be ligated to the Vial 5 ligation mix. If you are interested in using FlashTag for DNA labeling, please contact Genisphere Tech Support.

**What is Vial 3, ATP Mix?**

10mM ATP

**What is Vial 5, FlashTag Ligation Mix?**

Vial 5 contains Genisphere's proprietary 3DNA molecule conjugated with biotins. The 3DNA is attached to a poly(T) sequence that facilitates ligation to all poly(A) tailed RNA molecules.

**What is Vial 8, RNA Spike Control Oligos?**

Vial 8 consists of five oligos which are spiked into the RNA sample prior to FlashTag labeling. These oligos contain controls for the GeneChip miRNA array and the ELOSA QC Assay.

- Oligos 2, 23, and 29 are RNA, and confirm poly(A) tailing and ligation.
- Oligo 31 is poly(A) RNA, and confirms ligation.
- Oligo 36 is poly(dA) DNA, and confirms ligation and lack of RNAses in the RNA sample.

The Affymetrix library file lists the following names for these probe sets:

- spike in-control-2 st
- spike in-control-23 st
- spike in-control-29 st
- spike in-control-31 st
- spike in-control-36 st

Each probe set should show >1000 units (signal-background)

**How much Vial 8 is labeled and hybridized to the miRNA array?**

RNA oligo 2 @ 0.75ng/uL x 2uL = 1.5ng

RNA oligo 23 @ 0.75ng/uL x 2uL = 1.5ng

RNA oligo 29 @ 0.05ng/uL x 2uL = 0.1ng

Poly(A) RNA oligo 31 @ 0.05ng/uL x 2uL = 0.1ng

Poly(A) DNA oligo 36 @ 0.05ng/uL x 2uL = 0.1ng

**What is Vial 9, ELOSA Spotting Oligos?**

Vial 9 contains DNA oligos (complements to RNA oligos 2 & 23) at a concentration of 100ng/uL. These DNA oligos are used to coat the wells for the ELOSA QC assay.

**What is the concentration of Vial 10, ELOSA positive control?**

0.25ng/uL

**I've labeled my samples but I'm not ready to hybridize the miRNA arrays. What should I do?**

Store the biotin-labeled RNA samples on ice for up to 6 hours, or at -20°C for up to two weeks.

**Can PCR plates and thermalcyclers be used for the incubation steps in FlashTag HSR labeling?**

Yes.

**Are there any specific concerns for running FlashTag?**

All materials (tubes, tips, etc.) should be nuclease-free, and all reagents should be prepared with nuclease-free components.

**Can I label my samples in advance and hybridize arrays later?**

Yes, store at -20°C for up to two weeks.

**Do the Affymetrix miRNA Arrays require pre-hybridization?**

No.

**Is it acceptable to make a master mix for the array hybridization cocktail (everything except the labeled RNA) to add to the 21.5uL of labeled RNA?**

Yes.

**Why does the FlashTag product insert recommend a volume of 100uL for array hybridization?**

Genisphere compared 100uL to 80uL and obtained better data quality with 100uL. Therefore we recommend using 100uL as the hybridization volume.

**Is it OK to hybridize for 17 or 18 hours?**

Yes.

**Can I re-use my hybridization cocktail?**

Yes, and if necessary, add more volume of 1X hybridization mix prior to re-hybridization. Refer to Appendix B, Array Rehybridization procedure.

**I usually put wash buffer A on the chip after I remove the hyb cocktail. The FlashTag protocol says to add array holding buffer. What should I do?**

We recommend using array holding buffer.

**Can I make my own components for Hyb, Wash, and Stain?**

No, we recommend using HWS kits from Affymetrix.

**Can I use GCOS software for miRNA Arrays?**

No, we recommend using AGCC Software from Affymetrix.

**Is there a way to check for successful labeling prior to array hybridization?**

Successful biotin labeling is verified via a simple colorimetric ELOSA assay through the hybridization of the biotin-labeled RNA Spike Control Oligos (Vial 8) to complementary ELOSA Spotting Oligos (Vial 9) immobilized onto microtiter plate wells. Refer to Appendix A of the FlashTag product manual.

**Is it essential to run the ELOSA QC Assay procedure every time I use FlashTag?**

Yes, Genisphere recommends running the ELOSA for every labeled sample. The labeled sample may be stored at -20°C for up to two weeks prior to running the ELOSA.

**Is it acceptable to make a master mix for the ELOSA QC hybridization solution (5X SSC, 0.05% SDS, 0.005% BSA and 25% Dextran sulfate) to add to the 2uL samples?**

Yes.

**In the ELOSA, is mild shaking acceptable?**

Mild shaking (50-300RPM) is acceptable during sample hybridization and all other steps of the ELOSA, but is not required. Genisphere does not perform mild shaking during any of the steps of the ELOSA.

**What is the difference between the ELOSA plates sold by Nunc vs. Genisphere?**

There is no difference in these products; Genisphere sells the Nunc product in a package of five 96-well plates.

