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FINAL REPORT

Project Title: Assessment of the Added Value of New Quantitative Methodologies for the Analysis of Surface Soils in Forensic Soil Comparisons

Award Recipient Organization: North Carolina State University

Principal Investigator:
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1. PROJECT SUMMARY:

Goals and objectives:

The purpose of this proposed study was to assess the potential added value of new quantitative methodologies for the forensic analysis of surface soils. The methodologies selected for comparison are routinely used in ecology and geology, but seldom used in U.S. forensic soil examinations and might provide robust data for soil comparisons. To achieve this, we had four

GOAL 1: To collect forensic-like surface soils from across North Carolina (NC) for downstream analyses.
GOAL 2: To complete inorganic examinations of surface soils using current and new methods.
GOAL 3: To characterize biological taxa associated with the organic component of surface soils using DNA metabarcoding.
GOAL 4: To statistically determine the added value of new methodologies for the analysis of surface soils.

Summary of project design and methods:

Key steps in the project design and methods are broken down by subheadings. A schematic overview is provided in **Figure 1**.

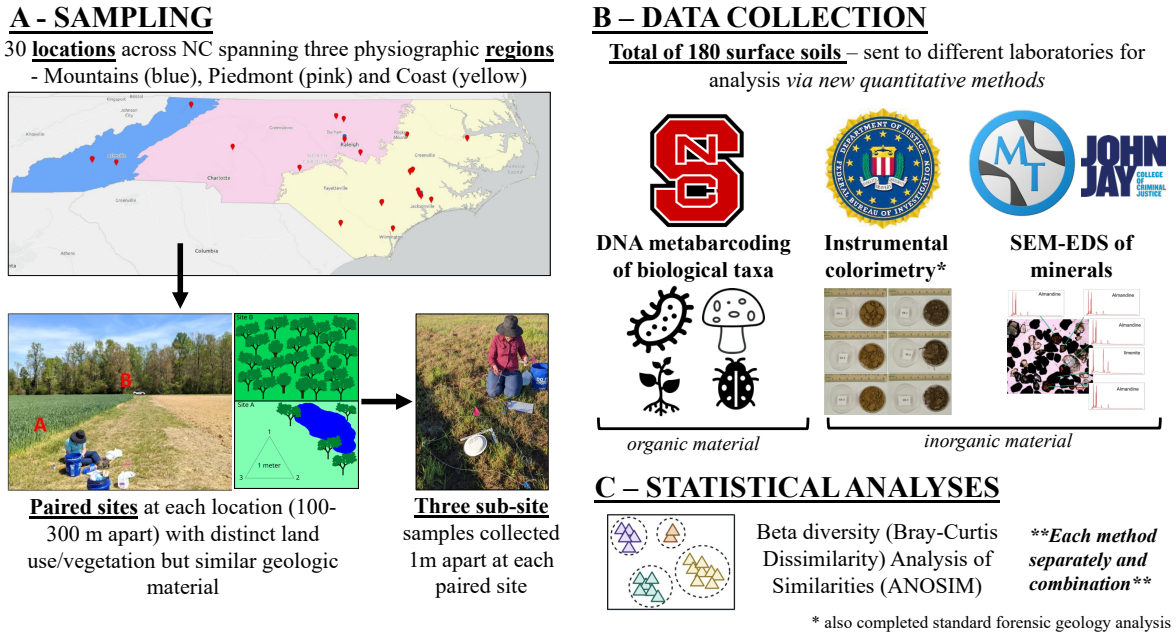

Figure 1. Schematic outlining the key steps in sample collection (A), the three new quantitative methods to be completed on all 180 surface soils samples (and the associated institution/laboratory who completed) (B), and the focus of the statistical analyses hinging on Bray Curtis Dissimilarity (C).

The following is a brief summary of the accomplishments for each of the goals outlined above.

i. *Surface soil collection:* Two types of surface soils representing scenarios that would potentially benefit the most from new quantitative methods were collected from across the state of North Carolina (mountains, piedmont and coastal plain), those with: a) similar inorganic content but with distinct land use (15 locations), and b) limited

inorganic content but recognizable organic fractions (15 locations). At each location, samples were collected from paired sites <500 meters apart (A and B). At each paired site, three sub-site samples were collected 1 meter apart to assess method reproducibility, accuracy and small-scale variation that might be realistically observed in questioned-to-known (Q-to-K) comparisons (total n, ~180).

ii. *Inorganic material analysis:*

a. *Standard forensic geology analysis (completed by the FBI Laboratory):* The examination scheme used for forensic soil comparisons by the FBI Laboratory varies from case-to-case due to case specific circumstances. The processes briefly summarized in **Figure 2** (black text) encompassed the examination scheme typically employed for casework samples and were employed in this study as a baseline for existing methods.

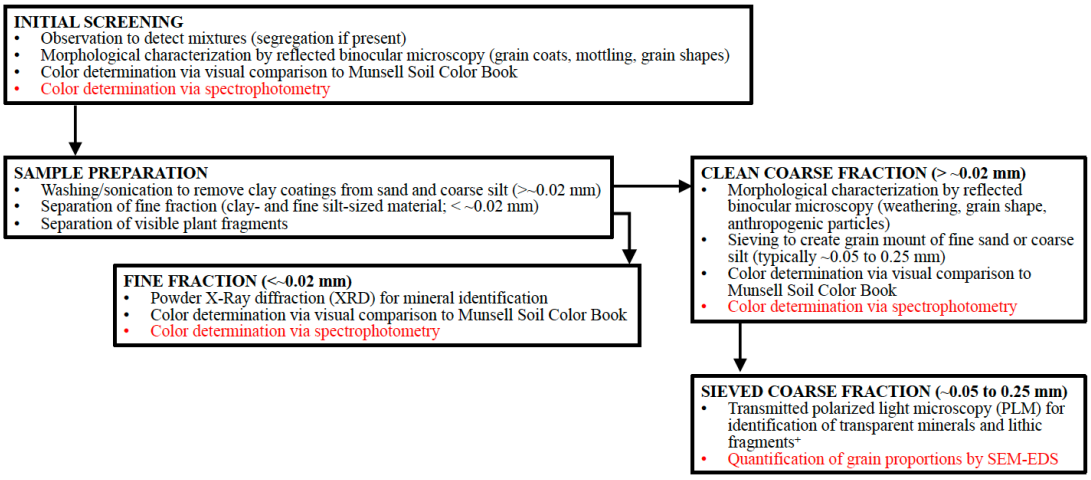

Figure 2. Typical examination scheme used to characterize soils at the FBI Laboratory. Analyses listed in red are new to forensic geology casework at the FBI Laboratory and were the focus of comparison in this study.

b. *Instrumental colorimetry (completed by the FBI Laboratory; new quantitative method):* The color of the soil samples was measured using a model CM600D hand-held spectrophotometer from Konica-Minolta, which is a commonly used in the soil science community. The measured color was recorded in the L*a*b* space for quantitative determination of color differences among samples (denoted by red text in **Figure 2**).

c. *Scanning electron microscopy – energy dispersive x-ray spectroscopy (SEM-EDS) of minerals (completed by Microtrace LLC and John Jay College of Criminal Justice; new quantitative method):* Minerals isolated from the soils were embedded in epoxy and subsequently ground and polished. After analysis by SEM-EDS, the resulting mineral spectra (n, ~1-3K mineral grains/sample) were searched against a custom-built reference library (currently consisting of ~650 mineral EDS spectra) which is derived from empirical data (i.e., from minerals of known identity) and idealized mineral formulas simulated using Desktop Spectrum Analyzer-II (DTSA-

II). The soil mineral EDS spectral searches were performed using customized code written in R, providing a list of identified minerals and their associated grain counts.

iii. *Organic material analysis:*

a. *DNA metabarcoding (completed by NC State University; new quantitative method):* Surface soil samples for DNA processing were stored at 4°C prior to DNA isolation. Total genomic DNA was isolated within 1 week of soil collection and subsequently subjected to DNA metabarcoding. This workflow involved: a) quantifying the total genomic DNA isolated from each sample, b) simultaneous amplification and library preparation of genetic regions that permit taxonomic assignment of bacteria (ribosomal 16S), arthropods (mitochondrial *COI*), fungi (nuclear *ITS1*) and plants (nuclear *ITS2* and chloroplast *trnL*), c) sequencing using Illumina chemistry, and d) taxonomic assignment of high quality sequences to appropriate sequence databases (GenBank, Silva, UNITE *etc*) (following methods developed by Meiklejohn laboratory in award 2020-R2-CX-0035 and published in Tiedge *et al.* 2025).

iv. *Statistical analyses:* For all three new quantitative methods, Bray-Curtis dissimilarity was used to measure the dissimilarity recovered between samples at varying spatial scales (sub-site samples, paired site samples, between locations and all samples). The Bray-Curtis value is calculated from 0 to 1, with 0 having all measured attributes in common and 1 having no common measured attributes. A Bray-Curtis inclusion/exclusion threshold for this dataset for each of the three new quantitative methods was assessed. Principal Components Analysis (PCA) plots based on Bray-Curtis dissimilarity was used to visualize differences between samples at varying spatial scales (primarily sub-site samples, paired site samples and between locations).

v. *Business Process Analysis*: We collated information on a) specific direct and indirect time/cost incurred, b) statistical contribution towards accuracy and reliability, and c) instrument cost to capture overhead, for each methodology.

Research hypotheses and questions:

Based on the experimental design, we tested the following hypotheses: 1) For soils with similar parent material in close proximity, biological taxa will provide more exclusionary differences than mineralogy; 2) Plant, arthropod and fungi species will be more consistently recovered (between sample replicates) than bacteria and indicative of land use; 3) Mineralogy will best distinguish soils developed on distinct parent material; and 4) At locations with limited inorganic content, only color and biological taxa will provide useful data for sample comparisons.

The key research questions of this study were as follows (for each new quantitative method both separately and in combination):

1. Is it possible to separate paired sites (A to B) at the same location?
2. For research stations where more than one location was sampled, is it possible to separate locations?
3. Is it possible to separate locations within the same physiographic region?

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Summary of results:

1) Sample collection

A total of 180 surface soils were successfully collected during April 2022 across North Carolina. Samples were collected with permission within 17 research stations managed and operated by NC State University. Organic rich (n, 90) and mineral rich (n, 90) surface soils were obtained for processing. **Figure 1** shows a map of the sampling locations.

2) Results from instrumental colorimetry

Color differences examined in the L*a*b color space (L*(lightness), a*(green to red), b* (blue to yellow)) are reported as ΔE_{00}. This approach gives a quantitative measure of how humans perceive and differentiate color. Prior studies (Dong et al. 2020; Liau et al. 2025) have suggested a threshold of ΔE_{00} ~5 as an indication of relevant difference for forensic soils comparison, but because color is used as a screening method, a more conservative value of ΔE_{00} >10 is also being considered. **Figure 3** shows the ΔE_{00} measured for samples collected at location 56 in this study. Sub site samples (56A-1 and 56A-3; 56B-1 and 56B-3) have an ΔE_{00} of <1.3, whereas the ΔE_{00} between the paired sites (A and B) are >6.1. These images suggest a ΔE_{00} of 5 would be appropriate, but in other subsite comparison this may increase the false exclusion rate, which is disadvantageous for using color as a "screening tool".

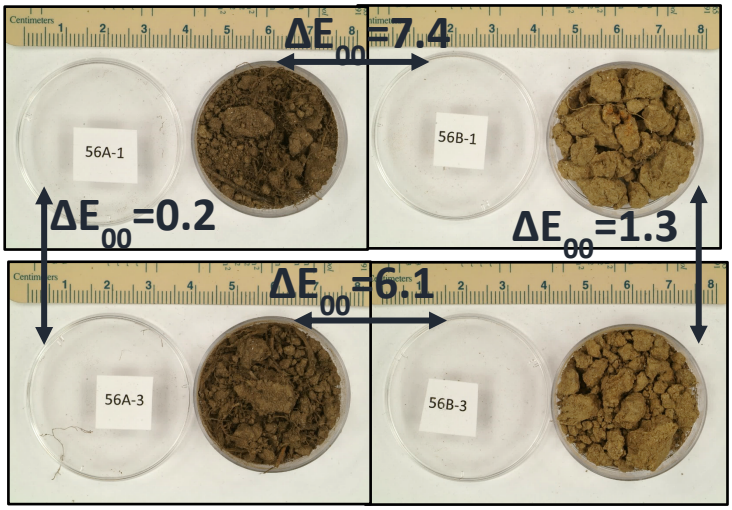


Figure 3. Color differences reported as ΔE_{00} between samples collected at location 56. Samples labelled 56A-1 and 56A-3 are sub-site samples collected at paired site A. Samples labelled 56B-1 and 56B-3 are sub-site samples collected at paired site B.

<u>*i) Sub-site comparisons:*</u> When applying a threshold of ΔE_{00} > 10 to sub-site samples, 5% have bulk soil colors difference above this value, whereas 3% of the fine fraction have color differences >10. The distribution of sub-site ΔE_{00} is given in **Figure 4A**.

<u>*ii) Paired sited comparison*</u>: When comparing soil color from paired sites (A and B), 21/19% (bulk/fine) exceeded ΔE_{00} = 10. The distribution of ΔE_{00} between paired sites is given in **Figure 4B.**

iii) Different site/location comparison: Comparing all soils collected from different sites/locations, 43/57% (bulk/fine) exceed ΔE00 = 10. The distribution of ΔE_{00} between all sites and locations (n, 180 samples) is given in **Figure 4C.**

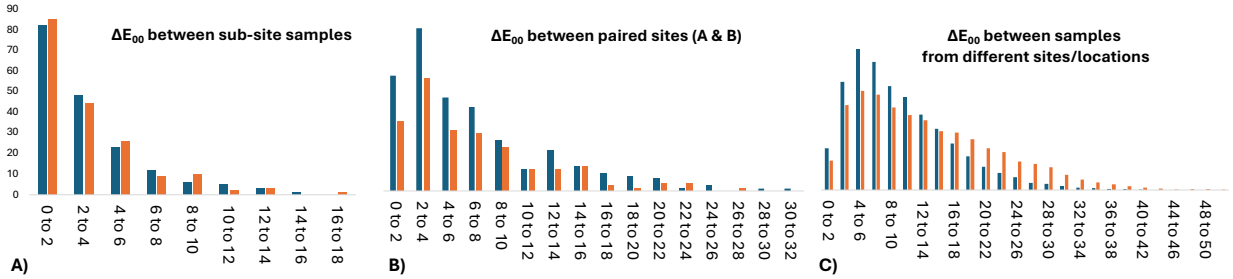


Figure 4. Color differences reported as ΔE_{00} (x-axis) between samples collected at varying spatial scales: A) sub-site samples collected within 1 meter, B) paired samples collected <500 meter apart, and C) samples from different locations collected across the state of North Carolina. Blue bars are color determined from the bulk surface soil fraction; orange bars are color determined from the fine surface soil fraction. Y-axis reflects the number of samples in that ΔE_{00} category.

iv) Color determination by traditional Munsell color chart versus instrumental methods: The handheld spectrophotometer can report color in the Munsell system, the method commonly used to document color of forensic soil samples (ASTM E3254). Given the soil color chart has discrete values the spectrophotometer produces continuous values, the results are not directly comparable for application of the exclusion criteria outlined in E3254.

3) Results from SEM-EDS

i) Method Development- Data Collection

For this research we investigated the potential to use automated SEM-EDS analysis to rapidly classify mineral particles in surface soils. Initial experiments were directed at optimizing the parameters for EDS collection. The instrument used was a JEOL JSM 7100 FT field emission microscope with an X-Max 50 mm^2 Oxford SDD detector. All samples analyzed by SEM-EDS were carbon coated. The method was optimized to balance the time needed to collect EDS spectra and the quality of the spectra. In addition, we investigated methods to set SEM imaging parameters to allow for useful and reproducible mineral particle detection (**Table 1**). We leveraged existing methods that are utilized for gunshot residue analysis to efficiently analyze the large number of soil samples.

Table 1. Summary of SEM-EDS parameters used for mineral analysis.

Parameter	Setting
Image Detector	Secondary electron
Magnification	50x (yielding approximately 80 fields-of-view/sample)
Brightness/ contrast	Calibrated using grey scale contrast for copper and epoxy
Vacuum	High vacuum mode
Spectrum Count Time	2 seconds (achieves as spectrum with approximately 250K counts)
Accelerating Voltage	15 kV

ii) Method Development- Data Analysis

The large datasets consisting of ~1-3K spectra per sample were exported from Oxford in the HDF5 file format. The spectra were standardized and the first-derivative was taken. The processed individual spectra were extracted and then compared to each of the ~650 database entries using Manhattan Distance. The unknown mineral spectrum was assigned to the class of mineral/ group that had the smallest Manhattan Distance.

iii) Analysis of Soil Samples

Detailed data analysis was undertaken using customized code written in R. For mineral profile comparisons, a matrix was developed using the Bray-Curtis similarity-dissimilarity distance metric. This matrix represents the Bray-Curtis (BC) scores for all pairwise comparisons (n=16,110) between the soil samples. We examined the distribution of BC scores at several spatial scales which include same-site (i.e., sub-site samples; **Figure 5A**), paired sites (i.e., A and B sites; **Figure 5B**), different locations in the same research station (**Figure 5C**), and different locations across the state (**Figure 5D**). We developed a receiver operator curve (ROC) using the distribution of scores for the same sites (**Figure 5A**) versus the scores for all other sites (the scores from **Figures 5B-D**). From the ROC plot it was determined that the threshold value (BC score) that provided the optimal true positive and true negative rates is 0.30375. This analysis provided an objective criterion in which to assess whether two samples are similar or not.

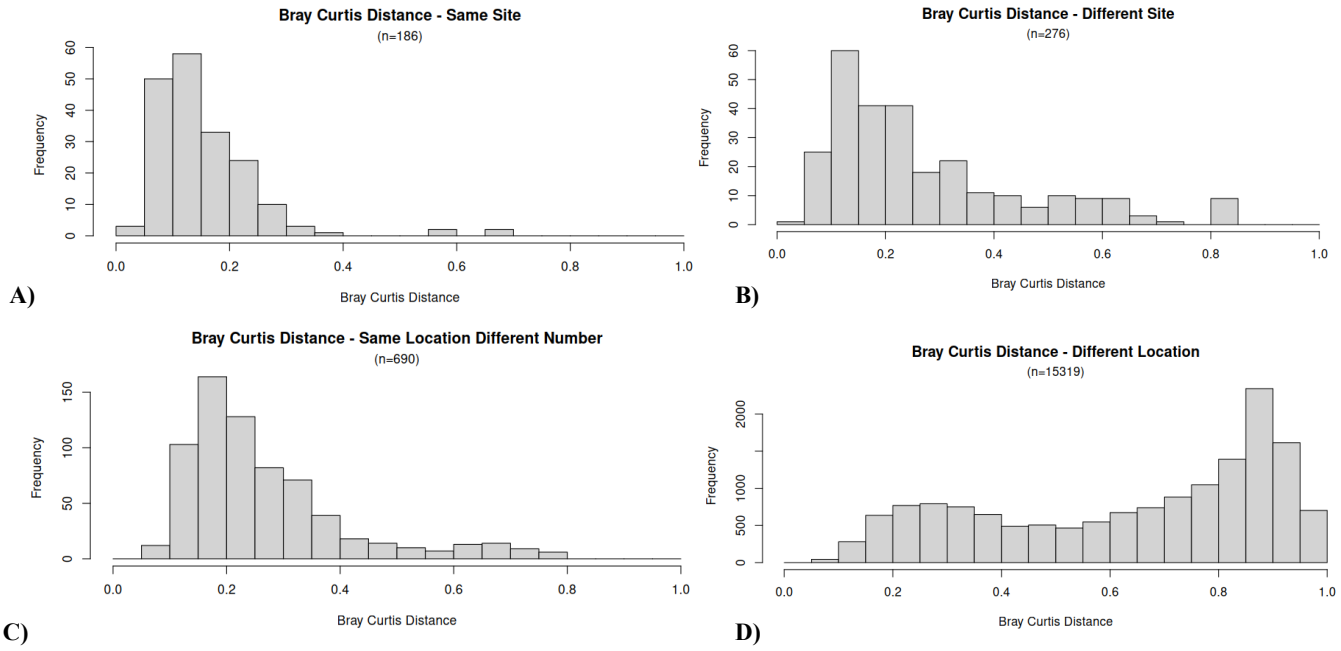


Figure 5. Histogram showing distribution of Bray-Curtis scores for the pairwise comparisons for samples from A) the same site, B) different sites, C) same research station but different locations, and D) different locations.

The overall performance of the SEM-EDS combined with the Bray Curtis Dissimilarity metric resulted in the following rates: 0.856 true positive rate, 0.167 false positive rate, 0.832 true negative rate, and 0.144 false negative rate. Notably, these were average values based on the entire dataset. The soils from the Coastal Plain are derived from well-mixed sands derived from the mountain

ranges to the west. Thus, the discrimination of these soils based on mineralogy was anticipated to be low.

4) Results from DNA metabarcoding

Wet-laboratory processing was successfully completed for all 180 samples following Tiedge et al. 2025. Raw sequence reads were taxonomically classified and partitioned into four data levels for downstream analysis: 1) *ASV 0.01* – Amplicon sequence variants (ASVs) filtered to retain only ASVs with an abundance >0.01%; 2) *ASV - 25%* – ASVs meeting the 0.01% lower limit threshold but also removing the top 25% most abundant ASVs; 3) *FAM 0.01* – families (FAM) filtered to retain only families with an abundance >0.01%; and 4) *FAM - 25%* – families meeting the 0.01% lower threshold but also removing the top 25% most abundant families. The number of ASVs and families varied substantially across the eight taxonomic combinations (**Table 2**), with the most granular dataset – ASV 0.01 – having the most data available for analysis. A Bray Curtis dissimilarity matrix was generated for each taxonomic combination and each data level (n, total 32) for all possible sample comparisons. Analysis of similarities (ANOSIM) was completed using the Bray Curtis dissimilarity matrix to assess whether differences between specific sample sets exist.

Table 2. Number of amplicon sequence variants (ASV) and families (FAM) available for analysis for each of the eight taxonomic combinations at varied data levels (ASV 0.01, ASV-25%, FAM 0.01, FAM -25%; see text above for detailed description). Shading reflects the number of ASVs or families available for analysis where green > light green > mustard > light mustard > red. Combinations of taxa denoted by icons as follows: = bacteria [16S]; = plants [*trnL* and ITS2]; = fungi [ITS1] and = arthropods [COI].

	ASV 0.01	ASV - 25%	FAM 0.01	FAM - 25%
	1,672	1,254	194	145
	1,887	1,415	98	89
	1,211	912	117	88
	2,883	2,166	311	233
	3,559	2,669	292	234
	3,098	2,327	215	177
	4,770	3,581	409	322
	4,928	3,700	425	334

i) Paired site comparison: Biological communities are different ($p \leq 0.099$) between paired sites; results differ based on a) target taxa and b) data level analyzed (**Table 3**). Datasets that included bacteria were better at separating paired sites. Utilizing data at the sequence (ASV) level is optimal. Notably, 72% of locations without paired-site separation, when utilizing data from any taxonomic combination or data level, were mineral rich.

ii) Within field station comparison: For this study, samples were collected from 30 locations across 17 NC State research field stations. Notably, from eight research field stations samples from two or more locations were collected. This sampling scheme allowed us to examine whether locations

within a field station could be separated based on the recovered biological communities. Spatial separation of locations was visible in PCA plots for some stations.

iii) Within region comparison: Within each of the three physiographic regions (coastal, mountains and piedmont), the biological communities were different ($p \leq 0.001$) for all datasets except for FAM -25% fungi. Spatial separation of locations was visible in PCA plots (**Figure 6**).

Table 3. Percentage of paired sites (A to B) that could be differentiated with DNA metabarcoding data based on varying combinations of taxa (columns) (icons denote the following = bacteria [16S]; = plants [*trnL* and ITS2]; = fungi [ITS1] and = arthropods [COI]) and different data levels (rows) (ASV 0.01, ASV - 25%, FAM 0.01 and FAM -25%; see text above for detailed description). Shading denotes the following: green = 100%, light yellow = 90-99%, no shading = 60-89%, red = <60%.

