STATISTICAL ANALYSIS PLAN (SAP)

FOR NON-INTERVENTIONAL STUDIES

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Non-Interventional Study Protocol
Protocol N° X9001083

Non-Interventional Multi-Center Study for Molecular Diagnostic Technologies Validation in NSCLC Patients

Statistical Analysis Plan (SAP)

Version: 2

Author: CEMP - Pfizer Chile

Date: 12- Jul-2019

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1 AMENDMENTS FROM PREVIOUS VERSION(S)

Version	Effective Date	Change Type (New, Revise, Admin)	Summary of Revisions
2.0	10-Jul-2019	Admin	New version signed to recover e-copy. Minor text revision included.
1.0	15-May-2018	Admin	Document signed and attached appendix 3 to 8.
1.0	05-Sep-2016	New	New Document.

2 INTRODUCTION

Note: in this document any text taken directly from the Non-Interventional (NI) study protocol is ***italicised***.

Non-small cell lung cancer (NSCLC) is a common cause of cancer mortality throughout the world. In 2007, there were 1.5 million new lung cancer cases diagnosed worldwide, including around 733,100 cases in the South American Region.6

Approximately 85% of lung cancer is histologically defined as non small cell and the remaining 14% as small cell. The majority of patients with NSCLC present with inoperable locally advanced (Stage IIIB) or metastatic (Stage IV) disease for which no curative treatment is yet available. 7, 8, 9, 10, 11, 12

The rapid and efficient identification of key driver genes in non-small-cell lung cancer (NSCLC) is becoming increasingly important.17 Clinical screening efforts have revealed that the most common mutations in lung cancer specimens involve EGFR and KRAS, along with 10 other genes that show a prevalence of mutation in 5% or less of tumors. The ALK gene is rearranged in around 3%-5% of patients with NSCLC and has been the focus of intense basic and clinical research, suggesting that the frequency of the gene rearrangement is similar in Asian and Western patients.

ROS1 is a receptor tyrosine kinase of the insulin receptor family. Chromosomal rearrangements involving the ROS1 gene were originally described in.16 More recently, ROS1 fusions were identified as potential driver mutations in an NSCLC cell line (HCC78; SLC34A2-ROS1) and an NSCLC patient sample (CD74-ROS1).18 These fusions led to constitutive kinase activity and were associated with sensitivity in vitro and in vivo to crizotinib.

The identification of these and other genes has challenged the current state of the art in molecular pathology from single genes test to multiplexed assays. The Ion Torrent Oncomine™ Focus Assay is a multi-biomarker next generation sequencing designed for nucleic acid extracted from Formalin Fixed Paraffin Embedded (FFPE) tissue. The assay detects multiples types of variants in 52 solid tumor genes analyzing simultaneously DNA and RNA.

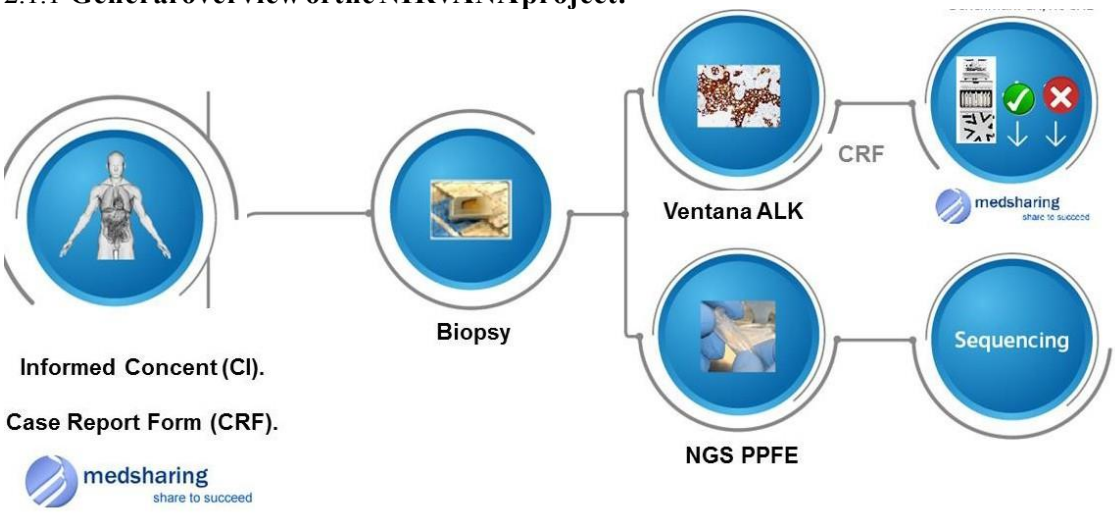
The methods describe here apply to Nº X9001083 clinical study protocol Non-Interventional Multi-Center Study for Molecular Diagnostic Technologies Validation in NSCLC Patients.

