

## Summary of Non-Clinical Studies

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### Introduction

The following is a selection of the non-clinical studies performed with the Veridex GeneSearch™ BLN Assay. This summary includes the Precision, Linearity and Limit of Detection, Interfering Substances, Microbial Limits and Microbial Challenge Testing, Preservative Effectiveness, and Guardband Studies conducted. Additionally, marker selection and a discussion of relevant literature associated with the selected markers are detailed.

The GeneSearch™ BLN Assay is a real time Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) assay that detects the presence of breast tumor cell metastasis in lymph nodes through the detection of gene expression markers present in breast tissue, but not in nodal tissue (cell type specific messenger RNA). The GeneSearch™ BLN Assay is composed of:

- GeneSearch™ BLN Test Kit: contains all the reagents required for performing RT-PCR (reverse transcription and amplification) and fluorescent detection of amplicon.
- GeneSearch™ RNA Sample Preparation Kit: contains reagents and spin columns for rapid lymph node tissue homogenization and RNA purification.
- GeneSearch™ BLN Assay Protocol Software compact disk (CD): includes parameters for performing and analyzing the GeneSearch™ BLN Assay

A picture of the reagents follows.



Veridex GeneSearch™ BLN Assay

## Precision

Precision is a measurement of the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple samples of a homogeneous sample. The degree of scatter in multiple measurements of a homogeneous sample is due to several factors such as the operator performing the measurement, the lot number of the material with which the sample is measured, the day on which the measurement is taken and the variability inherent in the measurement system itself. The precision of the GeneSearch™ BLN Test Kit was determined using a protocol similar to that recommended in the Clinical Laboratory Standards Institute Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline-Second Edition (EP05-A2). Two samples and two assay controls were tested on the GeneSearch™ BLN Test Kit using three operators testing both samples and controls on three lots of GeneSearch™ BLN Test Kits each day for eight days with two runs per day with two replicates of each sample and control tested on each run.

The Ct values obtained for each applicable marker in each sample were analyzed to determine the standard deviation (average variation from the mean) of the measurements. The standard deviation is divided by the mean Ct value for each marker in each sample and multiplied by 100 to determine the percent Coefficient of Variance (%CV), or degree of scatter in the data. All results for precision are presented as %CV.

$$\%CV = (\text{Standard Deviation}/\text{Mean}) \times 100$$

The Total Precision expresses the degree of scatter in the measurements on a given sample across all operators, all days, all lots and all runs.

Total assay precision was estimated using the model provided in EP05-A2. The formula used to estimate total precision was

$$S_T = \sqrt{S_{dd}^2 + S_{rr}^2 + S_{wr}^2}$$

where ST = total standard deviation, dd = between day, rr = between run and wr = within run. The results are provided in Table 1.

**Table 1. Total Precision Excluding Lot and Operator**

<b>Total Precision (%CV) Excluding Lot and Operator (<math>S_T</math>/Mean Ct)</b>				
	<b>Positive Sample</b>	<b>Negative Sample</b>	<b>Negative Control (NC)</b>	<b>Positive Control (PC)</b>
<b>PBGD</b>	2.9%	4.3%	1.9% (3.1%) <sup>c</sup>	NA <sup>b</sup>
<b>MG</b>	3.1%	NA <sup>a</sup>	NA <sup>b</sup>	1.6 % (6.0%) <sup>c</sup>
<b>CK19</b>	1.5%	NA <sup>a</sup>	NA <sup>b</sup>	1.6% (5.6%) <sup>c</sup>

- MG and CK19 were not analyzed as these markers are not expressed at appreciable levels in a negative sample. Negative results were obtained as expected in samples not containing these markers.
- MG and CK19 are not present the Negative Control. PBGD is not present in the Positive Control. Negative results were obtained as expected in samples not containing these markers.
- Values of results from samples where control was not added (user error) to the reaction are included in the analysis.

Lot-to-lot and operator-to-operator variability were also considered in the precision study design. The formula used to estimate total precision in this case was

$$S_T = \sqrt{S_{dd}^2 + S_{rr}^2 + S_{wr}^2 + S_{op}^2 + S_{lot}^2}$$

The assay results are reproducible; the values for each sample tested are provided in Table 2.

