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Appendix C

Pre-harvest Product Sampling and Testing Protocol

Version 8/27/2021

*This version supersedes the 8/9/2021 version.
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I. Introduction

Pre-harvest product testing is one of many tools that can help assess the potential for lot-specific produce contamination and assist in developing a long-term view of food safety system performance. This appendix provides guidance for pre-harvest product testing as specified in the Commodity Specific Food Safety Guidelines for the Production and Harvest of Lettuce and Leafy Greens (hereby referred to as the LGMA-approved Guidelines).

The purpose of this document is to provide guidelines to assist users in developing and executing:

- 1) A routine pre-harvest product testing protocol, and
- 2) A response to an observed and unexpected event judged as necessitating a risk-based pre-harvest product testing protocol.

These guidelines are founded on the best available science for designing and executing a robust sampling and testing plan that gives the user a high level of confidence in detecting contamination present at levels scientifically determined to increase the public risk of an outbreak. This document is based on three key assumptions:

- 1) The science-based design of preharvest testing can only be expected to address randomly distributed (systemic) contamination across a lot and would not be likely to detect highly clustered contamination within a field.
- 2) The pre-harvest testing is framed on scientifically derived models capable of detecting low but relevant levels (equal to or greater than 1 CFU/pound) of contamination within a lot.
- 3) The rationale for this calculated and weight-based metric is that it provides the required measurement standard relevant for leafy greens. This is strongly recommended rather than a testing confidence in detecting “% contamination”, which has been applied to-date.

The sampling and testing plans include both sampling protocol and target organism detection sensitivity (limit of detection) requirements, which are designed to provide a 95% confidence in rejecting a ≥ 1 CFU per pound (colony-forming unit of a living pathogen per pound of product) level of randomly distributed contamination in a lot.

Binary (yes/no) product testing standard operating procedures (SOPs) should be developed and implemented for routine lot-based and risk-based harvest acceptance. Going forward, the major value of a standard pre-harvest testing program will be trend analysis for improved risk assessment as data is collected and shared. Following this guidance and sharing of data and experiences throughout the industry is critical for refining and revising these sampling plans.

II. Routine Pre-harvest Product Sampling and Testing Protocol

1) Target organisms

- *E. coli* O157 and other pathotoxigenic STEC of clinical significance (including O26, O45, O103, O111, O121 and O145)
- *Salmonella enterica*

2) Measurement criteria

- The acceptable result for a defined lot is molecular non-detects for diagnostic PCR amplified products for:
 - *Salmonella* and
 - *E. coli* O157 and other pathotoxigenic STEC of clinical significance (including O26, O45, O103, O111, O121 and O145).
- In the event of a molecular detect indicating *E. coli* O157 or any sub-type of *stx* (1 or 2) along with additional virulence factors such as *eae*, molecular confirmation of non-detects for clinically significant STEC (O157, O26, O45, O103, O111, O121 and O145) from the original primary enrichment, may be acceptable.
- A qualified service laboratory or the developer of certified test kits or Performance Tested platforms (i.e. [AOAC PTM](#)) can explain the specific detection systems and platform(s) they offer and how these are validated or certified for these targets.

3) Timeline for sampling and testing

- Collect product samples ≤ 7 days prior to the scheduled harvest day. It is recommended that sampling be conducted as close as possible to harvest: the closer sampling is conducted to the actual initial harvest day, the more likely the lot harvest decision will be based on consequential contamination events associated with crop production.

4) Size of lot to be sampled

- This guidance considers a nominal area of ≤ 5 -acre increments for lot size as a “best practice” when conducting routine sampling. Other lot definitions may be individually defined and may vary depending on the ranch/farm.
- Based on practical interpretation of the available science and product sampling models, the maximum recommended lot size for sampling is 40 contiguous acres. This is also applicable to adjacent noncontiguous lots (up to 40 acres) on the same irrigation system node and within the same 7-day scheduled pre-harvest dates.
- When defining a lot size for sampling, keep in mind that one positive test result per designated lot will require destruction of the entire lot. Resampling of a positive lot is not allowed.
- Irrespective of the lot size, the sampling plan, in terms of being representative across the lot and for the minimum total product mass tested, remains the same.

