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## **16.5 BIOANALYSIS REPORTS**

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ABS\_09\_19 Nicotine Final Report

ABS\_10\_19 Met Salicylate Final Report



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## **Analytical Report**

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**Study Number: ABS/09/19**

**Determination of Nicotine in Human Plasma Samples from Swedish Match Clinical  
Protocol No. SM 18-01**

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**Sponsor:** Swedish Match  
SE-Box 17037  
104 62 Stockholm,  
Sweden

**Sponsor's Clinical Protocol Number:** SM 18-01, Final 3.0 16Jan2019

**Report Issue Date:** 17 May 2019

**Title:** Determination of Nicotine in Human Plasma Samples from Swedish Match Clinical Protocol No. SM 18-01

**ABS Report No.:** ABS/09/19

**ABS Study No.:** ABS/09/19

**Electronic filename:** ABS\_09\_19 Report Draft (1)

**Sponsor Study No:** SM 18-01, Final 3.0 16Jan2019

**Analytical Laboratory:** ABS Laboratories Ltd  
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**Sponsor:** Swedish Match  
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**Clinical Study Site:** CTC Clinical Trial Consultants AB  
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75237 Sweden

**Study Director:** (b) (4), (b) (6)

**Bioanalyst(s):**

**Report Author:**

**Sponsor's Study Monitor:**

**Experimental Phase Began:** 11 February 2019

**Experimental Phase Ended:** 14 March 2019

**No. of samples analysed:** 2347


### STUDY DIRECTOR'S STATEMENT

This study was conducted to the standards described in the United Kingdom Good Laboratory Practice Regulations (SI 1999 No. 3106 as amended SI 2004 No. 994) and the OECD Principles of Good Laboratory Practice 1997 (ENV/MC/CHEM(98)17).

This study was also conducted in compliance with the United Kingdom "The Medicines for Human Use (Clinical Trials) Regulations", (SI 2004 No. 1031 and subsequent amendments).

I declare that this report fully reflects the raw data generated during this study.

(b) (4), (b) (6)



Date: 17 May 2019



**QA STATEMENT**

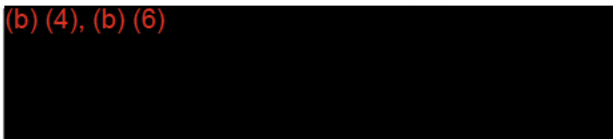
QA personnel have examined the raw data related to the analysis of the samples from study protocol number ABS/09/19 and their findings are detailed in ABS/09/19 QA-01 to 04. Their findings were reported to the Study Director and management on the following dates:

Dates of Audit	Date findings reported to the Study Director	Date findings reported to Management	Audit description
07-Feb-2019	07-Feb-2019	07-Feb-2019	Review of draft analytical protocol
07-Feb-2019	07-Feb-2019		Review of final analytical protocol
14-Feb-2019	14-Feb-2019	14-Feb-2019	Experimental audit sample preparation and result processing.
27-Mar-2019	27-Mar-2019	03-May-2019	Review of raw data to final results
25-Apr-2019	25-Apr-2019	03-May-2019	Review of draft report to raw data
16-May-2019	17-May-2019	17-May-2019	Review of final report after receipt of sponsor's comments.

In addition to the detailed study-based audits a series of routine facility and processed-based audits were also being conducted and reported to management during the course of this study. A full facility audit is conducted once a year whilst specified facilities are audited on a rolling schedule.

The raw data and the study report have been audited and the report accurately reflects the raw data.

(b) (4), (b) (6)



Date: 17 May 2019

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## 1. INTRODUCTION

ABS Laboratories has determined the concentrations of nicotine in human plasma (lithium heparin) samples, using high performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Study samples were received as part of clinical protocol number SM 18-01, entitled "Nicotine plasma concentrations and pharmacokinetics of single doses of a non-tobacco-based nicotine pouches (ZYN®) compared with conventional, tobacco-based Swedish snus and American moist snuff among current, daily snus users". This report provides the results and supporting documentation for the analysis of the study samples, as well as standard curve and quality control data.

A list of standard abbreviations used in this report is presented in [Appendix 1](#).

## 2. EXPERIMENTAL

The support to be provided to the clinical study was described in the analytical protocol, which is reproduced in [Appendix 2](#).

All temperatures referenced in this report are nominal temperatures.

### 2.1. Method and Materials

#### 2.1.1. Analytical Method

The analytical method was validated at ABS Laboratories, in accordance with the FDA Guidance for Industry<sup>1</sup> and the EMA Guideline on bioanalytical method validation<sup>2</sup>, in a previous study<sup>3, 4</sup>. Samples (100 µL) of Human Plasma (lithium heparin) containing the analyte and internal standard were extracted using a liquid-liquid extraction procedure. The extracted samples were analysed by an HPLC interfaced with an AB Sciex 4000 mass spectrometer. Positive ions were monitored in the multiple reaction ion-monitoring (MRM) mode. Quantification was performed by peak area ratio. Full details of the analytical procedure are documented in the method SOP<sup>5</sup>.

All documents referenced are on file at ABS Laboratories.

#### 2.1.2. Reference Standard

<i>Identity</i>	<i>Nicotine</i>
Source	Cerilliant
Supplier	Sigma Aldrich
Batch No.	FN05131604
ABS CSR No.	18005
Potency	100% Supplied as a 1.00 mg/mL solution in methanol
Expiry Date	31 May 2021
Storage Conditions	Nominally -20°C

The first page of the certificate of analysis is reproduced in [Appendix 3](#).

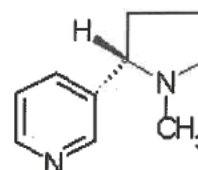
### 2.1.3. Internal Standard

<i>Identity</i>	<i>Nicotine-D<sub>4</sub></i>
Source	Cerilliant
Supplier	Yorlab
Batch/Lot No.	FN10221502
ABS CSR No.	18001
Potency	100% (Supplied as 100 µg/mL certified solution in acetonitrile)
Expiry Date	30 November 2020
Storage Conditions	Nominally -20°C

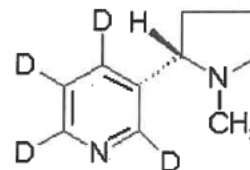
The first page of the certificate of analysis is reproduced in [Appendix 4](#).

### 2.1.4. Chemical Structures

Analyte: Nicotine  
 Formula: C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>  
 MW: 162.23 g/mol



Internal standard: Nicotine-D<sub>4</sub>  
 Formula: C<sub>10</sub>H<sub>10</sub>D<sub>4</sub>N<sub>2</sub>  
 MW: 166.26 g/mol



### 2.1.5. Biological Matrix

Human Plasma, with lithium heparin as anticoagulant, was obtained from healthy volunteers at ABS Laboratories. Human Plasma (lithium heparin) that was free of significant interference was used to prepare calibration standards and quality control (QC) samples.

### 2.1.6. Calibration Standards and Quality Control Samples

Calibration standards ranging from 0.500 – 50.0 ng/mL were prepared on 13, 19 and 26 February, 5, 6 and 11 March 2019, from standard spiking solutions prepared on 11 February 2019.

QC samples at three different concentrations (1.50, 15.0 and 40.0 ng/mL) were prepared on 13 February 2019 from quality control spiking solutions prepared on 11 February 2019 and subsequently stored at a nominal temperature of -20°C.

Copies of the batch record forms that document the preparation of these standard solutions and QC samples, are stored in the raw data for this study.

## 2.2. Study Samples

### 2.2.1. Sample Source and Date of Receipt

The first study samples were collected on 7 January 2019. Study samples were received at ABS, frozen on dry ice, on 13, 27 February and 12 March 2019 from Clinical Trial Consultants, (Dag Hammarskjölds väg 10B Uppsala 75237 Sweden).



### 2.2.2. Sample Storage

Study samples were stored at a nominal temperature of -20°C for a duration not exceeding 65 days, prior to analysis. The validation study<sup>3</sup> showed stability under these conditions for 246 days.

### 2.2.3. Sample Summary

The clinical protocol specified that a total of 36 subjects were to receive the following treatments in accordance to a pre-determined randomised order:

- 1 = ZYN Smooth containing 6 mg nicotine per portion
- 2 = ZYN Smooth containing 8 mg nicotine per portion
- 3 = ZYN Wintergreen containing 6 mg nicotine per portion
- 4 = ZYN Smooth containing 6 mg nicotine per portion (lower lip)
- 5 = General PSWL (8 mg nicotine/g) 2 x 1.0 g
- 6 = Longhorn Pouch Natural (12 mg nicotine/g) 1.5 g
- 7 = Longhorn Pouch Wintergreen (12 mg nicotine/g) 1.5g

Blood samples were scheduled to be taken at the following times:

Pre-dose (0), 5, 10, 15, 30, 60, 90, 120, 240, and 360 minutes post-dose.