ASV 0.01	100%	86%	75%	100%	97%	86%	97%	97%
ASV - 25%	100%	86%	34%	100%	100%	93%	100%	100%
FAM 0.01	76%	90%	59%	73%	86%	90%	86%	86%
FAM -25%	83%	76%	17%	83%	93%	76%	93%	93%

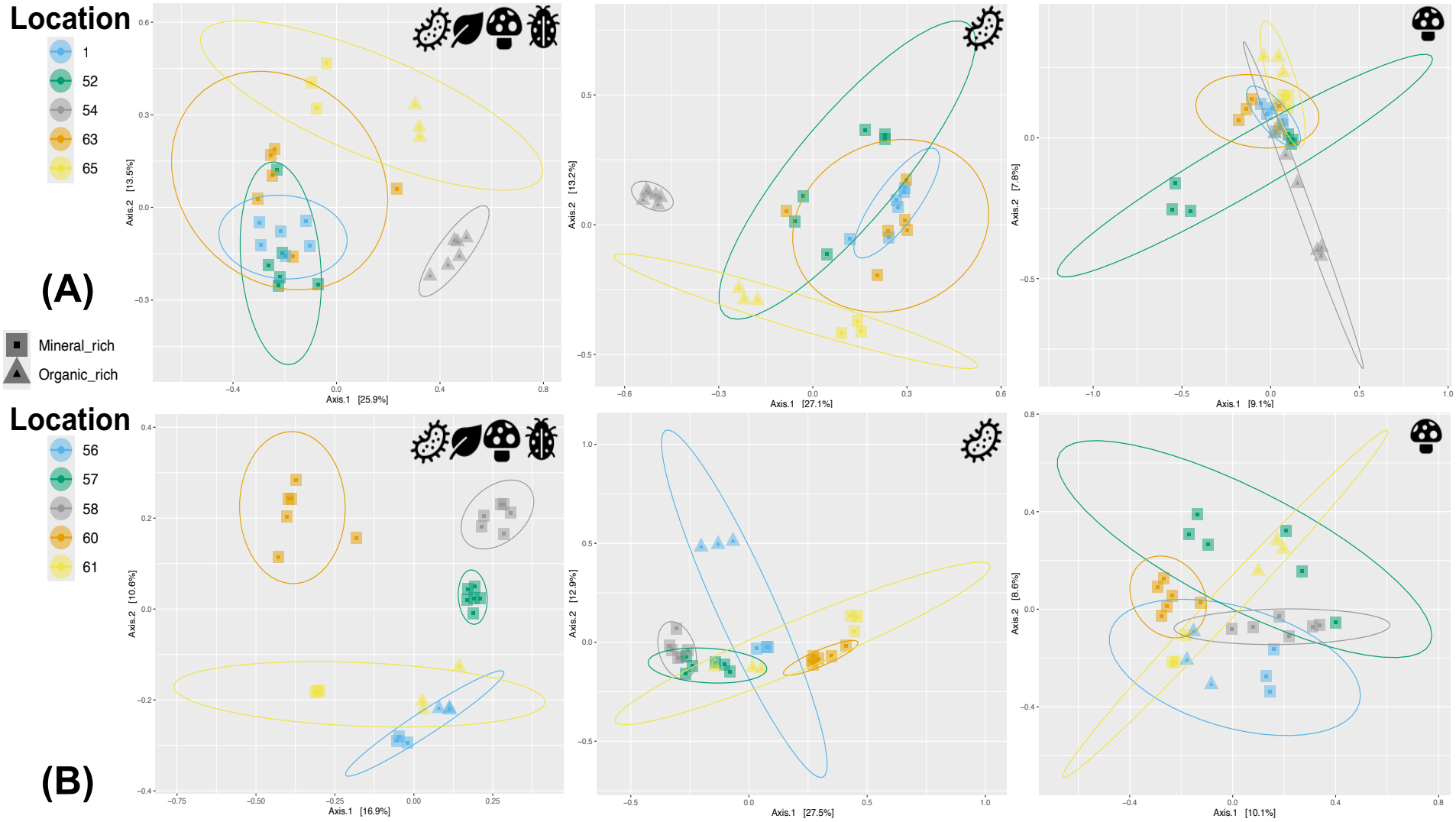


Figure 6. PCA Plots Comparing Separation of Locations Within a Given Region via Bray-Curtis Dissimilarity at the ASV 0.01 Level. (A) Piedmont region – with five locations (1, 52, 54, 63, and 65), and (B) Mountain region – with five locations (56, 57, 58, 60 and 61). Icons shown in the upper right corner of each plot denotes the taxa used to make the multidimensional plots. Ellipses are drawn around samples of a single location.

5) Results from Mineral ID and counting by optical microscopy

As a basis of comparison to methods in use by forensic examiners, the trace minerals present on grain mounts were identified and counted. For purposes of quantitative comparison, a threshold of five or more mineral grains identified in a single grain mount slide from one soil absent in a slide prepared from another was used as a basis of "exclusion."

i) Paired site comparison: About 50% of samples from paired sites could be differentiated by the criterion of the presence of five or more grains of some type in one sample and absence in the sample from the paired site.

ii) Within field station comparison: This threshold led to a 25% false exclusion.

iii) Differences between sites and locations: About 74% of samples could be differentiated from samples from the other 59 sites on the basis of the presence of five or more grains of some type in one sample and absence in the sample from another site.

6) Results from combined methods:

i) New inorganic methods: We successfully integrated the instrumental colorimetry data and the SEM-EDS mineral abundance data and assessed the combined performance of the two methods. It was determined that performance of the combined method has a true positive rate of approximately 80% and a true negative rate of approximately 91%.

It is important to note that there are several limitations to this study which may have influenced the overall performance of the quantitative color and mineralogy (SEM-EDS) method. These limitations include, but are not limited to: 1) the level of mineral classification/specificity can be limited when relying only on elemental composition (*e.g.,* polymorphs, groups with solid-solution, varietal types), 2) soil texture which was not compared, 3) mineral particle morphology which was not assessed, 4) presence of anthropogenic particles which were not identified (*e.g.,* glass, paint, plastics, metals, etc.), and 5) large-scale organic (*e.g.,* plant matter, pollen grains) was not taken into account. The above cited limitations, which are routinely performed in the forensic comparison of soils may have provided a higher level of sample differentiation.

ii) All three new quantitative methods combined: We examined the added value of each of the three new methods for paired site (A & B separation) and have provided the results for two exemplar locations (60 and 61) in this report (**Table 4; Figure 7**). These results highlight scenarios when the new quantitative methods both do and do not provide additional information for sample-to-sample comparisons at a small spatial scale. We are currently preparing a decision tree to complement methods documents that provide guidance as to which scenarios the new quantitative methods may provide exclusionary differences, depending on the spatial scale and soil composition (mineral rich *vs.* organic rich). This information will be included in a planned scientific manuscript that will report on the success and limitations of the combined new quantitative methods, but also will be uploaded as a separate document to NACJD.

Table 4. Performance of traditional forensic geology examination (PLM minerology) compared to new quantitative methodologies (denoted by *) for the analysis of surface soils collected from paired sites (A & B) approximately 300 meters apart in North Carolina.

	Location 60 (Mountain region, organic rich)		**Location 61** (Mountain region, organic/mineral rich)	
Analysis completed	***Separation?***	***Comment***	***Separation?***	***Comment***
PLM minerology	Yes	Zircon present in A but not B	No	Minerals similar
*Instrumental colorimetry**	No	100% have ΔE_{00} <10	Yes	100% have ΔE_{00} >10
*SEM-EDS of minerals**	No	Minerals similar	Yes	Bray-Curtis >0.30375
*DNA metabarcoding**	Yes	Only when using data at the sequence level with bacteria included	Yes	Any taxa at sequence or family level suitable

Figure 7. Exemplar locations for which the performance of new quantitative methodologies to separate paired sites (A and B) were assessed. Left most images (A and B) are from location 60. Right most images (A and B) are from location 61.

7) Business Process Analysis

Table 5 provides an initial business process analysis estimate based on the costs (capital and consumables) for processing 30 cases per year with 5 items per case, with both the existing and new quantitative methods.

Table 5. Business process analysis comparing current and new quantitative methods (denoted in red font) for surface soil analysis. Yellow highlighting denotes instruments possibly existing in forensic laboratories and used for existing analyses such that the capital outlay might not be necessary. # denotes if automated mineral identification software is available, high to develop in house. * denotes cost per sample based on sequencing plants, bacteria, fungi and arthropods, and is highly dependent on how many samples are pooled for sequencing (estimate based on 75 samples pooled per sequencing run, ~15 cases).

	Soil Color		**PLM**	**SEM**	**XRD**	**DNA Metabarcoding (4 taxa)**
	Visual	***Instrumental***	***Grain Mounts***	***Heavy Grains***		***MiSeq ****
Budgetary Costs						
Capital cost (instrumentation)	$ 275	$ 5,000	$ 50,000.00	$ 200,000.00	$ 205,000.00	$ 150,000.00
Lifetime of Capital (years)	5	10	30	20	20	8
Maintenance or calibration costs/year	$ -	$ 500	$ 100.00	$ 3,000.00	$ 15,000	$ 5,000
Consumables/item	$ -	$ -	$ 1.00	$ 3.00	$ -	$ 50.00
Cost/case (excluding labor) (based on 30 cases/year, 5 items/case)	**$ 55.00**	**$ 516.67**	**$ 1,675.00**	**$ 10,115.00**	**$ 10,750.00**	**$ 19,166.67**
Qualitative factors and Labor						
Labor/sample (hr)	0.1	0.1	4	4	1	8
Training Required	Minimal	Minimal	High	Moderate #	Moderate	High
Use of Hazardous Materials	No	No	Minimal	Yes (heavy liquids)	No	Minimal
Minimum soil required	~20mg	~20mg	~10 mg	~10 mg	100 mg	50 mg
Optimal soil required	~200mg	~200mg	~10 mg	~10 mg		100 mg

Applicability to criminal justice:
The implementation of these new methodologies into casework could drive forward the use and significance of forensic geology; greater discrimination between highly similar surface soils and those with limited mineral content, would be possible. In addition, these methods may provide the tools needed for additional crime laboratories to analyze soil in case work. The results may also permit the association of soil evidence to particular sites (geolocation) to aid in the investigative phases of criminal investigations and for intelligence purposes.

2. PRODUCTS:

List of all scholarly products:
None are published at the completion of the performance period.

List of all dissemination activities, including:

i. Conference presentations

- Hannah Dickson, Libby Stern, Jodi Webb, Kelly Meiklejohn and Jack Hietpas (2023). "*Assessment of methodologies for the analysis of surface soils in forensic soil comparisons"* (poster). Southeastern/Northeastern Joint Geological Society of America Meeting (Theme session: *T16. Geoscience for National Security and Law Enforcement)*. Reston, VA.
- Hannah Dickson, Libby Stern, Jodi Webb, Kelly Meiklejohn, Jack Hietpas, Ian Saginor (2023). "*Mineralogical and Color Variations Observed in Surficial Soils"* (oral). New Horizons in Forensic Geoscience: The Bedrock of International Security in Minerals, Mining, Metals, Murders and the Missing." A forensic geology joint meeting between Geological Society of London Forensic Geology Group (GSL-FGG) and International Union of Geological Sciences – Initiative on Forensic Geology (IUGS IFG). Burlington House, London
- Jack Hietpas, Ethan Groves, Chris Palenik. (2023). "*The development of an SEM-EDS based analytical routine for automated mineral identification*" (oral). Northeast Association of Forensic Scientists (NEAFS). Groton, CT.
- Jack Hietpas, Ethan Groves, Liam O'Callaghan, Chris Palenik, Skip Palenik. (2023). "*Investigating the Utility of Novel Techniques for the Forensic Characterization and Comparison of Surficial Soils*" (oral). International Microscopy Conference. Chicago, IL.
- Liam O'Callaghan, Ethan Groves, Jack Hietpas, Skip Palenik, Chris Palenik. (2023). "*Soil preparation for forensic research and analysis*" (oral). International Microscopy Conference. Chicago, IL.
- Ethan Groves, Jack Hietpas, Skip Palenik. (2023). "*Developing automated mineral identification by SEM-EDS for forensic laboratories*" (oral). International Microscopy Conference. Chicago, IL.
- Kelly A Meiklejohn, Jack Hietpas, Hannah Dickson, Melissa KR Scheible, Jodi B Webb, Libby A Stern. (2025). "*Assessment of the Added Value of New Quantitative Methodologies for the Analysis of Surface Soils in Forensic Soil Comparisons"* (oral). 2025 NIJ Forensic Science R&D Symposium. Baltimore, MD.
- Hannah Dickson, Libby A Stern, Jodi B Webb, Jack Hietpas, Kelly A Meiklejohn, Ian Saginor, Michael Smith. (2025). "*Differentiating soil by color and minerology*

for soil comparisons: an experiment using soil from North Carolina" (oral). American Academy of Forensic Sciences Meeting. Baltimore, MD.

- Kelly A Meiklejohn, Melissa KR Scheible, Tiffany Layne, Teresa M Tiedge, Jack Hietpas, Jodi B Webb, Libby A Stern. (2025). "*Assessing the added value of non-human DNA analysis for forensic soil analysis*" (poster). American Academy of Forensic Sciences Meeting. Baltimore, MD.

ii. Webinars, workshops
None.

iii. General press, podcasts, and other media
None.

3. APPENDIX

Documentation of methods

- The *DNA metabarcoding* methods used in this study were recently published in Tiedge et al. (2025) and are available to the public as open access. Notably, the method development was completed before the commencement of this study (such that no funds from this award were used in the method development). Funding from NIJ however through a Graduate Research Fellowship to T.M. Tiedge (2020-R2-CX-0035) was used for method development and was noted as a funding source in the paper.
- A methods paper outlining the *SEM-EDS methodology*, focused primarily on sample processing, reference spectral database and R script to characterize and quantify minerals, is currently in preparation for submission to a peer-reviewed journal. We anticipate submission in Summer 2025.
- The methods used for *instrumental colorimetry* are adapted from Dong et al. 2020. Methods used for PLM will be documented in a paper assessing the implications of stochastic "dropout" of minerals in standard PLM soil examinations. A manuscript has been partially drafted. This will need to be approved internally and we anticipate submission in late Spring 2025.

Standard operating procedures

- The methods papers articulated above contain the details needed for an analyst to complete sample processing. However, more detailed processing protocols for all three methods are available and can be found at the end of this report.

Validation summary

- No validation has taken place by the FBI Laboratory at this time of any of the three new quantitative methods.

4. REFERENCES

- Tiedge, T. M., Rabasco, J. T., & Meiklejohn, K. A. (2025). DNA Metabarcoding Using Indexed Primers: Workflow to Characterize Bacteria, Fungi, Plants, and Arthropods from Environmental Samples. *Diversity*, 17(2), 137. https://doi.org/10.3390/d17020137
- Liau, M., Low, J., Lee, K. H., & Lim, T. B. (2025). Instrumental color determination of local soils and its variation with elemental profiles. Forensic Chemistry, 100644.

- Dong, C. E., Webb, J. B., Bottrell, M. C., Saginor, I., Lee, B. D., & Stern, L. A. (2020). Strengths, limitations, and recommendations for instrumental color measurement in forensic soil characterization. Journal of Forensic Sciences, 65(2), 438-449.
- ASTM E3254 − 23 Standard Practice for Use of Color in the Visual Examination and Forensic Comparison of Soil Samples

V2: March 1, 2025

Guide for the use of the Konica Minolta CM-600d Spectrophotometer and the Konica Minolta CM-2100w SpectraMagic NX Color Data Software for forensic soil color measurement and comparison

1 Purpose

This document describes how to prepare the Konica Minolta CM-600d Spectrophotometer for instrumental color measurement of soil/sediment and how to perform color measurements and color difference calculations in conjunction with the Konica Minolta CM-S100w SpectraMagic NX Color Data Software (Lite Ver. 2.7).

This document also aims to outline instrument and software setup, care, and storage. For more detailed technical information, please see the full instrument manual "Konica Minolta Spectrophotometer CM-700d/600d Instruction Manual" and the full software manual "Konica Minolta Color Data Software CM-S100w SpectraMagic NX Professional/Lite Ver. 2.7 Instruction Manual" available from the links in the References section of this document.

Page 2 of this document, the Quick Guide, is intended as a standalone handbook for users familiar with the user of this instrument and software. Pages 3 through 43 are the full User Guide. It is recommended that the User Guide be read in full prior to use of the outlined procedures.

2 Quick Guide

The following is an abbreviated guide to using the Konica Minolta CM-600d Spectrophotometer for forensic soil color measurement and color difference calculation. This abbreviated guide is intended for quick reference after the user has already reviewed the full user guide. For full guide, see pages 3 through 43.

2.1 Prepare instrument and software

1. Check and clean Target Mask and White Calibration Cap
2. Connect SpectraMagic NX protect key (dongle) and CM-600d to computer USB ports, and connect CM-600d in SpectraMagic NX software
3. Open new document in software, and load template "forensic_comparison.mtp"
4. Perform Zero Calibration (optional) and White Calibration (required)
5. Check Auto Averaging

2.2 Prepare sample for measurement

1. Separate < 1 mm fraction (if possible and appropriate for sample)
2. Clean and fill aluminum sample holder, completely obscuring the sample well bottom (if applicable)

2.3 Make measurement

1. Select Target (questioned/evidentiary) or Sample (known/reference) from menu bar
2. Input soil name
3. Place instrument for measurement
4. Click OK
5. Repeat 2.2 and 2.3 for each soil, cleaning target mask in between soils

2.4 Review and analyze measurements

1. Link Samples to Target
2. Select Target under "Classification by Target" to view data in List Window
3. Edit Judgment and Warning if desired
4. Select Samples in the List Window to view data in graphic objects

2.5 Save data

1. Save measurement file (always perform)
2. Save measurement table (optional)
3. Print canvas (optional)

2.6 Cleanup

1. Clean Target Mask and White Calibration Cap (if necessary)
2. Replace White Calibration cap
3. Disconnect instrument and dongle from computer
4. Store instrument and cables in case

3 Overview of instrument

The Konica Minolta CM-600d Spectrophotometer is a hand-held instrument useful for rapid quantification of color (Figure 1, Figure 2). This instrument illuminates the sample with a known light spectrum and quantifies the light reflected/emitted from a material. It then reports back a color measurement in color spaces such as Munsell and L*a*b*. The instrument's capabilities are further enhanced by the companion software Spectramagic NX (Lite Ver. 2.7), which allows for easy calculation of color difference and visualization thereof.

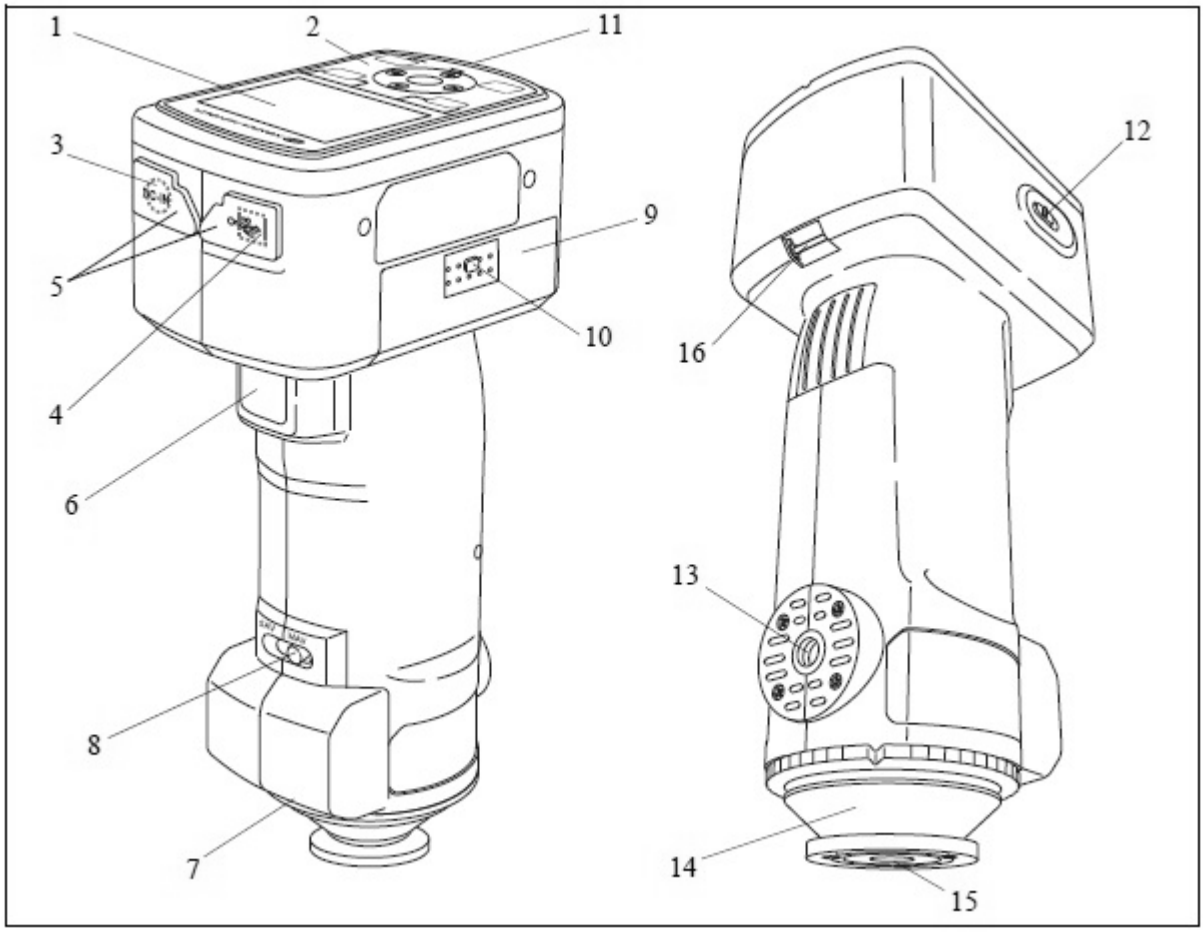


Figure 1: Schematic diagram of the CM-600d Spectrophotometer (from the instrument manual). Parts are as follows:

1. **LCD screen**
2. **Control panel**
3. **AC adapter terminal**
4. **USB connection terminal**
5. **Connector protection covers**
6. **Measuring button**
7. **Pairing No. label**
8. **Measurement area selector (not present in this model)**
9. **Battery chamber cover**
10. **Battery chamber cover button**
11. **READY Lamp**
12. **Power switch**
13. **Tripod mount**
14. **Target Mask**
15. **Specimen measuring port**
16. **Strap holder**

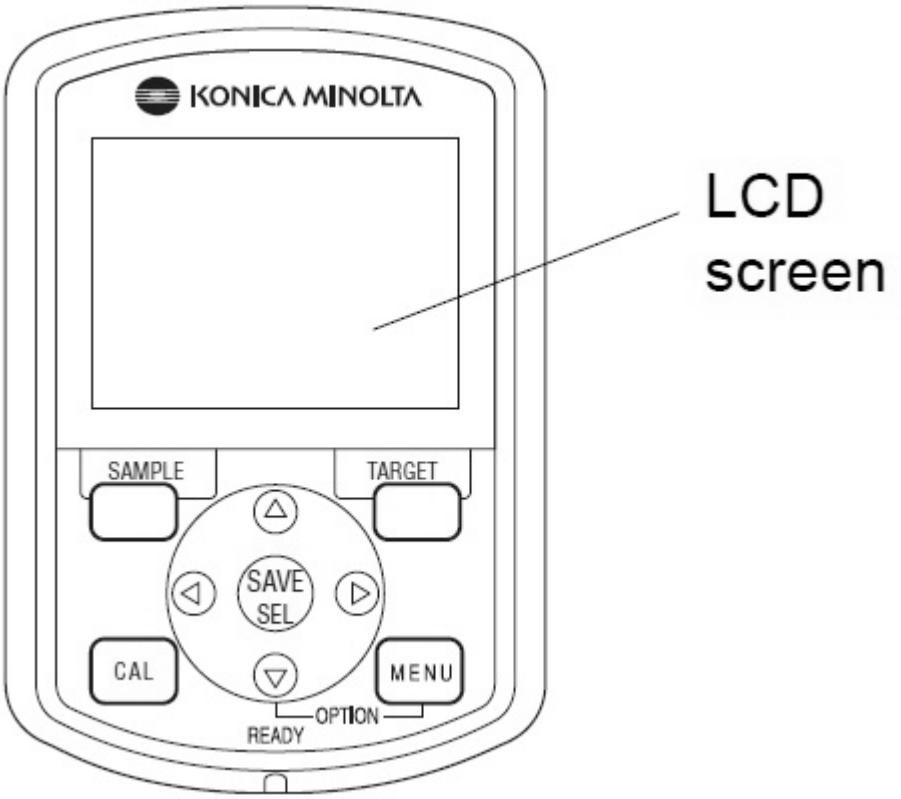


Figure 2: Schematic representation of the control panel and display of the CM-600d Spectrophotometer (from the instrument manual).

4 Preparing the instrument for measurement

4.1 Remove White Calibration Cap

Prior to use, remove the white calibration cap by pinching the lock buttons and pulling the cap straight from the instrument. (Figure 3)

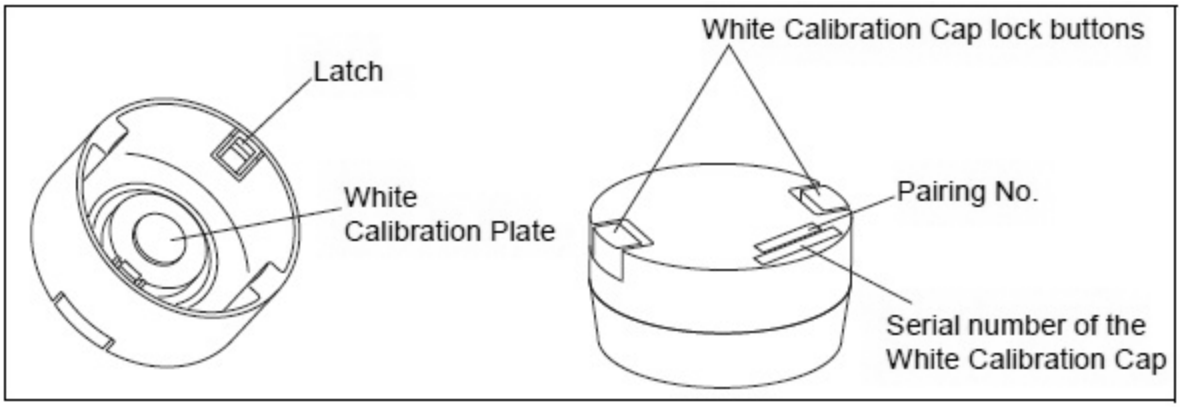


Figure 3: Diagram of the white calibration cap and its release buttons (from the instrument manual).

4.2 Clean and check target mask

Ensure that the CM-A183 target mask with glass plate is attached to the instrument (Figure 4). This instrument has other target masks without a glass covering the measurement opening, but to minimize contamination or damage of the instrument sensor by powders like soil, the CM-A183 mask is recommended.