The objective of this study is to determine concordance between the Vysis ALK Break Apart FISH test or the Ventana ALK IHC test and Thermofisher NGS technology
This statistical analysis plan (SAP) is, in general, consistent with the Statistical Methodology section of NIRVANA Protocol and includes additional details of data summaries and data presentations to be presented in the clinical study report (CSR). Details of the changes in statistical methods between the protocol and the SAP are provided in Section.

2.1 STUDY DESIGN

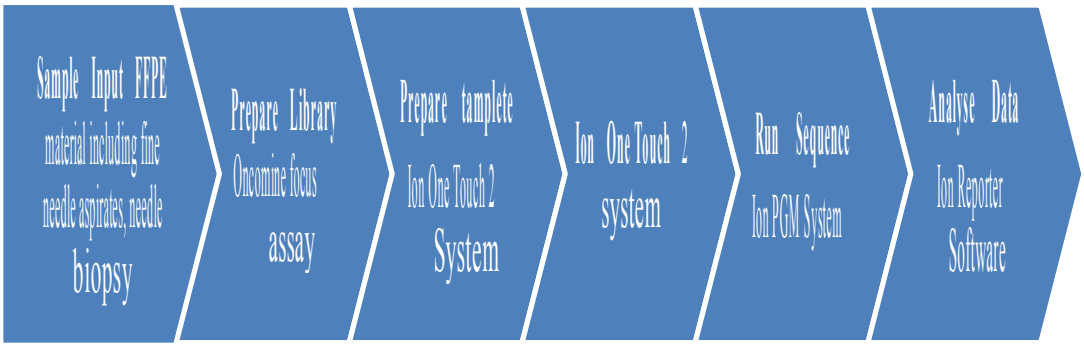
This is a non-interventional study. This study NOT designated as a Post- Authorizations Safety Study (PASS).

2.1.1 General overview of the NIRVANA project.



This protocol use a primary source FFPE biopsies and clinical records obtained from patients recruited in clinical sites distributed in Chile, Brazil, and Peru. The FFPE is used to evaluate the ALK biomarker using either Vysis ALK Break Apart FISH test or the Ventana ALK IHC test and to perform the Next generation sequencing in nucleotides prepared from NSCLC FFPE blocks.

2.1.2 General overview of NGS test.



Prior to performing the NGS Test, the FFPE clinical specimens are prepared for pathology review. A microtome is used to prepare 5µm FFPE sections. A 5µm FFPE section is stained using a standard hematoxylin and eosin (H&E) protocol. Stained tissue sections are visually examined by a pathologist for confirmation of tumor content area, tumor content per tissue section (TC), region(s) of interest (ROI), ROI tumor content (ROI TC), and percent necrosis are all recorded on the case report form (as described in Protocol Nº X9001083). If tumor content is greater than or equal to 20%, the tissue sections will be extracted. If tumor content is less than 20%, and ROI is greater than or equal to 10%, tumor areas will be macrodissected and enriched for tumor, in preparation for the extraction protocol.

