

Catalog No. 11832-050

50 Reactions

Overview and Intended Use

The *Vantage™* microRNA Detection Kits offer researchers a fast and simple method for profiling the expression levels of multiple microRNAs from many different sample types including total RNA, enriched low molecular weight (LMW) RNA, and degraded RNA. The assays are configured on the xMAP® bead array allowing for the detection of multiple microRNAs in one sample. In addition, the 96-well format allows many samples to be analyzed in one run.

MicroRNAs are a class of small molecules, about 21-23 nucleotides in length that regulate gene expression by various methods including translational repression, mRNA cleavage, methylation, and deadenylation. Differences in the expression levels of microRNAs have been associated with the pathogenesis of many diseases, including cancer (ref. 1-4). By measuring the expression levels of microRNAs, researchers obtain a better understanding of the processes involved in tumor development and progression. In addition, researchers can observe distinct expression patterns associated with particular stages of disease.

The specific microRNAs detected in the *Vantage™* Pancreatic Cancer Panel 1 are given in Appendix A. These microRNAs have been associated with pancreatic tumors (ref. 5-6).

Principle of Method

The *Vantage™* microRNA Detection Kit utilizes a simple, hybridization procedure where samples are ready for detection on the Luminex® reader within 90 minutes. The samples are first labeled with multiple biotin molecules using the *Vantage™* microRNA Labeling Kit (Kit sold separately. See Cat. No. 11820-025). The biotinylated samples are incubated with a Bead Mix containing a mixture of different fluorescently dyed xMAP® beads. Each distinct xMAP® bead is coupled with a unique probe that recognizes a specific microRNA. The beads and sample are incubated at 60°C allowing the microRNAs present in the sample to hybridize to the specific probes. Following hybridization, the samples are subjected to a high stringency wash to remove any non-specific binding. Finally, the samples are incubated with streptavidin-phycoerythrin (SAPE), which binds to the biotinylated microRNA hybridized to the xMAP® bead. The samples are read on Luminex® or Luminex-based instruments (e.g. BioPlex®) that detect the specific microRNAs present in the sample by their unique bead region and quantify the microRNAs by the intensity of the SAPE signal.

Terms and Conditions

By opening this Assay Product (which contains fluorescently labeled microsphere beads authorized by Luminex Corporation) or using this Assay Product in any manner, you are consenting to be bound by the following terms and conditions. You are also agreeing that the following terms and conditions constitute a legally valid and binding contract that is enforceable against you. If you do not agree to all of the terms and conditions set forth below, you must promptly return this Assay Product for a full refund prior to using it in any manner. You, the customer, acquire the right under Luminex Corporation's patent rights, if any, to use this Assay Product or any portion of this Assay Product, including without limitation the microsphere beads contained herein, only with Luminex Corporation's laser based fluorescent under the name Luminex Instrument.

Safety and Use Statement

All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of potentially infectious or hazardous agents.

This product is authorized for laboratory research use only. The product has not been qualified or found safe and effective for any human or animal diagnostic application. Uses other than the labeled intended use may be a violation of applicable law. If you have any questions concerning the use of this product, please contact Marligen Biosciences, Inc. at (866) 464 4990 or visit www.marligen.com.

Components included with this kit:

Component	Amount
Hybridization Buffer	1.25 mL
Pancreatic Cancer Panel 1 Bead Mix	400 µL
Detection Reagent	55 µL
Wash Buffer	2 x 10 mL
SAPE Diluent	25 mL
Aluminum Plate Sealers	2 Each
Filter Plate	1 Each

Storage Conditions:

Store all components at 2-8°C.

Handling Instructions: The kit is shipped on ice packs. Upon receipt, the components should be stored at 2-8°C.

Materials and Equipment Required But Not Supplied:

Nuclease-free PCR stripwell plate or nuclease-free PCR tubes
1.5 mL RNase free microfuge tubes
Plugged micropipette tips
Nuclease-Free water (Ambion Cat. No. AM9934 or equivalent)
Microcentrifuge
Thermocycler or heating block at 60°C
Plate Shaker
Vortex Mixer
Sonicating waterbath
96-well filter plate vacuum manifold
Luminex Instrument

Optional: RNase Inhibitor (Suprase-In, Ambion Cat. No. AM2694 or equivalent)

Important Information

READ ENTIRE PROTOCOL BEFORE USE

ADDITIONAL PRECAUTIONS SHOULD BE TAKEN TO PREVENT THE DEGRADATION OF RNA:

RNases are very stable and robust enzymes that degrade RNA. Autoclaving solutions and glassware is not always sufficient to actively remove these enzymes. The first step when preparing to work with RNA is to create an RNase-free environment. The following precautions are recommended as your best defense against these enzymes.