<b>Samples</b> (Identified as Subject ID, Visit, Timepoint)	<b>Subject Sample ID</b>	<b>No. of Samples</b>
Specified in protocol to be received	-	2520
Subject withdrew after the visit 2, therefore no visit 3 to 8 samples received	105 & 127	120
No samples received after visit 6, therefore no visit 7 and visit 8 samples received	129 & 134	40
No samples received after visit 7, therefore no visit 8 samples received	123	10
Individual samples not received: 129 Visit 4 15 minutes, 129 Visit 6 4 hours and 133 Visit 4 15 minutes	129, 133	3
Samples received	-	2347
Total number of study samples analysed		2347

All study samples will be retained for at least three months after issue of this report. After this period the Sponsor will be contacted for further sample retention or disposal instructions.

### 2.2.4. Sample Analysis

Samples for a given subject and visit were analysed in the same batch, except when samples had to be reanalysed. Each batch consisted of a duplicate set of double blanks and single blanks (zero standards), duplicate calibration standards containing 7 different non-zero concentrations, duplicate low, medium and high concentration QC samples (equal to at least 5% of the number of unknown samples in the batch).

### 2.2.5. Incurred Sample Reproducibility

To investigate incurred sample reproducibility approximately 10% of the analysed study samples up to 1000 and 5% thereafter were reanalysed. Approximately half were randomly

selected, the remainder were selected from the  $C_{\max}$  region (60 minutes post-dose) and from near the end of the elimination phase (4 hours post-dose). Full details of the sample selection and reporting are documented in an SOP<sup>6</sup>.

### 3. RESULTS

A summary of analysis batches performed in this study is presented (Table 1). Data from rejected batches are not included in the report, but remain on file at ABS Laboratories.

Due to rounding procedures, recalculations using the results presented in this report may differ slightly from the reported statistics.

#### 3.1. Batch Acceptance Criteria

An analysis batch was considered acceptable if the following criteria were met:

- The standard curve was constructed from at least 11 of the standard samples, excluding the zero concentration standards. The back calculated concentrations for the standard samples must be within  $\pm 15\%$  of the actual value, except at the lower limit of quantification where  $\pm 20\%$  is acceptable.
- The accuracy of at least two thirds of the quality control concentrations had to be within  $100 \pm 15\%$ . Half of the quality control samples at each concentration had to be within  $100 \pm 15\%$ .
- At least half of the blank samples with internal standard and half of the blank samples without internal standard, placed immediately before the calibration standards, had to be free of interference. Interference is defined as a detectable response, at the retention time of the analyte, greater than 20% of the mean response of the lowest concentration (LLOQ) standards.

Of the 38 analysis batches performed in this study 37 met the acceptance criteria.

#### 3.2. Quality Control Sample Analyses (Inter-Batch Precision and Accuracy)

Inter-batch precision (CV) and accuracy results for QC samples prepared at low, medium and high QC concentrations are summarised in Table 2. Precision was less than or equal to 3.2% and mean accuracy ranged from 99.9 to 102.1%.

#### 3.3. Calibration Standard Concentrations

Back-calculated calibration curve standard concentrations are provided in Table 3. Mean accuracy ranged from 96.6 to 102.0%.

#### 3.4. Standard Curve Parameters

Standard curve parameters from 37 successful analytical batches are provided in Table 4. The correlation coefficient ( $r$ ) was greater than or equal to 0.9986. A representative calibration curve is shown in Figure 1.

#### 3.5. Study Sample Concentrations

Study sample concentrations are provided in Table 5.

Study samples with determined concentrations below that of the LLOQ of the standard curve are reported as being <LLOQ ("Less than the Lower Limit of Quantification").

### **3.6. Reanalyses**

#### **3.6.1. Incurred Sample Reproducibility**

The incurred sample reanalysis results are shown in [Table 6](#). The differences between the original and repeat results of all 169 samples reanalysed were within 20% of the mean of the original and repeat results. The acceptance criterion, for incurred sample reanalysis, states that the differences for two thirds of the reanalysed samples have to be within 20% of the mean of the original and repeat results. This acceptance criterion was therefore met.

#### **3.6.2. Reanalyses for Analytical Reasons**

After initial analysis, study samples that were identified for reanalysis due to analytical reasons, were reanalysed singly. These samples are identified in [Table 7](#).

#### **3.6.3. Reanalyses for Non-analytical Reasons**

After initial analysis, study samples that were identified for reanalysis due to non-analytical reasons, were reanalysed in triplicate. These samples are identified in [Table 8](#).

### **4. RAW DATA AND CHROMATOGRAMS**

The individual result tables for all accepted analysis batches are presented in [Appendix 5](#). Chromatograms from a minimum of 5% of the subjects are provided in [Appendix 6](#), containing all the chromatograms from analysis batch IDs 20190304CF1 and 20190304CF2.

### **5. COMMENTS AND NOTES**

The protocol stated that a carryover blank sample, with no internal standard would be included after each of the highest calibration standards. After Batch 2 the carryover blanks were extracted as reagent blanks. The reagent blanks were used to avoid any issues with endogenous levels of nicotine present in the plasma.

### **6. ARCHIVES**

All raw data, associated data, and the report are archived at ABS Laboratories according to the SOP in effect during the conduct of the study.

### **7. COMPUTER APPLICATION PROGRAMS**

Computer application programs used to acquire and derive data for this study included NuGenesis 7.1 (Waters), AB Sciex Analyst® 1.6.1 (for API 4000), Waters Vision Publisher 7.1 SR6 and Microsoft® Excel.



## 8. REFERENCES

1. Guidance for Industry – Bioanalytical Method Validation (CDER, May 2001)
2. Guideline on bioanalytical method validation. 21 July 2011. EMEA/CHMP/EWP/192217/2009. Committee for Medical Products for Human Use (CHMP)
3. V/Nic/HP/A: The Partial Validation of an LC-MS/MS Method for the Determination of Nicotine in Human Plasma using Microtitre Injection Plates. Study Director: Paul Baker.
4. V/NIC/HP: The Validation of an Analytical Method for the Determination of Nicotine in Human Plasma using LC-MS/MS. Study Director: Laura McMeekin.
5. SOP No. 5-85.4 and subsequent amendments: Determination of Nicotine in Human Plasma by LC-MS/MS.
6. SOP No. 1-14.9: Guidelines for Performing Repeat Analyses

(b) (4)



Nicotine in Human Plasma

ABS Laboratories Study No. ABS/09/19



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Nicotine in Human Plasma

ABS Laboratories Study No. ABS/09/19



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**APPENDICES****Appendix 1. List of abbreviations**

<i>List of Abbreviations</i>	
ABS	Advanced Bioanalytical Service
AB	Applied Biosystems
API	atmospheric pressure ionisation
°C	degree Celsius (centigrade)
CDER	Center for Drug Evaluation and Research
CHEM	Testing of Chemicals
CHMP	Committee for Medical Products for Human Use
CSR	Compound storage record
CV	coefficient of variation (relative standard deviation)
EDTA	ethylenediaminetetraacetic acid
EMA/EMEA	European Medicines Agency
EWP	Ethics Working Party
ENV	Environment
FDA	US Department of Health and Human Services Food and Drug Administration
g	gram
GLP	Good Laboratory Practice
HP	Human Plasma
HPLC	high performance liquid chromatography
ID	identification
ISR	incurred sample reanalysis
L	litre
LC	liquid chromatography
LLOQ	lower limit of quantification
MC	Member Country
mg	milligram
mins	minutes
mL	millilitre
MRM	multiple reaction monitoring
MS	mass spectrometry
MW	molecular weight
n	number of data
ng	nanogram
NIC	nicotine
No.	number
OECD	Organization for Economic Cooperation and Development
QC	quality control
r	correlation coefficient
SI	Statutory Instrument
SOP	standard operating procedure
STD/Std	standard
Stds	calibration standards
µg	microgram
U.S./US	United States of America
V	Validation

Appendix 2. Analytical protocol



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**Analytical Protocol**

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**Study Number ABS/09/19**

**Determination of Nicotine in Human Plasma Samples from Swedish Match Clinical Protocol No. SM 18-01**

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**Sponsor:** Swedish Match  
SE-Box 17037  
104 62 Stockholm,  
Sweden

**Sponsor's Clinical Protocol Number:** SM 18-01, Final 3.0 16Jan2019

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Page 1 of 8

Detamination of nicotine in human plasma samples  
ABS Analytical Protocol ABS/09/19

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**SIGNATURE PAGE**

**ABS Laboratories**

Approved by the Study Director, (b) (4), (b) (6)

Signature (b) (6)

Date 7 Feb 2019

Approved by the Laboratory & QA Manager, (b) (4), (b) (6)

Signature (b) (6)

Date 07 Feb 2019

**Swedish Match**

Only subjects who give their informed consent will participate in the clinical trial. ABS Laboratories will be informed, as soon as practicable, of any subjects who withdraw their consent and do not wish any samples taken so far to be analysed.

The version of the clinical protocol specified on the title page is the most recent version and ABS Laboratories will be supplied with any updates or amendments and that these amendments will have received the necessary regulatory approvals (where applicable). I have reviewed this analytical protocol and confirm that the work described does not exceed or contradict the requirements set out in the clinical protocol. I will inform ABS Laboratories if relevant sections in the clinical protocol are updated or amended.