Two sections will be extracted in order to meet minimum tissue input requirements. The tissue (with or without macrodissection) is taken through sample processing to isolate gDNA and total cell RNA. cDNA prepared from Total RNA is

The prepared DNA or cDNA sample is made into a library of amplicons using the Ion PGM Library Kit. Each sample is given a unique, identifying sequence by the addition of a nucleic acid barcode adapter. Using these barcode adapters up to 6 specimens (1 DNA library and 1 RNA library from each sample) each with its own unique barcode can be templated and sequenced in the same system run with DNA, RNA and 2 NTC Controls.

The libraries are templated onto Ion Sphere Particles (ISP, which are proprietary beads) using the Ion OneTouch Template Kit and OneTouch instrument. Templated beads are enriched from un-templated beads on the OneTouch ES, and loaded onto the 318 chip with reagents to allow the sequencing reactions to take place.

The Ion PGM Sequencer uses the reagents provided in the Ion PGM Sequencing Kit to detect nucleotide incorporation on the chip. The signal generated by the sequencing reaction on the 318 chip is translated into base calls and then reads. The DNA reads are 'mapped' to the reference human genome (hg19) followed by detection of SNVs, Indels, and CNVs.

The RNA reads are ‘mapped’ to a reference containing control sequences and candidate gene fusion sequences. Gene fusions are detected as present if they map to these sequences and pass certain filtering criteria.

The end result of this workflow is a set of variant calls that correspond to the original sample. The Ion PGM Torrent Suite software will call out any variation between the Reproducibility Sample member sequence at the locations designated in the Hot Spot file. This process is executed using the Ion PGM Torrent Suite software which runs on the Ion Torrent Server. The Ion PGM Torrent Suite software manages the complete end-to-end workflow from sample to variant call.

The clinical data will be recorded in an electronic case report form (eCRF) managed digitally in the platform Medsharing. This system allows tracking and controlling the clinical

information associated to the specimen. It is based in an online interface that allows the centralized management of multiple clinical sites.

Study population

The study population includes all patients who satisfy all inclusion and do not present any of the exclusion criteria detailed in the protocol.

Inclusion Criteria (according with amendment 2, 23 November 2017)

1. *Men or women 18 years of age or older.*
2. *Patients with a diagnosis of non-small cell carcinoma (NSCLC) histologically or cytologically tested.*
3. *When a blood sample is donated, the individual must have an active tumor, i.e. the patient has a tumor in the moment of blood collection. The diagnosed is based on image test i.e.: X- RAY, Ct SCAN, RMN, PET Scan and among other, and biopsy (liquid or standard).*
4. *Informed consent form, signed and dated, stating that the patient (or the legal representative) has been informed of all relevant aspects of the clinical study before being incorporated into the study, when appropriate.*
5. *Patients must give consent for the research use of their tissues on file or fresh or 2 tubes of blood if available, or both.*

Exclusion criteria

Patients who meet some of the following criteria will not be included in the study:

1. *Previous chemotherapeutic treatment in the last six weeks before taking the sample (tumor tissue or blood or both)*
2. *Bone biopsy of non-small cell carcinoma (NSCLC)*
3. *Biopsy taken no longer than two years old*

The information collected in this will be related exclusively to the period time of diagnose of the patient.

Data source

The data sources for this study include:

1) *Medical records AND Patient interviews information storaged in Medsharing server.*
2) *Molecular profiling obtained from NGS test, storage in the CEMP servers.*

Treatment/cohort labels

No treatments are applied in this study. However, patients will be grouped base on their type of cancer, age, Ventana ALK test results and other variables described in their medical records. The objective of grouping the patients is to obtain the epidemiologic characteristics of NSCLC in the South American population. In particular the classification of patients between Ventana ALK positive vs negative will be used to make the statistical analysis to assess the agreement between NGS and Ventana ALK.

2.2 STUDY OBJECTIVES

1. To determine prevalence rate for ALK and ROS1 gene rearrangement in NSCLC patients from Latin America.
2. To determine concordance between the Ventana ALK IHC test and Thermo Fisher NGS technology.
3. To correlate the clinical data and the results from Ventana ALK and molecular diagnostics.
4. To evaluate the use of less invasive source of genomic material for the molecular classification of patients with NSCLC.