1. The RNA area should be located away from microbiological work stations.
2. Clean, disposable gloves should be worn at all times when handling reagents, samples, pipettes, disposable tubes, etc. It is recommended that gloves are changed frequently to avoid contamination.
3. There should be designated solutions, tips, tubes, lab coats, pipettes, etc. for RNA only.
4. All RNA solutions should be prepared using at least 0.05% DEPC-treated autoclaved water or molecular biology grade nuclease-free water.
5. Clean all surfaces with commercially available RNase decontamination solutions.
6. When working with purified RNA samples, ensure that they remain on ice during downstream applications.

Assay controls

1. Control 1 (bead 49) detects 5.8S RNA that is ubiquitously expressed in mammalian cells and is selected as a house-keeping gene for the *Vartage*TM Detection Kits. A signal of 5000-10000 MFI is typically observed when using 0.5-1 µg of high quality (RIN >8) total RNA.
2. An additional assay control is available from Marligen. The *Vartage*TM MiR-plex Control (Cat. No. 11830-001) contains 7 synthetic biotinylated RNAs including 5.8S, miR-21, and miR-107 that are detected by the *Vartage*TM Pancreatic Cancer

Panel 1. Add 20 µL of the *Vartage*TM miR-plex Control into 33 µL of the Hybridization/Bead Mix at **Hybridization Step 6**, then follow protocol as described. Expected results for this assay control are shown in Appendix B.

Note: There is no need to label this sample as the MiR-plex Control is already biotinylated.

Set-up Prior to Starting Detection Protocol

1. Prepare labeled RNA. Prior to using this detection kit the microRNAs present in the samples must be labeled with biotin. To obtain optimal results, it is recommended that the *Vartage*TM microRNA Labeling Kit (Cat. No. 11820-025) is used to label samples.
2. Use 0.5-1 µg of labeled RNA per reaction. If duplicates are to be performed in the detection assay, double the amount of input RNA to be labeled and split the sample accordingly.

Notes: Less than 0.5 µg/reaction may be used for most samples. However, it is recommended that a pilot study is carried out to determine the optimal amount of labeled RNA for a particular sample type. Refer to the protocol for *Vartage*TM microRNA Labeling Kit for further details on sample labeling.

3. Set thermocycler or heating block to 60°C.
4. Warm up the Luminex or Luminex-based instrument.

Luminex Instrument Setup

A. Set up the instrument as described in the user's manual.

Setup details specific to this kit are described below:

1. The XY platform heater should be off.
2. Set the events/bead to 50.
3. Set the minimum events to 20.
4. Enter the number of samples.
5. Set the sample size to 50 µL.
6. Set the flow rate to Fast.
7. Enter the bead region numbers as indicated in the table in Appendix A.
8. Check the probe height and adjust it, if necessary, to accommodate the filter plate.
9. Perform 1 prime with sheath fluid, 1 alcohol flush, and 2 sheath fluid washes.

B. Adjust Luminex Instrument to High Gain Setting

A high gain setting for the Luminex instrument is recommended to provide the best results. Each specific software used with the Luminex or Luminex-based instrument may have different instructions for obtaining the high gain setting. Below are instructions using the Luminex 2.3TM software. Please see manufacturer's guidelines for instrument/software specific instructions (e.g. BioPlex®).

1. Create a new lot number for CAL2 and enter lot number with an HG at the end to designate High Gain.

- Record the CAL2 Calibrator target "RP1" which is usually around 3832.
- Multiply the CAL2 Calibrator target "RP1" by 4.55 to get a new target value of approximately 17,436.
- Enter the new Calibrator target "RP1" as the value for your New CAL2 lot.
- Run the CAL2 calibration.

Detection Protocol

Part I: Hybridization

During this step the microRNAs present in the sample are hybridized to their complimentary sequences on the xMAP® beads.

- Vortex the **Bead Mix** vigorously for 20 seconds.
- Sonicate the Bead Mix in a sonicating waterbath for 2 minutes.
- Prepare the **Hybridization/Bead Mix** based on the number of reactions to be run in the assay as illustrated in Table 1.