Sponsor, Mikael Staaf

Signature (b) (6)

Date 11 Feb 2019

Determination of nicotine in human plasma samples  
ABS Analytical Protocol ABS/09/19



## 1 CONTACT DETAILS

Study Director

(b) (4), (b) (6)

Test Facility Management

Sponsor's Study Monitor

Receipt of Data

## 2 QUALITY STATEMENT

This study will be conducted to the standards described in the United Kingdom Good Laboratory Practice Regulations (SI 1999 No. 3106 as amended SI 2004 No. 994) and the OECD Principles of Good Laboratory Practice 1997 (ENV/MC/CHEM(98)17).

This study will also be conducted in compliance with the United Kingdom "The Medicines for Human Use (Clinical Trials) Regulations", (SI 2004 No. 1031 and subsequent amendments).

Study based inspections will be carried out on this study by ABS Laboratories Quality Assurance as follows:

1. The experimental phase inspection for this study will be the analysis of one batch of samples.
2. Raw data to final results tables will be audited prior to the issue of any final results to the sponsor.
3. The analytical report and the study file will be audited.
4. Various aspects of this study may also be audited during process audit inspections.
5. Inspections and audits will be carried out by personnel independent of the staff involved in this study.

## 3 OBJECTIVE

The determination of nicotine in human plasma samples from Swedish Match Protocol No. SM 18-01 entitled "Nicotine plasma concentrations and pharmacokinetics of single doses of a non-tobacco-based nicotine pouches (ZYN®) compared with conventional, tobacco-based Swedish snus and American moist snuff among current, daily snus users."

## 4 SCHEDULE

Anticipated Experimental Start Date:	February 2019
Anticipated Experimental Completion Date:	April 2019
Anticipated Draft Report Date:	May 2019

Determination of nicotine in human plasma samples  
ABS Analytical Protocol ABS/09/19



## 6 EXPERIMENTAL

### 5.1 Test Method

Concentrations of nicotine will be determined in the samples using an analytical method developed and validated by ABS Laboratories<sup>1,2</sup>. The method is documented in SOP No. 5-85.4<sup>3</sup> (and subsequent amendments).

### 5.2 Reference Compounds

Identity	Nicotine
Source	Cerilliant
Supplier	Sigma Aldrich
Batch No.	FN05131604
ABS CSR No.	18003
Potency	100% (supplied as a 1.00 mg/mL solution in methanol)
Expiry	31 May 2021
Storage Conditions	Nominally -20°C

Other appropriate in-date batches of reference compounds may be used. Full details including certificates of analysis of the actual reference compounds used will be included in the final report.

### 5.3 Internal Standard

Identity	Nicotine-D <sub>3</sub>
Source	Cerilliant
Supplier	Yorlab
Batch No.	FN10221502
ABS CSR No.	18001
Potency	100% (supplied as 100 µg/mL certified solution in acetonitrile)
Expiry	30 November 2020
Storage Conditions	Nominally -20°C

Other appropriate in-date batches of compound may be used. Full details including certificates of analysis of the actual reference compounds used will be included in the final report.

### 5.4 Biological Matrix

Blank human plasma, with lithium heparin as anticoagulant, will be supplied from healthy volunteers by ABS Laboratories or another appropriate source. Human plasma (lithium heparin), free of significant interference, will be used to prepare calibration standards and quality control (QC) samples.

### 5.5 Test Samples

This study design is open, randomized, seven-way cross-over, single dose administration. The study is therefore not blinded.

Number of samples expected: Approximately 2520 human plasma samples

Determination of nicotine in human plasma samples  
 ABS Analytical Protocol ABS/09/19

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 LABORATORIES

Study design: 36 subjects are expected to receive the following:

- 1 = ZYN Smooth containing 6 mg nicotine per portion
- 2 = ZYN Smooth containing 8 mg nicotine per portion
- 3 = ZYN Wintergreen containing 6 mg nicotine per portion
- 4 = ZYN Smooth containing 6 mg nicotine per portion (lower lip)
- 5 = General PSWL (8 mg nicotine/g) 2 x 1.0 g
- 6 = Longhorn Pouch Natural (12 mg nicotine/g) 1.5 g
- 7 = Longhorn Pouch Wintergreen (12 mg nicotine/g) 1.5 g

Blood samples are scheduled to be taken at the following times:

Pre-dose (0) and at 5, 10, 15, 30, 60, 90, 120, 240 and 360 minutes post-dose.

All samples received will be analysed. Samples will be shipped by the clinic on dry ice and on arrival at ABS Laboratories will be stored at a nominal temperature of -20°C, until analysis.

#### 5.6 Calibration Standards and Quality Control (QC) Samples

Calibration standards will be prepared and used which contain the following nominal concentrations, in pooled human plasma:

Concentration of nicotine, ng/mL							
0.00	0.500	1.00	2.00	5.00	10.0	20.0	50.0

Calibration standards may be prepared in bulk and stored in portions under conditions of known stability. Calibration standards at each concentration will be analysed in duplicate in each analysis batch.

Quality control (QC) samples will be used which contain the following nominal concentrations, in pooled plasma:

Concentration of nicotine, ng/mL		
1.50	15.0	40.0

If the concentrations of the QC samples prove to be inappropriate, then QC samples at additional concentrations will be prepared, including a dilution QC sample if required. Quality control samples will be stored in portions at a nominal temperature of -20°C. Duplicate quality control samples will be included in each analysis batch.

#### 5.7 Analysis Batches

Study samples will be analysed in separate uniquely labelled batches. Samples will be analysed in time profile order. Each analysis batch will include the following:

1. A blank sample without IS (double blank)
2. A set of calibration standards (including single blank)
3. A carry-over blank
4. A QC sample
5. Study samples with QC samples interspersed
6. A QC sample



Determination of nicotine in human plasma samples  
ABS Analytical Protocol ABS/09/19

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7. A blank sample without IS (double blank)
8. A set of calibration standards (including single blank)
9. A carry-over blank

Initially the samples will be analysed once. If the sample volume is small or the sample concentration is believed to be over-range, the initial analysis may be done using a diluted sample.

Samples may be re-analysed for the reasons listed in SOP 1-14.

#### 5.8 Incurred Sample Reproducibility

To investigate incurred sample reproducibility 10% of the analysed study samples up to 1000 and 5% thereafter will be reanalysed. As 2520 samples are anticipated, 176 samples are anticipated for reanalysis. These samples will be selected and reported according to ABS SOP 1-14.

#### 5.9 Data Handling

Applied Biosystems Analyst 1.6.1 will be used for peak integration and for calculation of concentrations. The concentrations of the analyte in the samples will be determined using a weighted least squares ( $1/x^2$ ) linear regression on the peak area ratios from the calibration standards. The purity of the test material will be taken into account. Zero concentration calibration standards will not be included in the construction of the standard curve. Concentrations will be reported to 3 significant figures.

#### 6 ANALYSIS BATCH ACCEPTANCE CRITERIA

The calibration standards must have a back-calculated accuracy within  $100 \pm 15\%$ , except at the lower limit of quantification (LLOQ) where it must be within  $100 \pm 20\%$ . The standard curve must be constructed from at least three quarters (i.e. 11) of the calibration standards, excluding the zero concentration calibration standards. If both of the calibration standards at the lower limit of quantification are rejected then this particular analytical batch will have a raised LLOQ, corresponding to the lowest acceptable calibration standard. Samples with determined concentrations below the raised LLOQ will be re-analysed, if sufficient sample remains.

Duplicate quality control samples at low, medium and high concentrations will be included in each analysis batch. The accuracy of at least two thirds of the quality control samples must be within  $100 \pm 15\%$ . Half of the quality control samples at each concentration must be within  $100 \pm 15\%$ .

At least half of the blank samples with internal standard and half of the blank samples without internal standard, placed immediately before the calibration standards, must be free of interference. Interference is defined as a detectable response, at the retention time of the analyte, greater than 20% of the mean response of the lowest concentration (LLOQ) standards.

#### 7 RECORDS

Documentation at the test facility will include, but not be restricted to, the following records:

1. Sample receipt records
2. Weighing and solution preparation records
3. LC-MS/MS conditions
4. Chromatograms



Determination of nicotine in human plasma samples  
ABS Analytical Protocol ABS/09/19



5. Raw data entered on study specific proformas
6. Derived data
7. Plasma control data
8. Calibration data

## 8 REPORTING

The sponsor will be provided with a draft report in order to make comments. The report will include, but will not be restricted to, the following:

1. The title of the study
2. The objectives stated in the approved protocol
3. The identity of the reference compounds including details of their origin, purity and stability
4. The name and address of the sponsor
5. The name and address of the test facility and the dates on which the study was started and completed
6. The signature of the Study Director accepting responsibility for the validity of the report
7. Any unforeseen circumstances, which may have affected the quality or integrity of the study
8. A description of the method and materials used
9. A summary of results
10. Results including QC data, calibration data and determined concentrations in the study samples
11. A discussion of the results
12. The location of all the raw data and the final report
13. Data on the analytical method conditions used including representative chromatograms from a minimum of 5% of the subjects
14. A Quality Assurance statement

When the sponsor's comments are received a final report will be produced. The sponsor will receive one bound and one electronic (PDF) copy of the final report. Although procedures are in place to make sure that the PDF copy of the final report is a true copy the insertion of the signatures does not comply with 21CFR.11.