5) Sampling plan: Sample number and size

- Samples are broken down as follows: total sample size (the amount of leafy greens taken from a lot) > subsamples (a composite of leaves (grab specimen) that will be tested as a unit) > grab specimen (the number of approximate mass from each randomly selected plants sampled).
- The total sample mass of leafy greens per the designated lot must equal approximately 1,500 grams ($\pm 2\%$ 30 g) weighed and recorded by the third-party service lab. The total sample mass of 1,500 grams should be divided into

subsamples within a lot in a sampling configuration that adequately represents the designated lot (see examples in Figures 1 and 2).

- Flexibility in total mass sampled and tested is allowed if based on a scientifically developed and validated protocol which provides an equivalent detection limit.
- The recommended best practice is a minimum of $n = 60$ sampling plan, which consists of at least 60 samples for a nominal total mass of 1,500 grams representing a given designated lot. Samples are most reasonably collected in multiple composite subsamples (see examples in Figures 1 and 2). Grab specimens may be composited for analysis up to the maximum allowable analytical portion specified in the method. Maximum mass for any one (composite of grab specimens or) subsample is 375 grams, which, for a total sample mass of 1,500 grams, would result in 4 composite subsamples. In an $n = 60$ sampling plan, each of the 4 composite subsamples of 375 grams would include a minimum of 15 grab specimen (Table 1). Each grab specimen of 25 grams should include tissue collected from multiple heads/plants. Collecting and testing samples in multiple composite subsamples allows for area tracking, if desired, for root cause and investigative analysis.

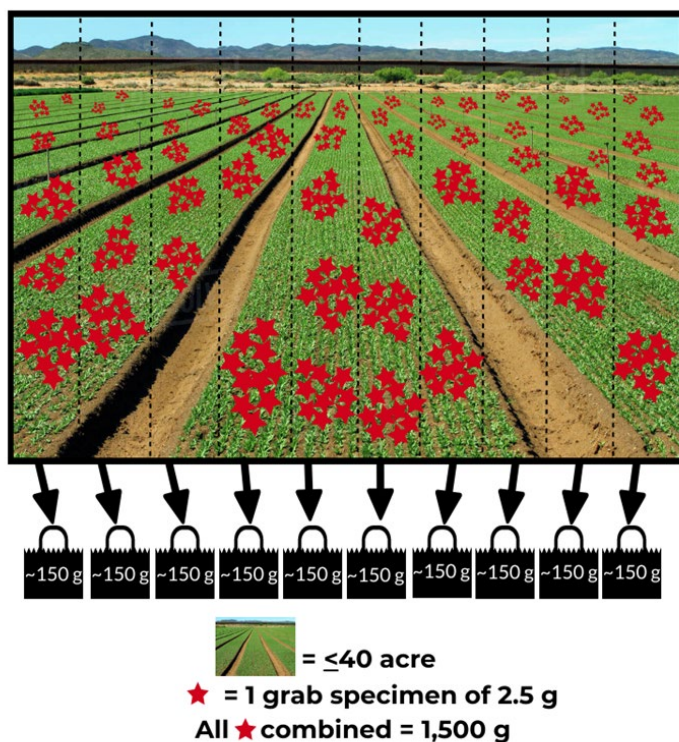
Table 1. Sampling plan examples

Routine Testing	Timeline	Maximum lot size (acre)	Total sample size (grams) per lot	Required # of grab specimen per subsample	Approximate grab specimen size (grams)	Required # of subsamples	Subsample size (grams)	Sampling location
	Maximum 7 days prior to harvest	≤ 40	1,500	15	25	4	375	Randomized and evenly distributed over the entire lot
				10	25	6	250	
				60	2.5	10	150	