Table 1 Hybridization/Bead Mix Preparation

Component	Volume per reaction	Volume per 25 reactions	Volume per 50 reactions
Hybridization Buffer	25 µL	625 µL	1250 µL
Bead Mix	8 µL	200 µL	400 µL

- Vortex the Hybridization/Bead Mix to ensure that it is fully mixed.
- Add 33 µL of the Hybridization/Bead Mix into each well of a nuclease-free PCR stripwell plate or into each nuclease-free PCR tube.
- Transfer 20 µL of the labeled RNA sample (prepared using *Vantage™* microRNA Labeling Kit) into the 33 µL of the **Hybridization/Bead Mix** in the PCR wells or tubes, mix by pipeting up and down.

Note: If duplicates are to be performed, add 10 µL of the labeled RNA sample to the 33 µL of the Hybridization/Bead Mix in the PCR wells or tubes, bring the volume to 53 µL by adding 10 µL nuclease-free water, and mix by pipeting up and down.

IMPORTANT: As noted in the Set-up, duplicates should have double the amount of input RNA labeled with the *Vantage™* microRNA Labeling Kit.

- Hybridize the reactions at 60°C by using a thermocycler or heating block for one hour with continuous shaking at 400 rpm. Protect the reactions from light during this incubation.

Note: If shaking is not possible during this step, the MFI signals may be slightly reduced. However the overall results will not be affected.

Part II: Detection

During this step, non-specific binding is removed by subjecting the reactions to high stringency washes. The specific microRNAs present in the sample are then detected by labeling the biotins with SAPE.

Usage Notes

- IMPORTANT:** Do not allow the filter membrane to dry throughout the transfer, wash and detection steps.
- It is highly recommended to add 1 unit/µL of RNase Inhibitor to the **SAPE Diluent**.
- It is important to apply a slight vacuum of ~2-3 in Hg during all wash steps. Higher vacuum may result in the loss of beads and reduce bead count.
- During all wash steps, cover unused wells with a plate sealer to ensure a seal necessary to pull a vacuum.

A. Washes

- Pre-wet the wells in filter plate with 100 µL **Wash Buffer**.
- Transfer the hybridized reactions to each pre-wet well, cover unused wells with a plate and apply vacuum to evacuate.
- Remove vacuum and immediately add 100 µL of Wash Buffer to each well and apply vacuum to remove buffer. Repeat this wash step for total of 3 washes.

Optional: For customer convenience, all washes are performed at room temperature. As shown in Appendix B, increased assay specificity may be achieved by increasing temperature of wash buffer to 60°C. However, please note that increasing the wash temperature may reduce overall assay signal.

- Remove vacuum and add 100 µL SAPE Diluent to each well and apply vacuum to remove diluent. Remove plate from manifold.
- Blot the bottom of the filter plate dry on a clean paper towel.

B. SAPE Detection

- Prepare **SAPE Detection Reagent** as shown in Table 2.

Table 2 SAPE Detection Reagent Preparation

Component	Volume per reaction	Volume per 25 reactions	Volume per 50 reactions
Detection Reagent	1 µL	25 µL	50 µL
SAPE Diluent	100 µL	2.5 mL	5 mL

Optional: Add 1 unit of RNase Inhibitor (e.g. Superase-In, Ambion Cat. No. AM2694 or equivalent) per microliter of **SAPE Diluent**.

- Mix the SAPE Detection Reagent by vortexing.
- Add 100 µL of SAPE Detection Reagent into the washed well of the filter plate.
- Incubate the filter plate in dark for 30 minutes at room temperature.

Note: To increase MFI signal, the plate may be shaken at 400 rpm during this incubation. Protect the reactions from light.

5. Add 100 µL of SAPE Diluent to each well and apply vacuum to remove buffer. Repeat this wash step for total of 3 washes.
6. Blot the bottom of the filter plate dry on a clean paper towel.
7. Add 100 µL of SAPE Diluent into each well to resuspend the beads in the filter plate by shaking for 2 minutes at 400 rpm or by pipeting up and down.
8. Read the filter plate in Luminex instrument at high gain setting (see Luminex Instrument Set-up).