3 INTERIM ANALYSES

Given an estimated prevalence of ROS1 at 1-2% and ALK at 3-5% for NSCLC, an initial set of 1000 patients will be screened. An interim analysis will be performed at the first 1000 NSCLC samples to evaluate prevalence rates.

The first interim analysis will be carried out for the purpose of monitoring the overall progress of the study and to define the sample size of the study.

We expect that after the interim analysis changes in the final total number of recruited patient occur.

If anticipated prevalence rates do not align with determined prevalence rates at this stage, an additional 2000 patients will be screened for a potential total of 3000 patients.

A subsequent interim analysis will be performed at the 3000 NSCLC samples to evaluate prevalence rates. The main objective of this interim analysis is to determine de number of total recruited patients needed to get the statistical power.

4 HYPOTHESES AND DECISION RULES

4.1 STATISTICAL HYPOTHESES

This study have several aims however just the aim 2 has a tested hypothesis.

a. Null Hypothesis (H_0) :

H_0 : "The results of the detection of ALK gene re-arrangements obtained using NGS are not equal to those obtained from using Ventana®".

b. Alternative Hypothesis (H_1) :

H_1 : "The results of the detection of ALK gene re-arrangements obtained using NGS are equal to those obtained from using Ventana®".

4.2 STATISTICAL DECISION RULES

In case of multiple testing P values thresholds will be computed with Benjamini- Hoshberg criterion and FDR≤0.05. For other cases P value ≤0.05

5 ANALYSIS SETS/ POPULATIONS

The study population includes all patients who satisfy all inclusion and exclusion criteria.
To test the hypothesis the results in NGS for ALK will be compared with those obtained in patients that present a positive results for Ventana®-ALK. Those NSCLC patients that are negative for Ventana®-ALK will be used to stablish the levels of false positive. In general the level of concordance between both methods will be calculated.

5.1 FULL ANALYSIS SET

The final study population will include any NSCLC patients who satisfy all inclusion and exclusion criteria.

5.2 SAFETY ANALYSIS SET
Not Applicable

5.3 OTHER ANALYSIS SET
Not Applicable

5.4 SUBGROUPS
- The patients will be considered **RETROSPECTIVE** in those whose sample was taken before the date of signature of the IC. Meanwhile, patients whose samples were taken after the IC will be called **PROSPECTIVE**

- Based on their smoking habits, patients will be grouped into smoker or no-smokers.
- Subgroups will be considered depending on the characteristics of the samples concerning the location of the tumors, their stage and the histological classification of the biopsy.
- Different subgroups will be considered according to their gender (female/male) and different age ranges (18-30 / 31-40 / 41-60 / 60 and more).

6 ENDPOINTS AND COVARIATES

a. Primary Endpoint.

- **Primary endpoint 1**: Obtaining results / diagnosis of NGS and Ventana for the ALK biomarker.
- **Primary endpoint 1**: Analysis of NGS y Ventana for ALK.

b. Secondary Endpoints

Secondary endpoint 1: PE1 in prospective and retrospective patients.
Secondary endpoint 2: PE1 in smokers and non-smokers.
Secondary endpoint 3: PE1 depending on the location, stage and histological classification of the biopsy
Secondary endpoint 4: PE1 in patients of different gender and age.

6.1 EFFICACY/ EFFECTIVENESS ENDPOINT(S)
Not Applicable

6.2 SAFETY ENDPOINTS

Since NIRVANA X9001083 is a NI study without study drug and randomization procedures we are using the Adverse Event Report Form Completion Guide, for a Therapeutic Areas, For Investigators Conducting Pfizer-Sponsored Non-Interventional Clinical Studies, For Protocols with Stipulated Active Collection of Adverse Events (Including Pragmatic Clinical Studies [Non-Medicinal Intervention]) according CT24-GSOP-SD-GL25 version 4.0, effective date 01-Aug-2016.