Figure 1. Illustration of n = 60 sampling scheme in head lettuce



Figure 2. Illustration of n = 600 sampling scheme for baby spinach



- All collected product tissue must be used, according to the validated test method, in the pre-enrichment phase prior to a pathogen detection test. Removing and testing only a portion from the total sample is not consistent with this guidance. Based on long-established guidance, statistical assumption requires that all sample material (i.e., $n = 60$ totaling 1,500 grams) be used for lab analysis.
- Plant density may be considered in an individual product testing SOP. However, the foundation for sample number and lot size must meet the current equivalent acceptance criteria (see workbook) for achieving a standardized recommended confidence in detecting target contaminants (*E. coli* O157/other pathotoxigenic STEC and *Salmonella*) at the level predicted to result in an outbreak.
- Samples should be collected according to stratified randomized and representative locations within a designated lot. For example:
 - Walk the designated lot in a pattern that results in samples inclusive of all length and width of the beds within a designated lot. There is no current single statistically valid or fixed sampling configuration. Sampling efficiencies should determine the path taken to reach randomized locations representative of the designated lot. For instance, recent research suggested that, while preferential to alternate-bed sampling plans, the Z sampling pattern will miss in-field contaminants if they are not dominant on the field's border/edges that are sampled, especially if contamination is dominant on the edges that are never tested. Approximate sampling locations may be assigned within readily defined spacing (i.e., every two sprinkler line joints).
- The greater the number and tissue mass of individual samples, the greater the confidence in the likelihood to detect the target organisms - in other words, a greater confidence that a product lot is unlikely to have what has been termed "systemic risk of contamination". However, increasing sample number to improve confidence in lot acceptance decisions rapidly becomes unrealistic and economically unfeasible.
- If a sample from a lot tests positive during routine testing, **re-testing of that lot or a sub-lot area in response is not a valid application of this guidance**. It is recommended that a response involving re-testing of a lot that tests positive, other than for investigative root cause analysis purposes, be considered a non-conformance (i.e., outside the guidelines in this appendix as well as the LGMA-approved Guidelines). Surrounding lots, on the other hand, are subject to risk-based testing as described in section III.

6) Sample collection

- Using reasonable aseptic sample collection techniques utilizing sterile gloves for each new lot, select leaves from the edible portion of plants. Focus on leaves that would contact harvest tools, mechanized harvest equipment, or harvest workers' gloves and apparel.
- Incorporate basic crop characteristics into tissue sampling strategies such as tender leaf crops vs. head lettuce (e.g., romaine). Sample tender leaf crops such as baby spinach to include the full leaf blade and basal petiole. Sampling should include full leaves or sections of a full head rather than pinching off the upper quarter of a single leaf or leaves on an individual plant (Kroupitski, 2011; Van der Linden, 2016).

- Place each sample in a sterile container or sealable sample bag and include the specific sampling location in documentation, either by a planned randomized location on a field map or by operator point-to-point or app-based walking GPS-time-tracked tagging. If using multiple sample collection containers or bags, make sure to label them in a way that the laboratory can identify all containers/bags that are part of the same lot.
- Place samples in a cooler with adequate ice packs, but do not freeze. If using water-based ice (not recommended), ensure the product is protected from potential cross-contamination from melting ice/water.
- Fill out the chain of custody form with the sample collection information.
- Select a qualified third-party service or laboratory for sample analysis. It is in your best interest to select a validated or performance tested method for pathogen testing (AOAC Certified or Performance Tested method, AFNOR certification, etc.) that the laboratory is qualified / accredited to perform.
- Confirm that the service laboratory utilizes validated methods for sample mass to enrichment buffer ratios and time for pre-enrichment, matched to the target detection platform. You should understand the general specifications and basics of the test method you have selected, focusing on detection limits and time to results.
- Samples must be transported promptly and at the right temperature as required under your specific sampling method protocol. Service laboratories generally specify this transfer time to be consistent with test method certification. For instance, within 48 hours if the arrival temperature is assured to be between 33°F and 41°F.
- Make sure deviations from these recommendations for investigative purposes are communicated and documented on Chain of Custody forms.

7) Remedial actions

Remedial actions may vary depending on how sampling lots are defined and the outcome of a root cause analysis (RCA).

- Conduct RCA to make a concerted effort to determine what may have led to the detectable contamination on product. Based on the findings of your RCA:
 - Consider the potential for recurrence of the hazard or associated risk identified through the RCA. How likely is it that future plantings might be affected by the same hazard?
 - Consider the suitability/safety of the area where a pathogen was detected for replanting a fresh consumed leafy green crop for the remainder of the season.
- Do not harvest produce from the lot where a pathogen was detected. Destroy the crop in this area.
- Clean and sanitize all equipment utilized to destroy the crop upon exiting the field. Consider swabbing equipment after crop destruction as part of your RCA effort.
- Document all remedial actions including both considerations adopted and those evaluated but not implemented. All documentation must be available for verification from the responsible grower.