## 9 PATIENT SAFETY AND EXPEDITED REPORTING OF ANOMALOUS RESULTS

Since the samples are being analysed after the administration to each subject is complete, it is not considered necessary to expedite the reporting of any anomalous results.

If urgent analysis of any samples is required due to an unexpected patient safety issue, then such samples may be analysed outside of the requirements of this protocol, if meeting the requirements of the protocol would cause an unacceptable delay. The requirements for such analysis and the reporting of results will be agreed with the sponsor when the request for the urgent analysis is made.

## 10 ARCHIVE

The study file, containing all the study raw data and a copy of the final report, will be archived within 3 months of issuing the final report. After approximately two years (one MHRA inspection cycle), the study file will be transferred to the sponsor for storage. Some of the items will be supplied electronically on CD in PDF format.

Determination of nicotine in human plasma samples  
ABS Analytical Protocol ABS/09/19

 **ABS**  
LABORATORIES

When the study file has been transferred to the sponsor, ABS Laboratories will only have a copy of the clinical protocol and the final analytical report in its GLP archive.


The remaining test samples and stock solutions will be stored under appropriate conditions and retained for 3 months after submission of the final report. At this time the sponsor will be contacted to ascertain whether continued storage is necessary. ABS Laboratories reserves the right to charge for sample storage after this time.

#### 11 REFERENCES

- 1 Validation Report: V/NIC/HP/A - The Partial Validation of an LC-MS/MS Method for the Determination of Nicotine in Human Plasma using Microtitre Injection Plates. Study Director: Paul Baker.
- 2 Validation Report: V/NIC/HP - The Validation of an Analytical Method for the Determination of Nicotine in Human Plasma using LC-MS/MS. Study Director: Laura McMeekin.
- 3 SOP-5-85.4: Determination of Nicotine in Human Plasma by LC-MS/MS.
- 4 SOP 1-14: Guidelines for Performing Repeat Analyses.

Appendix 3. Certificate of analysis for nicotine (ABS CSR No. 18005)

18005



**Cerilliant®**  
Analytical Reference Standards  
a SIGMA-ALDRICH company

N-008  
 FN05131604  
 Revision 00  
 Page 1 of 7  
 Product of USA

### Certified Reference Material - Certificate of Analysis

#### (S)-(-)-Nicotine, Primary Standard

**Catalog Number:** N-008

**Lot:** FN05131604

**Expiration:** May 2021

**Description:** (S)-(-)-Nicotine in Methanol.

**Packaging:** Solution in 2 mL amber USP Type I glass ampoule containing not less than 1 mL of certified solution.

**Storage:** Store unopened in freezer (-10 °C to -25 °C)

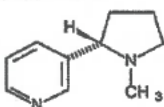
**Shipping:** Ambient. See Stability Section.

**Intended Use:** This Certified Reference Material is suitable for the *in vitro* identification, calibration, and quantification of the analyte(s) in analytical and R&D applications. Not suitable for human or animal consumption.

**Instructions for Use:** Users should quantitatively transfer desired volume using established good laboratory practices to spike into matrix or to dilute to the desired concentration. Each ampoule is intended for one-time use.

**Safety:** Danger. See Safety Data Sheet.

- Expiration date has been established through real-time stability studies.
- Ampoules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette.
- For quantitative applications the minimum sample size for introduction is 1 µL.



Analyte	Certified Concentration Value
(S)-(-)-Nicotine	1.000 ± 0.003 mg/mL

Uncertainty of the concentration is expressed as an expanded uncertainty to achieve with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k = 2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the main balance purity factor, material density, balance, and weighing technique.

This standard is prepared gravimetrically and purity results are reported on the conventional basis for weighing in air. Nominal concentration is calculated based on the actual measured mass; Mass Balance Purity Factor (20.0000) is the measured mass of the solution and the density of the pure solvent at 20 °C.


Concentration is corrected for chromatographic purity, residual solvent, and residual byproducts. No adjustment required before use.

Additional certification information available upon request.

#### Metrological Traceability

- This standard has been prepared and certified under the ISO Guide 34, (ISO/IEC 17025: ISO 9001) and ISO 13485 standards. This standard meets the requirements of a Certified Reference Material and a Primary Standard as defined by ISO and is traceable to the SI and higher order standards through an unbroken chain of comparisons.
- This standard has been gravimetrically prepared using facilities that have been fully qualified and calibrated to ISO 17025 requirements. All calibrations utilize NIST traceable weights which are calibrated externally by a qualified ISO 17025 accredited calibration laboratory to NIST standards. Qualification of each balance includes the assignment of a minimum weighing by a qualified and ISO 17025 accredited calibration laboratory taking into consideration the balance and installed environmental conditions to ensure compliance with USP tolerances of 0.04% relative error. Balance calibration adjustments are performed weekly utilizing the balance's internal adjustment mechanism. Calibration verifications are performed pre-use. Weight data from the calibration verification are included in the production batch record for this standard. Production data package available upon request.
- Fill volume is gravimetrically verified throughout the dispensing process using qualified and calibrated balances.
- Concentration is verified against an independently prepared calibration solution gravimetrically prepared.
- Each raw material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided on subsequent pages of this COA. The density and material Mass Balance Purity Factor is traceable to the SI and higher order reference standards through mass measurement and instrument qualification and calibrations.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.



(b) (6)


Darron Ellsworth, Quality Assurance Manager

July 22, 2016

Date

Cerilliant Corporation 811 Polomo Drive, Suite A, Round Rock, TX 78665 800-848-7837 / 512-238-9974

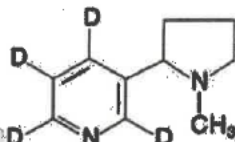
Appendix 4. Certificate of analysis for nicotine-D<sub>4</sub> (ABS CSR No. 18001)



**Certified Reference Material - Certificate of Analysis**  
**(±)-Nicotine-D<sub>4</sub>, Primary Standard**

**Catalog Number:** N-048  
**Lot:** FN10221502  
**Expiration:** November 2020  
**Description:** (±)-Nicotine-D<sub>4</sub> in Acetonitrile  
**Packaging:** Solution in 2 mL amber USP Type I glass ampoule containing not less than 1 mL of certified solution.  
**Storage:** Store unopened in freezer (-10 °C to -25 °C).  
**Shipping:** Ambient. See Stability Section.  
**Intended Use:** This Certified Reference Material is suitable for the *in vitro* identification, calibration, and quantification of the analyte(s) in analytical and R&D applications. Not suitable for human or animal consumption.  
**Instructions for Use:** Users should quantitatively transfer desired volume using established good laboratory practices to spike into matrix or to dilute to the desired concentration. Each ampoule is intended for one-time use.  
**Safety:** Danger. See Safety Data Sheet.

- Expiration date has been established through real time stability studies.
- Ampoules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette.
- For quantitative applications, the minimum sample size for intended use is 1 µL.
- For MS Applications, we advise laboratories not to mix lot during a single sequence.



N-048  
FN10221502  
Revision 00  
Page 1 of 10  
Product of USA


**Certified Quality**  
ISO 9001:2015  
ISO/IEC 17025  
ISO 13485  
ISO 15189  
ISO 9001  
GMP/GIP

Analyte	Certified Concentration Value
(±)-Nicotine-D <sub>4</sub>	100.0 ± 0.5 µg/mL
<p>• Uncertainty of the concentration is expressed as an expanded uncertainty in accordance with ISO 17025 and Guide 34 at the approximate 95% confidence interval using a coverage factor of k=2 and has been calculated by statistical analysis of our production system and incorporates uncertainty of the mass balance purity factor, material density, balance, and weighing techniques.</p> <p>• This standard is prepared gravimetrically and mean results are reported on the conventional basis for weighing in air. Nominal concentration is calculated based on the constant measured mean Mass Balance Purity Factor of the individual individual mass of the solution and the density of the pure solvent at 20 °C.</p> <p>• Concentration is corrected for chromatographic purity, residual volume, residual solvent and residual impurities. No adjustment required before use.</p> <p>• Additional certification information available upon request.</p>	

**Metrological Traceability**

- This standard has been prepared and certified under the ISO Guide 34, ISO/IEC 17025, ISO 9001 and ISO 13485 standards. This standard meets the requirements of a Certified Reference Material and a Primary Standard as defined by ISO and is traceable to the SI and higher order standards through an unbroken chain of comparisons.
- This standard has been gravimetrically prepared using balances that have been fully qualified and calibrated to ISO 17025 requirements. All calibrations utilize NIST traceable weights which are calibrated externally by a qualified ISO 17025 accredited calibration laboratory to NIST standards. Qualification of each balance includes the assignment of a solution weighing by a qualified and ISO 17025 accredited laboratory which takes consideration the balance and installed environmental conditions to ensure compliance with USP tolerance of 0.10% relative error. Balance calibration adjustments are performed weekly utilizing the balance's internal adjustment mechanism. Calibration verification are performed pre-use. Weight tapes from the calibration verification are included in the production batch record for this standard. Production data package available upon request.
- Fill volume is gravimetrically verified throughout the dispensing process using qualified and calibrated balances.
- Concentration is verified against an independent prepared calibration solution gravimetrically prepared.
- Each raw material received has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided in subsequent pages of this COA. The density and material Mass Balance Purity Factor is traceable to the SI and higher order reference standards through metrological traceability and instrument qualification and calibrations.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/retest date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules stored after opening.