6.3 OTHER ENDPOINTS

Establish the incidence of ROS1 alterations in South American populations
Assessment of inter-laboratory reliability of NGS methods to realized molecular diagnostics in cancer patients.

6.4 COVARIATES

a. Patient Strata.

The strata of the patient will be reported as a frequency count. The categories are Prospective Patients and Patients Retrospective. Smoker history

The smoking history will be reported as a categorical variable. The categories of smoker to consider are the following:

1. Never Smoker: No smoking exposure

2. Current Smoker: Currently uses tobacco in either cigarette, cigar or similar method (tobacco chewers excluded)

3. Former Smoker: Documented that the patient at one time smoked but then later quit. Covers instances where patients start smoking and then quit smoking multiple times. If a patient is a current smoker at the time of the study it will be considered as a “current smoker”

4. Smoking status unknown: For any patient that has no smoking status done.

b. Stage at diagnosis:

Stage will define at time of initial diagnosis of NSCLC and patients were staged according to the guidelines set by the NCCN version 7.2015. Patient stage will be categorized as:

1. STAGE O
2. STAGE 1A
3. STAGE 1B
4. STAGE 2A
5. STAGE 2B
6. STAGE 3A
7. STAGE 3B
8. STAGE 4

7 HANDLING OF MISSING VALUES

- **Clinical Data**:
  - Retrospective patients without information whether or not Smoker -> Patient is eliminated from the secondary analysis (of the smokers' subgroup).
  - Patients without PI data (the PI did not provide the information) -> Patient is removed from the secondary analysis (of the subset)
  - Patient without ALK data -> Patient is eliminated from primary analysis.

- **Sequencing:**
  - Insufficient sample: If at least 20 ug of DNA and 20 ug RNA or both are not obtained, the sample is eliminated from the primary analysis. The data will be used to establish pre-analytical QC parametric.
  - Samples within the sequencing flow: if BAM files have not been generated, the sample is eliminated from the primary analysis. For the final analysis just samples with BAM files will use to perform the genomic analysis, those samples without BAM files but that are not within the sequencing flow will be considered for the QC analytics.

- **Bioinformatics:**
  - Sample without cellularity, 100% cellularity will be assumed; the missing data will be recorded, the sample is included in all analyzes, except those where this variable is considered for QC analytics.
  - Sample without gender, UNK gender will be assumed (The team in case of UNK assumes as MALE), the missing data will be recorded, the sample is included in all analyzes. Except those where the subgroup is used for analysis.
  - DNA sample fails in QC parameters, it is eliminated from DNA dependent analysis, but it is included in primary analysis
  - RNA sample fails in QC parameters, primary analysis is eliminated.
- ** There are special cases where the primary analysis could be answered with defective samples, should it continue? Remove from the primary analysis, but group in special cases to analyze later.

8 STATISTICAL METHODOLOGY AND STATISTICAL ANALYSES

8.1 STATISTICAL METHODS

a. Sample Size Calculation:

The sample size can be estimated according to a desired sensitivity or specificity, and the choice of the N obtained will be defined by privileging to maximize the sensitivity (cut-off value defined> 0.8). The sample size necessary to demonstrate hypothesis will be defined. This calculation will be made at each of the interim analysis, to evaluate the total number of patients to be recruited. The sample size calculation formulas are defined

Formula of the sample size calculation dependent on Sensitivity

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[redacted]

$$N(sN)=\frac{TP+FN}{P}$$

Where,

$$TP+FN=z^2\times\frac{(SN(1-SN))}{W^2}$$

TP : Prevalence of ALK rearrangements in the population and / or in the data set of the interim analysis.