**Table 2. Total Precision Including Lot and Operator**

<b>Total Precision (%CV) Including Lot and Operator (<math>S_T</math>/Mean Ct)</b>				
	<b>Positive Sample</b>	<b>Negative Sample</b>	<b>Negative Control (NC)</b>	<b>Positive Control (PC)</b>
<b>PBGD</b>	5.6%	5.2%	2.5% (3.4%) <sup>c</sup>	NA <sup>b</sup>
<b>MG</b>	5.5%	NA <sup>a</sup>	NA <sup>b</sup>	1.8% (6.1%) <sup>c</sup>
<b>CK19</b>	2.5%	NA <sup>a</sup>	NA <sup>b</sup>	1.9% (5.6%) <sup>c</sup>

- MG and CK19 were not analyzed as these markers are not expressed at appreciable levels in a negative sample. Negative results were obtained as expected in samples not containing these markers.
- MG and CK19 are not present the Negative Control. PBGD is not present in the Positive Control. Negative results were obtained as expected in samples not containing these markers.
- Values if results from samples where control was not added (user error) to the reaction are included in the analysis.

## Linearity and Limit of Detection

The linearity of an analytical or biological test method is its ability (with a given range) to obtain results that are directly proportional to the concentration of analyte in the sample. The linearity of the GeneSearch™ BLN Test Kit was assessed by preparing samples containing known amounts of in vitro transcript (IVT) RNA for each marker, testing these samples on the GeneSearch™ BLN Test Kit and directly comparing the Ct values obtained for each marker in each sample to the concentration of each marker in each sample by regression analysis.

Linearity is expressed by the equation of the line ( $Y=mx+b$ ) which results from plotting the Ct value of each marker (Y) versus the concentration of target ('x' expressed as the  $\log_{10}(\text{copies}/\mu\text{L})$ ) in each sample (regression analysis). The regression line expresses the best prediction of the Ct value based on the concentration. The closeness of the observed measurements to the resultant line is demonstrated by the  $R^2$  value. An  $R^2$  value of 100% indicates values that lie perfectly on the line.

A theoretical limit of detection was calculated using the equation generated by regression analysis during linearity testing. The limit of detection is defined as the number of copies of the target sequence detected at 35.9 Ct, the highest value that can be obtained with the GeneSearch™ BLN Assay thermal cycling protocol.

The initial study was completed using serial dilutions of a specific number of copies of in vitro transcript (IVT) RNA (not control plasmids) in water. [REDACTED]

the study was repeated using a specific number of copies of IVT RNA in a background of porcine lymph node RNA, as suggested. *(FDA suggested serially diluting the control plasmids for MG and CK19 into a consistent concentration of RNA from a negative lymph node instead of buffer, as it would more closely mimic a natural sample. These studies would best be performed with each target individually, as well as measuring all three targets together in each sample. By measuring each target separately and then together in a multiplex reaction, results should show the effect of the amplification of the other targets on each other. Because the PBGD would be found in a normal lymph node, performing the studies using RNA for the dilution from some other source such as non-human RNA is acceptable).* Each marker was tested alone and in combination (sample comprised of a mix of IVT RNA for all three markers) with the results detailed in Table 3 below [REDACTED]

[REDACTED]

[REDACTED]

[REDACTED] The

results are summarized in Table 3.

**Table 3: Analysis of Data from 36 Cycles of PCR**

<b>Regression Analysis for Linearity Determination of Individual IVTs in Porcine Lymph Node RNA</b>					
	<b>Equation of the Line</b> <b>Y = b-m * (log<sub>10</sub>(copies/μL))</b>	<b>p-value</b> (<0.05) <sup>a</sup>	<b>R<sup>2</sup> value</b> (>95%) <sup>a</sup>	<b>Correlation Coefficient</b>	<b>Analytical Detection Limit</b>
PBG D	PBGD Ct=44.50-3.571* log <sub>10</sub> (copies/μL)	0.000	99.7%	0.998	10 <sup>2.4</sup>
MG	MG Ct= 41.62 – 3.265* log <sub>10</sub> (copies/μL)	0.000	99.3%	0.997	10 <sup>1.8</sup>
CK1 9	CK19 Ct = 43.62 – 3.559* log <sub>10</sub> (copies/μL)	0.000	99.9%	0.999	10 <sup>2.2</sup>
<b>Regression Analysis for Linearity Determination of IVTs (Mix of all 3 Markers) in Porcine Lymph Node RNA</b>					
	<b>Equation of the Line</b> <b>Y = b-m * (log<sub>10</sub>(copies/μL))</b>	<b>p-value</b> (<0.05) <sup>a</sup>	<b>R<sup>2</sup> value</b> (>95%) <sup>a</sup>	<b>Correlation Coefficient</b>	<b>Analytical Detection Limit</b>
PBG D	PBGD Ct=43.58-3.419* log <sub>10</sub> (copies/μL)	0.000	99.5%	0.997	10 <sup>2.3</sup>
MG	MG Ct= 41.7 – 3.269* log <sub>10</sub> (copies/μL)	0.000	99.4%	0.997	10 <sup>1.8</sup>
CK1 9	CK19 Ct = 43.38 – 3.521* log <sub>10</sub> (copies/μL)	0.000	99.9%	0.999	10 <sup>2.1</sup>

<sup>a</sup>Acceptance Criteria: p-value<0.05 and R<sup>2</sup> value >95%

**Individual Marker Sample Results:**

**PBGD Ct Values in PBGD-only sample:** The diluent-only samples were all negative for all markers as expected. All samples below 10<sup>3</sup> copies/μL had cycle threshold values of 36 (maximum number of cycles run for this study) so linearity was assessed between 10<sup>8</sup> to 10<sup>3</sup> copies/μL.