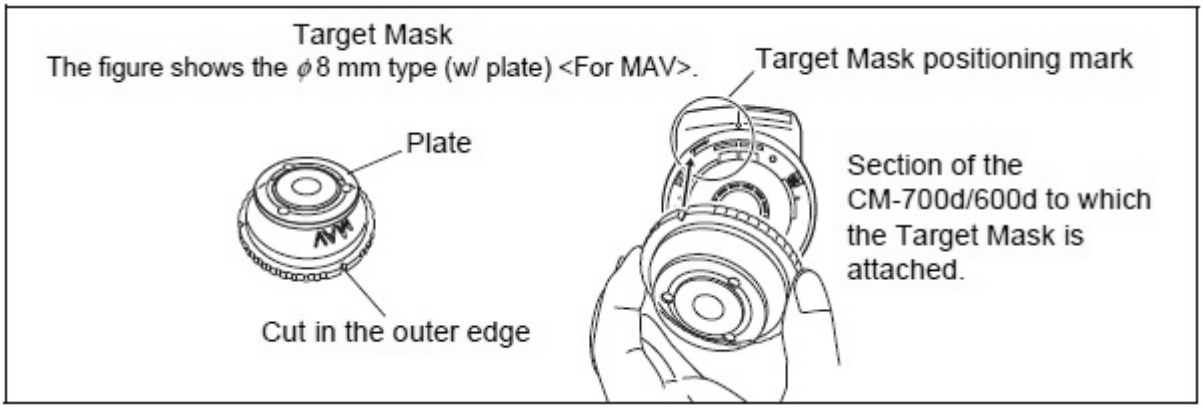
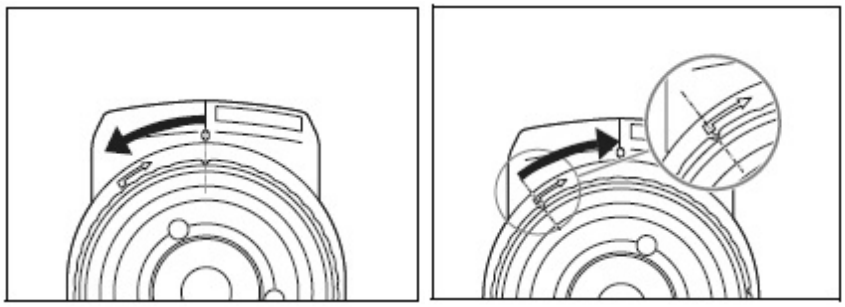


Figure 4: Diagram of a target mask and how it attaches to the instrument (from the instrument manual).

If a different target mask is attached to the instrument, twist counter clock-wise until it releases and pull away to remove (Figure 5 left). To attach CM-A183 target mask, align the mark on the end of the mask with the Target Mask positioning mark and twist clock-wise until it clicks into place (Figure 5 right).



Remove Mask (left) **Attach Mask (right)**

Figure 5: Attachment and removal of a target mask (from the instrument manual).

Before using the instrument, clean the target mask using a moistened tech wipe or cloth, ensuring that the target mask is completely dry before use. Also, check that the target mask does not have visible marks or scratches. If the glass plate on the target mask is scratched, it can't be used because these may interfere with measurement.

4.3 Clean and replace White Calibration Cap

Before replacing the white calibration cap, gently clean the white calibration plate and the glass plate of the target mask with a dry cloth, tech wipe, and/or blower. Lens cleaning solution can be used if necessary. These should be cleaned before and after each use of the instrument, and the target mask should also be cleaned in between samples. Replace white calibration cap

by squeezing the lock buttons, placing the Cap over the target mask, and releasing the buttons. Turn off the instrument whenever the instrument is not in use.

4.4 Software and instrument startup and calibration procedure

4.4.1 Connect cables

The instrument must have a power source in order to function, and must be connected via USB cable to the computer to make use of the software. Since the internal batteries do not currently have the necessary power to run the instrument (Figure 1, item 9), plug the (black) AC adapter into the AC adapter terminal (Figure 1, item 3) and into a wall outlet. Plug the (tan) USB cable into the USB connection terminal (Figure 1, item 4) and into a USB port on the Soil Lab computer.

4.4.2 Connect protect key (dongle)

Connect the protect key to a USB port on the computer.

Figure 6: Protect key

4.4.3 Connect instrument to software

Open Spectramagic NX (Lite Ver 2.7) software using the Desktop shortcut.

Figure 7: Desktop icon for SpectraMagic NX (Lite Ver. 2.7)

Click the “New (Default template)” button to open a new document.

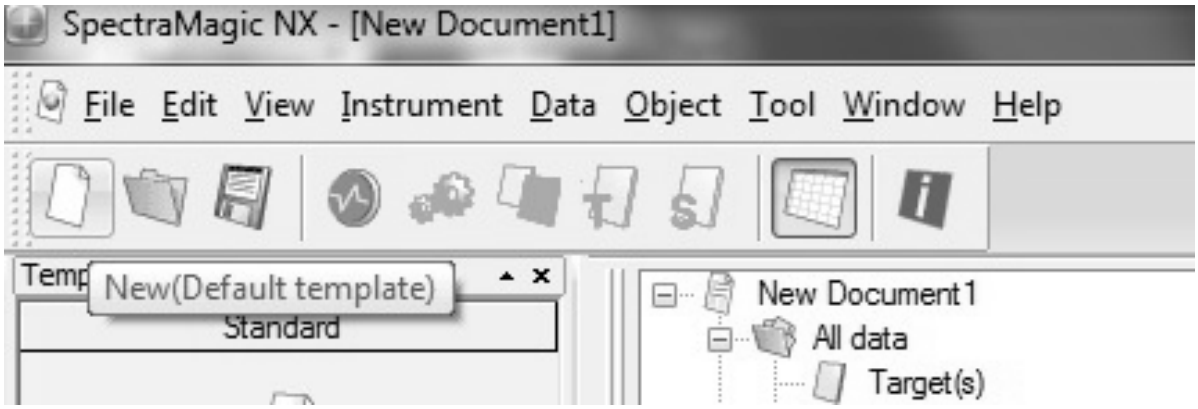


Figure 8: Opening a new document

Figure 9 shows the labeled software interface that will appear upon opening a new document.

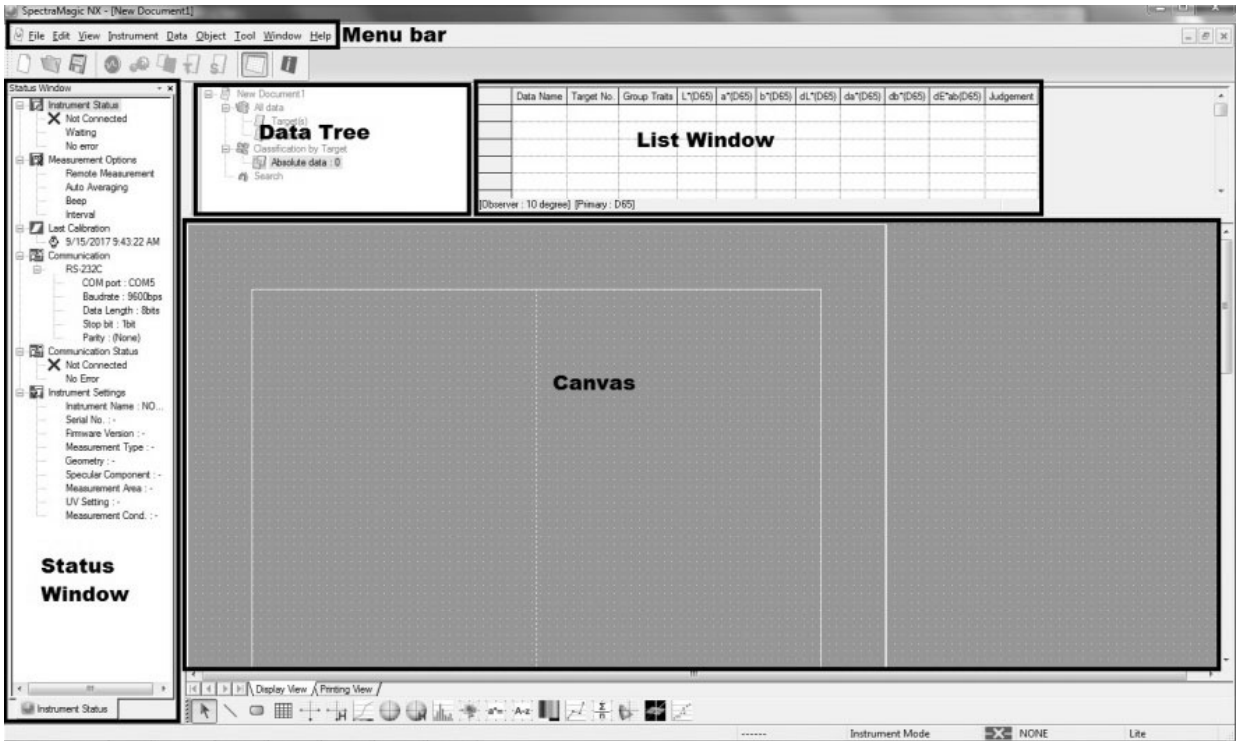


Figure 9: Labeled SpectraMagic NX (Lite Ver. 2.7) interface

Switch on the instrument (Figure 1, item 12). Once the instrument has started up, the message "COMMUNICATING" will appear across the top of the instrument's LCD screen. Select Instrument and then Connect from the menu bar to connect the instrument (Figure 10).

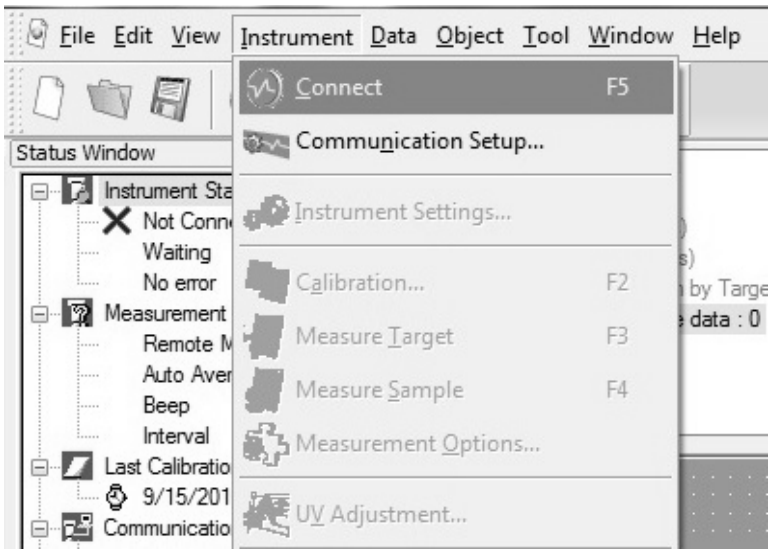


Figure 10: Menu selections to connect instrument

If the message "CALIBRATION RECOMMENDED" appears (Figure 11), the instrument has properly connected; click OK.

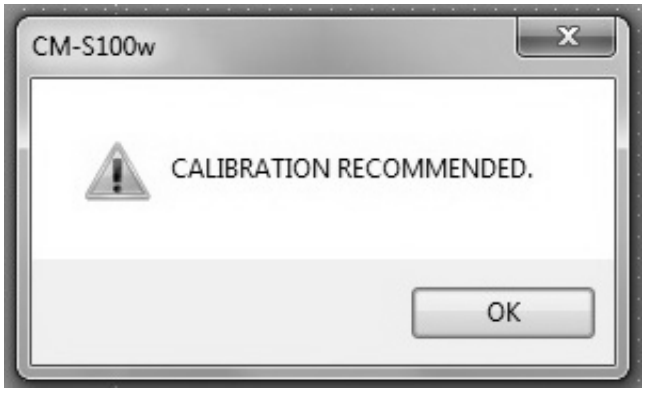


Figure 11: Calibration recommended message, indicates that instrument has properly connected

If the messages in Figure 12 appear, the software is not checking the correct USB port for the instrument.

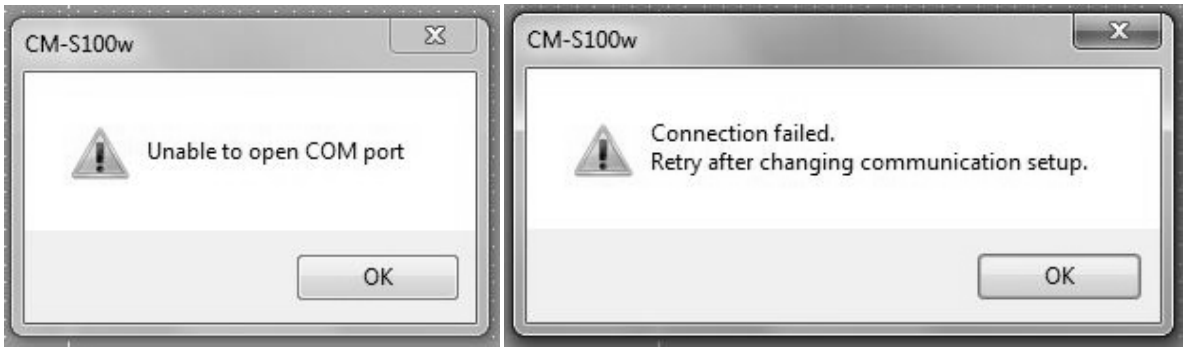


Figure 12: Messages when software is not checking correct USB port upon initial connection attempt

Clicking OK through these messages will open Communication Setup (Figure 13).

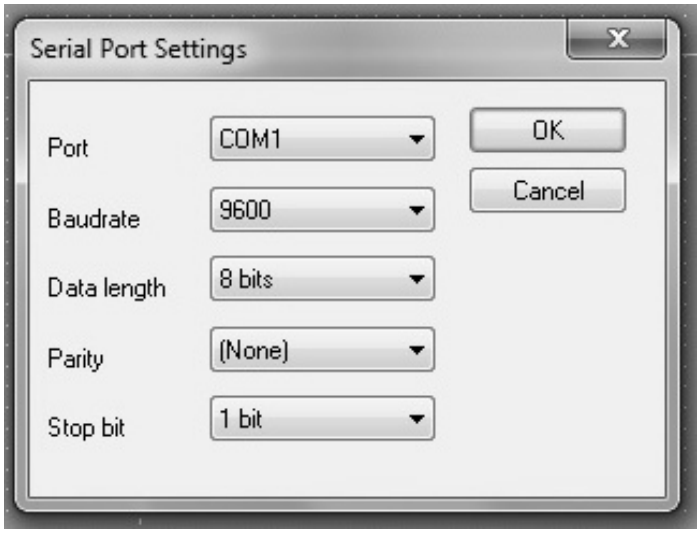


Figure 13: Communication Setup

Click the drop down menu next to Port (Default is COM1) and select the correct port according to what USB port the instrument is connected to (Figure 14).

Figure 14: USB ports on front of Soil Lab computer labeled by COM port

All other options should be kept default. Click OK. Select Instrument and Connect from the menu bar once again to connect the instrument. When "CALIBRATION RECOMMENDED" appears (Figure 11) click OK. If the messages in Figure 15 appear, the wrong port was selected in the Communication Setup.

Figure 15: Messages when wrong USB port is selected

If this happens, click OK through the messages, and then reopen Communication Setup by selecting Instrument and Communication Setup from the menu bar (Figure 16).

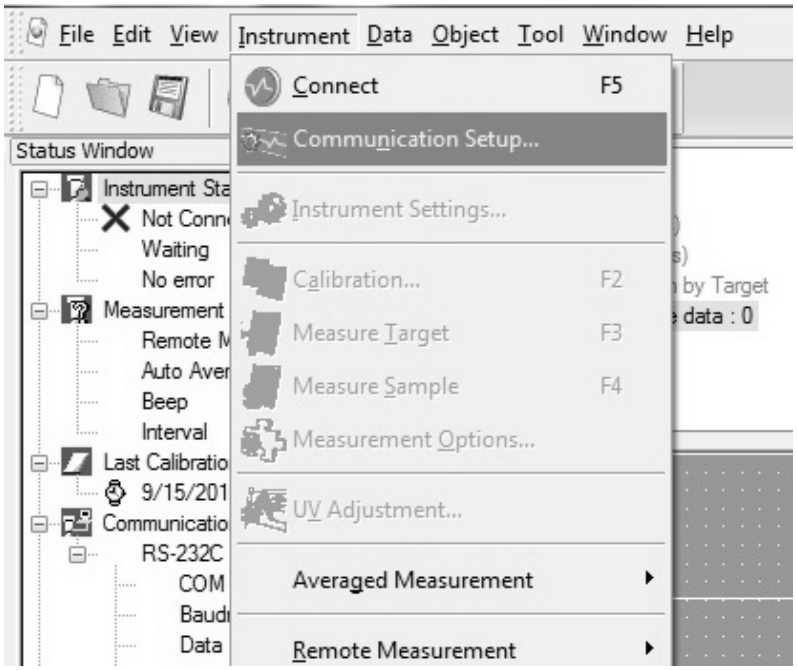


Figure 16: Menu selections to open communication setup

Change the port, click OK, and then connect again (Figure 10).

4.4.4 Load Template

Select File, Template, and Load Template from the menu bar (Figure 14).

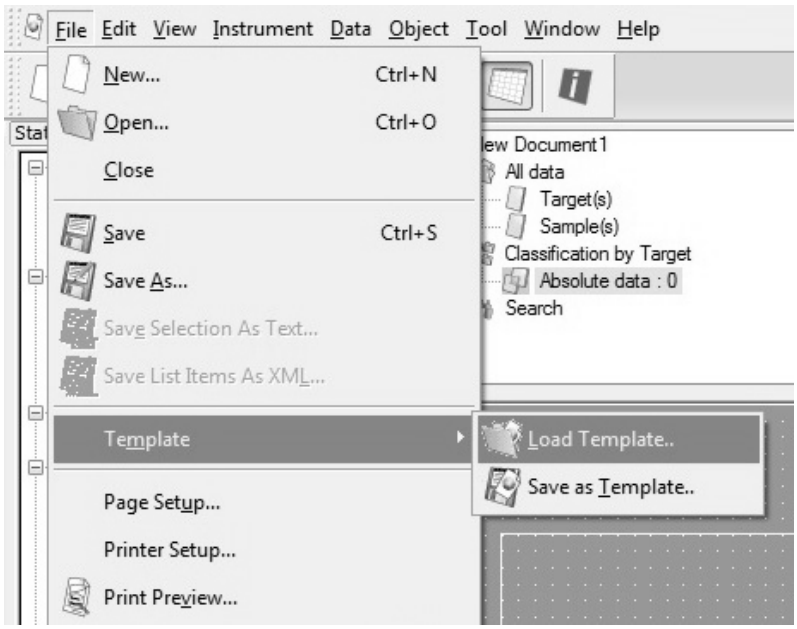


Figure 17: Menu selections to load template

Select the template "forensic_comparison.mtp" from the default template folder. Once the template is loaded, the screen will look like Figure 18. This template adds pre-set graphic objects to the canvas and configures settings and the list window.

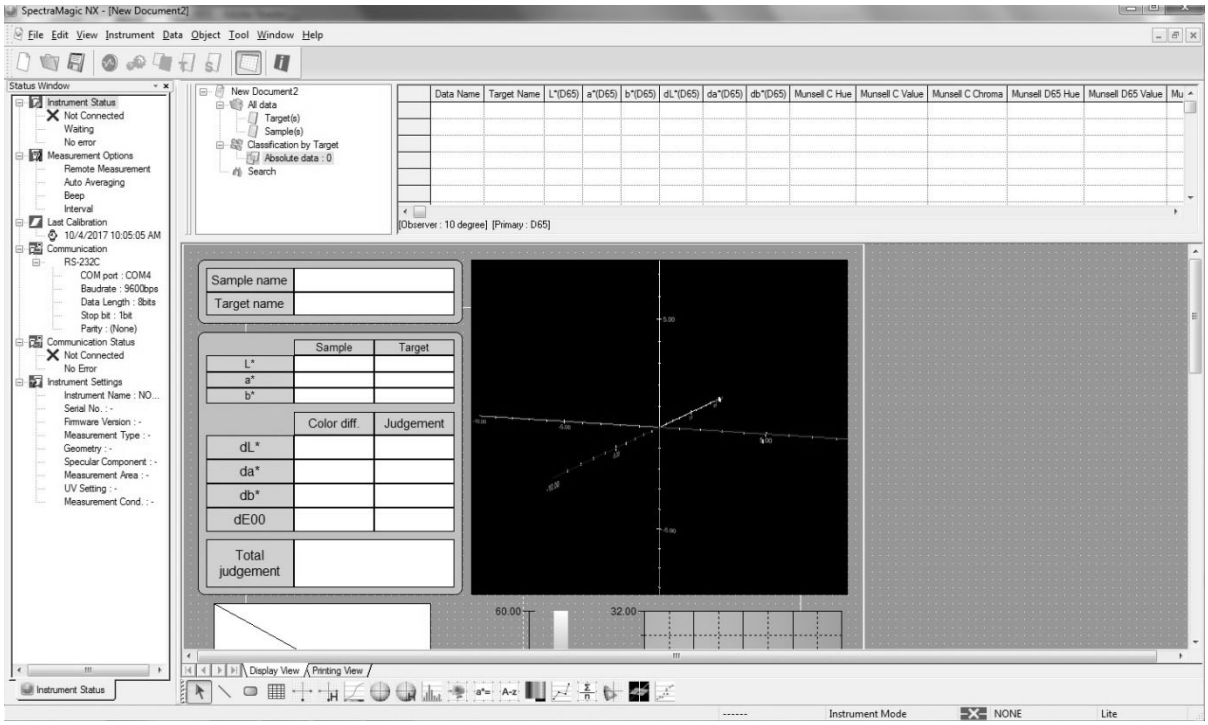


Figure 18: Interface for forensic_comparison.mtp template

4.4.5 Perform a Zero Calibration (occasional procedure)

Select Instrument and then Calibration from the menu bar.

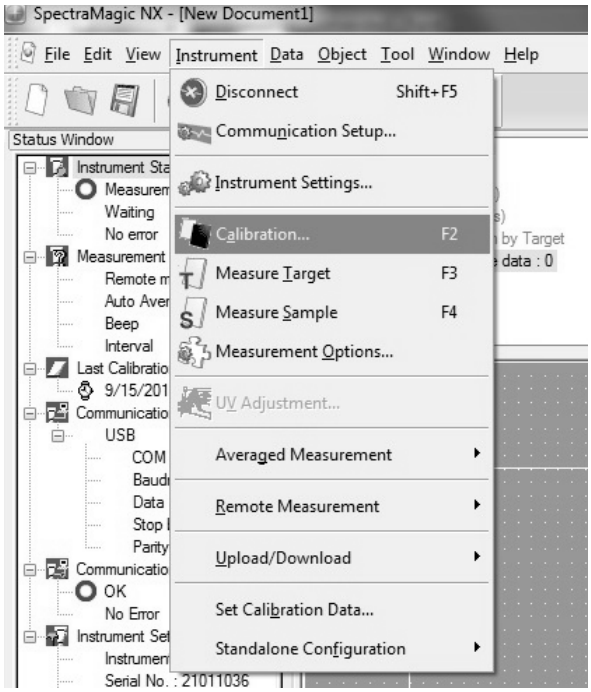


Figure 19: Menu selections to start calibration

This will cause the Zero Calibration dialog box to appear. **DO NOT CLICK ANY BUTTONS YET.**

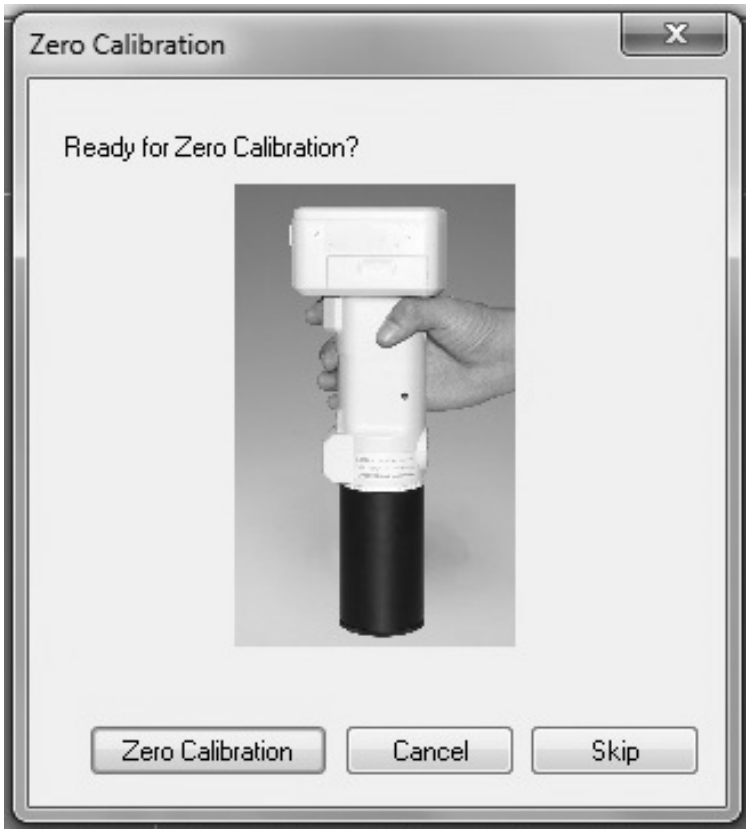


Figure 20: Zero calibration dialog box

Remove the White Calibration Cap. Remove the lid of the CM-A182 Zero Calibration Box and place it on the table top. Place the target mask in the CM-A182 Zero Calibration Box such that the instrument is vertical and resting in the Box (photograph within Figure 20). Click the Zero Calibration button pictured in Figure 20, keeping the instrument as still as possible. The instrument will calibrate in 5 replicates. When this calibration is complete, the clicking sounds will stop, the Ready light will stop flashing and stay green (Figure 1, item 11), and the White Calibration dialog box will appear on the computer screen.

Zero calibration does not need to be performed every time the instrument is turned on. It should be performed any time the measurement conditions change (location, temperature, humidity, etc.) and at least once per month. Always calibrate with the CM-A183 target mask with glass plate on the instrument. Always replace lid of Zero Calibration Box when not in use.

4.4.6 Perform White Calibration (required procedure)

If Zero Calibration was not performed, select Instrument and then Calibration from the menu bar (Figure 19), and click the "Skip" button. If Zero Calibration was performed, the White Calibration dialog box will have appeared (Figure 21). **DO NOT CLICK ANY BUTTONS YET.**

Figure 21: White Calibration dialog box

Replace White Calibration Cap on instrument. Rest the Cap on a table top (photograph within Figure 21). Click the White Calibration button (Figure 21), keeping the instrument as still as possible. The instrument will calibrate in 5 replicates. When this calibration is complete, the

clicking sounds will stop, the Ready light will stop flashing and stay green (Figure 1, item 11), and the dialog box will disappear.