III. Risk-based Pre-harvest Product Sampling and Testing Protocol

SOPs should be developed and applied, as needed, for risk-based observations and events including, but not limited to:

- During pre-harvest environmental assessments, when a potential risk that was mitigated during the pre-plant assessment, changes such that the likelihood of contamination now warrants increased testing.
- When irrigation water that exceeds generic *E. coli* water quality standards was inadvertently used on the unharvested crop or when Type B → A water treatment fails to achieve acceptance criteria as established by the LGMA-approved Guidelines and that water was inadvertently applied to unharvested crop.
- When there are hazards with uncertain risk associated with adjacent land conditions, features, or uses (e.g., runoff, potential windborne contamination from animal holding/transfer/feeding operations, composting operations, or staging/application of compost).
- Other unforeseen sources or incidents potentially resulting in crop contamination.
- Situations described in the California LGMA pre-harvest testing guidance, which lists elevated risk factors that can trigger pre-harvest testing.¹

1) Target organisms

- *E. coli* O157 and other pathotoxigenic STEC (including O26, O45, O103, O111, O121 and O145)
- *Salmonella enterica*

2) Measurement criteria

- The acceptable result for a defined lot is molecular non-detects for diagnostic PCR amplified products for:
 - *Salmonella* and
 - *E. coli* O157 and other pathotoxigenic STEC (including O26, O45, O103, O111, O121 and O145).
- In the event of a molecular detect indicating *E. coli* O157 or any sub-type of *stx* (1 or 2) along with additional virulence factors such as *eae*, molecular confirmation of non-detects for clinically significant STEC (O157, O26, O45, O103, O111, O121 and O145 from the original primary enrichment, may be acceptable.
- An accredited service laboratory or developer of certified test kits or Performance Tested platforms (i.e. [AOAC PTM](https://www.aoac.org/ptm/)) can explain the specific detection systems and platform(s) they offer and how these are validated or certified for these targets.

¹ <https://lgmatech.com/wp-content/uploads/2021/04/Pre-Harvest-Testing-Guidance-20210416.2.pdf>

3) Timeline for sampling and testing

- Conduct risk-based product testing as soon as an unanticipated or previously unrecognized hazard is first observed to establish whether detectable contamination has occurred (1st round of testing).
- Conduct routine product testing at the scheduled product harvest date (2nd round of testing) within 4-7 days of harvest) only if:
 - the hazard is observed prior to routine sampling,
 - the initial risk-based test format (1,500g per ac) result is negative, and
 - the crop is not destroyed.
- Conduct a root cause analysis to determine what may have led to the unforeseen or unaccounted for hazard (i.e., a contamination risk from a recognized adjacent or seasonal hazard judged to be acceptable within established guidelines or an actual risk exposure resulting in detectable contamination of the harvested or unharvested product).

4) Size of lot to sample

- For risk-based testing purposes, **lot size may not exceed one acre.**
- Sampling of less than one acre should follow the same sampling plan as one acre.

5) Sampling plan: Sample number and size

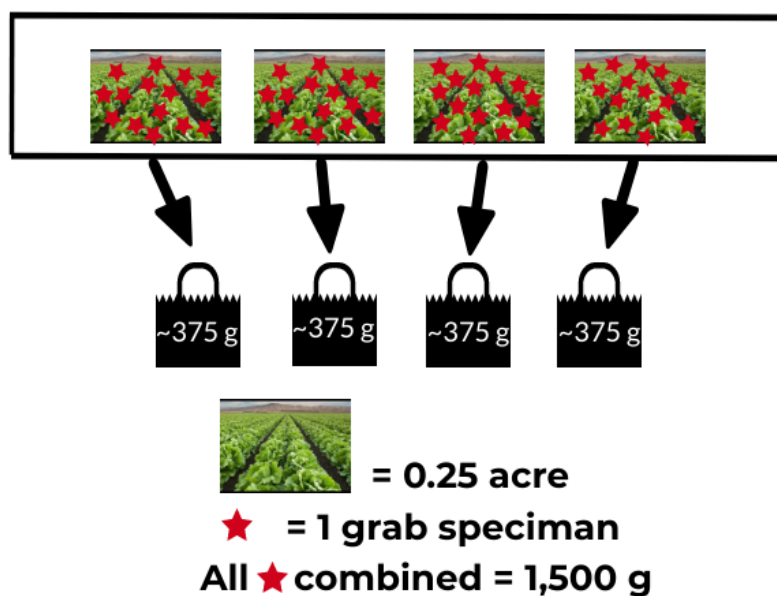
Samples are broken down as follows: total sample size (the amount of leafy greens taken from a lot) > subsamples (a composite of leaves (grab specimen) that will be tested as a unit) > grab specimen (the number of plants sampled).