**MG Ct Values in MG-only sample:** The diluent-only samples were all negative for all markers as expected. All samples below 10<sup>2</sup> copies/μL had cycle threshold values of greater than 36, so linearity was assessed between 10<sup>8</sup> to 10<sup>2</sup> copies/μL.

**CK19 Ct Values in CK19-only sample:** All samples below 10<sup>3</sup> copies/μL had cycle threshold values of greater than 36, so linearity was assessed between 10<sup>8</sup> to 10<sup>3</sup> copies/μL.

The p-values of 0.000 indicate that there is correlation between RNA concentration and Ct value, as expected. The R<sup>2</sup> value was greater than 99% indicating that there is a relationship between RNA concentration and marker Ct value at concentrations ranging from 10<sup>8</sup>-10<sup>3</sup> copies/μL for each marker (see Table 3).

**Mix Sample Results:**

The following results were obtained from a sample composed of IVT RNA for each marker combined to a final concentration of 10<sup>8</sup> copies/μL for each marker diluted in porcine lymph node RNA.

**PBGD Ct Values in Mix sample:** The diluent-only samples were all negative as expected. All samples below  $10^3$  copies/ $\mu$ L had cycle threshold values of greater than 36 so linearity was assessed between  $10^8$  to  $10^3$  copies/ $\mu$ L.

**MG Ct Values in Mix sample:** The diluent-only samples were all negative as expected. All samples below  $10^2$  copies/ $\mu$ L had cycle threshold values of greater than 36 so linearity was assessed between  $10^8$  to  $10^2$  copies/ $\mu$ L.

**CK19 Ct Values in Mix sample:** All samples below  $10^3$  copies/ $\mu$ L had cycle threshold values of greater than 36, so linearity was assessed between  $10^8$  to  $10^3$  copies/ $\mu$ L.

The p-values of 0.000 for all markers indicate that there is correlation between RNA concentration and Ct value, as expected. The  $R^2$  values of greater than 99% for all markers indicate that there is a relationship between RNA concentration and marker Ct value at the concentrations discussed above.

**Conclusions:** The Ct values obtained from the BLN assay are linear following 36 cycles of PCR for RNA concentrations between  $10^8$ - $10^3$  copies/ $\mu$ L for PBGD and CK19 both individually and when combined together. For MG the range of linear response is  $10^8$ - $10^2$  copies/ $\mu$ L. The results obtained with IVT individually and in combination are very similar.

## **Interfering Substances Studies**

Interfering substances were evaluated in order to identify potential limitations to GeneSearch™ BLN Assay performance. The Product Risk Analysis was the primary source used to identify potential interfering substances to be evaluated. The GeneSearch™ BLN Test Kit Instructions for Use and the Design Input Requirements were also reviewed as a gap analysis of the parameters chosen for the evaluations.