White calibration must be performed every time the instrument is turned on for use, every time measurement conditions change (location, temperature, humidity, etc.), and at least every 20 minutes. Always calibrate with the CM-A183 target mask with glass plate on the instrument.

4.4.7 Check Auto Averaging

Check the status window to ensure that Auto Averaging is set to 5 times (Figure 22).

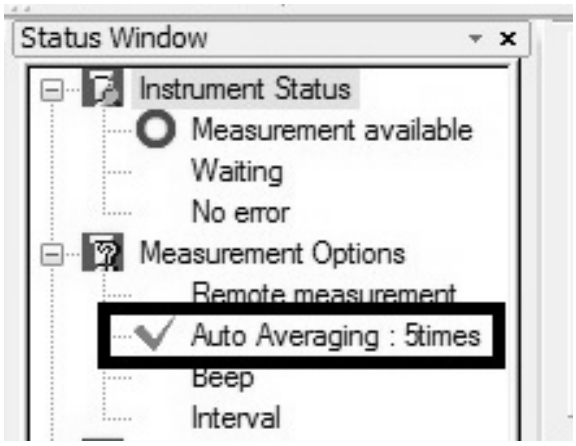


Figure 22: Checking that Auto Averaging is correctly set

If Auto Averaging is not set to 5 times, open Measurement Options by selecting Instrument and Measurement Options from the menu bar (Figure 23).

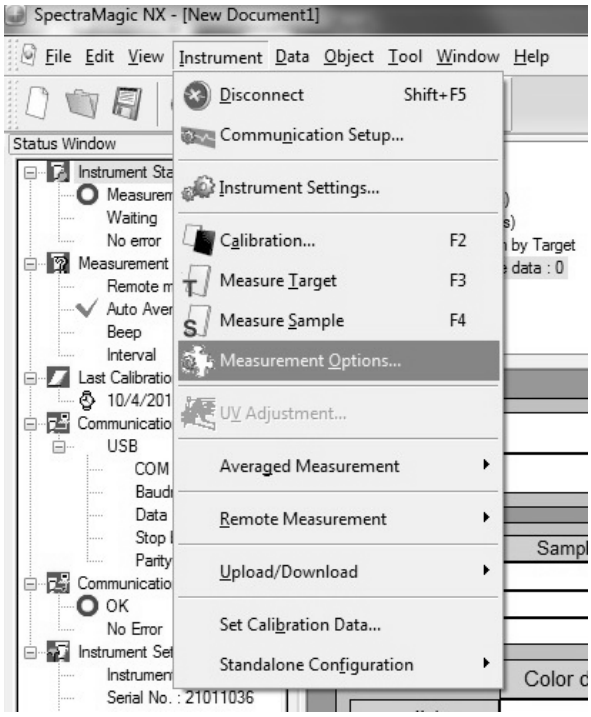


Figure 23: Menu selections to open measurement options

Check the box next to Enable Averaging if necessary and change Number to 5 (Figure 24). Click OK.

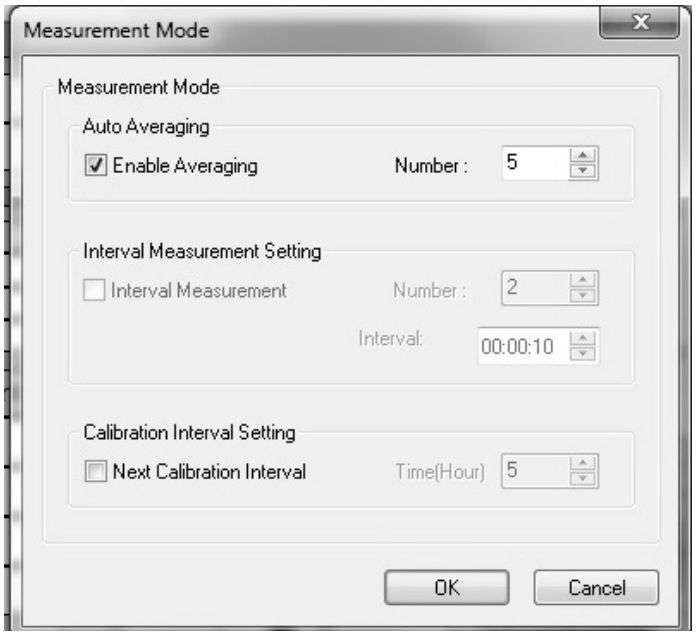


Figure 24: Enabling Auto Averaging

The instrument is now ready for measurement.

5 Sample preparation and measurement procedure

For each sample, follow section 5.1 to prepare the software for measurement. After preparing the software, follow one of the following sections according to the material being measured to prepare the sample: 5.2 (fine particles in the aluminum sample holder), 5.3 (large intact aggregates), or 5.4 (sample in evidence container). After preparing the sample, follow section 5.5 to perform the measurement. The sample preparation procedure in section 5.2 is the preferred procedure over those in sections 5.3 or 5.4 for samples of limited quantity.

5.1 Choose appropriate measurement type and enter data name

For questioned soils, select Instrument then Measure Target from the menu bar (Figure 25 Left). For known soils, select Instrument then Measure Sample from the menu bar (Figure 25 Right). These Target and Sample designations can be changed later if necessary.

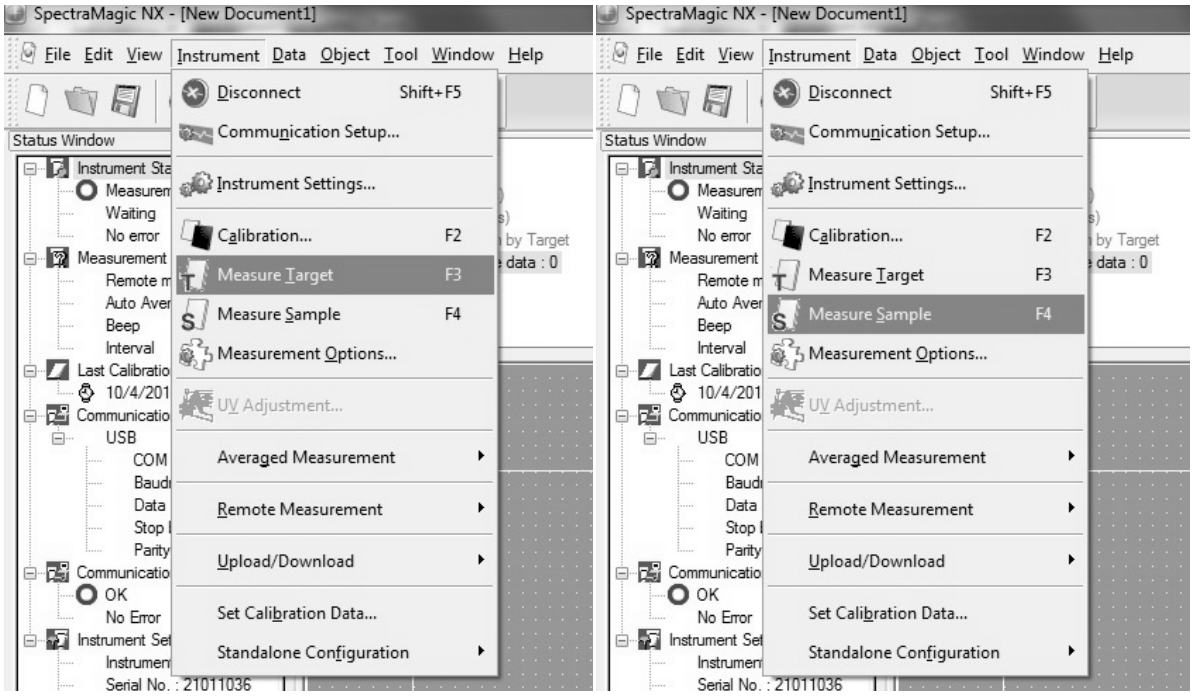


Figure 25: Menu selections to measure a questioned soil as a Target (Left) and to measure a known soil as a Sample (Right)

For both Targets (questioned soils) and Samples (known soils), enter the identifier of the soil in the Name box and any further information you would like to attach to the measurement in the Comment box (Figure 26).

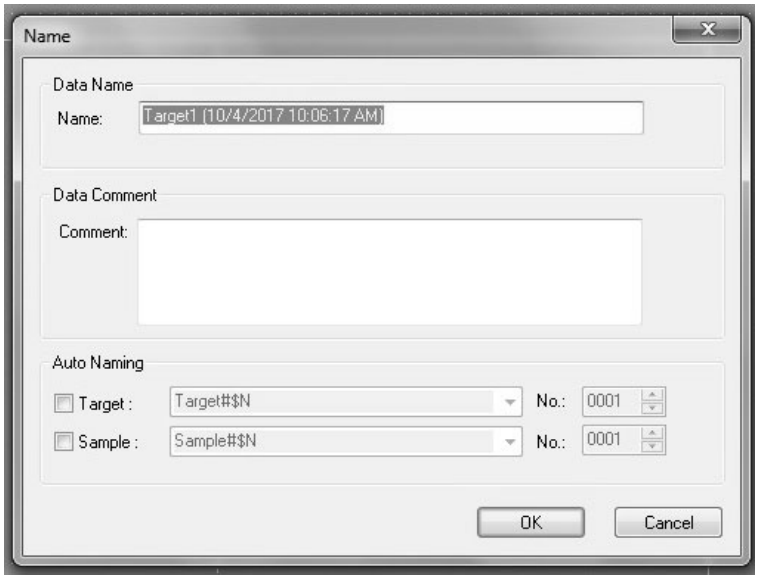


Figure 26: Sample/Target measurement and name input

These can also be edited later. **DO NOT CLICK "OK" YET**; clicking OK starts the measurement, so the instrument must be placed first.

5.2 Measuring in the sample holder

Section 5.2 describes how to measure soils and sediments in the custom aluminum sample holders.

5.2.1 Clean the sample holder

Clean the sample holder prior to use and in between samples. This can be cleaned using either water or alcohol and a tech wipe. Ensure that the sample holder is completely dry before proceeding.

Figure 27: Custom aluminum sample holder, 1mm deep

5.2.2 Separate < 1 mm size fraction (optional)

If possible, separate the < 1 mm size fraction of the sample. This can be done via gentle sieving or via gentle shaking of the sample in a petri dish and removing the fines using a spatula. If enough soil is present, using multiple subsamples can minimize error.

5.2.3 Fill sample holder

For samples with particle sizes less than the depth of the sample holder(s), transfer the sample to the sample well on the aluminum sample holder. For soils with a significant < 1 mm size fraction, the 1 mm deep sample holder should be used. For potentially translucent sands and coarser soils (samples dominated by 1 to 3 mm aggregates), the deepest sample holder that can be completely filled should be chosen; aluminum sample holders with wells 1 and 3 mm deep have been made. Note that all samples to be measured must be dry. Fill the sample well as full as possible without filling over the edge of the well. Use at least 0.020 g of organic soils and at least 0.040 g of mineral horizon soils. This quantity of soil or sediment should visibly obscure the bottom of the sample well and therefore be sufficient for an accurate color measurement. Gently shake the sample holder horizontally to distribute the sample evenly in the well and flatten the sample surface. Ensure that the surface of the subsample in the sample holder is as representative as possible of the sample overall.

5.2.4 Place instrument in sample holder

Remove White Calibration Cap. Place the target mask attached to the instrument in the indentation in the sample holder (Figure 28). This will center the target mask aperture over the sample well. The instrument's weight should rest on the target mask.

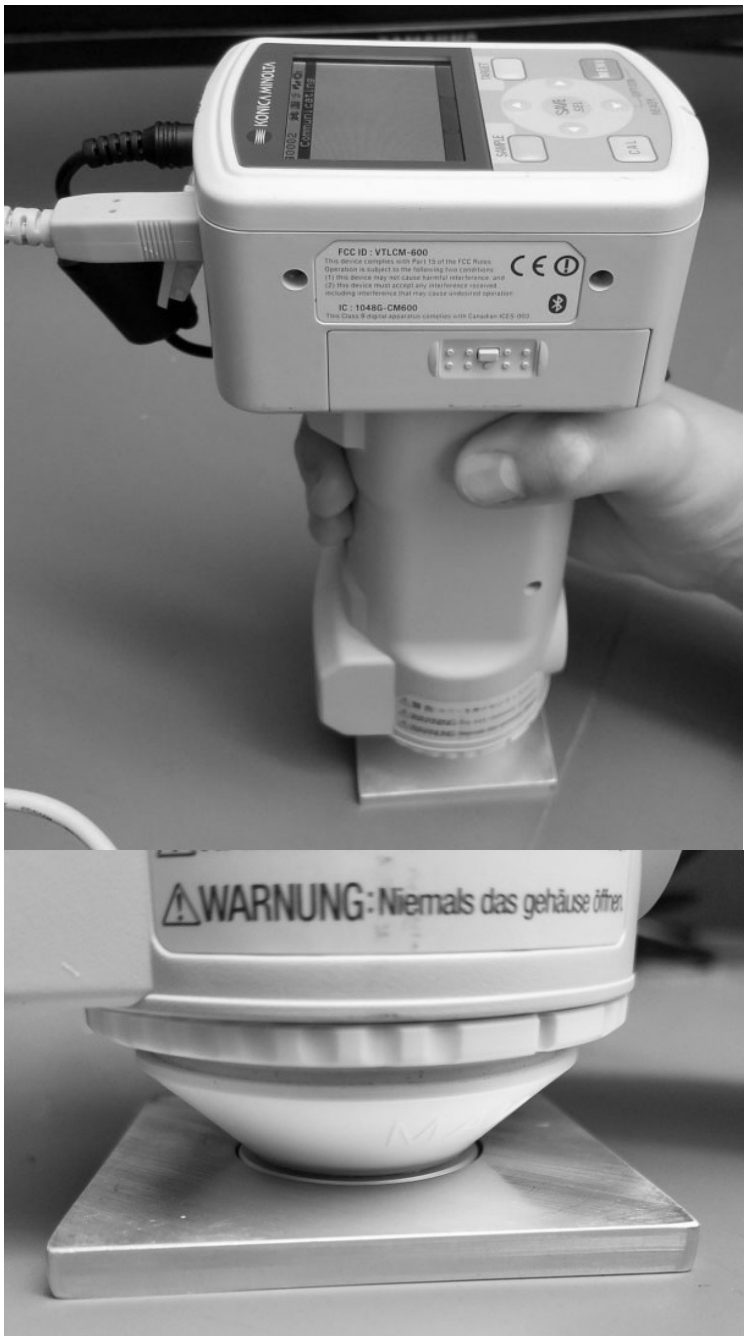


Figure 28: Placing instrument in sample holder for color measurement

5.3 Measuring aggregates

Section 5.3 describes how to measure soil aggregates ≥ ~ 8 mm in diameter which the user wishes to keep intact.

Rest the instrument on its side on the table top. Choose a relatively flat face of the aggregate and hold it against the target mask, covering as much of the aperture as possible. Measuring in this way prevents the weight of the instrument from breaking the aggregate.

Figure 29: Placing aggregate for color measurement

5.4 Measuring from evidence container

Section 5.4 describes how to measure soil directly from the soil's evidence container, provided the container and the sample itself are large enough to allow such measurement (for example, see Figure 30).

Choose or create an even surface in the evidence container large enough to place the target mask flat without interference from the sides of the container. Ensure that this surface is uninterrupted by plant matter, etc. that would interfere with an accurate color measurement. If possible, choose a surface with finer particles to ensure a more precise measurement. Rest the target mask of the instrument gently on the even surface. Avoid pressing the mask into sandy material; this might scratch the glass of the target mask.

Figure 30: Placing instrument in evidence container for color measurement

5.5 Perform measurement

When the instrument is placed and you are ready to begin the measurement, click the OK button in the dialog box (Figure 26) to begin measurement. The instrument will measure in 5 replicates with a dialog box on the screen recording its progress.

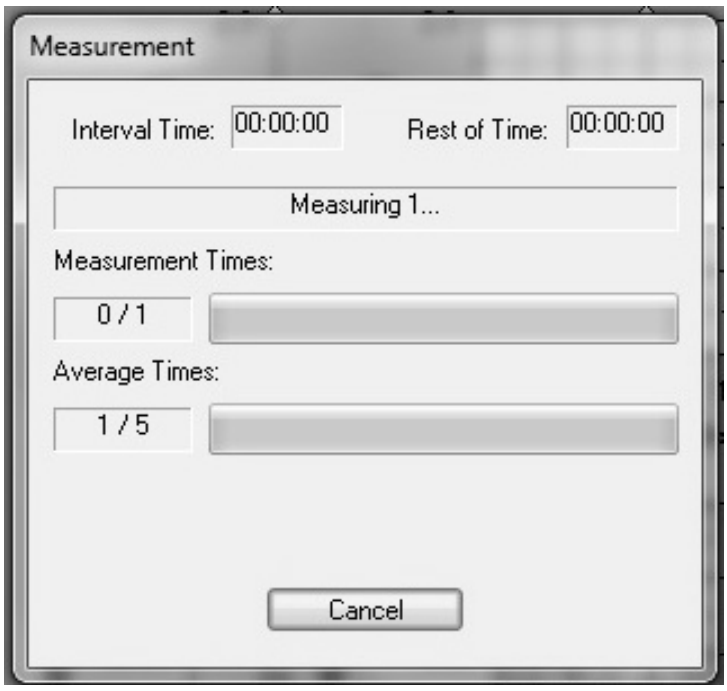


Figure 31: Measurement dialog box

Keep the instrument as still as possible during measurement, and do not move it until it has made 5 click sounds, the READY light stops flashing and stays green, and the dialog box disappears. There will be approximately 2 seconds in between measurements. Repeat section 5 for all soils, choosing the appropriate measurement type for each soil and ensuring that the sample holder and target mask are clean and dry before use.

6 Viewing and comparing measurements

Measurements can be viewed in the List Window (Figure 32).

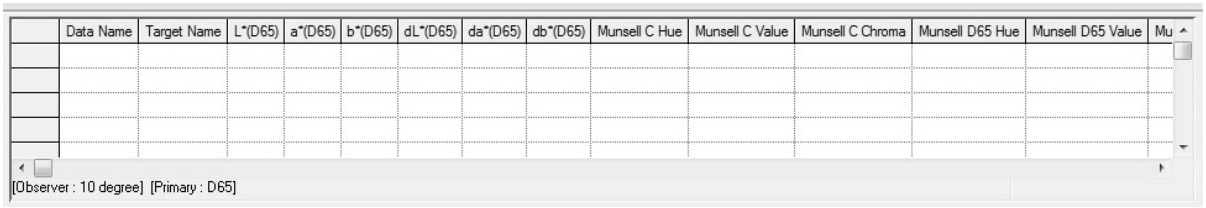


Figure 32: SpectraMagic NX List Window

Which measurements are displayed in the List Window can be changed by clicking on different items in the Data Tree (Figure 33).

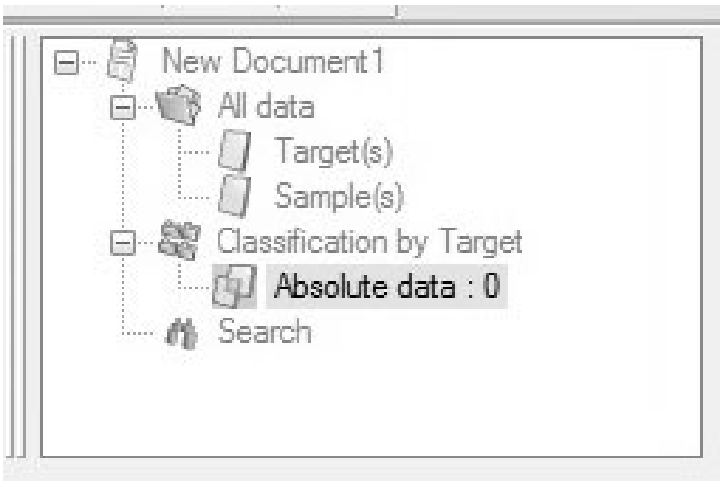


Figure 33: SpectraMagic NX Data Tree

6.1 Procedure for a single questioned soil sample

Follow this procedure if known soils are being compared to only one questioned soil.

6.1.1 Display samples

Click Sample(s) in the Data Tree to display all Samples.

6.1.2 Select samples

Click the box at the top left of the List Window to select all Samples (Figure 34).

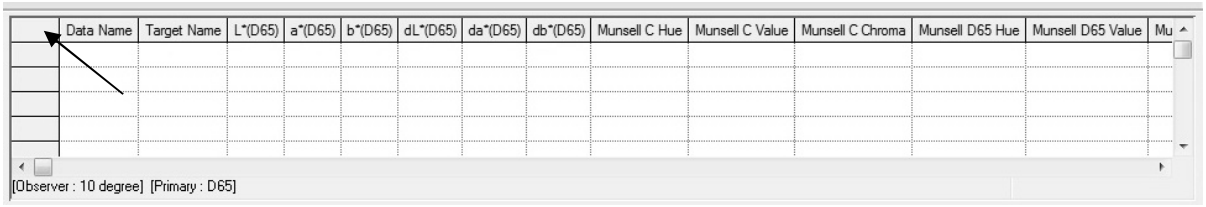


Figure 34: Selecting all data in the List Window

Samples may also be selected by clicking the box to the left of the Sample in the List Window. To select multiple Samples, Shift-click (select all records in between the clicked and Shift-clicked record) or Control-click (select all records that are Control-clicked).

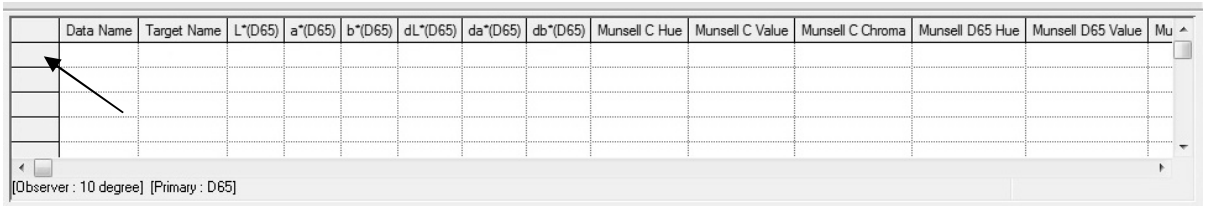


Figure 35: Selecting individual record(s) in the List Window

6.1.3 Link to Target (to compare known(s) to questioned)

Right click a selected Sample. Select Tool then Change Target from the menu that appears (Figure 36).

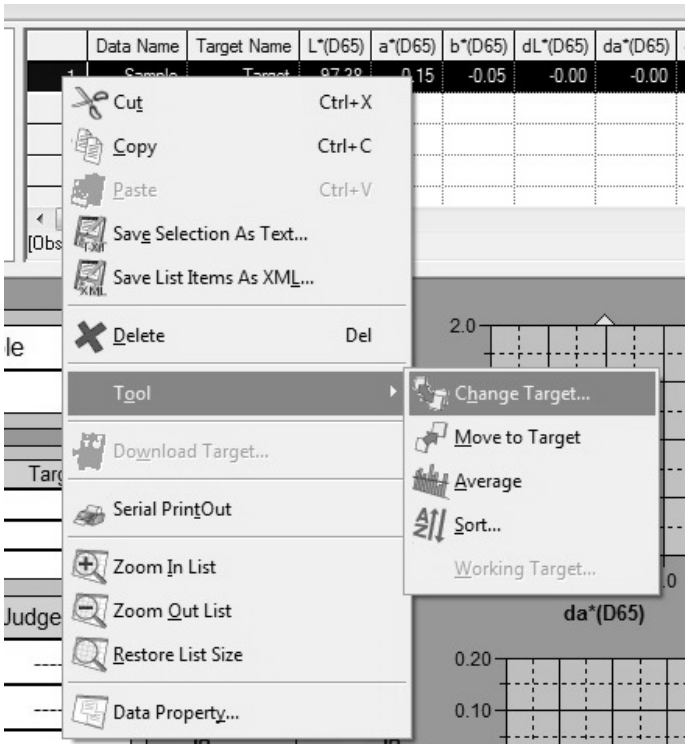


Figure 36: Selections to link Target

In the Target Linkage dialog box that appears, select Link to Specified Target if necessary and select the questioned soil from the Target drop-down menu (Figure 37). Click OK.

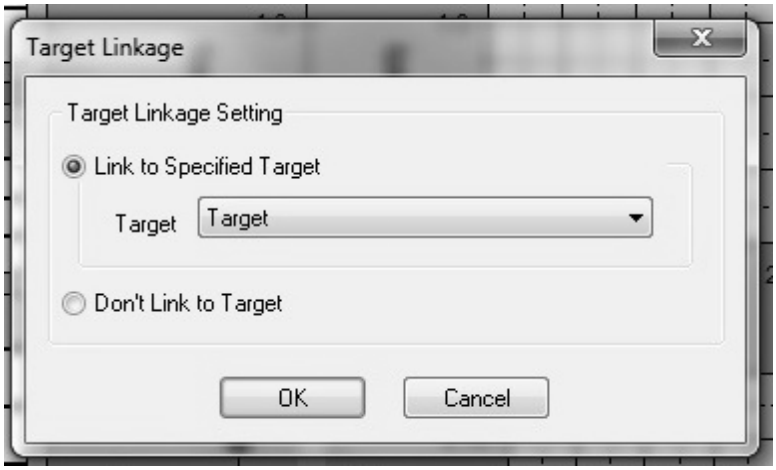


Figure 37: Selecting the questioned soil to link to known soil(s)

6.1.4 Display measurements and color differences

Click on the name of the Target (questioned soil) in the Data Tree under the Classification by Target level (Figure 38). The number next to the Target's name is the number of Samples linked to it. A Sample (known) must be linked to a Target (questioned) in order for the color difference to be calculated and displayed.