DATA Analysis

1. Use the MFI data output from the Luminex Instrument to collect the raw MFI.
2. Subtract the background MFI in negative control from the Sample MFI. Note: If after subtraction of background MFI signal is negative, regard results as zero i.e., that specific microRNA is not detectable in the sample.
3. Control 1 (bead 49) detects 5.8S RNA that is ubiquitously expressed in mammalian cells and is selected as a house-keeping microRNA gene for the *Vartage*TM Pancreatic Cancer Panel. When comparing two samples, normalize the MFI with Control 1.

$$\text{Normalization factor} = \frac{\text{MFI of Control 1 in Control Sample}}{\text{MFI of Control 1 in Tumor or Treated Sample}}$$

For example:

Control 1 in normal tissue: MFI = 20000

Control 1 in tumor tissue: MFI = 16000

$$\text{Normalization factor} = \frac{20000}{16000} = 1.25$$

4. Then multiply Tumor or Treated Sample MFI by the normalization factor 1.25.
5. The fold change in microRNA expression can be calculated by dividing the normalized MFI for the Tumor or Treated Sample by the MFI for the Control Sample. Plot results using Excel or equivalent. Note that if an MFI value is zero (or close to 0 {<10}), the microRNA is not detectable in the sample and should be considered as not expressed.
6. To determine assay precision, calculate standard deviation (SD) and assay coefficient of variation (CV). [%CV =SD/mean x 100%]. Assay CVs are typically less than 5% for technical replicates.

Technical Support

For further technical assistance please contact us at (866) 464.4990.ext.102 or technical.support@marligen.com.

Technical support and troubleshooting guides for these products can also be found on our website at www.marligen.com.

Related Products:

To see our full line of *Vartage*TM microRNA analysis products visit our website at www.marligen.com.

Limited Use License and Use Restrictions

A limited, research use only, license is conveyed to the purchaser of this product.

Trademarks

*Vartage*TM is a trademark of Marligen Biosciences, Inc. Luminex® and xMAP® are trademarks of Luminex Corporation.

References

1. Calin G.A., et al. (2004). Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc. Natl. Acad. Sci.*, 104(19) pp8017-8022.
2. Volinia S., et al. (2006). A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci.*, 103(7) pp2257-2261.
3. Blower P.E., et al. (2007). MicroRNA expression profiles for the NCI-60 cancer cell panel. *Mol. Cancer Ther.* 6pp1483-1491.
4. Gaur A., et al. (2007). Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. *Cancer Res.* 67pp2456-2468.
5. Lee, E.J. et al. (2007) Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer.*;120(5);pp1046-54.
6. Bloomston M, et al. (2007) MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* ;297(17);pp1901-8.

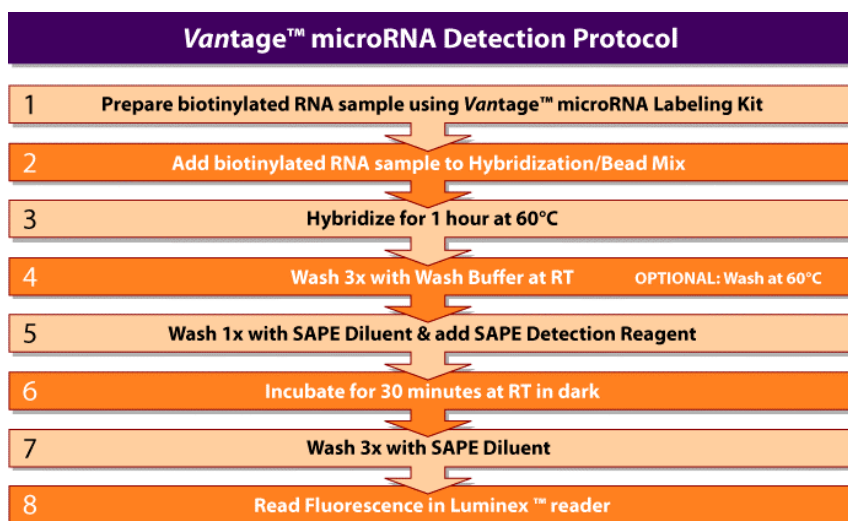
APPENDIX A: TABLE OF microRNAs AND xMAP® BEAD REGIONS

The sequences and nomenclature of the mature microRNAs are extracted from The miRBase Sequence Database version 12.0, released in September 2008, in Sanger Institute in UK. The nomenclature of the sequences detected by Vantage™ Pancreatic Cancer Panel is designated using the human sequence nomenclature. The equivalent mouse and rat sequences are indicated in the table below. For more information on species homology, please contact technical.support@marligen.com.