- The total sample mass of leafy greens per the designated lot must equal approximately 1,500 grams ($\pm 2\%$ 30 g). The total sample mass of 1,500 grams should be divided into subsamples within a lot in a sampling configuration that adequately represents the designated lot (see example in Figure 3).
- A sampling plan of minimum $n = 60$ would consist of 15 grabs from 10 different plants across 10 locations that are evenly distributed across an affected area for a nominal total mass of 1,500 grams per one acre lot. Samples are most reasonably collected in multiple composite subsamples (see example in Figure 3). Grab specimens may be composited for analysis up to the maximum allowable analytical portion specified in the method. Maximum mass for any one (composite of grab specimens or) subsample is 375 grams, which, for a total sample mass of 1,500 grams, would result in 4 composite subsamples. In an $n = 60$ sampling plan, each of the 4 composite subsamples of 375 grams would include a minimum of 15 grab specimen (Table 2). Each grab specimen of 25 grams should include plant tissue collected from multiple heads/plants. Collecting and testing samples in multiple composite subsamples allows for area tracking, if desired, for root cause and investigative analysis.

Table 2. Sampling plan examples

	Timeline	Maximum lot size (acre)	Total sample size (grams) per lot	Required # of grab specimen per subsample	Approximate grab specimen size (grams)	Required # of sub-samples	Subsample size (grams)	Sampling location
Risk-based Testing	ASAP	≤ 1	1,500	15	25	4	375	the suspect area of the field believed to be affected by the potential or observed risk factor
				10	25	6	250	
				60	2.5	10	150	

Figure 3. Illustration of n = 60 sampling scheme



- All collected product tissue must be used, according to the validated test method, in the pre-enrichment phase prior to a pathogen detection test. Removing and testing only a portion from the total sample is not consistent with this guidance. Based on long- established guidance, statistical assumption requires that all sample material (i.e., n = 60 totaling 1,500 g) be used for lab analysis.
- Plant density may be considered in an individual product testing SOP. However, the foundation for sample number and lot size must meet the current equivalent acceptance criteria (see workbook) for achieving a standardized recommended confidence in detecting target contaminants (*E. coli* O157/other pathotoxigenic STEC and *Salmonella*) at the level predicted to result in an outbreak.

The following methods are recommendations for sampling plans and not requirements.

- To help develop the greatest level of confidence in detecting non-uniformly distributed contamination, if present: Divide a 1-acre lot or field-level block into a grid and conduct systematic sampling within each grid starting at a randomized location with a predetermined spacing basis. For example, every third bed and approximately every quartered position of the bed length within each grid.²
- In the case of directional risk, biased sampling of a field's edge/border beds may be appropriate. But experience informs us that contaminant deposition may not be uniquely defined by edge proximity. For instance, when bioaerosols drift from a point source, deposition may be more central than strictly at the field edge closest to the source.