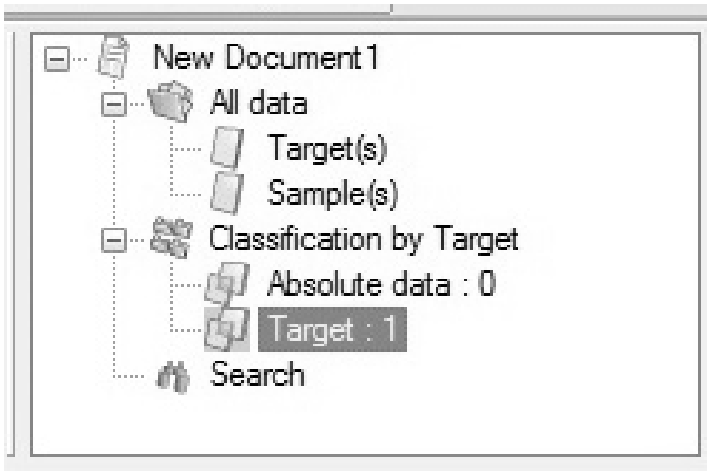


Figure 38: Selecting Target in the Data Tree

6.2 Procedure for multiple questioned soils

For cases in which there is more than one questioned soil, Samples must be linked to each Target (as in section 6.1.3) separately; Samples cannot be linked to more than one Target at a time, so if multiple questioned soils are being compared to the same known soils, the comparisons for each questioned soil must be done one at a time. Repeat section 6.1 for each questioned sample, saving the measurement table in between if desired for later viewing (see section 7.3).

6.3 Interpreting the List Window

As mentioned in section 6.1.4, to view the Target-Sample pair, click the name of the Target under the Classification by Target level in the Data Tree. The Target measurement will

be displayed at the top of the List Window with the Samples underneath. Table 1 shows the columns displayed in the List Window and their meanings. Note that illuminant C is daylight proxy without ultraviolet and illuminant D65 is daylight proxy with ultraviolet.

Table 1: List Window columns

Column	Meaning
Data Name	The assigned name of the measurement
Target Name	The name of the Target to which the measurement is linked
dE00	ΔE (perceptible color difference) between the sample and the linked Target calculated using the ΔE00 algorithm (called dE00 in this document as that is how it is referred to in the instrument and software, but usually referred to as ΔE00)
Judgment	Whether the sample is within the dE00 thresholds of the Target (Pass/Warning/Fail)
L*(D65)	L* parameter of the measurement (illuminant D65), dark to light (0 to 100)
a*(D65)	a* parameter of the measurement (illuminant D65), green to red/magenta (-128 to 127)
b*(D65)	b* parameter of the measurement (illuminant D65), blue to yellow (-128 to 127)
dL*(D65)	The difference between L* for the Sample and the linked Target (illuminant D65)
da*(D65)	The difference between a* for the Sample and the linked Target (illuminant D65)
db*(D65)	The difference between b* for the Sample and the linked Target (illuminant D65)
Munsell C Hue	Munsell hue for the measurement (illuminant C)
Munsell C Value	Munsell value for the measurement (illuminant C)
Munsell C Chroma	Munsell chroma for the measurement (illuminant C)
Munsell D65 Hue	Munsell hue for the measurement (illuminant D65)
Munsell D65 Value	Munsell value for the measurement (illuminant D65)
Munsell D65 Chroma	Munsell chroma for the measurement (illuminant D65)

The forensic_comparison.mtp template will give Munsell measurements in both illuminant C and illuminant D65, allowing for determination of the presence of metamerism.

The dimensionless parameter dE00 quantifies the difference between two color measurements. Lower dE00 values indicate less perceptible difference and higher values indicate more perceptible difference with 0 being the lowest possible value and 100 being the highest possible value. This parameter is not efficient to calculate by hand and therefore must be calculated in the software. Based on the dE00 calculations in the validation study as well as outside assessments of the color difference marking the limits of human perception (Ikeda et al., 2003; Ishikawa-Nagai et al., 2009; Mokrzycki and Tatol, 2011; Ocean Optics, 2015),

Table 2 was devised proposing provisional thresholds for dE00, including what these thresholds indicate, how these thresholds relate to Munsell, the corresponding recommendation, and the default Judgment given by the software (see section 6.4). These thresholds do not apply to circumstances in which the color of an evidentiary soil might be expected to differ from known exemplars

Table 2: Provisional dE00 value thresholds

~dE00	Indication	Relation to Munsell	Recommendation
<1	Color difference is most likely not perceptible by the human eye	Samples will appear to be the same color	The possibility that the soils are derived from the same source cannot be eliminated; conduct further examinations.
1-2	Color difference is perceptible when observed side by side by a trained professional	Color difference is almost definitely within one chip	The possibility that the soils are derived from the same source cannot be eliminated; conduct further examinations.
2-3.5	Slight but apparent color difference that is perceptible to both the untrained and the trained, still most likely similar enough in color to warrant further comparison	Color difference is most likely within one chip, assuming difference in hue exclusively	Soil color is sufficiently similar that the possibility that the soils are derived from the same source cannot be eliminated; conduct further examinations.
3.5-6	Apparent color difference between samples, may be different enough in color to not warrant further comparison	Color difference is most likely approaching or is greater than or equal to one chip in one or more dimensions, especially in hue and chroma	Color differences are minor and might indicate soils originated from distinct sources.
6-10	Distinct color difference between samples, most likely different enough in color to not warrant further comparison	Color difference is most likely at least one chip in at least one dimension, including value	Color differences likely soils originated from distinct sources, but additional soils assessments are recoemmended
>10	Samples are clearly different in color	Difference is almost definitely at least one chip in at least one dimension	Color differences indicate soils originated from distinct sources; no further examinations needed.

Applying prior forensic decision practices that used Munsell color criteria to ΔE00 color difference is complicated by the fact that the different Munsell parameters all have different magnitudes of impact on perceptible color difference. A single Munsell chip offset is the nominal color resolution, but a one chip offset in value produces a significantly larger difference in dE00 (and thus larger perceptible color difference) than differences in the other two Munsell dimensions, especially hue. Thus, using dE00 to guide decision criteria in forensic soil comparisons must undergo further testing to refine appropriate decision thresholds.

6.4 Judgment

The forensic_comparison.mtp template sets a dE00 Judgment tolerance of 10 and a Warning tolerance of 5 by default. This means that Samples that have a dE00 of greater than 10.00 will be designated as Fail (outside of the tolerance), Samples that have a dE00 of 5 to 10 will be designated as Warning (inside of the tolerance but approaching the tolerance threshold), and Samples that have a dE00 of less than 5.00 will be designated as Pass (well within the tolerance). The Judgment can be disabled or edited by the user.

6.4.1 Disabling Judgment

Disabling Judgment will cause Judgments to not be displayed in the List Window and in the table on the canvas (there will be nothing displayed in the Judgment column/boxes), and will affect how the measurement points will appear in the plots on the canvas. To disable it for future Targets, select Data and then Default Tolerance Setting from the menu bar (Figure 39). To disable it for the current Target, select Data and then Tolerance Setting (Figure 40).

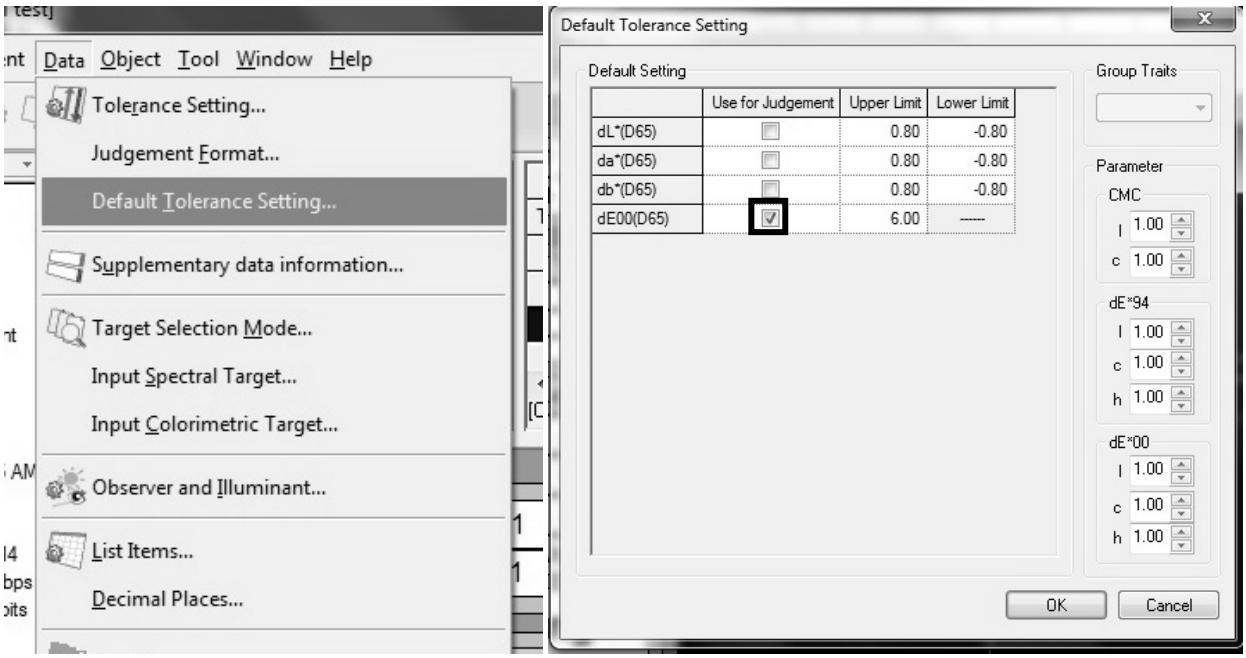


Figure 39: (Left) Menu selections to open Default Tolerance Settings and (Right) Default Tolerance Setting Window

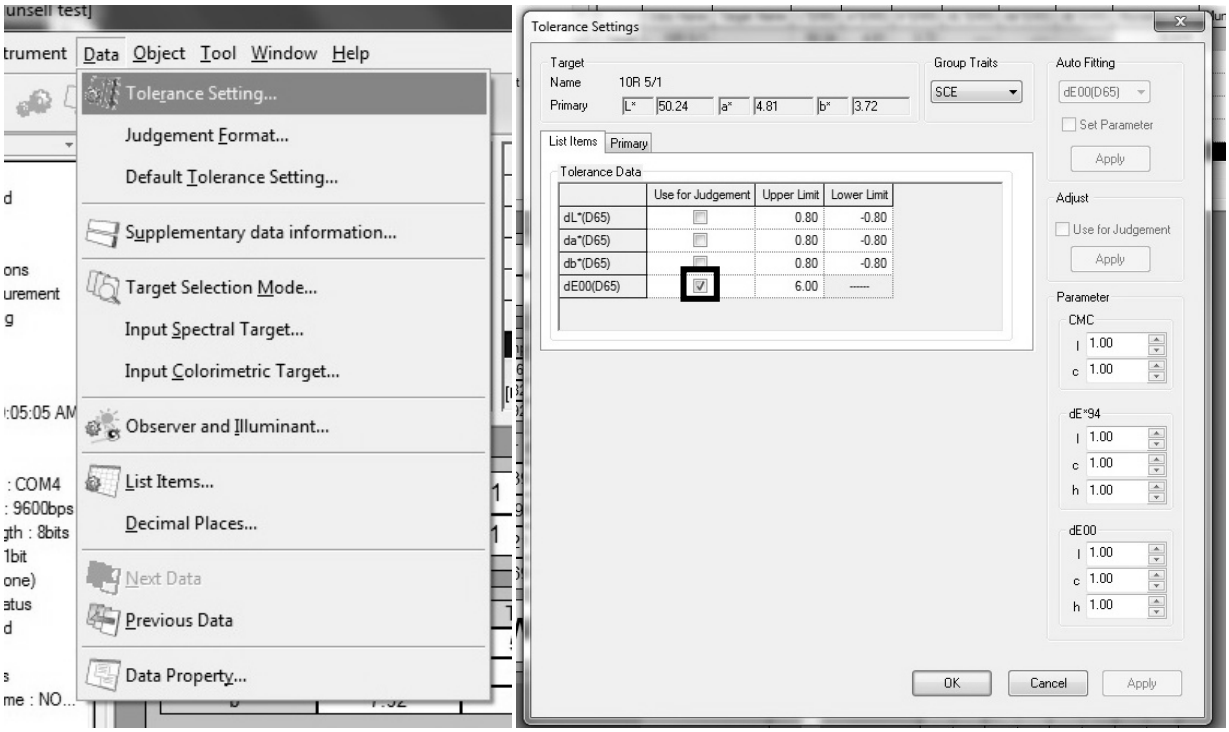


Figure 40: (Left) Menu selections to open Tolerance Settings and (Right) Default Tolerance Setting Window

Uncheck the box next to dE00 to disable Judgment in the Default Tolerance Setting or Tolerance Settings box (Figure 41). Click OK.

	Use for Judgement	Upper Limit	Lower Limit
dL*(D65)	☐	0.80	-0.80
da*(D65)	☐	0.80	-0.80
db*(D65)	☐	0.80	-0.80
dE00(D65)	☑	6.00	------

Figure 41: Editing Tolerance settings

6.4.2 Editing the Judgment tolerance

To edit the tolerance settings for all future Targets, open the Default Tolerance Setting window (Figure 39), and to edit the tolerance setting for the current Target, open the Tolerance Settings window by selecting Data and then Tolerance Setting in the menu bar (Figure 40). For both Default Tolerance Settings and Tolerance Settings, to change the dE00 tolerance, edit the number in the Upper Limit box in the dE00 row to the right of the check box. To incorporate dL*, da*, or db* into the Judgment, check the corresponding box(es) and edit the corresponding numbers in the Upper Limit and Lower Limit boxes. You only need to change settings in the part of the window that is pictured in Figure 41.

6.4.3 Editing or disabling the Warning threshold

Select Data and then Judgment format from the menu bar (Figure 42). The percentage in the Warning Level box means the percentage of the tolerance that will be considered the Warning threshold (e.g. 3.50 is ~58% of 6.00, the default tolerance). Edit the percentage in this box to change the Warning threshold, or uncheck the Show Warning Level box to disable it (Figure 42).

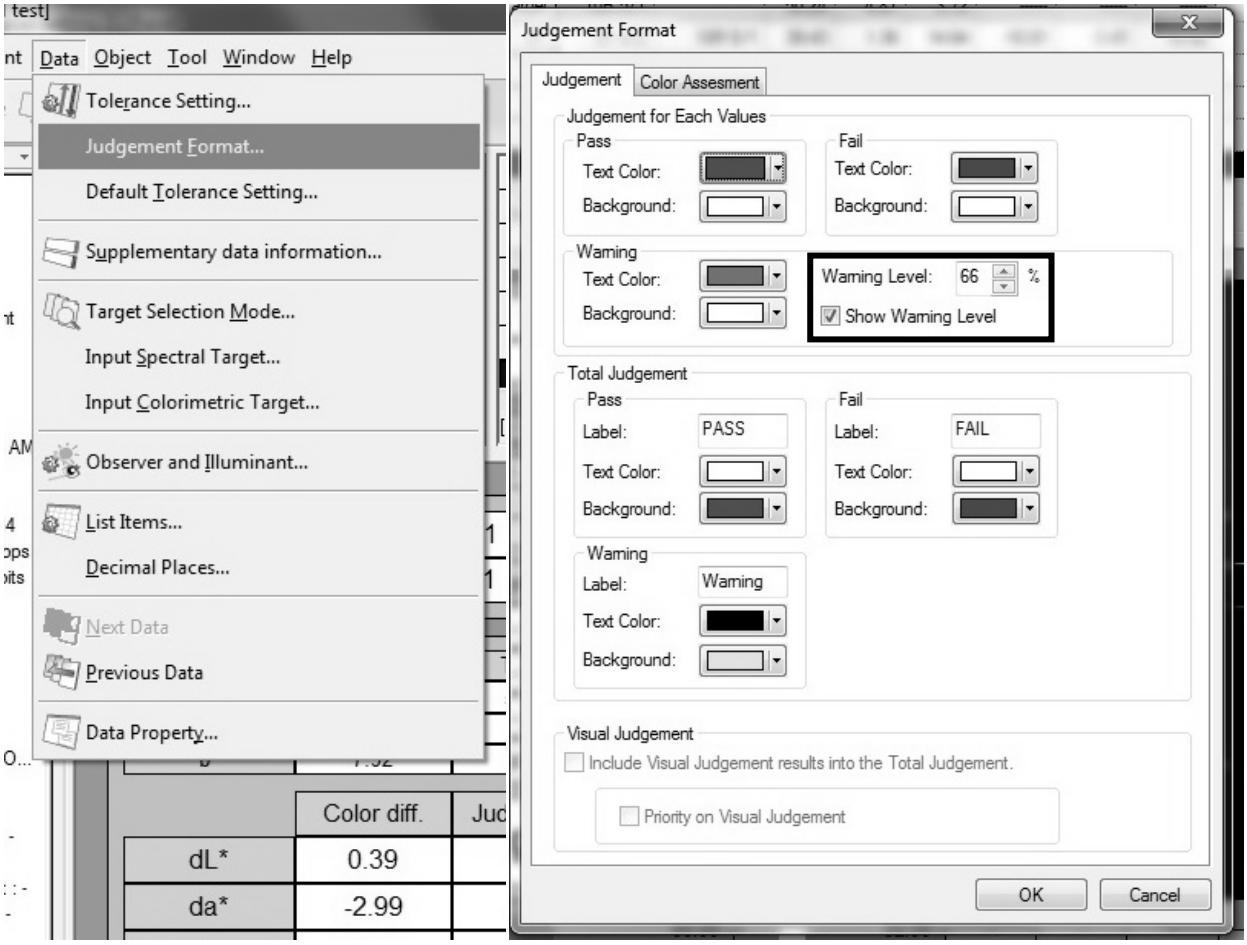


Figure 42: (Left) Menu selections to open Judgment Format and (Right) Editing or disabling Warning Level in the Judgment Format window

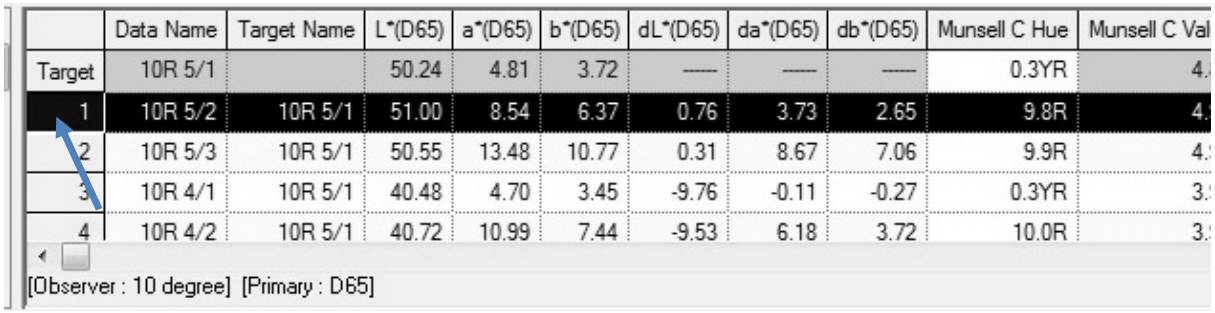
6.5 Graphic object canvas

To properly use the graphic objects on the canvas (Figure 18), you will need to select a Target under "Classification by Target" in the Data Tree (Figure 38). The plot objects will by default display all of the data in the level of the Data Tree that is selected (e.g. "Sample(s)," the Target). Selecting Samples will alter how the objects display data (see sections 6.5.1 through 6.5.5). This can be done by Shift clicking (selecting all Samples in between the first click and the second click) or Control clicking (selecting only clicked Samples).

6.5.1 Comparison Table

The Comparison Table displays the L*a*b* colors of the selected Target (questioned soil) and one selected Sample (known soil) and the color difference and color difference

Judgments between the two. The color of the Target will populate the Comparison Table on Target selection (described in 6.1.4), and the color of the Sample, dE00, and Judgments will populate the Comparison Table on Sample selection. The Sample can be selected from the List Window by clicking the box to the left of the row (Figure 43). The selected Sample can be changed by clicking on the box next to different samples.

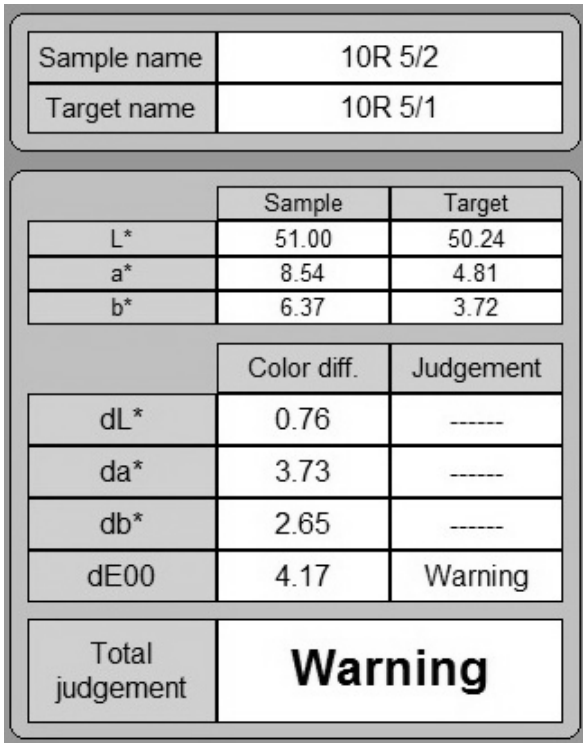


	Data Name	Target Name	L*(D65)	a*(D65)	b*(D65)	dL*(D65)	da*(D65)	db*(D65)	Munsell C Hue	Munsell C Val
Target	10R 5/1		50.24	4.81	3.72	-----	-----	-----	0.3YR	4.
1	10R 5/2	10R 5/1	51.00	8.54	6.37	0.76	3.73	2.65	9.8R	4.
2	10R 5/3	10R 5/1	50.55	13.48	10.77	0.31	8.67	7.06	9.9R	4.
3	10R 4/1	10R 5/1	40.48	4.70	3.45	-9.76	-0.11	-0.27	0.3YR	3.
4	10R 4/2	10R 5/1	40.72	10.99	7.44	-9.53	6.18	3.72	10.0R	3.

[Observer : 10 degree] [Primary : D65]

Figure 43: Selecting a sample in the List Window.

This table shows the L*a*b* measurements for each of the two measurements, the differences between these parameters, the dE00 between the two colors, and (if enabled) the Pass/Warning/Fail Judgment(s) (Figure 44).



Sample name	10R 5/2
Target name	10R 5/1

	Sample	Target
L*	51.00	50.24
a*	8.54	4.81
b*	6.37	3.72

	Color diff.	Judgement
dL*	0.76	------
da*	3.73	------
db*	2.65	------
dE00	4.17	Warning
Total judgement	**Warning**	

Figure 44: Comparison table graphic object comparing measurements of Target and one selected Sample.

6.5.2 Pseudo Colors

The Pseudo Color graphic objects display the measured colors of the Target and the selected Sample side by side (Figure 45). The Target and Sample displayed are the same as

those displayed in the Comparison Table and are selected in the same way (6.5.1). The objects are labeled with the Target/Sample names upon Target and Sample selection.

Figure 45: Pseudo Color graphic objects comparing colors of Target and Sample.

6.5.3 3D L*a*b* plot

The 3D L*a*b* plot shows each of the Samples plotted in 3D space using the differences from the Target in L*, a*, and b* (dL*, da*, and db*). The Target is at the origin of the plot. When Judgment is enabled, the Sample points are represented by squares colored according to their Judgment status: green for PASS, yellow for WARNING, and red for FAIL. Selected Samples are represented in cyan. A wire frame ellipsoid (shown in blue-gray) shows the dE00 tolerance; Samples that are within the ellipsoid have a dE00 below the tolerance and those outside of it have a dE00 above the tolerance. Note that the shape of the ellipsoid will adjust slightly for the Sample(s) that is/are selected; this is to be expected and is not indicative of any errors (Figure 46).

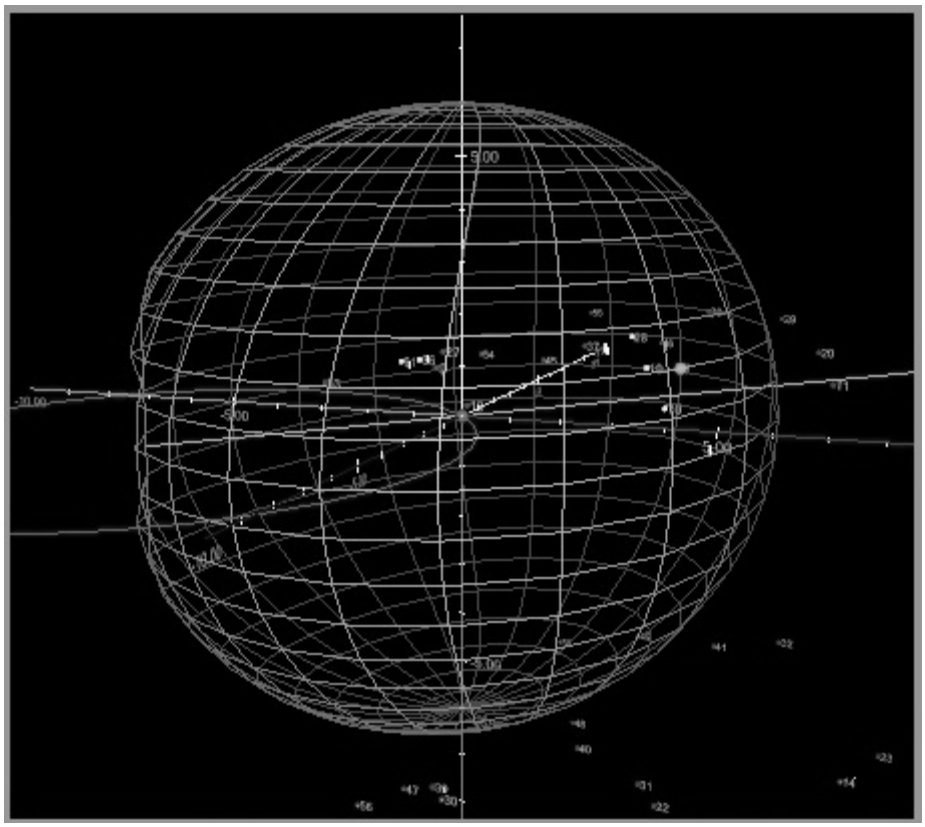


Figure 46: 3D L*a*b* plot graphic object, Judgment enabled

When Judgment is disabled, the wire frame is not displayed and the Sample points are colored according to their Pseudo Color (RGB representation of the Sample's measured color, as in section 6.5.2). Selected Samples are represented in cyan (Figure 47).