SN : Minimal acceptable sensitivity

W : Desired confidence interval, also called α (here a 99 % is desired)

z : Constant dependent on the desired confidence interval, this value is obtained from the table of the normal distribution. For a 95 % confidence interval the value of z is 1.96, and for a range of 99 %, it is 2.58.

i. Formula of the calculation of the sample size dependent on the Specificity:

$$N(sP)=\frac{FP+TN}{(1-P)}$$

Where,

$$FP+TN=z^2\times\frac{(SP(1-SP))}{W^2}$$

P : Prevalence of ALK rearrangements in the population and / or in the data set of the interim analysis.

SP : Minimal acceptable specificity

W : Desired confidence interval, also called α (here a 99 % is desired)

z : Constant dependent on the desired confidence interval, this value is obtained from the table of the normal distribution. For a 95 % confidence interval the value of z is 1.96, and for a range of 99 %, it is 2.58

b. Concordance test:

i. Test type:

The agreement between Ventana ALK and NGS assay for ALK will be evaluated based on the Binary Qualitative type (Two tails), where the results can only have two values (concordance, discordance). The following metrics will be evaluated from the results:

- $\underline{Sensitivity}: \frac{TP}{(TP+FN)}$
- $Especificity: \frac{TN}{(TN+FP)}$

- $Presision: PPV = \frac{TP}{(TP+FP)}$
- $Accuracy: \frac{TP+TN}{TP+TN+FP+FN}$
- Limit of detection (LOD): They will be established a priori by the reference values delivered by Thermo Fisher. After the interim analysis, these will be re-evaluated and / or modified. Among the influential values in the limits of detection are: the coverage

ii. Concordance methods:

Each of the following methods could be affected differently by the proportion of positive and negative cases; therefore both methods will be used.

Simple Agreement: Simple agreement will be calculated as the concordance proportion for the positive cases (PPA), and the concordance proportion for the negative cases (NPA).

Specific Agreement:

To stablish the specific agreement we will use two methods **McNemar's Chi-Square** and **Cohen's Kappa**. The objective is to stablish the statistical significance (P-value) of the correlation between the results obtained for ALK using NGS y Ventana®. However, previous investigation stablish that these methods tend to reject the hypothesis H0.

- **McNemar's Chi-Square:** This method is easier to calculate and more precise when the discordance is small compared with the concordance agreement**.**

For the following variables:

	Test 2 Positive	**Test 2 Negative**	**Totals**
Test 1 Positive	a	b	$n_1=a+b$
Test 1 Negative	c	d	$n_2=c+d$
Totals	$m_1=a+c$	$m_2=b+d$	$N=n_1+n_2$

Where, the null hypothesis is H_0: $p_b=p_c$ and the alternative hypothesis is H_1: $p_b \neq p_c$

The McNemar's test statistic with Yates' continuity correction is:

$X^2 = \frac{(|b-C|-1)^2}{b+c}$, with degree of freedom =1

Under the null hypothesis, with a sufficiently large number of discordant (cells b and c), X^2 has a chi-squared distribution with 1 degree of freedom. If the X^2 result is significant, this provides sufficient evidence to reject the null hypothesis, in favour of the alternative hypothesis that $p_b \neq p_c$, which would mean that the marginal proportions are significantly different from each other. If either b or c is small (b + c < 25) then X^2 is not well-approximated by the chi-squared distribution and a modification at this method will be required.

Cohen's Kappa: Kappa measures the percentage of data values in the main diagonal of the table and then adjusts these values for the amount of agreement that could be expected due to chance alone.

For the following variables:

	Test 2 Positive	**Test 2 Negative**	**Totals**
Test 1 Positive	a	b	n_1=a+b
Test 1 Negative	c	d	n_2=c+d
Totals	m_1=a+c	m_2=b+d	N=n_1+n_2

The table below contains cell probabilities (P) of each type of results in 2 by 2 table.

	Test 2 Positive	**Test 2 Negative**	**Totals**
Test 1 Positive	P_a	P_b	
Test 1 Negative	P_c	P_d	
	$P_{(a+c)}$	$P_{(b+d)}$	1

To calculate Kappa, first is necessary to calculate the observed level of agreement $P0 = P_a + P_d$

P0 is then compared to the value obtained if both methods were totally independent $Pe = (P_{(a+c)})^2 + (P_{(b+cd)})^2$

$$K = \frac{((P_0 - P_e))}{((1 - P_e))}$$

8.2 STATISTICAL ANALYSES

This study plan to perform the following statistical analysis:

1- Prevalence de ALK and ROS1 mutation
2- Concordance between Ventana ALK and NGS ALK assay.