**(b) (6)**

Darren Ellsworth, Quality Assurance Manager

**December 14, 2015**

Date

Cerilliant Corporation 811 Pelonia Drive, Suite A, Round Rock, TX 78665 800.646.7837 / 512.238.0074

## Appendix 5. Result tables

The batch ID is given at the top left hand corner of the page for each table.

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Nicotine in Human Plasma

ABS Laboratories Study No. ABS/09/19



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Fax: +44 (0) 1707 358667

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## **Analytical Report**

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**Study Number: ABS/10/19**

**Determination of Salicylic Acid in Human Plasma Samples from Swedish Match  
Clinical Protocol No. SM 18-01**

---

**Sponsor:** Swedish Match  
SE-Box 17037  
104 62 Stockholm,  
Sweden

**Sponsor's Clinical Protocol Number:** SM 18-01, Final 3.0 16Jan2019

**Report Issue Date:** 30 May 2019

**Title:** Determination of Salicylic Acid in Human Plasma Samples from Swedish Match Clinical Protocol No. SM 18-01

**ABS Report No.:** ABS/10/19

**ABS Study No.:** ABS/10/19

**Electronic filename:** ABS\_10\_19 Report Draft

**Sponsor Study No:** SM 18-01, Final 3.0 16Jan2019

**Analytical Laboratory:** ABS Laboratories Ltd  
BioPark  
Broadwater Road  
Welwyn Garden City  
Herts  
AL7 3AX  
United Kingdom

**Sponsor:** Swedish Match  
SE-Box 17037  
104 62 Stockholm,  
Sweden

**Clinical Study Site:** CTC Clinical Trial Consultants AB  
Dag Hammarskjölds väg 10B  
Uppsala  
75237 Sweden

**Study Director:** (b) (4), (b) (6)

**Bioanalysts:**

**Report Author:**

**Sponsor's Study Monitor:**

**Experimental Phase Began:** 20 March 2019

**Experimental Phase Ended:** 28 March 2019

**No. of samples analysed:** 518


### STUDY DIRECTOR'S STATEMENT

This study was conducted to the standards described in the United Kingdom Good Laboratory Practice Regulations (SI 1999 No. 3106 as amended SI 2004 No. 994) and the OECD Principles of Good Laboratory Practice 1997 (ENV/MC/CHEM(98)17).

This study was also conducted in compliance with the United Kingdom "The Medicines for Human Use (Clinical Trials) Regulations", (SI 2004 No. 1031 and subsequent amendments).

I declare that this report fully reflects the raw data generated during this study.

(b) (4), (b) (6)



Date: 30<sup>th</sup> May 2019

**QA STATEMENT**

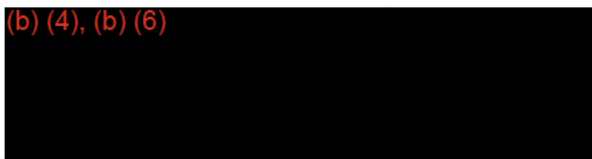
QA personnel have examined the raw data related to the analysis of the samples from study protocol number ABS/10/19 and their findings are detailed in ABS/10/19 QA-01 to 03. Their findings were reported to the Study Director and management on the following dates:

Dates of Audit	Date findings reported to the Study Director	Date findings reported to Management	Audit description
07-Feb-2019	12-Feb-2019	12-Feb-2019	Review of draft analytical protocol
12-Feb-2019	12-Feb-2019		Review of final analytical protocol
09-Apr-2019	09-Apr-2019	25-May-2019	Review of raw data to final results
02-May-2019	02-May-2019	25-May-2019	Review of draft report to raw data
30-May-2019	30-May-2019	30-May-2019	Review of final report as no changes requested by sponsor.

In addition to the detailed study-based inspections a series of routine facility and processed-based inspections were also being conducted and reported to management during the course of this study. A full facility audit is conducted once a year whilst specified facilities are audited on a rolling schedule.

The raw data and the study report have been audited and the report accurately reflects the raw data.

(b) (4), (b) (6)



Date: 30-May-2019



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## 1. INTRODUCTION

ABS Laboratories has determined the concentrations of salicylic acid in human plasma (lithium heparin) samples, using high performance liquid chromatography with tandem mass spectrometric detection (LC-MS/MS). Study samples were received as part of clinical protocol number SM 18-01 entitled "Nicotine plasma concentrations and pharmacokinetics of single doses of non-tobacco based nicotine pouches (ZYN®) compared with conventional, tobacco-based Swedish snus and American moist snuff among current, daily snus users". This report provides the results and supporting documentation for the analysis of the study samples, as well as standard curve and quality control data.

A list of standard abbreviations used in this report is presented in [Appendix 1](#).

## 2. EXPERIMENTAL

The support to be provided to the clinical study was described in the analytical protocol, which is reproduced in [Appendix 2](#).

All temperatures referenced in this report are nominal temperatures.

### 2.1. Method and Materials

#### 2.1.1. Analytical Method

The analytical method was validated at ABS Laboratories, in accordance with the FDA Guidance for Industry<sup>1</sup> and the EMA Guideline on bioanalytical method validation<sup>2</sup>, in a previous study<sup>3</sup>. Samples (50 µL) of human plasma (lithium heparin) containing the analyte and internal standard were extracted using a protein precipitation extraction procedure. An aliquot of the supernatant was diluted with mobile phase A and analysed by an HPLC interfaced with an AB Sciex API4000 mass spectrometer. Negative ions were monitored in the multiple reaction ion-monitoring (MRM) mode. Quantification was performed by peak area ratio. Full details of the analytical procedure are documented in the method SOP<sup>4</sup>.

All documents referenced are on file at ABS Laboratories.

#### 2.1.2. Reference Standard

<i>Identity</i>	<i>Salicylic acid</i>
Source	Cerilliant
Supplier	Sigma-Aldrich
Batch No.	FN01141601
ABS CSR No.	18027
Potency	1 mg/mL solution
Expiry Date	31 January 2021
Storage Conditions	-20°C

The certificate of analysis is reproduced in [Appendix 3](#).

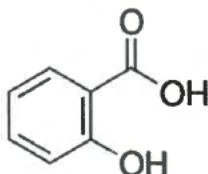
**2.1.3. Internal Standard**

Identity	Salicylic acid-D4
Source	Cerilliant
Supplier	Sigma-Aldrich
Batch/Lot No.	FN08011801
ABS CSR No.	18028
Potency	100 µg/mL solution
Expiry Date	31 October 2019
Storage Conditions	-20°C

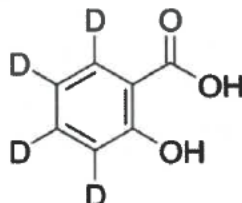
The certificate of analysis is reproduced in [Appendix 4](#)

**2.1.4. Chemical Structures**

Analyte: Salicylic acid  
Formula:  $C_7H_6O_3$   
MW: 138.12 g/mol



Internal standard: Salicylic acid-D4  
Formula:  $C_7H_2D_4O_3$   
MW: 142.15 g/mol

**2.1.5. Biological Matrix**

Human plasma, with lithium heparin as anticoagulant, was obtained from volunteers at ABS Laboratories. Human plasma (lithium heparin) was used to prepare quality control (QC) samples, calibration standards were prepared in surrogate matrix (5% BSA in PBS).

**2.1.6. Calibration Standards and Quality Control Samples**

Calibration standards ranging from 10.0 – 1000 ng/mL were prepared fresh each day of analysis on 20, 21, 22, 25 and 28 March 2019, from standard spiking solutions prepared on 18 March 2019.

QC samples at three different concentrations (30.0, 400 and 800 ng/mL) were prepared on 19 March 2019 and subsequently stored at -20°C. The standard spiking solutions used were prepared on 18 March 2019.

Copies of the batch record forms that document the preparation of these standard solutions and QC samples, are stored in the raw data for this study.

## **2.2. Study Samples**

### **2.2.1. Sample Source and Date of Receipt**

The first study samples were collected on 14 January 2019. Study samples were received at ABS, frozen on dry ice, 13 February 2019, 27 February 2019 and 12 March 2019 from CTC Clinical Trial Consultants AB, (Dag Hammarskjölds väg 10B, SE-752 37 Uppsala, Sweden).

### **2.2.2. Sample Storage**

Study samples were stored at a nominal temperature of -20°C for a duration not exceeding 73 days, prior to analysis. The validation study<sup>3</sup> showed stability under these conditions for 97 days.