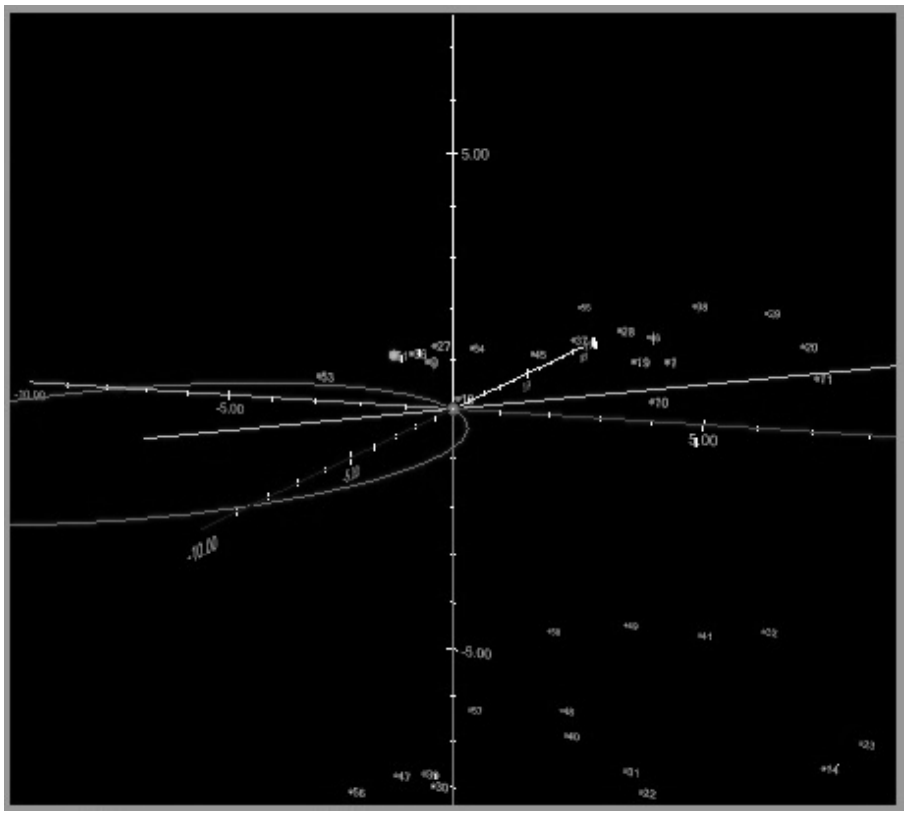


Figure 47: 3D L*a*b* plot graphic object, Judgment disabled

6.5.4 Absolute L*a*b* plot

The absolute L*a*b* plot consists of two parts: a bivariate plot of a* versus b* and a univariate plot showing L*, each with the Target as a red circle and Samples as squares. When distinguishing between populations of Samples from different sites, Pye et al. (2006) report that the a* versus b* plot will be the most useful. The background of the a* versus b* plot is colored to give a viewer an approximation of the hue of the points in the plot.

When Judgment is enabled, Samples will be represented as squares and colored according to their Judgment status as with the 3D L*a*b* plot in section 6.5.3. Selected Samples will be circled in blue (Figure 48)

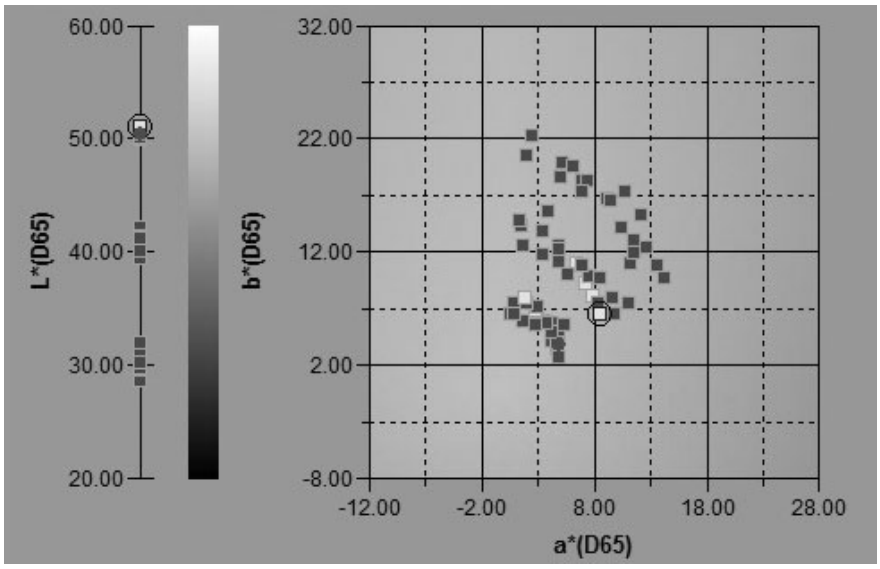


Figure 48: Absolute L*a*b* graphic object, Judgment enabled

When Judgment is disabled, non-selected Samples are displayed in gray and selected Samples are circled and displayed in blue (Figure 49).

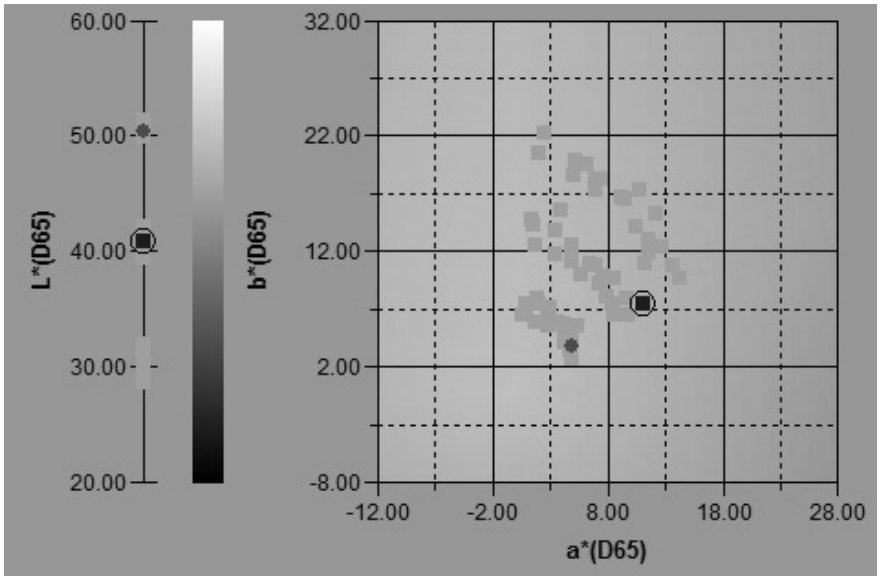


Figure 49: Absolute L*a*b* plot graphic object, Judgment disabled

6.5.5 L*a*b* differences plot

Like the absolute L*a*b* plot, the L*a*b* differences plot consists of two parts: a bivariate plot of da* (difference in a* between the Target and the Sample) versus d b* (difference in b* between the Target and the Sample) and a univariate plot showing dL* (difference in L* between the Target and the Sample. The Target is a red circle at the origin of the plots. Samples are represented as squares. When distinguishing between populations of Samples from different sites, the da* versus db* plot will be the most useful (Pye et al., 2006).

When Judgment is enabled, Samples will be colored according to their Judgment status as with the 3D L*a*b* plot in section 6.5.3. Selected Samples will be circled in blue. The colored

arrows on the axes of the two plots are indicative of the color that the axis progresses towards, allowing the user to visualize the relationship between two points. For example, the selected Sample is yellower and redder than the Target. The da* versus db* plot will have added contours to help the user interpret the results. The magenta ellipse is the constant chroma locus, meaning that all points on that ellipse have the same chroma. The cyan line is the constant hue locus, meaning that all points on that line have the same hue. The yellow ellipse is the Warning threshold for dE00; Samples that are within that circle are below the threshold. Similarly, the red ellipse is the Fail threshold for dE00. These ellipses are intended only for reference purposes, and in cases of low saturation colors, ellipses may be distorted such that data points may incorrectly fall inside or outside of the ellipses (the color of the Sample's data point will correctly reflect the Judgment status for that point). The shapes of the ellipses will change shape slightly for the Sample(s) that is/are selected, similar to the 3D plot in section 6.5.3. This is normal and not indicative of error (Figure 48).

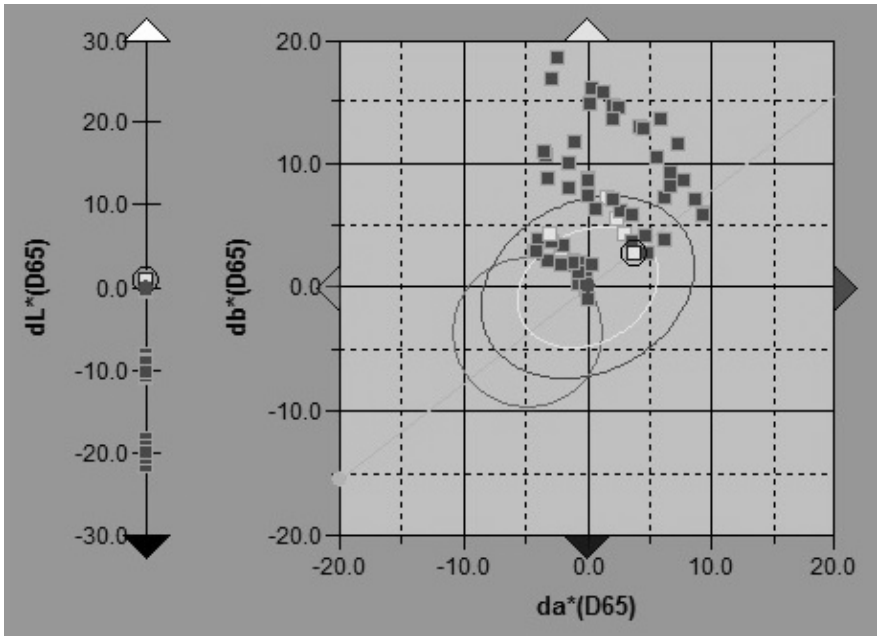


Figure 50: L*a*b* differences plot graphic object, Judgment enabled

When Judgment is disabled, non-selected Samples are displayed in gray and selected Samples are displayed in blue. This plot has the constant chroma and hue loci mentioned previously Figure 51.

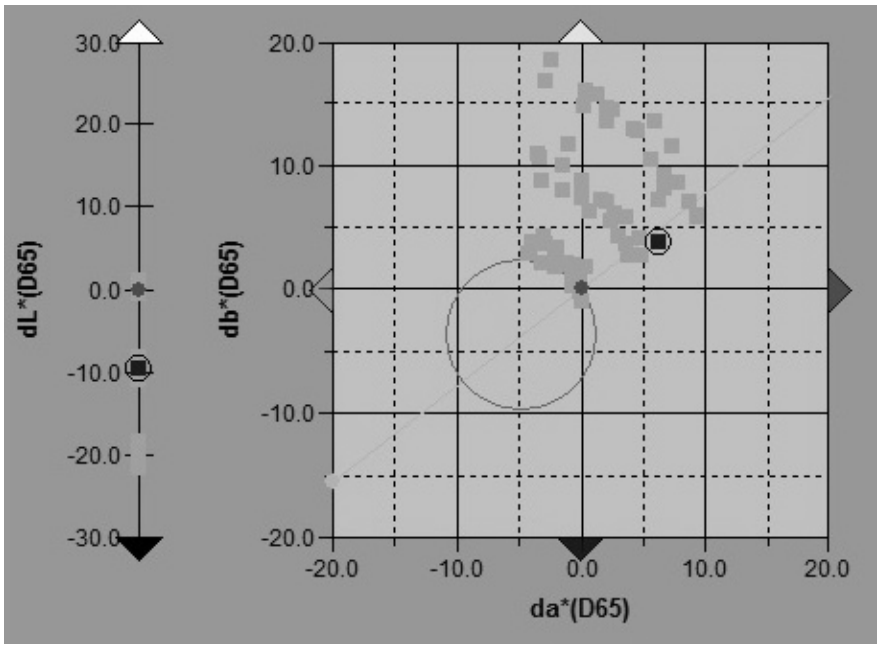


Figure 51: L*a*b* differences plot graphic object, Judgment disabled

6.5.6 L*a*b* statistics

The L*a*b* statistics object group shows the maximum, minimum, range, and mean for the differences between the Target and the Samples linked to it for each of the L*a*b* parameters as well as for ΔE (dE00).

Figure 52: L*a*b* statistics graphic object

7 Saving and printing

7.1 Save measurement file (always perform)

Select File then Save As from the menu bar (Figure 53).

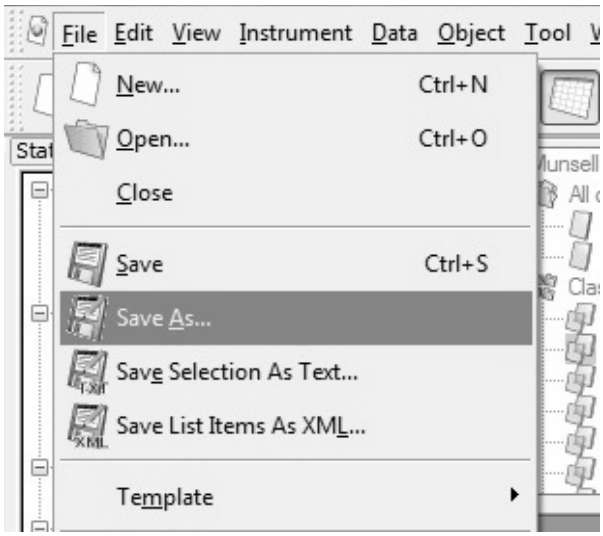


Figure 53: Menu selections to save measurement file

Name and save your measurement file in the desired location. It will have the file extension ".mes." This file will save the measurements, calculations, and graphic object canvas, and can be opened later for viewing and/or addition of measurements. Any time the file is altered (adding more measurements, editing the canvas, etc.), create a new file with Save As or overwrite the existing file with Save as desired.

NOTE: measuring while connected to the software causes the measurements to not save in the instrument memory, so **ALWAYS SAVE THE MEASUREMENT FILE.**

7.2 Print canvas (optional)

To print the graphic object canvas, select File and Print from the menu bar (Figure 54). Follow the prompts to print your canvas to a connected printer.

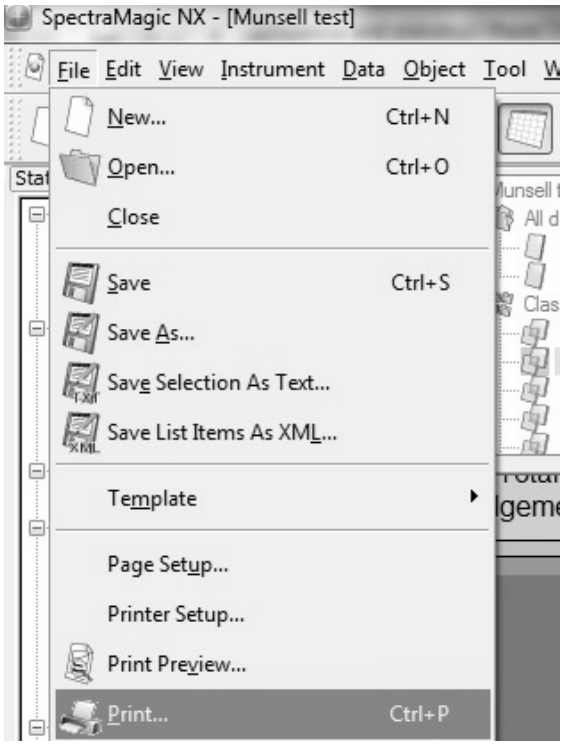


Figure 54: Menu selections to print

You can also preview what will be printed before printing without leaving the software. Select File and then Print Preview from the menu bar (Figure 55).

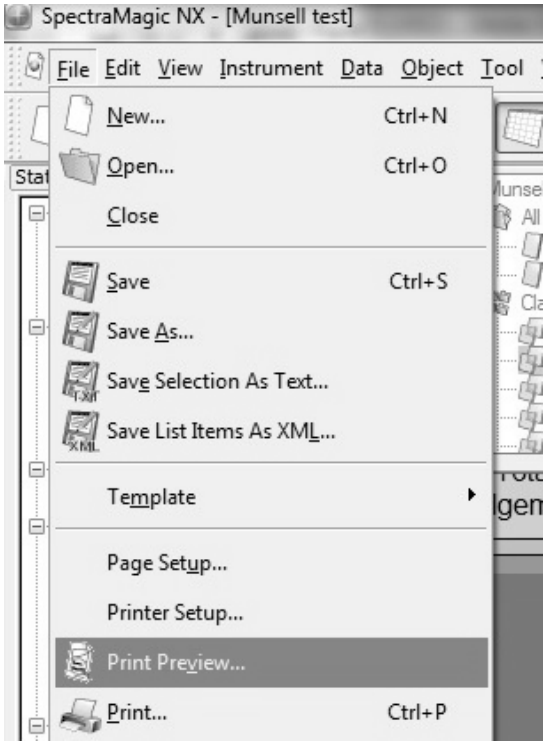


Figure 55: Menu selections to open print preview

The Print Preview will look like Figure 56.

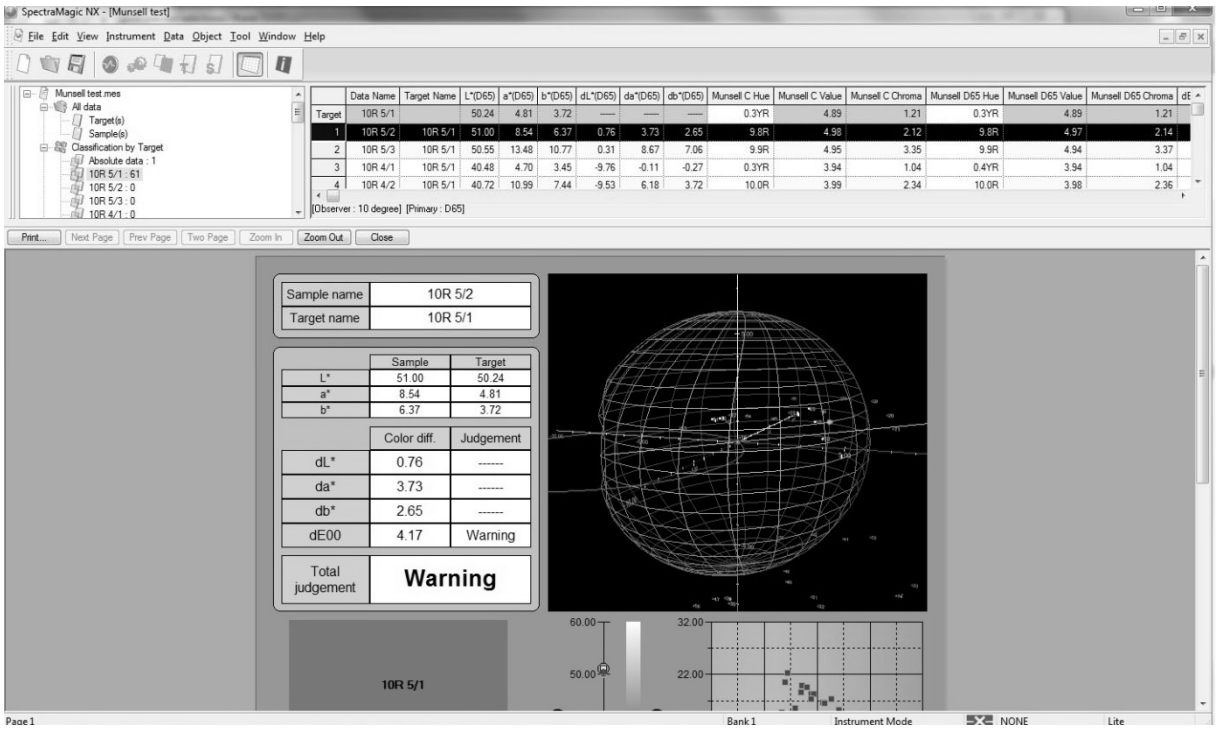


Figure 56: Print Preview interface

Use the buttons at the top of the Print Preview menu and the scroll bar at the right to print, navigate, or close the preview.

7.3 Save measurement table (optional)

Follow this procedure to save a table with your measurements and color difference calculations.

7.3.1 Select Target

If desired, select the desired Target (questioned soil) in the Data Tree under the "Classification by Target" level (Figure 38).

7.3.2 Select data

Click the box at the top left of the "List Window" (Figure 34) to select the questioned measurement and all of the known measurements being compared to it. It is also possible to select only some of the data. This can be done by Shift clicking (selecting all Samples in between the first click and the second click) or Control clicking (selecting only clicked Samples).

7.3.3 Copy and Paste

Right click the selected data in the List Window and select copy.

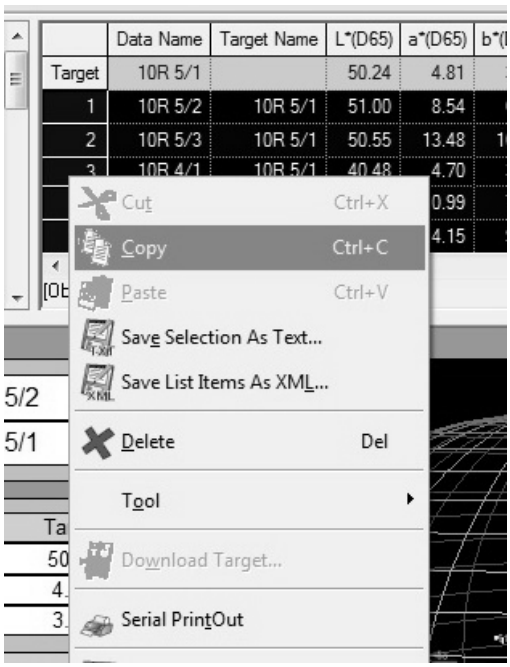


Figure 57: Selections to copy data

Open Excel and paste the data into a worksheet.

8 Instrument cleanup, transportation, and storage

8.1 Switch off the instrument

Switch off the instrument using the power switch (Figure 1, item 12).

8.2 Disconnect cables

Disconnect and unplug all cables. Close the instrument's connector protection covers.

8.3 Clean

Clean the target mask with a tech wipe. Also clean the plate in the White Calibration Cap if necessary.

8.4 Store with White Calibration Cap.

Squeeze the White Calibration Cap lock buttons, place the Cap on the target mask, and release the buttons to lock the Cap in place. Always keep the White Calibration Cap on the instrument when it is not in use.

8.5 Place in carry case

Place instrument in designated slot in carry case with the measurement button pointing down. There are also slots where the user can place the coiled cables.

Figure 58: Instrument and cables placed in carry case

Keep the carry case shut and in a safe, climate-controlled place when the instrument is not in use. The carry case can be used for instrument transportation, though care should be taken to not jostle or knock the case during transport.

9 Limitations and appropriate use for instrumental soil color measurement

Instrumental colorimetry using the instrument and methods described in this user guide is recommended for comparing the color of visibly homogeneous, opaque soils or sediment and may be used in addition to (and possibly eventually in lieu of) color determination by visual comparison to the Munsell soil color book. Measurements will be the most accurate on samples with particle sizes < 1 mm. For soil comparison, color measurement of all samples should be performed on the same size fraction and following identical pre-treatments, if performed (e.g. cleaning with detergent, ashing).

For very translucent sands (e.g. quartz with minimal surface texture), this procedure may produce slightly biased color measurement due to light transmitting through the entire thickness of the sample. This effect is mitigated by sample thickness, so when comparing potentially translucent sands, the same sample holder and quantity should be used. For typical, light color beach sands, this is not necessarily a problem.

For samples which are visibly heterogeneous, instrumental color measurement is recommended when the average color of the sample is desired, but not when measurements of the individual components are desired (e.g. soil from multiple sources mixed together). For these samples, visual color comparison to a Munsell soil color book is recommended to

characterize the colors of the different components. Likewise, for very small soil samples or those which are difficult to separate from a substrate or from a distinct soil source, visual comparison to a Munsell soil color book is recommended.

9.1 Advantages of instrumental colorimetry

Instrumentally measured soil color is more precise and more objective than comparison to a Munsell soil color book and it permits sub-chip Munsell color determination and comparison. It also has the ability to obtain an average color of a heterogeneous sample. The objectivity of instrumental measurement makes this method well suited for the rare situation when subsequent laboratory submissions are received after the initial soil evidence is returned to the submitter, preventing side-by-side comparisons. Accompanying software allows for the visualization and objective calculation of color differences between samples. Thus, for soil samples of suitable size and characteristics, this approach adds values to a soil examination.

9.2 Advantages of visual colorimetry

Characterization of soil color by comparison to a Munsell soil color book is better suited than instrumental measurement to characterization of soil colors in situ, in mixed samples, and small samples. In addition, while the spectrophotometer is suitable for comparisons, it may not, however, be recommended for cases in which soil provenance needs to be determined from Soil Survey data; the soil conditions (e.g. moisture) under which the instrument measures Munsell color and under which the Soil Survey measures color are not completely equivalent, and thus instrumental color measurements for provenance determination should be interpreted with caution. For cases involving soil provenance, measurements taken using illuminant D65 should be used to make the instrumental conditions as representative as possible of the Soil Survey measurement conditions.