8.2.1 Safety Analyses

Not Applicable

8.2.2 Analyses of endpoint

Not Applicable

[redacted]

[redacted]

9 LIST OF TABLES AND TABLE SHELLS

10 REFERENCES

[redacted]

[redacted]

11 APPENDICES

All the decision made base on the results of each interim analysis will be included as appendices

11.1 APPENDIX 1: DATA DERIVATION DETAILS

NIRVANA protocol deviations will be documented on a Protocol Deviation Form customized for CEMP protocol. Deviations from the protocol should be discussed immediately with the Study Clinician, Dr. PPD who will determine if the deviation affects the safety of the operator or the validity of the study data. Deviations will be documented in the study report.

11.2 APPENDIX 2: ADDITIONAL STATISTICAL METHODOLOGY DETAILS

Not Applicable

A2.1 Further Details of the Statistical Methods
Not Applicable

11.3 11.3 APPENDIX 3: SUMMARY_UPDATE_20171017.DOCX
11.4 11.4 APPENDIX 4: NIRVANA SAMPLE SIZE RE-ASSESSMENT – SEPTEMBER 23RD, 2017.DOCX
11.5 11.5 APPENDIX 5: NIRVANA SAMPLE SIZE RE-ASSESSMENT – AUGUST 2017.XLSX
11.6 11.6 APPENDIX 6: SUMMARY_UPDATE_20180131.DOCX
11.7 11.7 APPENDIX 7: NIRVANA SAMPLE SIZE RE-ASSESSMENT – JANUARY 31ST, 2018.DOCX
11.8 11.8 APPENDIX 8: NIRVANA SAMPLE SIZE RE-ASSESSMENT – JANUARY 2018.XLSX

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12 SIGNATURE REGISTRATION

Author PPD Clinical Data & Bioinformatics 8	PPD	10 Jul 2019 16:08:046-0400
Reviewed by PPD Scientific Project Manager	PPD	10 Jul 2019 16:01:056-0400
Approved by PPD Senior Researcher	PPD	10 Jul 2019 16:05:000-0400

[redacted]

[redacted]

MSB V3.12

Validation Report as of: 10 Jul 2019 16:09:032-0400

1

Subject CN	MSB
Subject DN	EMAILADDRESS=operations@msbdocs.com,CN=MSB,OU=MSB,O=TAIGLE LLC,L=Irvine,ST=California,C=US
Email	operations@msbdocs.com
Serial #	103155442024134641897105422308128156249
Issuer DN	CN=Entrust Class 3 Client CA - SHA256,OU=(c) 2015 Entrust, Inc. - for authorized use only,OU=See www.entrust.net/legal-terms,O=Entrust, Inc.,C=US
Signing Time	10 Jul 2019 16:08:046-0400

The Certificate chain was successfully built to a Trusted Root Certificate.

The Signer's identity is valid.

Audit Trail Report

Date	PPD	Document	Action	Details
10 Jul 2019 16:00:033-0400			Started	The custodian composed the ePak succesfully. User UUID : a87e3eea-4baf-4d27-aa0a-2ca18a0af244
10 Jul 2019 16:00:034-0400			RequestSent	Sign request sent to ePak recipient. User UUID : 88d8a066-d960-467a-9ded-10684f5df214
10 Jul 2019 16:00:034-0400			RequestSent	Sign request sent to ePak recipient. User UUID : a87e3eea-4baf-4d27-aa0a-2ca18a0af244
10 Jul 2019 16:00:034-0400			RequestSent	Sign request sent to ePak recipient. User UUID : 36081703-c94b-4281-98ef-01b11b4bef50
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