**Can I perform a gel-shift assay instead of the ELOSA?**

No, because FlashTag reactions are not purified, the same amount of biotin ligation mix (Vial 5) would be detected by the gel-shift assay, whether the labeling was successful or failed.

**Are there any specific concerns for running the ELOSA?**

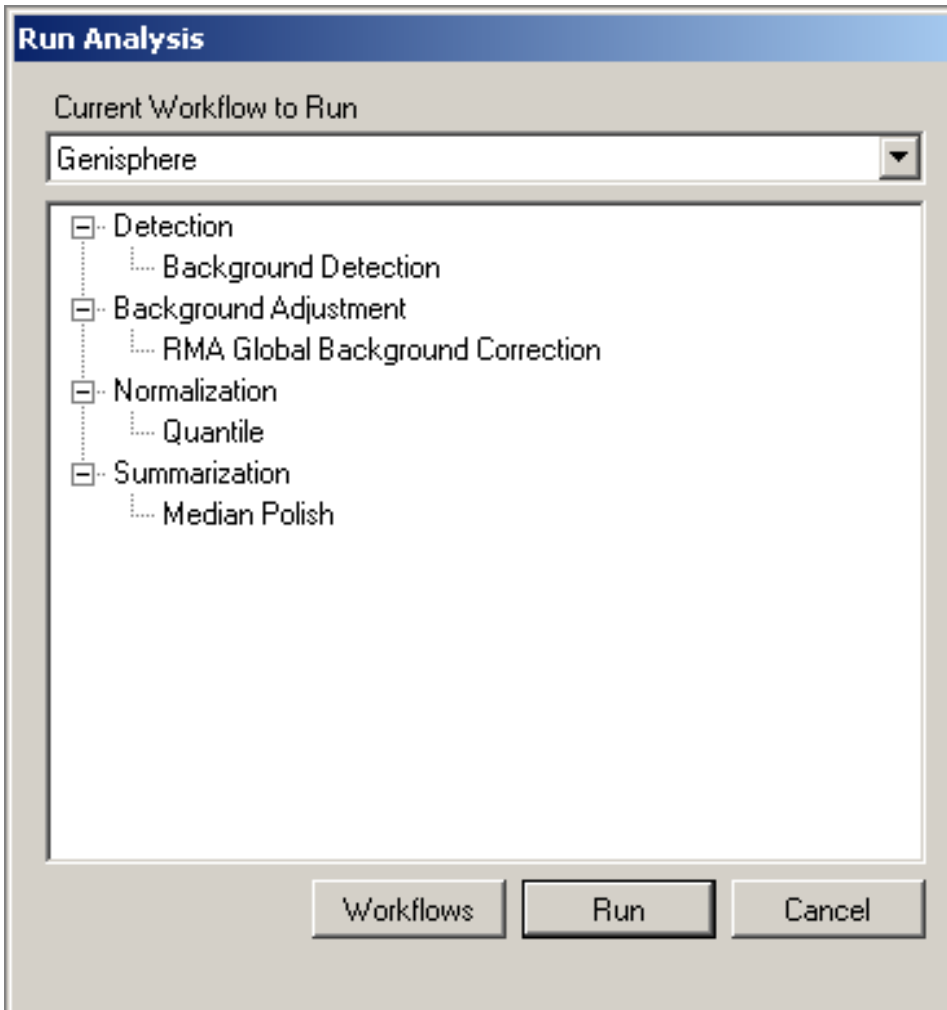
Store all buffers that contain BSA at either 4°C or -20°C. All materials (tubes, tips, etc.) should be nuclease-free, and all reagents should be prepared with nuclease-free components.

**Why is there signal in my negative control ELOSA wells?**

The most common cause of signal in negative control ELOSA wells is the source of BSA. We highly recommend BSA from Sigma, catalog number A3294. In addition, use fresh pipet tips to add reagents to each individual well of the ELSOA plate, to avoid carryover from one well to the next.

**What workflow is recommended in miRNA QC Tool?**

Genisphere recommends the following workflow in miRNA QC Tool:



### **What are the definitions of the steps in the recommended workflow?**

#### Background Detection:

Refer to “Appendix A – Algorithms” in the Affymetrix miRNA QC Tool User’s Guide

#### RMA Global Background Correction, Quantile Normalization, and Median Polish:

- Rafael A. Irizarry, Bridget Hobbs, Francois Collin, Yasmin D. Beazer-Barclay, Kristen J. Antonellis, Uwe Scherf and Terence P. Speed. Exploration, Normalization, and Summaries of High Density Oligonucleotide Array Probe Level Data. Biostatistics, April 2003; Vol. 4; Number 2: 249–264.
- <http://bmbolstad.com/misc/ComputeRMAFAQ/ComputeRMAFAQ.html>

### **How do I edit the workflow in miRNA QC Tool?**

Open QCtool software.

Click Cancel (do not load any CEL files).

Select Tools, Workflow Editor.