10 Reproducibility and Uncertainty

Instrumental color measurements using the CM-600d have excellent reproducibility. The reproducibilities of measurements of different materials (expressed as the standard deviation of replicate measurements) are summarized in

Table 3. Reproducibility of color measurements of mineral soil mixtures were calculated from only one subsample of each, while for all others, reproducibility was calculated using measurements of three subsamples.

Table 3: Reproducibility of spectrophotometer measurements of soils and sands measured. Reproducibility is given as standard deviations in the following format: mean, maximum, minimum.

	Munsell			L*a*b*		
Material	**Hue number**	**Value**	**Chroma**	**L***	**a***	**b***
< 1 mm soil fraction in custom aluminum sample holder	0.05, 0.09, 0.00	0.03, 0.06, 0.01	0.04, 0.09, 0.02	0.33, 0.56, 0.13	0.12, 0.30, 0.05	0.19, 0.36, 0.08
~2 – 3 mm soil aggregates in black and white sample holders	0.07, 0.10, 0.06	0.05, 0.07, 0.03	0.04, 0.10, 0.01	-	-	-
Mixture of fine soil of different colors	0.01, 0.06, 0.00	0.01, 0.03, 0.00	0.02, 0.06, 0.00	-	-	-

Large aggregates held to target mask	0.15, 0.18, 0.10	0.25, 0.37, 0.13	0.12, 0.16, 0.09	-	-	-
Light colored sands	0.10, 0.21, 0.00	0.04, 0.14, 0.01	0.04, 0.09, 0.01	0.43, 1.35, 0.04	0.11, 0.32, 0.01	0.24, 0.60, 0.04
Visibly heterogeneous sands and sediments	0.17, 0.24, 0.13	0.08, 0.11, 0.04	0.09, 0.11, 0.07	0.82, 0.18, 0.34	0.23, 0.33, 0.17	0.52, 0.58, 0.46

Soils dominated by ~2 – 3 mm aggregates have additional uncertainty (≤ 0.16 Munsell chips) from the potential for light passing in between aggregates and the measurement thus partially reflecting the sample holder. Translucent sands have an additional uncertainty (≤ 0.50 Munsell chips) from the potential for light passing through the entire depth of the sample and the measurement thus partially reflecting the sample holder.

11 Notes and Acknowledgments

This document was initially drafted by Christine E. Dong, Visiting Scientist in the FBI Laboratory, CFSRU, in aid of TEU forensic geology program with funding provided by the Oak Ridge Institute for Science and Education. Eric Crandall of the Operational Projects Unit at the FBI Laboratory assisted in design and completed the construction of the intermediate prototypes and facilitated production of the aluminum sample holders by the FBI Operational Technology Division. Use of the CM-600d for instrumental colorimetry is not an endorsement of this product, but rather this instrument was owned by the FBI Laboratory and was suitable for this task; names of commercial products are provided for identification only and inclusion does not imply endorsement by the FBI.

12 References

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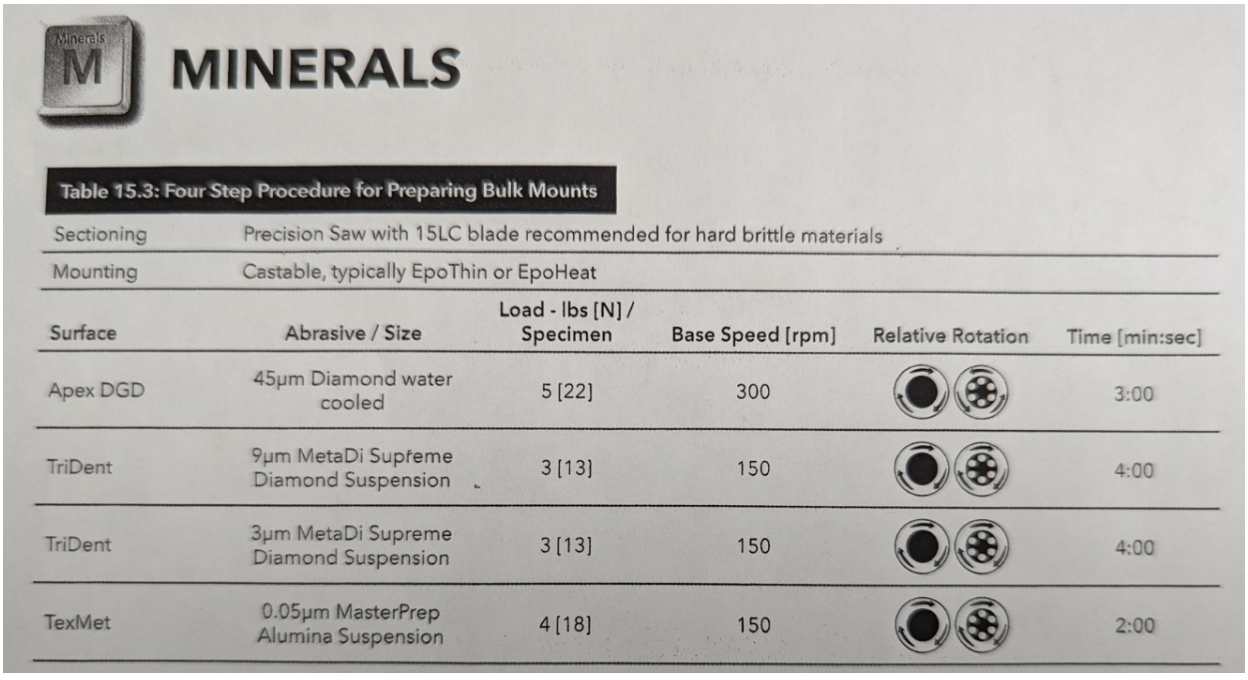
Method for preparing and analyzing the mineral fraction of soils by SEM-EDS

Sample Preparation

The sand fraction of the soils is isolated by washing and decanting with reverse osmosis water. The sand is then passed through a nest of sieves: 5 mm, 250 µm, and 105 µm. All fractions are dried and stored in clean glass vials. The heavy mineral fractions for each of the samples were isolated using bromoform. Both the heavy and light fractions are dried and stored in new glass vials of appropriate size.

The heavy mineral fraction from each sample is "sprinkled" through a piece of 250 µm sieve cloth onto 3M packaging tape. The sieve is then removed and two copper and one aluminum sphere are placed onto the mounts. These spheres serve as fiducial marks. Buehler 1 inch OD reusable ring-forms are placed over the samples and they are embedded using Buehler Epo-Qwik 2-part epoxy. The mounts are allowed to cure overnight before grinding/ polishing.

The sample mounts can be polished either using the Buehler EcoMet 30 or by hand. Up to six mounts can be ground/ polished simultaneously using the EcoMet 30. The following method will be used for all sample mounts:



MINERALS

Table 15.3: Four Step Procedure for Preparing Bulk Mounts

Sectioning	Precision Saw with 15LC blade recommended for hard brittle materials
Mounting	Castable, typically EpoThin or EpoHeat

Surface	Abrasive / Size	Load - lbs [N] / Specimen	Base Speed [rpm]	Relative Rotation	Time [min:sec]
Apex DGD	45µm Diamond water cooled	5 [22]	300		3:00
TriDent	9µm MetaDi Supreme Diamond Suspension	3 [13]	150		4:00
TriDent	3µm MetaDi Supreme Diamond Suspension	3 [13]	150		4:00
TexMet	0.05µm MasterPrep Alumina Suspension	4 [18]	150		2:00

1. Grind with 600-grit for approximately 3 minutes to expose the mineral grain interiors. Grind at 25 pounds of pressure at 300 / 150 rpm (for 6 mounts). Note this is a critical step and should be monitored closely to ensure the grinding isn't too aggressive as significant grain loss can occur.

2. The mounts are then gently ground with 9 µm diamond paste for 4 minutes at 20 pounds of pressure at 150 / 80 rpm (for 6 mounts).
3. The mounts are then polished with 3 µm diamond paste for 4 minutes at 22 pounds of pressure at 150 / 80 rpm (for 6 mounts).
4. The mounts then undergo a final polishing with 1 µm diamond paste for 2 minutes at 25 pounds of pressure at 150 / 80 rpm (for 6 mounts).

Following grinding and polishing the mounts, they are then sonicated twice in reverse osmosis water for ~15 minutes each round. The mounts are then gently patted dry with paper towel and dried in the laboratory oven at 60 °C.

Carbon Coating

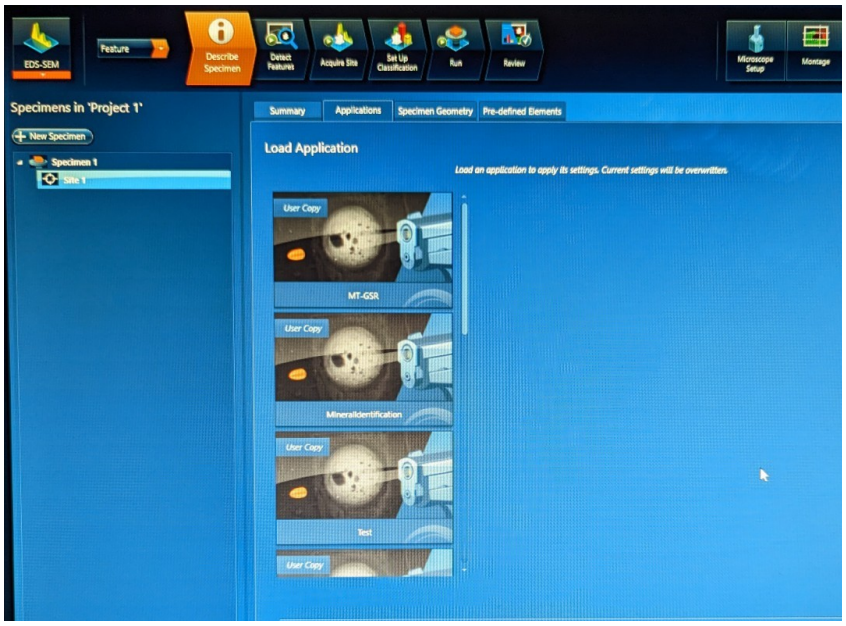
All mounts are coated with ~15 nm of carbon.

SEM Conditions:

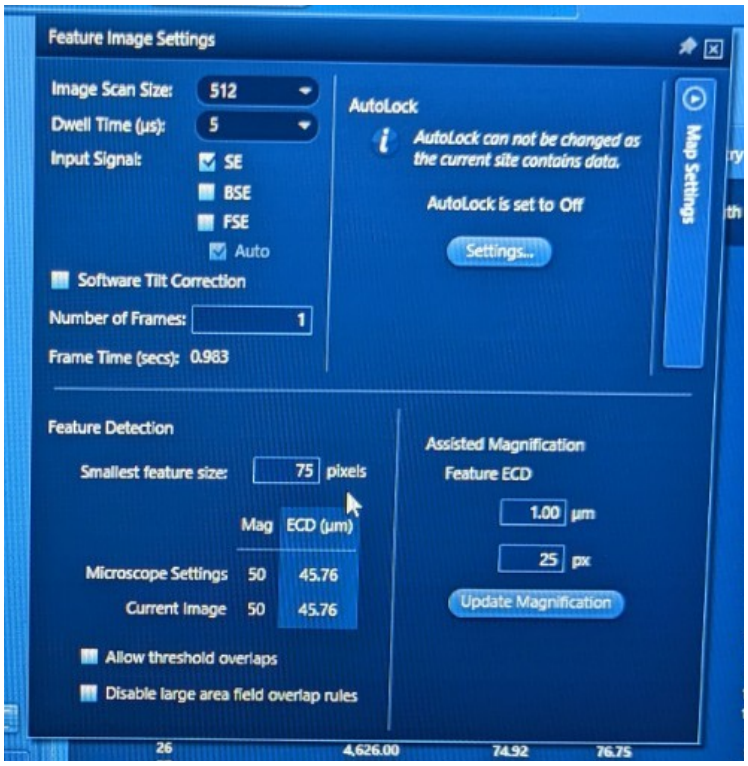
1. Up to six sample pucks can be loaded in to the stage mount.
2. Set the stage surface offset to 9.0 mm.
3. The analysis will be done using secondary electron imaging. Adjust the SE detector to have a -1 bias (this enhences the contrast by increasing backscatter electron contribution to the image).
4. Set the accelerating voltage to 15 kV.
5. Set probe current to 12.
6. Set working distance to 10 mm.
7. The magnification for the analyses will be done at 50x.
8. Locate one of the copper spheres on one of the samples mounts.

EDS conditions:

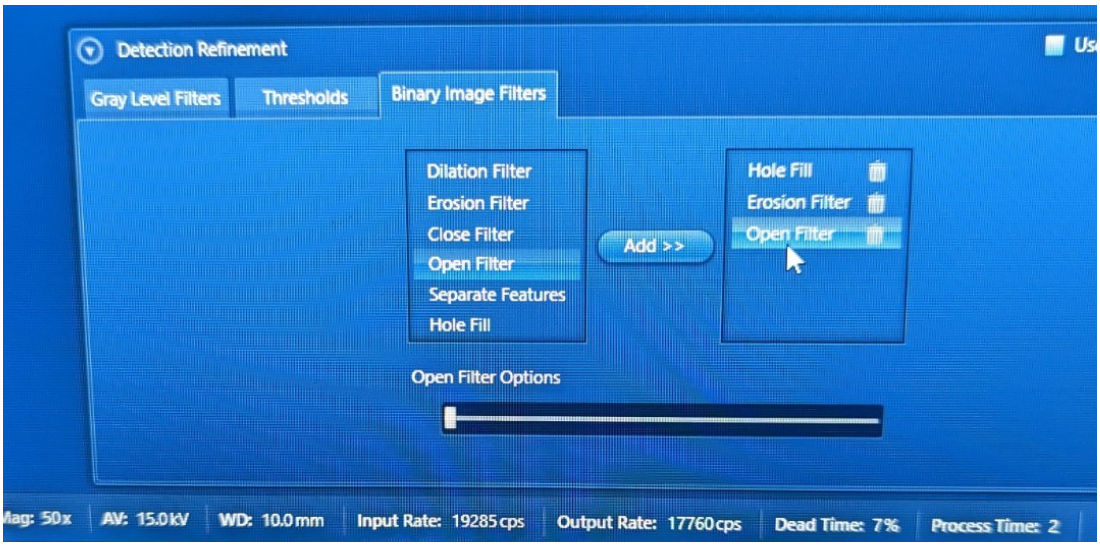
1. The analysis of the mineral grains will be done using Oxford Feature. Load the “MineralIdentification” application.



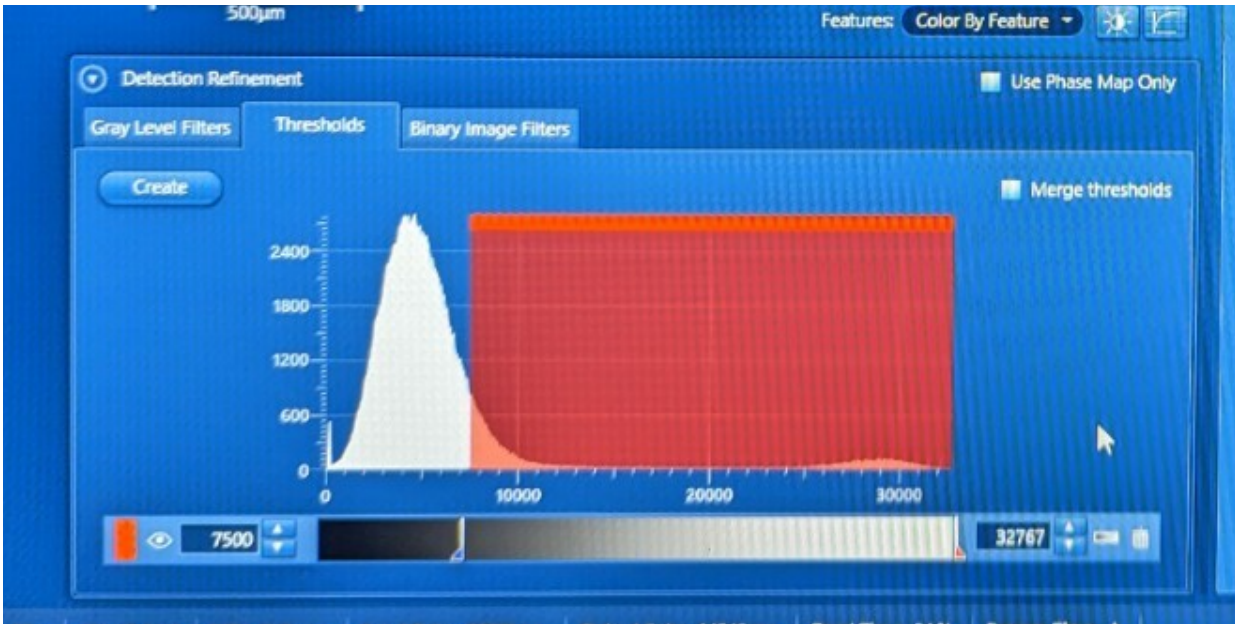
2. The EDS process time is set to 2.
3. Set energy resolution to 10 ev/channel (default).
4. Under the Detect Features tab, select Feature Image Settings. Adjust values as shown in the image below:



5. To optimize mineral grain detection, use the following Binary Image Filters: 1. Hole Fill, 2. Erosion Filter, and 3. Open Filter. Note the order of that these filters are selected matters. All three of these filters are set to their lowest value (furthest left).



6. The threshold range to detect minerals is **7500 – 32767 (max)**:



7. Feature analysis settings are set as shown below. Note the scan mode is set to spot and the live time acquisition is set to 2 seconds.

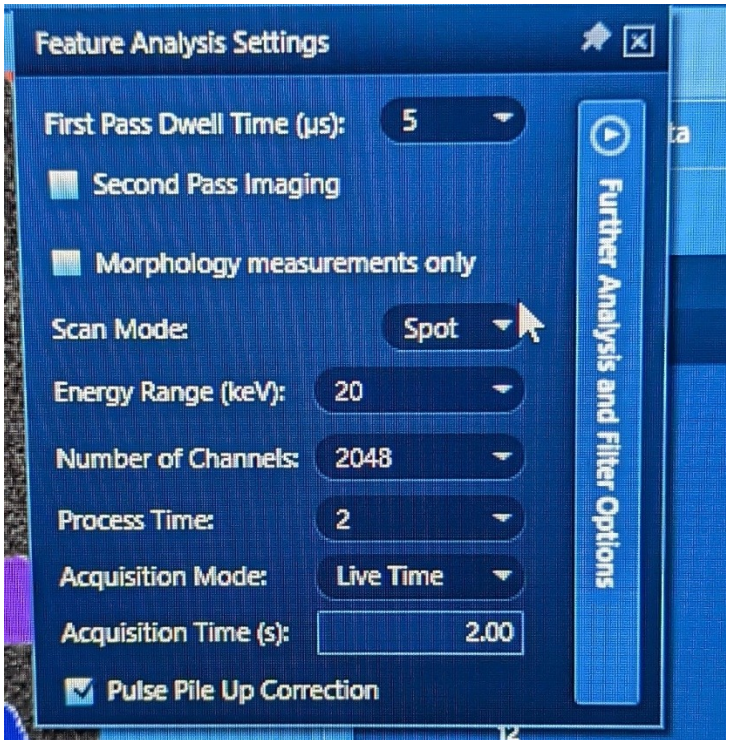
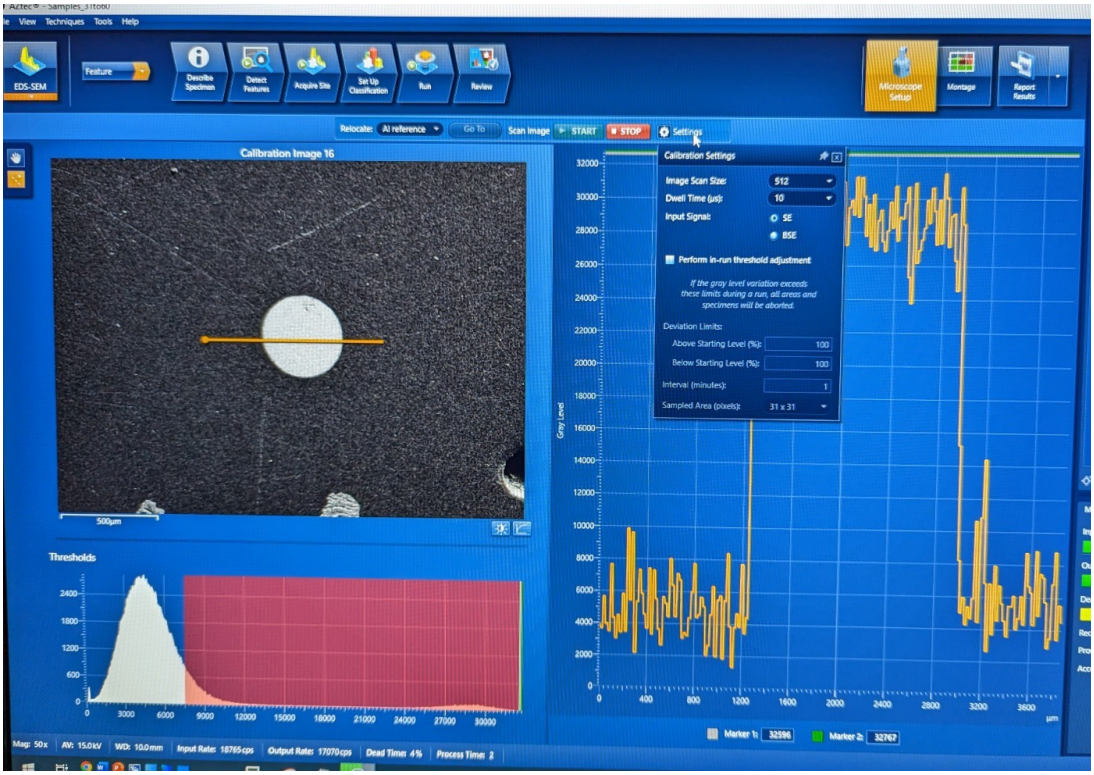


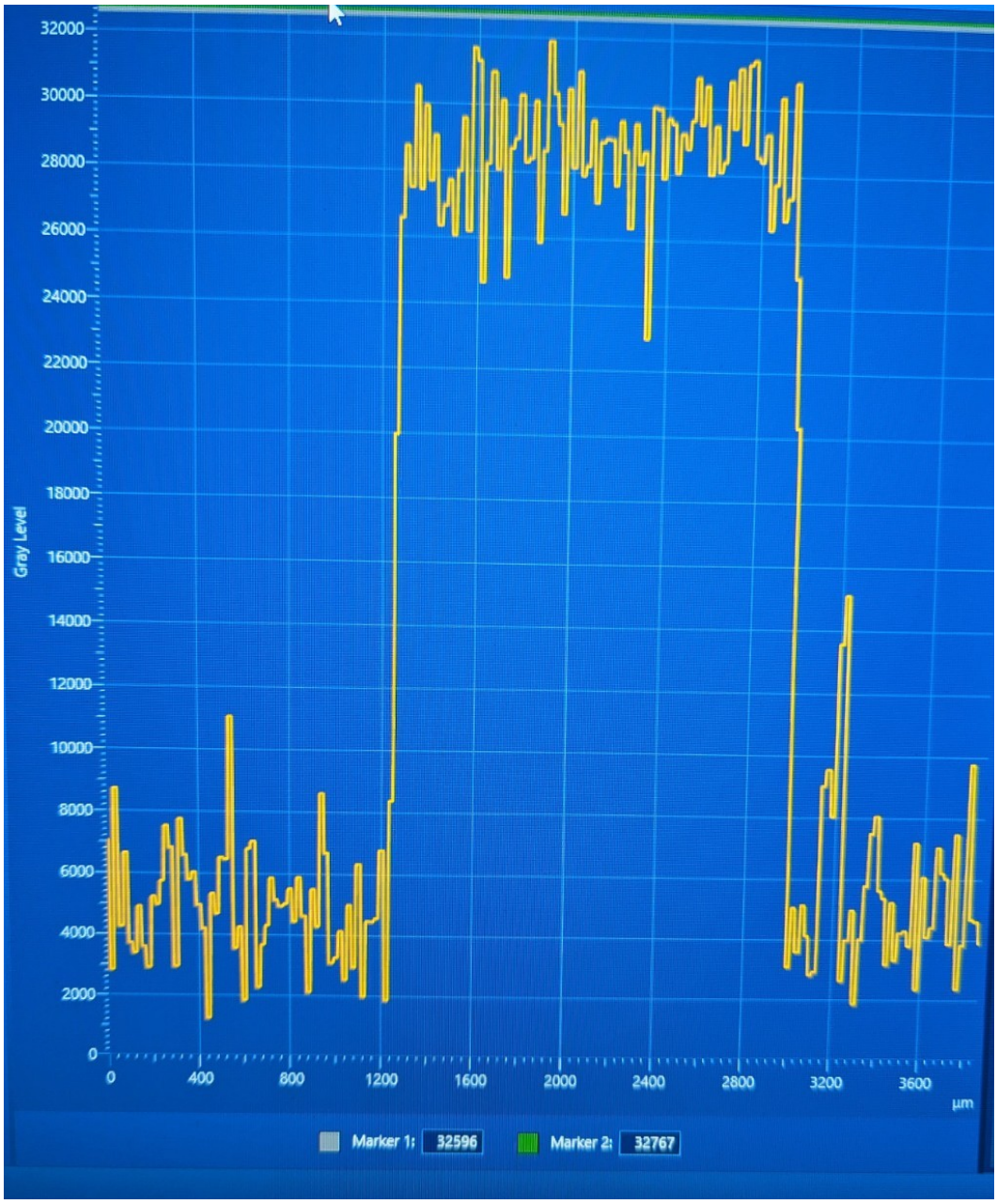
Image calibration:

This step is required because the brightness and contrast settings on the SE detector can change over time. To control for this, we are incorporating a variation of the routine used for GSR image calibration (although this method is not done by Microtrace for GSR analysis).