### **2.2.3. Sample Summary**

The clinical protocol specified that a total of 36 subjects were to receive the following treatments:

36 subjects were expected to receive the following:

- 1 = ZYN Smooth containing 6 mg nicotine per portion
- 2 = ZYN Smooth containing 8 mg nicotine per portion
- 3 = ZYN Wintergreen containing 6 mg nicotine per portion
- 4 = ZYN Smooth containing 6 mg nicotine per portion (lower lip)
- 5 = General PSWL (8 mg nicotine/g) 2 x 1.0 g
- 6 = Longhorn Pouch.Natural (12 mg nicotine/g) 1.5 g
- 7 = Longhorn Pouch Wintergreen (12 mg nicotine/g) 1.5g

Blood samples were scheduled to be taken at the following times:

Pre-dose (0), 5, 10, 15, 30, 60, 90, 120, 240, and 360 minutes post-dose.

Samples received from the ZYN Wintergreen and Longhorn Pouch Wintergreen dosing occasions were analysed for salicylic acid. The following time-points were selected for salicylic acid analysis:

Pre-dose (0) and at 15, 30, 60, 90, 120, 240 and 360 minutes post-dose.



<b>Samples</b> <b>(Identified as Subject, Time, Period)</b>	<b>Subject</b> <b>Sample ID</b>	<b>No. of</b> <b>Samples</b>
Specified in protocol to be received	-	576
Subject withdrew after visit 2, therefore no samples received	105 & 127	32
No samples received after visit 6, therefore no visit 7 and 8 samples received	129 & 134	16
No samples received after visit 7, therefore no visit 8 samples received	123	8
Individual samples not received: 129 and 133 Visit 4, 15 minute	129, 133	2
Samples received	-	518
Total number of study samples analysed		518

All study samples will be retained for at least three months after issue of this report. After this period the Sponsor will be contacted for further sample retention or disposal instructions.

#### 2.2.4. Sample Analysis

All samples, for a given subject, were analysed together in a single batch except when samples had to be reanalysed. Each batch consisted of a duplicate set of double blanks and single blanks (zero standards). Duplicate calibration standards containing 8 different non-zero concentrations and duplicate low, medium and high concentration QC samples (equal to at least 5% of the number of unknown samples in the batch).

#### 2.2.5. Incurred Sample Reproducibility

Approximately 10% of the analysed samples (53) were selected for reanalysis. Approximately half of the samples were randomly selected. The remainder were selected from the  $C_{max}$  region (60 minutes post-dose) and from near the end of the elimination phase (6 hours post-dose).

### 3. RESULTS

A summary of analysis batches performed in this study is presented (Table 1). Data from rejected batches are not included in the report, but remain on file at ABS Laboratories.

Due to rounding procedures, recalculations using the results presented in this report may differ slightly from the reported statistics.

#### 3.1. Batch Acceptance Criteria

An analysis batch was considered acceptable if the following criteria were met:

- The standard curve was constructed from at least 75% (12) of the standard samples, excluding the zero concentration standards. The back calculated concentrations for the standard samples must be within  $\pm 15\%$  of the actual value, except at the lower limit of quantification where  $\pm 20\%$  is acceptable.
- The accuracy of at least two thirds of the quality control concentrations had to be within  $100 \pm 15\%$ . Half of the quality control samples at each concentration had to be within  $100 \pm 15\%$ .
- At least half of the blank samples with internal standard and half of the blank samples without internal standard, placed immediately before the calibration standards, had to be free of interference. Interference is defined as a detectable response, at the retention

time of the analyte, greater than 20% of the mean response of the lowest concentration (LLOQ) standards.

Of the 10 analysis batches performed in this study all 10 met the acceptance criteria.

### **3.2. Quality Control Sample Analyses (Inter-Batch Precision and Accuracy)**

Inter-batch precision (CV) and accuracy results for QC samples prepared at low, medium and high QC concentrations are summarised in [Table 2](#). Precision was less than or equal to 5.3% and mean accuracy ranged from 101.3 to 104.4%.

### **3.3. Calibration Standard Concentrations**

Back-calculated calibration curve standard concentrations are provided in [Table 3](#). Mean accuracy ranged from 99.1 to 101.0%.

### **3.4. Standard Curve Parameters**

Standard curve parameters from the 10 successful analytical batches are provided in [Table 4](#). The correlation coefficient (r) was greater than or equal to 0.9992. A representative calibration curve is shown in [Figure 1](#).

### **3.5. Study Sample Concentrations**

Study sample concentrations are provided in [Table 5](#).

Study samples with determined concentrations below that of the LLOQ of the standard curve are reported as being <LLOQ ("Less than the Lower Limit of Quantification").

### **3.6. Reanalyses**

#### **3.6.1. Incurred Sample Reproducibility**

The incurred sample reanalysis results are shown in [Table 6](#). The differences between the repeat and original results for all of the 53 samples reanalysed was within 20% of the original result. The acceptance criterion for incurred sample reanalysis states that the differences for two thirds of the reanalysed samples have to be within 20% of the original results, was therefore met.

#### **3.6.2. Reanalyses for Analytical Reasons**

There were no reanalyses for analytical reasons.

#### **3.6.3. Reanalyses for Non-analytical Reasons**

After initial analysis, study samples that were identified for reanalysis due to non-analytical reasons, were reanalysed in triplicate. These samples are identified in [Table 7](#).

## **4. RAW DATA AND CHROMATOGRAMS**

The individual result tables for all accepted analysis batches are presented in [Appendix 5](#). Chromatograms from a minimum of 5% of the subjects are provided in [Appendix 6](#), containing all the chromatograms from analysis batch ID 20190320CF2.

## **5. COMMENTS AND NOTES**

The protocol incorrectly stated that calibration standards would be prepared in control human plasma. As salicylic acid is an endogenous compound the calibration standards were prepared in surrogate matrix (5% BSA in PBS), as detailed in the analytical method<sup>4</sup>.

For clarity blank samples without IS (double blanks), single blanks and carryover blank samples, were prepared using surrogate matrix as described above.

## **6. ARCHIVES**

All raw data, associated data, and the report are archived at ABS Laboratories according to the SOP in effect during the conduct of the study.

## **7. COMPUTER APPLICATION PROGRAMS**

Computer application programs used to acquire and derive data for this study included NuGenesis 7.1 (Waters), AB Sciex Analyst® 1.6.1, Waters Vision Publisher 7.1 SR6 and Microsoft® Excel.

## **8. REFERENCES**

1. Guidance for Industry – Bioanalytical Method Validation (CDER, May 2001)
2. Guideline on bioanalytical method validation. 21 July 2011. EMEA/CHMP/EWP/192217/2009. Committee for Medical Products for Human Use (CHMP)
3. Validation Protocol: V/SAC/HP - The Validation of an Analytical Method for the Determination of Salicylic Acid in Human Plasma using LC-MS/MS. Study Director: John Allanson.
4. SOP-5-155.0: Determination of Salicylic Acid in Human Plasma using LC-MS/MS.



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**APPENDICES**

## Appendix 1. List of abbreviations

<i>List of Abbreviations</i>	
ABS	Advanced Bioanalytical Service
AB	Applied Biosystems
API	atmospheric pressure ionisation
BRF	Batch record form
BSA	Bovine Serum Albumin
°C	degree Celsius (centigrade)
CFR	Code of Federal Regulations
CSR	Compound storage record
CV	coefficient of variation (relative standard deviation)
EMA	European Medicines Agency
FDA	US Department of Health and Human Services Food and Drug Administration
GCLP	Good Clinical Laboratory Practice
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
h	hour
HPLC	high performance liquid chromatography
ID	identification
IS	internal standard
ISR	incurred sample reanalysis
L	litre
LBN	Laboratory batch number
LC	liquid chromatography
LLOQ	lower limit of quantification
mg	milligram
mL	millilitre
MRM	multiple reaction monitoring
MS	mass spectrometry
MW	molecular weight
n	number of data
N/A	not applicable
ng	nanogram
No.	number
Nos.	numbers
OECD	Organization for Economic Cooperation and Development
PK	pharmacokinetic
PBS	Phosphate Buffered Saline
QC	quality control
r	correlation coefficient
SD	standard deviation
SI	Statutory Instrument
SOP	standard operating procedure
STD	standard
Temp	temperature
µg	microgram

Appendix 2. Analytical protocol



ABS Laboratories Ltd  
BioPark  
Broadwater Road  
Welwyn Garden City  
Herts AL7 3AX  
United Kingdom  
www.abslabs.com  
Tel: +44 (0) 1707 358666  
Fax: +44 (0) 1707 358667

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**Analytical Protocol**

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**Study Number ABS/10/19**

**Determination of Salicylic Acid in Human Plasma Samples from Swedish Match  
Clinical Protocol No. SM 18-01**

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**Sponsor:** Swedish Match  
SE-Box 17037  
104 62 Stockholm,  
Sweden

**Sponsor's Clinical Protocol Number:** SM 18-01; Final 3.0 16Jan2019

Determination of salicylic acid in human plasma samples  
ABS Analytical Protocol ABS/10/19



**SIGNATURE PAGE**

**ABS Laboratories**

Approved by the Study Director, (b) (4), (b) (6)

Signature .. (b) (6) ..... Date 12 February 2019

Approved by the Laboratory & QA Manager, (b) (4), (b) (6)

Signature .... (b) (6) ..... Date 12 Feb 2019

**Swedish Match**

Only subjects who give their informed consent will participate in the clinical trial. ABS Laboratories will be informed, as soon as practicable, of any subjects who withdraw their consent and do not wish any samples taken so far to be analysed.