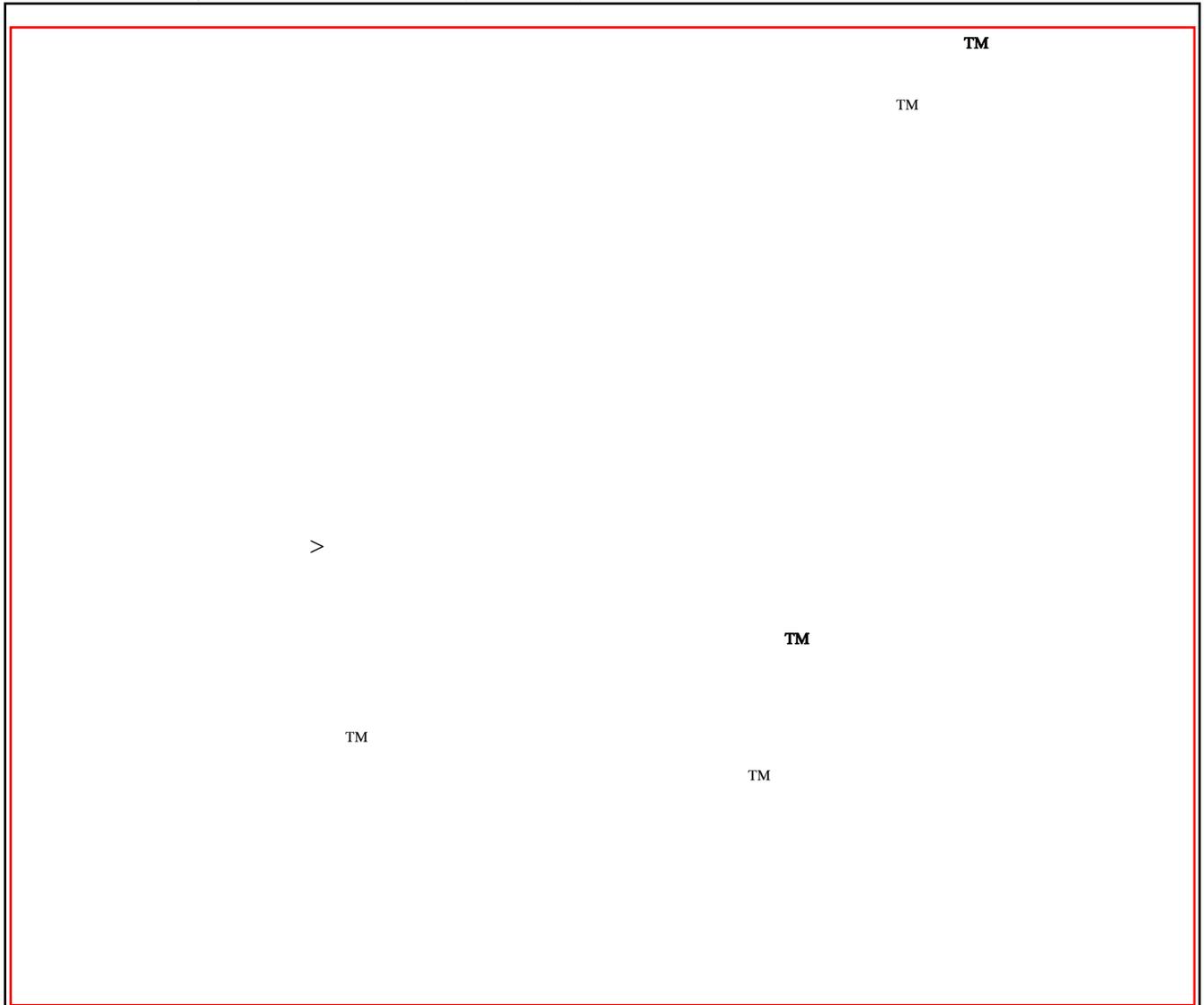
The following potentially interfering substances were evaluated: fat, lymphoma, fixed tissue (formalin fixed and frozen tissue), bleach, breast cells, tissue marking dyes, blood, tracing dyes and the pooling of nodes.

Results of the studies follow:

- A mixture of fat and positive lymph node tissue were processed at different ratios (Fat:Positive Lymph Node 100:0, 75:25, 50:50, 25:75 and 0:100). Fat was found to have an effect on the Assay Result when present in a higher amount than the positive lymph node sample in the mixture (25:75). Fat, if present, should be trimmed from tissue sections being used in the assay to avoid erroneous results. This suggestion is clinically practical and was done during the pivotal clinical trial.
- Negative lymph nodes were obtained from patients diagnosed with lymphoma and tested using the GeneSearch™ BLN Assay. A false positive result was observed with most of these lymph nodes. Patients diagnosed with any other type of cancer such as lymphoma may not be good

candidates for the GeneSearch™ BLN Assay, as lymph nodes from these patients may generate a false positive result for the assay.

- Fixation of lymph nodes with formalin was found to interfere with assay results. No result was obtained from any tissue fixed with formalin. Non-fixed tissue must be used with the assay.
- Contamination of the reagents with bleach will affect assay results and should be avoided.
- GeneSearch™ BLN Assay performance was analyzed in the presence of blood, tissue marking dyes, tracing dyes and technitium99 (Tc99). Assay performance was not affected by the presence of any of these materials.
- Primary tumor tissue was found to interfere with assay results. Contamination with primary tumor may cause false positive or invalid test results.
- Contamination with breast tissue may cause a false positive result.
- Pooling nodes can decrease assay sensitivity and should be avoided.



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[Redacted Title]					
Process Step	Objective	Parameter Tested	Sample Type Utilized	Result	Mitigation

[Redacted Title]					
Process Step	Objective	Parameter Tested	Sample Type Utilized	Result	Mitigation
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[Redacted Title]					
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Process Step	Objective	Parameter Tested	Sample Type Tested	Result	Mitigation
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[Redacted]					
Process Step	Objective	Parameter Tested	Sample Type Tested	Result	Mitigation
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[Redacted Header]					
Process Step	Objective	Parameter Tested	Sample Type Tested	Result	Mitigation
	®				

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