1. Locate one of the copper spheres on the sample mounts. Ensure 50x magnification.
2. Click on the Microscope Setup tab.
3. Under settings select an image size of 512 and a 10 µs dwell time.
4. Using the line scan tool draw a line across the Cu sphere and includes the background epoxy.

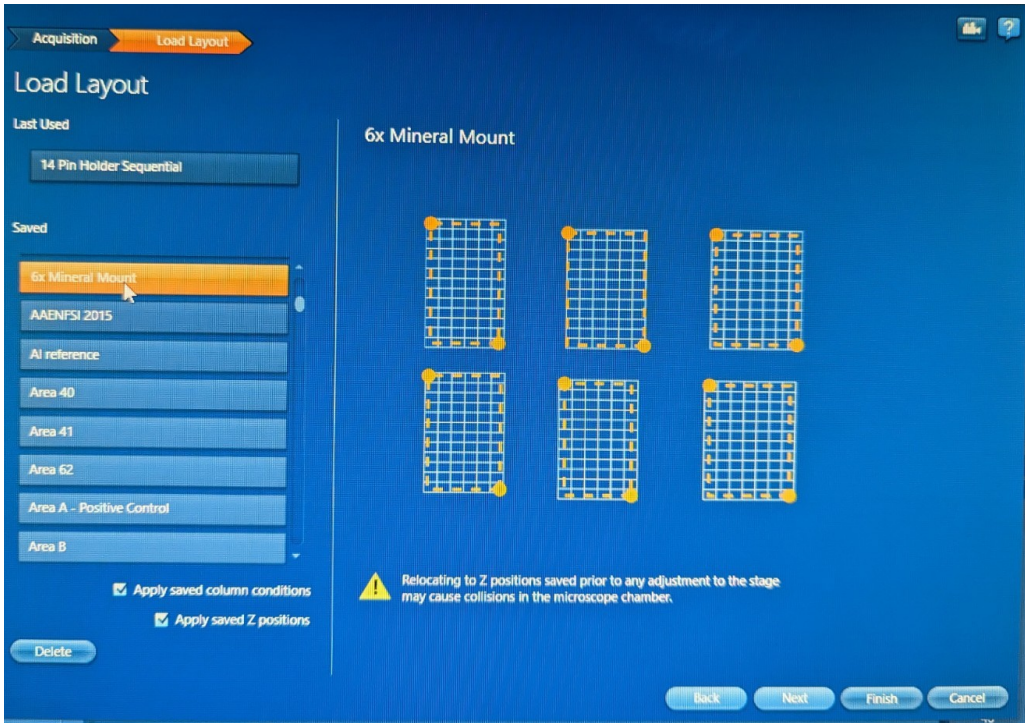


5. Adjust the brightness and contrast values on the microscope to achieve a mean pixel intensity of 29,000 on copper and 5,000 on the epoxy background. This results in a "contrast step" of ~6 which was empirically shown to provide optimal particle detection.
    a. Currently (17MAR2023) the contrast value is ~2680 and the brightness value is ~1410.


32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Gray Level
0
400
800
1200
1600
2000
2400
2800
3200
3600
µm
Marker 1: 32596
Marker 2: 32767

Setup Automated Run:

1. Load the 6-mount template called “6xMineralMount”. This will have 6 rectangular regions of interest already saved. The two sets of x-y-x coordinates will need to be updated for each sample mount. These coordinates define the region contains the minerals in each mount.



2. The runs take approximately 2 hours per mount (given approximately 2000 grains present).
3. After the autoruns are completed, the data needs to be processed to remove duplicate analyses (grains that appeared in multiple fields-of-view). To perform this clean-up, click on Montage on the Review screen.
4. Using the selector tool manually align at least 3 FOVs. The FOVs must form an “L” shape.
5. Once aligned, click and highlight the 3 FOVs, then right click and select “guided align”.
6. Under the Review page select “Reconstruct”. This process should have removed the duplicate analyses and thus reduced the total number of spectra.
7. As a final step, the data from each mount is exported as a HDF5 file. This can take several minutes depending on the size of the file.

DNA Metabarcoding Standard Operating Procedure

Developed by laboratory of Dr. Kelly Meiklejohn at North Carolina State University

1. DNA Isolation

Supplies required

- DNeasy® PowerSoil® Pro Kit, Qiagen (Cat # 47014 / 47016)
- Vortex Adapter for 24 (1.5 – 2.0 mL) tubes, Qiagen (Cat # 13000-V1-24)
- Vortex-Genie 2, Scientific Industries SI-0236, or similar
- Microcentrifuge
- Pipettors (50 – 1000 µL)

Procedure (Taken directly from DNeasy PowerSoil Pro Kit Handbook v06/2023 accessed on 03/07/2025; modifications noted in yellow font).

1. Spin the PowerBead Pro Tube briefly to ensure that the beads have settled at the bottom. Add up to 100 mg of soil and 800 µL of Solution CD1. Vortex briefly to mix.
2. Secure the PowerBead Pro Tube horizontally on a Vortex Adapter for 1.5–2 ml tubes. Vortex at maximum speed for 10 minutes. ***Note:*** If using the Vortex Adapter for more than 12 preps simultaneously, increase the vortexing time by 5–10 minutes.
3. Centrifuge the PowerBead Pro Tube at 15,000 x g for 1 minute.
4. Transfer the supernatant to a clean 2 mL Microcentrifuge Tube (provided). ***Note***: Expect 500–600 µL. The supernatant may still contain some soil particles.
5. Add 200 µL of Solution CD2 and vortex for 5 seconds.
6. Centrifuge at 15,000 x g for 1 minute. Avoiding the pellet, transfer up to 700 µL of supernatant to a clean 2 mL Microcentrifuge Tube (provided). ***Note:*** Expect 500–600 µL.
7. Add 600 µL of Solution CD3 and vortex for 5 seconds.
8. Load 650 µL of lysate to an MB Spin Column. Centrifuge at 15,000 x g for 1 minute.
9. Discard the flow-through and repeat step 8 to ensure that all of the lysate has passed through the MB Spin Column.
10. Carefully place the MB Spin Column into a clean 2 mL Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.
11. Add 500 µL of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 1 minute.
12. Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.
13. Add 500 µL of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 1 minute.
14. Discard the flow-through and place the MB Spin Column into a new 2 mL Collection Tube (provided).
15. Centrifuge at up to 16,000 x g for 2 minutes. Carefully place the MB Spin Column into a new 1.5 mL Elution Tube (provided).
16. Add 50 µl of Solution C6 to the center of the white filter membrane.
17. Centrifuge at 15,000 x g for 1 min. Discard the MB Spin Column. The DNA is now ready for downstream applications. ***Note:*** We recommend storing the DNA frozen (–30 to –15°C or –90 to –65°C) as Solution C6 does not contain EDTA.

2. Amplification

Supplies required

- KAPA3G Plant PCR Kit, Roche (Cat # 08041091001)
- Thermal cycler
- Plate centrifuge
- PCR plates and microcentrifuge tubes
- Microcentrifuge
- Molecular biology grade water
- Low EDTA TE Buffer
- Custom primers (see https://doi.org/10.3390/d17020137 for sequence details)
- 100% Dimethyl sulfoxide (DMSO)
- Pipettors (10 – 1000 µL) and multichannel pipettors recommended

Procedure

- Dilute primer stocks if needed:
  - (100 µM)(x) = (2.67 µM)(100 µL) → 2.67 µL of stock + 97.33 µL Low EDTA TE Buffer/Molecular biology grade water
- Prepare amplification master mixes for full 96-well plate of samples (overages provided to account for pipetting error):
  - All forward primers (stored in plate format) are added individually to amplification plate using a multichannel pipettor and are excluded from the master mix.

a) *Bacteria* (16S amplifications)

- Master mix components sufficient for duplicate amplifications for each sample

Component	**1X volume (µL)**	**200X volume (µL)**
KAPA Plant PCR Buffer	6.25	1250
KAPA3G Plant DNA Polymerase	0.1	20
Forward primer – 515F (2.67 µM)	1.41	-
Reverse primer – 806R (2.67 µM)	1.41	282
Molecular biology grade water	1.34	268
DNA extract	2	-

- Cycling conditions

Cycle step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	94	3:00	1
Denaturation	94	0:45	40
Annealing	50	1:00	
Extension	72	1:30	
Final extension	72	10:00	1

b) *Fungi* (ITS1 amplifications)

- Master mix components sufficient for duplicate amplifications for each sample

Component	**1X volume (µL)**	**200X volume (µL)**
KAPA Plant PCR Buffer	6.25	1250
KAPA3G Plant DNA Polymerase	0.1	20
Forward primer – ITS1-F (2.67 µM)	1.41	-
Reverse primer – ITS2 (2.67 µM)	1.41	282
Molecular biology grade water	1.34	268
DNA extract	2	-

- Cycling conditions

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	94	1:00	1
Denaturation	94	0:30	40
Annealing	52	0:30	
Extension	68	0:30	
Final extension	68	10:00	1

c) Plants (ITS2 – p3/u4 master mix)

- Master mix components sufficient for <u>single</u> amplification for each sample

Component	1X volume (µL)	100X volume (µL)
KAPA Plant PCR Buffer	6.25	625
KAPA3G Plant DNA Polymerase	0.1	10
Forward primer – ITSp3 (2.67 µM)	1.41	-
Reverse primer – ITSu4 (2.67 µM)	1.41	141
Molecular biology grade water	1.34	134
DNA extract	2	-

- Cycling conditions

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	94	4:00	1
Denaturation	94	0:30	40
Annealing	55	0:40	
Extension	72	0:20	
Final extension	72	10:00	1

d) Plants (ITS2 – 2F/p4 master mix)

- Master mix components sufficient for <u>single</u> amplification for each sample

Component	1X volume (µL)	100X volume (µL)
KAPA Plant PCR Buffer	6.25	625
KAPA3G Plant DNA Polymerase	0.1	10
100% DMSO	0.5	50
Forward primer – ITS2F (2.67 µM)	1.41	-
Reverse primer – ITSp4 (2.67 µM)	1.41	141
Molecular biology grade water	0.84	84
DNA extract	2	-

- Cycling conditions

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	94	4:00	1
Denaturation	94	0:30	40
Annealing	55	0:40	
Extension	72	0:20	
Final extension	72	10:00	1

e) *Plants* (*trnL* master mix)

- Master mix components sufficient for duplicate amplifications for each sample

Component	**1X volume (µL)**	**200X volume (µL)**
KAPA Plant PCR Buffer	6.25	1250
KAPA3G Plant DNA Polymerase	0.1	20
Forward primer – trnLc (2.67 µM)	1.41	-
Reverse primer – trnLh (2.67 µM)	1.41	282
Molecular biology grade water	1.34	268
DNA extract	2	-

- Cycling conditions

Cycle step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	94	2:00	1
Denaturation	94	1:00	40
Annealing	55	0:30	
Extension	72	0:30	
Final extension	72	5:00	1

f) *Arthropods* (COI – ZBJ master mix)

- Master mix components sufficient for single amplification for each sample

Component	**1X volume (µL)**	**100X volume (µL)**
KAPA Plant PCR Buffer	6.25	625
KAPA3G Plant DNA Polymerase	0.1	10
Forward primer – ZBJArtF1c (2.67 µM)	1.41	-
Reverse primer – ZBJArtR2c (2.67 µM)	1.41	141
Molecular biology grade water	1.34	134
DNA extract	2	-

- Cycling conditions

Cycle step	**Temperature (°C)**	**Time**	**Cycles**
Initial denaturation	94	5:00	1
Denaturation	94	0:30	40
Annealing	45	0:45	
Extension	72	0:45	
Final extension	72	10:00	1

g) *Arthropods* (COI – mLepF1/LepR1 master mix)

- Master mix components sufficient for single amplification for each sample

Component	**1X volume (µL)**	**100X volume (µL)**
KAPA Plant PCR Buffer	6.25	625
KAPA3G Plant DNA Polymerase	0.1	10
Forward primer – mLepF1 (2.67 µM)	1.41	-
Reverse primer – LepR1 (2.67 µM)	1.41	141
Molecular biology grade water	1.34	134
DNA extract	2	-

- Cycling conditions

Cycle step	Temperature (°C)	Time	Cycles
Initial denaturation	95	2:00	1
Denaturation	94	0:30	5
Annealing	45	0:40	
Extension	72	1:00	
Denaturation	94	0:30	35
Annealing	51	0:40	
Extension	72	1:00	
Final extension	72	5:00	1

3. Post-amplification purification

Supplies required

- KAPA Pure Beads, Roche (Cat # 07983271001 / 07983280001 / 07983298001)
- Ethyl alcohol/ethanol, 100%
- Molecular biology grade water
- 1.5 mL microcentrifuge tubes
- Magnetic stand for 1.5 mL microcentrifuge tubes
- Elution buffer, Buffer EB (Qiagen Cat # 19086 recommended)
- Pipettors (10 – 1000 µL), multichannel pipettors recommended

Procedure

- Allow KAPA Pure Beads to come to room temperature before purification. Make sure they are thoroughly mixed.
- Prepare 80% ethanol for 800 µL per sample in two separate 50 mL conicals
    - (100)(x) = (80)(50) → 40 mL ethyl alcohol + 10 mL molecular biology grade water
    - (100)(x) = (80)(50) → 40 mL ethyl alcohol + 10 mL molecular biology grade water

- Purification:
    - Following amplification of all targets indicated above, amp product will be pooled and purified with KAPA Pure Beads using a 0.9X ratio (125 µL * 0.9 = 112.6 µL of KAPA Pure Beads)
        - 0.9X bead ratio selected based on Tiedge *et al.* 2025 (https://doi.org/10.3390/d17020137)
        - Combine the amplified product from nine plates into the tenth plate using a multichannel pipettor. For example, all samples in column 1 across all plates should be added to column 1, etc. Once everything is transferred to a single plate, transfer the contents of each well (125 µL total) to individual 1.5 mL microcentrifuge tubes.
            - ***Note****:* Libraries must be purified in tubes with this volume as adding the KAPA Pure Beads will exceed the volume amount the plates can hold.

    - Vortex the KAPA Pure Beads and immediately add 112.6 µL to 125 µL of sample. Mix by pipetting up and down multiple times.
    - Incubate the tubes at room temp for 5 min to bind DNA.
    - Place tubes on magnet and incubate until the liquid is clear. Remove and discard the supernatant into the appropriate waste accumulating container.
        - Leave behind some supernatant (~10 µL), as the viscosity pulls beads off the magnet.

    - While on the magnet, add 400 µL of 80% ethanol and incubate for 30 seconds. Remove the ethanol and keep samples on the magnet.
    - While on the magnet, add 400 µL of 80% ethanol and incubate for 30 seconds, then remove the ethanol without disturbing beads.
    - Dry the beads for 3-5 minutes or until ethanol has evaporated.
        - If needed, spin tubes down briefly to collect ethanol droplets. Place back on the magnet and remove any remaining ethanol droplets with a 10 µL tip to speed up drying.

    - Remove the samples from the magnet.
    - Add 100 uL of elution buffer and vortex briefly to resuspend the beads and incubate for 2 minutes.
    - Place tubes on the magnet until clear.
    - Transfer the supernatant to a new plate, maintaining the same plate location as the original DNA extract plate to help with sample tracking.
        - Plate can remain at 4°C for short term storage prior to qPCR but should be placed in -20°C for long term storage.

4. Library Quantification

Supplies required

- KAPA Library Quantification Kit Complete kit (Universal), Roche Cat # 07960140001
- Pipettors (10 – 1000 µL), multichannel pipettors recommended
- Real-Time PCR System, QuantStudio™ 5 Real-Time PCR System, ThermoFisher Scientific Cat # A34322 recommended
- Optical plates and seals compatible with Real-Time PCR System
- Additional 96-well PCR plates for dilutions
- 1.5 mL microcentrifuge tubes
- Molecular biology grade water

Procedure (Modified from KAPA Library Quantification Kit Illumina® Platforms Technical Data Sheet v11.20 l accessed on 03/07/2025. Modifications highlighted in yellow font).

- Dilute libraries 1:1,000,000 by completing the following in separate 96-well plates using the multichannel pipette:
    - Plate 1 (1:100) = Combine 2 µL of purified amplicon library and 198 µL of DNA Dilution Buffer.
        - Seal, vortex, and centrifuge plate.
    - Plate 2 (1:10,000) = Combine 2 µL of the 1:100 dilution and 198 µL of DNA Dilution Buffer.
        - Seal, vortex, and centrifuge plate.
    - Plate 3 (1:1,000,000) = Combine 2 µL of the 1:10,000 dilution and 198 µL of DNA Dilution Buffer.
        - Seal, vortex, and centrifuge plate.

- If the KAPA Library Quantification Kit is being used for the first time, add 1 mL of 10X Primer Premix to the 5 mL bottle of 2X KAPA SYBR FAST qPCR Master Mix. Mix thoroughly via vortexing.
- Prepare the qPCR Master mix as follows (enough for two 96-well plates of purified amplicon libraries along with standards):

Component	**20 µl reaction**	**N = 240**
KAPA SYBR FAST qPCR Master Mix (2X) + Primer Premix (10X)	12	2880
50X ROX Low	0.4	96
Molecular Grade Biology water	3.6	864
Purified amplicon libraries	4	-

- Mix and briefly centrifuge the reagent master mix and dispense 16 µl to each designated well of a 96-well optical PCR plate.
- Add 4 µL of each DNA Standard (a total of 6 standards for this kit) in duplicate, starting with the least concentrated (Standard 6) to the most concentrated (Standard 1).
- Add 4 µL molecular biology grade water to designated "No Template Control" wells in duplicate.
- Add 4 µL of each purified amplicon library (1:1,000,000 dilution) to designated wells.
- Seal with an optical seal and centrifuge
- Place plate on Real-Time PCR System
    - Designate DNA Standard, NTC and sample wells and set known concentrations of DNA Standards.
    - Run the following program:

Step	Temp	Duration	Cycles
Initial denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	35
Annealing/Extension/ Data acquisition	60 °C	45 sec[1]	
Melt curve analysis[2]	65 – 95 °C		

- Once complete, the software program will calculate quantities of the purified amplicon libraries.
    - Calculate the concentration of the undiluted library by multiplying by the dilution factor (in this case 1,000,000 and the size adjustment factor of 1.02 (452 bp/445 bp); see Tiedge *et al.* 2025 for details (https://doi.org/10.3390/d17020137))
- Pool samples to be sequenced on a single sequencing run together with differing volumes to achieve an equimolar pool.
    - Purify the library pool as described in section 3 using as ratio of 0.9X.
    - Once samples have been pooled, dilute the library pool as noted above (1:100, 1:10,000 and 1:1,000,000) and quantify the 1:1,000,000 dilution as the only unknown sample. Ensure that the DNA Standards and NTC are also quantified in duplicate.
- Based on the calculated concentration of the pool, prepare at least 30 µL of a 10 nM dilution for sequencing.

5. Sequencing using an Illumina MiSeq

Supplies required

- Appropriate MiSeq Reagent Kit
- Denature and Dilute Libraries Protocol for the MiSeq System (https://support-docs.illumina.com/IN/MiSeq_DnD/Content/MiSeq/DnD-MiSeq.htm?protocol=standard)
- Pipettors (10 – 1000 µL)
- 1 N Sodium hydroxide (NaOH), molecular biology grade
- Molecular biology grade water
- Microcentrifuge tubes
- 10 mM Tris-HCl, pH 8.5 with 0.1% Tween 20
- Illumina PhiX Control, Illumina (Cat # FC-110-3001)
- Heat block
- Custom sequencing primers (see https://doi.org/10.3390/d17020137 for sequence details)

Procedure

- Follow the manufacturer's recommendations for preparation of sequencing libraries, with the following modifications

    - Loading & Concentration
        - When using MiSeq Reagent Kit v2, use 8 pM as the loading concentration
        - When using MiSeq Reagent Kit v3, use 10 pM as the loading concentration

    - PhiX
        - PhiX should be spiked in at 10% to increase sequence diversity in the run

    - Heat denaturation
        - Once the pool has been diluted to the desired loading concentration (following the Denature and Dilute Libraries Protocol for the MiSeq System guide), the pool must undergo a heat denature step to help with GC rich templates.
        - Based on Illumina guidance:
            - After NaOH denaturation of the sequencing library and dilution in HT1 to the final loading concentration, incubate the diluted library at 96°C for 2 minutes using a heat block.
            - Invert the tube 1-2 times to mix.
            - Quickly move the library to an ice bath for 5 minutes. The quick cooling step helps lock the library in its single stranded form.

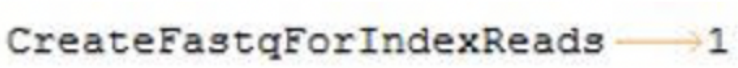
            https://support.illumina.com/bulletins/2016/06/how-to-achieve-more-consistent-cluster-density-on-illumina-sequencing-platforms.html

    - Prepare sequencing primer pools
        - Add 5 µL of each Read 1 sequencing primer to a 1.5 mL tube.
        - Add 5 µL of each Read 2 sequencing primer to a 1.5 mL tube.

    - Spiking custom primers into the sequencing cartridge
        - When preparing the sequencing cartridge, pierce the foil with a 1000 µL pipette tip and add 600 µL of the denatured library plus PhiX in the "Load Sample" well.
        - Next, pierce the foil seals with a pipette tip and add 3.4 µL of Index Sequencing Primer (100 µM) to reservoir 13.
        - Add 23.8 µL of pooled Read 1 Sequencing Primers (100 µM) into reservoir 12.
        - Add 23.8 µL of pooled Read 2 Sequencing Primers (100 µM) into reservoir 14.

        - Mix the contents of each of the reservoirs (12, 13, and 14) with a different Pasteur pipette to ensure that the primers added by the user are mixed with the standard Illumina cocktail already in the reservoirs.
    - Read confirmation
        - Paired end sequencing should be selected, using the confirmation 27 x 12 x 563.

- MiSeq setup
    - Ensure the option to generate a fastq file for the index is selected in Local Run Manager or MiSeq Reporter.
        - Local Run Manager
            - Navigate to the Local Run Manager analysis module folder.
                - Frameworks versions 1 and 2: **C:\Illumina\Local Run Manager\Modules\{module}\{version}**
                - Framework version 3: **C:\Program Files\Illumina\Local Run Manager\Modules\{module}\{version}**
            - Edit the file **IsisConfigSettings.tsv** in a plain text editor like Notepad.
                - Note: If you receive permissions errors editing or saving the file, run Notepad in Administrator mode.
            - Add a line and enter the value **CreateFastqForIndexReads**, enter tab, and then enter the value **1**
              The line should look like below, where the arrow indicates a tab:



            - **Note:** The option is case sensitive and must be entered exactly as written. Save the IsisConfigSettings.tsv file, making sure it remains a tab-separated value text file. Restarting the Local Run Manager services is not necessary.
            - **IMPORTANT**: Change **CreateFastqForIndexReads** back to **0** after successful analysis, otherwise future analyses will also generate index read FASTQs.
        - **MiSeq Reporter**
            - Navigate to **C:\Illumina\MiSeqReporter**
            - Make a backup copy of **MiSeq Reporter.exe.config**
            - Edit the original **MiSeq Reporter.exe.config** using Notepad
            - Add the following to the top portion of the file (in the **<appSettings>** section)
                - **<add key="CreateFastqForIndexReads" value="1" />**
            - Save the file
            - Restart the MiSeq Reporter service
                - Start the Windows Task Manager by pressing Ctrl-Alt-Delete on the keyboard and then selecting **Start Task Manager**
                - Select the **Services** tab
                - Find **MiSeqReporter** on the list of services
                - Right-click on **MiSeqReporter** and select **Stop Service**
                - Right-click on **MiSeqReporter** again and select **Start Service**
            - **IMPORTANT**: Change **<add key="CreateFastqForIndexReads" value="1" />** to **<add key="CreateFastqForIndexReads" value="0" />** after successful analysis, otherwise future analyses will also generate index read FASTQs.
    - **Creating a run**
        - The MiSeq uses a "Sample Sheet" .csv file set-up through the Illumina Experiment Manager on a separate PC to dictate the parameters of each run. These instructions are based on version 1.0.31 of the Illumina Experiment Manager. When creating the sample sheet for this run, under "Select Workflow" choose "MiSeq Reporter" and then select "de novo Assembly"."Metagenomics" can also be selected at this stage. The important point is to select "MiSeq Reporter" as this will allow you to obtain sequences that are not demultiplexed, so

demultiplexing can be performed with QIIME. This is important because QIIME can correct barcode errors while the MiSeq instrument software does not attempt to correct barcode errors.)

- Under the field "Select Compatible Assay" select "TruSeq DNA/RNA." Fill in the number listed on the cartridge you'll be using in the "Sample Sheet Name*" adding two zeros before the 300 (e.g. MS0002657-00300). Then, select "Paired End," 1 Index Read, Index Cycles 6, and **27x563**bp. Note that despite the barcodes being 12 bases, you should set Index Cycles to 6 in this step – this will be corrected manually in a subsequent step. On the next screen, fill in a Sample ID and select one of the standard barcodes Illumina provides (e.g. A001). Once the required columns have been filled-in, click in any box to see the word "valid" in green and then proceed.
- Once this .csv file is created, it will need to be edited manually to instruct the MiSeq to conduct a 12bp index read. This is achieved by opening the appropriate sample sheet for the run in a text editor (e.g. Notepad on Windows, TextEdit on Mac, or gedit on Linux). The columns in this sheet are comma separated, so it is crucial to include/remove the appropriate amount of commas when editing the file. On the line directly under [Settings] include the command OnlyGenerateFASTQ,1**.** Next, under [Data] replace the 6bp barcode with a 12bp barcode (use: ACGTAACCACGT) to indicate to the instrument you want a 12bp index read. You will also need to remove the field 17_Index_ID**,** by removing both the column name and comma. In order to check that the columns appropriately align in your edited .csv, open the Sample Sheet in Excel. In Excel, you should see that the columns line-up and that the column containing I7_Index_ID is gone (see Screenshot, which is an example of what the csv should look like after you've edited it).