The version of the clinical protocol specified on the title page is the most recent version and ABS Laboratories will be supplied with any updates or amendments and that these amendments will have received the necessary regulatory approvals (where applicable). I have reviewed this analytical protocol and confirm that the work described does not exceed or contradict the requirements set out in the clinical protocol. I will inform ABS Laboratories if relevant sections in the clinical protocol are updated or amended.

Sponsor, Mikael Staaf

Signature ... (b) (6) ..... Date 14 Feb 2019

Determination of salicylic acid in human plasma samples  
ABS Analytical Protocol ABS/10/19

 **ABS**  
LABORATORY

## 1 CONTACT DETAILS

Study Director

(b) (4), (b) (6)

Test Facility Management

Sponsor's Study Monitor

Receipt of Data

## 2 QUALITY STATEMENT

This study will be conducted to the standards described in the United Kingdom Good Laboratory Practice Regulations (SI 1999 No. 3106 as amended SI 2004 No. 994) and the OECD Principles of Good Laboratory Practice 1997 (ENV/MC/CHEM(98)17).

This study will also be conducted in compliance with the United Kingdom "The Medicines for Human Use (Clinical Trials) Regulations", (SI 2004 No. 1031 and subsequent amendments).

Study based inspections will be carried out on this study by ABS Laboratories Quality Assurance as follows:

1. The experimental phase inspection for this study will be the analysis of one batch of samples.
2. Raw data to final results tables will be audited prior to the issue of any final results to the sponsor.
3. The analytical report and the study file will be audited.
4. Various aspects of this study may also be audited during process audit inspections.
5. Inspections and audits will be carried out by personnel independent of the staff involved in this study.

## 3 OBJECTIVE

The determination of salicylic acid in human plasma samples from Swedish Match Protocol No. SM 18-01 entitled "Nicotine plasma concentrations and pharmacokinetics of single doses of a non-tobacco-based nicotine pouches (ZYN®) compared with conventional, tobacco-based Swedish snus and American moist snuff among current, daily snus users."

## 4 SCHEDULE

Anticipated Experimental Start Date:	February 2019
Anticipated Experimental Completion Date:	April 2019
Anticipated Draft Report Date:	May 2019



Determination of salicylic acid in human plasma samples  
ABS Analytical Protocol ABS/10/19



## 5 EXPERIMENTAL

### 5.1 Test Method

Concentrations of salicylic acid will be determined in the samples using an analytical method developed and validated by ABS Laboratories<sup>1</sup>. The method is documented in SOP No. 5-155.0<sup>2</sup> (and subsequent amendments).

### 5.2 Reference Compounds

Identity	Salicylic acid
Source	Cerilliant
Supplier	Sigma-Aldrich
Batch No.	FN01141601
ABS CSR No.	18027
Concentration/Potency	1 mg/mL
Expiry	31 January 2021
Storage Conditions	-20°C

Other appropriate in-date batches of reference compounds may be used. Full details including certificates of analysis of the actual reference compounds used will be included in the final report.

### 5.3 Internal Standard

Identity	Salicylic acid-D4
Source	Cerilliant
Supplier	Sigma-Aldrich
Batch No.	FN08011801
ABS CSR No.	18028
Potency	100 µg/mL
Expiry	31 October 2019
Storage Conditions	-20°C

Other appropriate in-date batches of compound may be used. Full details including certificates of analysis of the actual reference compounds used will be included in the final report.

### 5.4 Biological Matrix

Blank human plasma, with lithium heparin as anticoagulant, will be supplied from healthy volunteers by ABS Laboratories or another appropriate source. Human plasma (lithium heparin), free of significant interference, will be used to prepare calibration standards and quality control (QC) samples.

### 5.5 Test Samples

This study design is open, randomized, seven-way cross-over, single dose administration. The study is therefore not blinded.

Approximately 2520 human plasma samples are expected to be received, all samples will initially be analysed for nicotine<sup>3</sup> prior to selected samples being analysed for salicylic acid.

Determination of salicylic acid in human plasma samples  
 ABS Analytical Protocol ABS/10/19


**ABS**  
 LABORATORY

**Study design:** 36 subjects are expected to receive the following:

- 1 = ZYN Smooth containing 6 mg nicotine per portion
- 2 = ZYN Smooth containing 8 mg nicotine per portion
- 3 = ZYN Wintergreen containing 6 mg nicotine per portion
- 4 = ZYN Smooth containing 6 mg nicotine per portion (lower lip)
- 5 = General PSWL (8 mg nicotine/g) 2 x 1.0 g
- 6 = Longhorn Pouch Natural (12 mg nicotine/g) 1.5 g
- 7 = Longhorn Pouch Wintergreen (12 mg nicotine/g) 1.5 g

Blood samples are scheduled to be taken at the following times;

Pre-dose (0) and at 5, 10, 15, 30, 60, 90, 120, 240 and 360 minutes post-dose.

Samples received from the ZYN Wintergreen and Longhorn Pouch Wintergreen dosing occasions will be analysed for salicylic acid. The following time-points have been selected for salicylic acid analysis:

Pre-dose (0) and at 15, 30, 60, 90, 120, 240 and 360 minutes post-dose.

Therefore a total of 576 samples are expected for salicylic acid analysis.

Samples will have been previously analysed for nicotine and will be stored at a nominal temperature of -20°C, until analysis.

#### 5.6 Calibration Standards and Quality Control (QC) Samples

Calibration standards will be prepared and used which contain the following nominal concentrations, in pooled human plasma:

Concentration of salicylic acid, ng/mL								
0.00	10.0	20.0	50.0	100	250	500	800	1000

Calibration standards may be prepared in bulk and stored in portions under conditions of known stability. Calibration standards at each concentration will be analysed in duplicate in each analysis batch.

Quality control (QC) samples will be used which contain the following nominal concentrations, in pooled plasma:

Concentration of salicylic acid, ng/mL		
30.0	400	800

If the concentrations of the QC samples prove to be inappropriate, then QC samples at additional concentrations will be prepared, including a dilution QC sample if required. Quality control samples will be stored in portions at a nominal temperature of -20°C. Duplicate quality control samples will be included in each analysis batch.

#### 5.7 Analysis Batches

Study samples will be analysed in separate uniquely labelled batches. Samples will be analysed in time profile order. Each analysis batch will include the following:

Determination of salicylic acid in human plasma samples  
ABS Analytical Protocol ABS/10/19



1. A blank sample without IS (double blank)
2. A set of calibration standards (including single blank)
3. A carry-over blank
4. A QC sample
5. Study samples with QC samples interspersed
6. A QC sample
7. A blank sample without IS (double blank)
8. A set of calibration standards (including single blank)
9. A carry-over blank

Initially the samples will be analysed once. If the sample volume is small or the sample concentration is believed to be over-range, the initial analysis may be done using a diluted sample.

Samples may be re-analysed for the reasons listed in SOP 1-14.

**5.8 Incurred Sample Reproducibility**

To investigate incurred sample reproducibility 10% of the analysed study samples (anticipated to be 58) will be reanalysed. These samples will be selected and reported according to ABS SOP 1-14<sup>4</sup>.

**5.9 Data Handling**

Applied Biosystems Analyst 1.6.1 will be used for peak integration and for calculation of concentrations. The concentrations of the analyte in the samples will be determined using a weighted least squares ( $1/x^2$ ) linear regression on the peak area ratios from the calibration standards. The purity of the test material will be taken into account. Zero concentration calibration standards will not be included in the construction of the standard curve. Concentrations will be reported to 3 significant figures.

**6 ANALYSIS BATCH ACCEPTANCE CRITERIA**

The calibration standards must have a back-calculated accuracy within  $100 \pm 15\%$ , except at the lower limit of quantification (LLOQ) where it must be within  $100 \pm 20\%$ . The standard curve must be constructed from at least three quarters (i.e. 12) of the calibration standards, excluding the zero concentration calibration standards. If both of the calibration standards at the lower limit of quantification are rejected then this particular analytical batch will have a raised LLOQ, corresponding to the lowest acceptable calibration standard. Samples with determined concentrations below the raised LLOQ will be re-analysed, if sufficient sample remains.

Duplicate quality control samples at low, medium and high concentrations will be included in each analysis batch. The accuracy of at least two thirds of the quality control samples must be within  $100 \pm 15\%$ . Half of the quality control samples at each concentration must be within  $100 \pm 15\%$ .

At least half of the blank samples with internal standard and half of the blank samples without internal standard, placed immediately before the calibration standards, must be free of interference. Interference is defined as a detectable response, at the retention time of the analyte, greater than 20% of the mean response of the lowest concentration (LLOQ) standards.