xMAP® Bead Number	Human microRNA Nomenclature	Human microRNA mature sequences	Equivalent Mouse (Mus musculus) Nomenclature and Percent Equivalency		Equivalent Rat (Rattus norvegicus) Nomenclature and Percent Equivalency	
5	hsa-miR-100	AACCCGUAGAUCGGAACUUGUG	mmu-miR-100	100%	rno-miR-100	100%
18	hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	mmu-miR-16	100%	rno-miR-16	100%
27	hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA	mmu-miR-21	100%	rno-miR-21	100%
47	hsa-miR-221	AGCUACAUUGUCUGCGGGUUUC	mmu-miR-221	100%	rno-miR-221	100%
50	hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA	mmu-miR-15b	100%	rno-miR-15b	100%
53	hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU	mmu-let-7d	100%	rno-let-7d	100%
56	hsa-miR-181c	AACAUUCAACCUUGUCGGUGAGU	mmu-miR-181c	100%	rno-miR-181c	100%
62	hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA	mmu-miR-107	100%	rno-miR-107	100%
65	hsa-miR-23a/b	AUCACAUUGCCAGGGAUUUCC	mmu-miR-23a/b	100%	rno-miR-23a/b	100%
72	hsa-miR-155	UUA AUGCUAAUCGUGAUAGGGGU	NA	-	NA	-
75	hsa-miR-92a	UAUUGCACUUGUCCGGCCUGU	mmu-miR-92a	95%	rno-miR-92a	95%
92	hsa-miR-181a	AACAUUCAACGUCUGCGGUGAGU	mmu-miR-181a	100%	rno-miR-181a	100%
49	5.8S control	na				

APPENDIX B: PERFORMANCE CHARACTERISTICS OF VANTAGE™ PANCREATIC CANCER PANEL

1. Protocol Overview



Note that the wash temperature at Step 4 may be increased to 60°C. By increasing the wash temperature the specificity of the assay may be increased. See **Assay Specificity** to determine if this is an appropriate modification for your assay.

2. Assay Specificity

microRNA Sequences		let-7d	miR-100	miR-107	miR-155	miR-15b	miR-16	miR-181a	miR-181c
		% Cross-Reactivity							
let-7d	AGAGGUAGUAGGUUGCAUAGUU	100.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
miR-100	AACCCGUAGAUCCGAACUUGUG	0.3%	100.0%	0.0%	1.1%	2.7%	0.1%	0.1%	0.0%
miR-107	AGCAGCAUUGUACAGGGCUAUCA	0.0%	0.0%	100.0%	0.7%	0.0%	0.0%	0.0%	0.0%
miR-155	UUA AUGCUAAUCGUGAUAGGGGU	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%	0.0%
miR-15b	UAGCAGCACAUCAUGGUUUACA	0.0%	0.0%	0.0%	0.0%	100.0%	0.0%	0.0%	0.0%
miR-16	UAGCAGCACGUAAAUAUUGGCG	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	0.5%	0.0%
miR-181a	AACAUUCAACG CUGUCGGUGAGU	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	100.0%	8.3%
miR-181c	AACAUUCAAC-CUGUCGGUGAGU	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	8.1%	100.0%

In separate reactions, 200 attomoles of synthetic biotinylated let-7a, miR-100, miR-155, miR-15b, miR-16, miR-181a, or miR-181c were incubated with the *Vantage*TM Pancreatic Cancer Panel 1 (Cat. No. 11832-050). The assay was performed using the protocol. With the exception of the closely related sequences of miR-181a and miR-181c (sequence differences indicated in red), all miR sites in the assay showed negligible cross-reactivity under standard protocol conditions. Specificity for miR-181a and miR-181c may be increased by increasing the wash temperature in Part II, Step A to 60°C.

3. Assay Sensitivity

To determine the multiplex assay sensitivity, the limit of detection was determined using synthetic microRNAs and total RNA extracted from tissues and cells.

a. LOD determined using synthetic miRs

Synthetic miRs were labeled using the *Vantage*TM microRNA Labeling Kit (11820-025) and then titrated in various *Vantage*TM Detection Kits starting from a concentration of 25 pgs. The limit of detection was calculated using 2*blank as the y intercept value.

Limit of Detection Range : 0.03-0.1 pg (5-15 attomoles)