Select File, New.

Type a name for the new workflow.

Add the procedures (listed above) by selecting them on the left, and clicking the middle “arrow” button.

Select File, Save.

Click OK.

Click Close to close the editor.

### **I downloaded the .cel files and opened them with miRNA QC tool. How do I export to 3<sup>rd</sup> party software for data analysis?**

In miRNA QC Tool software, data is displayed in the window as an "intensity" table.

You can re-generate this table by Clicking Tables -> Intensities. Save the data by clicking on the Save button on the bottom right of the table. This will save the table as a CSV (comma separated value) file that can be imported into 3<sup>rd</sup> party software. You can save the data at various “points” in the workflow by checking the "show details" box in the lower left of the intensity data view, and then clicking on the point in the workflow (now displayed in the upper left of the screen) that you want to work from. For example, you can click on raw intensity or background adjustment and save the data at that point to be imported into 3<sup>rd</sup> party software.

### **In miRNA QC tool, is the Detection (True/False) based on the P-value, and does True mean Present?**

Yes, the detection is based on the p-value. True does mean present, relative to the p-value. For example: if a miR has low signal and high p-value, it will probably be FALSE. But if a miR has low signal (but still above background) and low p-value, it might be TRUE. Refer to the miRNA QC Tool User’s Guide for more detail.

### **In miRNA QC tool, how is the P-value calculated?**

The p-value is calculated using the 4 probe replicates for each miRNA, and the corresponding variance for each set. Detection means: probe performance/detection using an algorithm and comparing to background.

### **What is the normalization control (gi555853 probes)?**

The normalization control (gi555853 probes) is human 5.8S rRNA. The 5.8S rRNA control probe is the complement to 5.8S rRNA in the sample. If there is any human 5.8S rRNA in the sample, it will be labeled with FlashTag, and hybridize to the miRNA array. When total RNA is titrated, the average signal of these probes also titrates. The rRNA content is variable across samples, and does not necessarily imply performance issues. Signal intensity in rRNA probes is not used for QC as the signals are highly variable between sample types and experimental conditions.

**Should I be concerned if the signal intensity of 5.8S rRNA probes is lower in some arrays, compared to others?**

No, the rRNA content is variable across samples, and does not necessarily imply performance issues. Signal intensity in rRNA probes is not used for QC as the signals are highly variable between sample types and experimental conditions.

**What are some possible reasons for relatively lower 5.8S rRNA signals?**

- Less total RNA was loaded on those arrays
- Those RNA samples were a little degraded
- Those RNA samples were not human (even mouse would have lower signal intensity – homology is not perfect to human)
- Those RNA samples were significantly enriched for 22mer / microRNA, removing the 5.8S rRNA which is around a 150mer

**Should I disregard all array data for probes that are not the same species as my RNA sample?**

Most researchers eliminate the irrelevant species from analysis, because the extra data can be cumbersome. However, looking at multi-species probes may provide an increased confidence in the data. Genisphere typically observes a clustering of cross-species miRs that are identical, or nearly identical.

**How can I determine the limit of detection?**

In Partek, the limit of detection = 2 Standard Deviations over background.

In miRNA QC Tool, click 2<sup>x</sup> to subtract the background. At this point, anything above background (as defined by the project description table) is significant, if the p-value is also significant (as determined by the researcher).

**Are there any articles which describe miRNA array data analysis?**

Yes, and please share new articles with Genisphere, as you find them or publish them.

- F Sato. Intra-Platform Repeatability and Inter-Platform Comparability of microRNA microarray technology. PLoS ONE May 2009, volume 4, issue 5, e5540.
- D Sarkar. Quality Assessment and data analysis for microRNA expression arrays. Nucleic Acids Research 2009, vol. 37, no. 2, e17 (doi: 10.1093/ner/gkn932).

**Can Genisphere explain correlation of miRNA array data to miRNA QPCR data?**

QPCR is not yet the gold standard for microRNA validation. Unlike mRNA validation, in which the amplicon is already present in the sample, microRNA qPCR requires the amplicon to be synthesized by combining the sample with either a specially designed hairpin molecule, adding a 3' polyA tail, or some other manipulation of the microRNA sample. The amplicon-building process will be different from sample to sample and will result in variability in the PCR results. However, it is still very important to validate the array results with another method, like PCR. The trends in up and down regulation should match in direction, even if they do not match in magnitude.

**I'd like to check my miRNA array data with qRT-PCR. In addition to my miRs of interest, what other qRT-PCR controls should I run?**

At least 5 miRs or SnoRNAs should be used to normalize:

- RU44, RU48, and/or U6
- microRNAs that are not changed among your samples, and are at least 5X over background, according to your microarrays. These miRs might include miR 15, 16, 17, or let 7a, let 7b, let7c

All 5 (or more) of these RNAs should not show any change among the samples. Average some or all of these to get the normalization factor, and apply to your qRT-PCR data.

**How do I contact Genisphere Technical Support?**

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