6) Sample collection

- Using reasonable aseptic sample collection techniques, select leaves from the edible portion of plants. Focus on leaves that would contact harvest tools, mechanized harvest equipment, or harvest workers' gloves and apparel.
- Incorporate basic crop characteristics into tissue sampling strategies such as tender leaf crops vs. head lettuce (e.g., romaine). Sample tender leaf crops such as baby spinach to include the full leaf blade and basal petiole. Sampling should include full leaves or sections of a full head rather than pinching off the upper quarter of a single leaf or leaves on an individual plant.
- Do not trim and discard leaves that would not be included with harvested product but focus on the areas of the plant/field that would be at greatest risk for crop contamination including but not limited to the following: inner leaves, outer leaves, and wrapper leaves. Additionally, when assessing the possibility of contamination via furrow irrigation water or animal intrusion, collect leaf samples from beds at the irrigation discharge point of the field - the head row area.
- Place each sample in a sterile container or sealable sample bag and include the specific sampling location in documentation, either by a planned randomized location on a field map or by operator point-to-point or app-based walking GPS-time-tracked tagging. If using multiple sample collection containers or bags, make sure to label them in a way that the laboratory can identify all containers/bags that are part of the same lot.
- Place samples in a cooler with adequate ice packs, but do not freeze. If using water-based ice (not recommended), ensure the product is protected from potential cross-contamination from melting ice/water.
- Fill out the chain of custody form with the sample collection information.
- Select a qualified third-party service or laboratory for sample analysis. It is in your best interest to select a validated or performance tested method for pathogen testing (AOAC, Performance Tested Certification, etc.) that the laboratory is qualified / accredited to perform.

² Adopted from the Interstate Technology and Regulatory Council (IRTC) recommended soil sampling practices.

- Confirm the service laboratory utilizes validated methods for sample mass to enrichment buffer ratios and time for pre-enrichment, matched to the target detection platform. You should understand the general specifications and basics of the test method you have selected, focusing on detection limits and time to results.
- Samples must be transported promptly and at the right temperature as required under your specific sampling method protocol. Service laboratories generally specify this transfer time to be consistent with test method certification. For instance, within 48 hours if the arrival temperature is assured to be between 33°F and 41°F.
- Make sure deviations from these recommendations for investigative purposes are communicated and documented on Chain of Custody forms.

7) Remedial actions

Remedial actions may vary depending on how sampling lots are defined and the outcome of a root cause analysis (RCA).

- Conduct an RCA to make a concerted effort to determine what may have led to the detectable contamination on product. Based on the findings of your RCA:
 - Consider the potential for recurrence of the hazard or associated risk identified through the RCA. How likely is it that future plantings might be affected by the same hazard?
 - Consider the suitability/safety of the area where a pathogen was detected for replanting a fresh consumed leafy green crop for the remainder of the season.
- Do not harvest from the lot where a pathogen was detected. Destroy the crop in this area.
- Clean and sanitize all equipment utilized to destroy the crop upon exiting the field. Consider swabbing equipment after crop destruction as part of your RCA effort.
- Document all remedial actions including both considerations adopted and those evaluated but not implemented. All documentation must be available for verification from the responsible grower.

IV. Rationale

This document contains guidelines to consider for product testing. It was originally developed in 2019 and most recently updated in June 2021. The protocol is based on current scientific knowledge, feedback from food safety experts and industry members. Ongoing and future research efforts should assist in refining this document in the future. Below are the technical basis and rationale behind the above guidelines.

Broth to product ratios – Laboratories must use a validated protocol for broth to product ratios (generally 4:1 or 5:1) in the pre-enrichment phase when sub-sample mass units are greater than 25 grams (Lopez, 2015).

Grab specimen – Indicates the lowest sampling unit in a sample collection event; includes plant tissue from multiple heads/plants and not just one head/plant or point location.

Lot size – Statistical analysis informs us that area sampled has much less of an impact on finding contamination vs total product mass (weight) and/or number of individual samples when they are representative of the whole. We recognize that the allowance for individual lot designation, up to 40 acres, may provide sufficient confidence in an operational absence of a consequential contamination level within a tested lot. The revisions to Appendix C are an effort to support this statistically based and standardized approach and allow for better data analysis towards continual technical and practical refinements.

Qualified laboratory – Laboratories that are accredited to the ISO 17025 standard and accredited to perform validated microbiological testing.

Sampling pattern – A peer-reviewed publication (Xu & Buchanan, 2019) indicates that the commonly applied Z sampling plan will miss contaminants in fields due to over emphasis on horizontal or vertical field edge dimensions. Additional field research examples are available in the field report available on the Center for Produce Safety's website (Rock, 2019; Stasiewicz, 2021).

Target organism selection – *E. coli* O157 and other pathotoxigenic STEC and *Salmonella* are selected for the current protocol. In the future, particular local conditions may indicate the need for risk-based testing for other foodborne pathogens.

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