b. LOD determined using total RNA

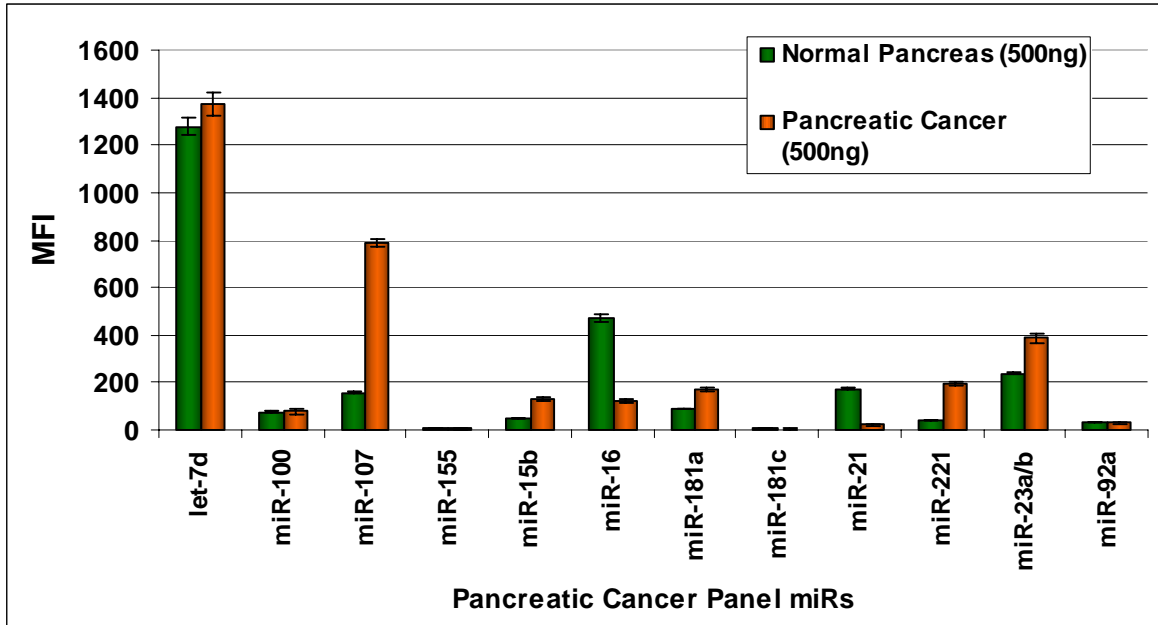
Total RNA was extracted from normal pancreatic tissue. The total RNA of each was labeled using the *Vantage*TM microRNA Labeling Kit (11820-025) and then titrated in the *Vantage*TM Pancreatic Cancer Panel starting from a concentration of 500 ngs. The limit of detection was calculated using 2*blank as the y intercept value.

microRNA	Limit of Detection (ng Total RNA)
	Normal Pancreas
5.8S	1.5
let-7d	2.4
miR-100	56.4
miR-107	23.1
miR-155	329.5
miR-15b	39.3
miR-16	7.1
miR-181a	68.3
miR-181c	1460.1
miR-21	6.4
miR-221	16.4
miR-23a/b	13.1
miR-92a	n.d.

The limit of detection in tissues and cells is dependent upon expression levels. In some cases the miRs limit of detection could not be determined in the total RNA samples because the miR was expressed at too low levels. This is indicated by n.d. in the chart. 5.8S is ubiquitously expressed in mammalian cells and tissues. The Limit of detection of 5.8S in normal pancreatic tissue is 1.5 ng total RNA.

4. MicroRNA profiles in normal versus pancreatic cancer

Total RNA was extracted from normal pancreas and pancreatic tumor tissue*. 500ng of total RNA from each extract was labeled using the *Vantage*TM microRNA Labeling Kit (11820-025) and then detected in the *Vantage*TM Pancreatic Cancer Panel 1. The MFI signals from 5.8S were used to normalize the pancreatic tumor MFIs. The results show that microRNAs are differentially expressed between normal and tumor samples.



*Note: Samples are not from matched patient samples.

5. *Vantage*TM MiR-plex Control (Cat. No. 11930-001) Expected Results

<i>Vantage</i> TM microRNA Detection Kit	Oncology Detection Panel 1 (47-plex)	Breast Cancer Panel 1 (8-plex)	Pancreatic Cancer Panel 1 (12-plex)	Ovarian Cancer Panel (8-plex)	Cardiac Panel 1 (10-plex)	Diabetes Panel 1 (11-plex)	Hypoxia Panel 1 (10-plex)
Cat. No.	11831-050	11833-050	11832-050	11834-050	11835-050	11836-050	11837-050
microRNA	Expected MFI Values						
miR-1	-	-	-	-	Low	Low	-
miR-107	-	-	Low	-	-	-	Low
miR-126	-	Low	-	-	-	-	-
miR-203	-	-	-	Low	-	-	-
miR-21	Medium	Medium	Medium	Medium	Medium	-	Medium
miR-9	High	-	-	-	-	High	-
5.8S	High	High	High	High	High	High	High