**7 RECORDS**

Documentation at the test facility will include, but not be restricted to, the following records:

Determination of salicylic acid in human plasma samples  
ABS Analytical Protocol ABS/10/19



1. Sample receipt records
2. Weighing and solution preparation records
3. LC-MS/MS conditions
4. Chromatograms
5. Raw data entered on study specific proforma
6. Derived data
7. Plasma control data
8. Calibration data

#### 8 REPORTING

The sponsor will be provided with a draft report in order to make comments. The report will include, but will not be restricted to, the following:

1. The title of the study
2. The objectives stated in the approved protocol
3. The identity of the reference compounds including details of their origin, purity and stability
4. The name and address of the sponsor
5. The name and address of the test facility and the dates on which the study was started and completed
6. The signature of the Study Director accepting responsibility for the validity of the report
7. Any unforeseen circumstances, which may have affected the quality or integrity of the study
8. A description of the method and materials used
9. A summary of results
10. Results including QC data, calibration data and determined concentrations in the study samples
11. A discussion of the results
12. The location of all the raw data and the final report
13. Data on the analytical method conditions used including representative chromatograms from a minimum of 5% of the subjects
14. A Quality Assurance statement

When the sponsor's comments are received a final report will be produced. The sponsor will receive one bound and one electronic (PDF) copy of the final report. Although procedures are in place to make sure that the PDF copy of the final report is a true copy the insertion of the signatures does not comply with 21CFR.11.

#### 9 PATIENT SAFETY AND EXPEDITED REPORTING OF ANOMALOUS RESULTS

Since the samples are being analysed after the administration to each subject is complete, it is not considered necessary to expedite the reporting of any anomalous results.

If urgent analysis of any samples is required due to an unexpected patient safety issue, then such samples may be analysed outside of the requirements of this protocol, if meeting the requirements of the protocol would cause an unacceptable delay. The requirements for such analysis and the reporting of results will be agreed with the sponsor when the request for the urgent analysis is made.

Determination of salicylic acid in human plasma samples  
ABS Analytical Protocol ABS/10/19



## 10 ARCHIVE

The study file, containing all the study raw data and a copy of the final report, will be archived within 3 months of issuing the final report. After approximately two years (one MHRA inspection cycle), the study file will be transferred to the sponsor for storage. Some of the items will be supplied electronically on CD in PDF format. When the study file has been transferred to the sponsor, ABS Laboratories will only have a copy of the clinical protocol and the final analytical report in its GLP archive.


The remaining test samples and stock solutions will be stored under appropriate conditions and retained for 3 months after submission of the final report. At this time the sponsor will be contacted to ascertain whether continued storage is necessary. ABS Laboratories reserves the right to charge for sample storage after this time.

## 11 REFERENCES

- 1 Validation Protocol: V/SAC/HP - The Validation of an Analytical Method for the Determination of Salicylic Acid in Human Plasma using LC-MS/MS. Study Director: John Allanson.
- 2 SOP-5-155.0: Determination of Salicylic Acid in Human Plasma by LC-MS/MS.
- 3 Study Number ABS/09/19: Determination of Nicotine in Human Plasma Samples from Swedish Match Clinical Protocol No. SM 18-01. Study Director: John Allanson.
- 4 SOP 1-14: Guidelines for Performing Repeat Analyses.



Appendix 3. Certificate of analysis (first page) for salicylic acid (ABS CSR No. 18027)



**Certified Reference Material - Certificate of Analysis**

**Salicylic acid, Primary Standard**

5-019  
7700141601  
Revision 00  
Page 1 of 6  
Product of USA



<b>Catalog Number:</b>	5-019
<b>Lot:</b>	FN01141601
<b>Expiration:</b>	January 2021
<b>Description:</b>	Salicylic acid in Acetonitrile
<b>Packaging:</b>	Solution in 2 mL amber USP Type I glass ampoules containing not less than 1 mL of certified solution.
<b>Storage:</b>	Store unopened in freezer (-18 °C to -25 °C).
<b>Shipping:</b>	Ambient. See Stability Section.
<b>Intended Use:</b>	This Certified Reference Material is suitable for the identification, calibration, and quantification of the analyte(s) in analytical and R&D applications. Not suitable for human or animal consumption.
<b>Instructions for Use:</b>	Users should quantitatively transfer desired volume using established good laboratory practices to spike into matrix or to dilute to the desired concentration. Each ampoule is intended for one-time use.
<b>Safety:</b>	Danger: See Safety Data Sheet


- + Expiration date has been established through and from stability studies.
- + Ampoules are overfilled to ensure a minimum 1 mL volume can be transferred when using a 1 mL Class A volumetric pipette.
- + For quantitative applications, the minimum sample size for intended use is 1 µL.

Analyte	Certified Concentration Value
Salicylic acid	1,000 ± 0.002 mg/mL

**Metological Traceability**

- + This standard has been prepared and certified under the ISO Guide 34, ISO/IEC 17025, ISO 9001 and ISO 13485 standards. The standard meets the requirements of a Certified Reference Material and a Primary Standard as defined by ISO and is suitable for the SI and higher order standards through an unbroken chain of comparisons.
- + This standard has been prepared using balances that have been fully qualified and calibrated to ISO 17025 requirements. All calibrations within NIST traceable weights which are calibrated annually by a NIST-404 ISO 17025 accredited calibration laboratory to NIST standards. Qualification of each balance includes the assignment of a calibration weighting by a qualified and ISO 17025 accredited calibration vendor taking into consideration the balance and installed air buoyancy adjustment characteristics. Calibration verification and performance are performed weekly utilizing the balance's assigned adjustment characteristics. Calibration verification and performance are included in the production batch record for the standard. Production date package is affixed upon request.
- + Fill volume is gravimetrically verified throughout the dispensing process using certified and calibrated balances.
- + Concentration is verified against an independently prepared calibration and using gravimetrically prepared.
- + Each raw material utilized has been identified and thoroughly characterized through the use of multiple analytical techniques. Spectral data is provided in subsequent pages of this CSR. The purity and material Mass Balance Party Power is traceable to the SI and higher order reference standards through mass measurement and calibration.

Cerilliant certifies that this standard meets the specifications stated in this certificate and warrants this product to meet the stated acceptance criteria through the expiration/test date when stored unopened as recommended. Product should be used shortly after opening to avoid concentration changes due to evaporation. Warranty does not apply to ampoules opened after opening.



(b) (6)


Daron Ellsworth, Quality Assurance Manager

February 17, 2016

Date

Cerilliant Corporation    811 Paloma Drive, Suite A, Round Rock, TX 78665    800-846-7837 / 512-236-9974

Appendix 4. Certificate of analysis (first page) for salicylic acid-D<sub>4</sub> (ABS CSR No. 18028)



**Cerilliant**  
Analytical Reference Standards  
A SIGMA-ALDRICH COMPANY

## Certified Reference Material - Certificate of Analysis

### Salicylic acid-D<sub>4</sub>, Primary Measurement Standard

*2-Hydroxybenzoic-3,4,5,6-D<sub>4</sub> Acid*

**Cerilliant Quality**

ISO GUIDE 34

ISO/IEC 17025

ISO 12485

ISO 15194

ISO 9001

GMP/OLP

**Product No.:** S-042-1ML

**Lot No.:** FN08011801

**Description of CRM:** Salicylic acid-D<sub>4</sub> in Acetonitrile (Solution)

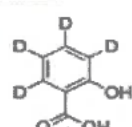
**Retest Date:** October 2019 See Section "Stability Assessment".

**Storage:** Store unopened in freezer (-10 °C to -25 °C).

**Shipping:** Ship Cold See Section "Stability Assessment".

**Chemical formula:** C<sub>7</sub>D<sub>4</sub>H<sub>2</sub>O<sub>3</sub>

**CAS No.:** 78646-17-0



Analyte	Certified Concentration ± associated uncertainty U, u=k*u (k=2)
Salicylic acid-D <sub>4</sub>	100.0 ± 0.6 µg/mL

**Metrological traceability:** Traceable to the SI and higher order standards from NIST through an unbroken chain of comparisons. See "Details on metrological traceability" on page 2.

**Measurement method:** The certified value is calculated from high precision weighing of thoroughly characterized starting material. See "Details about certification process" on page 2.


**Intended use:** This Certified Reference Material is suitable for the in vitro identification, calibration, and quantification of the analyte(s) in analytical and R&D applications. Not suitable for human or animal consumption.

**Minimum sample size:** 1 µL for quantitative applications

**Instructions for handling and correct use:** Concentration is corrected for chromatographic purity, residual solvents and residual inorganics. No adjustment required before use.  
Users should quantitatively transfer desired volume using established good laboratory practices to spike into matrix or to dilute to the desired concentration. Each ampoule is intended for one-time use.  
For MS Applications, we advise laboratories not to mix lots during a single sequence.

**Health and safety information:** Danger. Please refer to the Safety Data Sheet for detailed information about the nature of any hazard and appropriate precautions to be taken.

**Accreditation:** Cerilliant Corp. is accredited by the US accreditation authority ANAB as registered reference material producer AR-1353 in accordance with ISO Guide 34 and registered testing laboratory AT-1352 according to ISO/IEC 17025.



(b) (6)

\_\_\_\_\_  
Darron Ellsworth, Quality Assurance Manager

October 29, 2018

\_\_\_\_\_  
Issue Date

Certificate Page 1 of 10

Cerilliant Corporation, 811 Paloma Drive, Suite A Round Rock, TX 78665, USA, Tel: 800-048-7837 / 512-238-9974

S-042-1ML  
Revision 01

**Appendix 5. Result tables**

The batch ID is given at the top left hand corner of the page for each table.



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