

# SAMPLING CONSIDERATIONS FOR DETECTION OF GENETICALLY ENGINEERED (GE) TRAITS IN ALFALFA HAY

Daniel H. Putnam, PhD<sup>1</sup>

## ABSTRACT

Sampling principles for analysis of alfalfa hay quality have been worked out by scientists and industry members over decades of experience. These principles include 1) Proper identification of hay lots, 2) Multiple combined samples from a proper coring device using a defined technique across a random sample of bales within a lot, and 3) correct handling of samples before analysis. Sampling for detection of a GE trait requires many of the same elements. However, purpose of GE sampling is to detect a small amount (e.g. <5% or <0.1%) of an unwanted trait, vs. sampling for quality where analysis of the average quality across a lot is desired. For GE trace sampling, the sampling protocol will depend upon the level of detection required and the source of the Low Level Presence (LLP). For a LLP trait distributed throughout the crop mass at levels that may be detectable by PCR (0.1% or lower), it is recommended to collect 30 samples per lot at 12-14" depth, using a proper sampling implement (core device) and composite those samples. The entire sample should then be ground and sub-sampled for analysis. Higher threshold detection limits require fewer samples to detect. For double-compressed bales, common for export, power-assist or other sampling techniques are feasible. If the trait is distributed throughout the mass, it is not necessary to sample all bales, but a high number of samples are needed for low level detection. Non-endemic LLP (e.g. a random bale or partial bale) is not likely to be reliably detected using any systematic method—since such sources are non-uniform in the hay mass. In this case, quality assurance processes on the part of hay growers and handlers are more important. Prevention of unwanted LLP in alfalfa hay requires a combination of good stewardship by hay farmers and handlers (and possible certification of methods using a ‘process-based’ approach), combined with testing to confirm Non-GE status of hay.

## INTRODUCTION

Proper hay sampling has been an important consideration for determining quality of alfalfa hay for decades. Typically, the objective is to predict feeding value from a lab analysis (e.g. ADF, NDF, CP, NDFD) and the sample must fairly represent the average quality of the hay mass. The purpose is to predict feeding value and economic worth. Sampling must represent the variation in leaf, stem, weeds, as well field variation. Principles of proper sampling have been developed over decades by scientists and experienced hay handlers and promoted by University scientists, USDA, and representative hay groups, NFTA, NAFA, NHA and AFGC (Putnam, 2002). Proper sampling technique is likely the most important determinant of accuracy and repeatability of hay quality tests. Details and certification processes for proper hay sampling can be found at: [www.foragetesting.org](http://www.foragetesting.org). and <http://alfalfa.ucdavis.edu>.

---

<sup>1</sup> D. H. Putnam (dhputnam@ucdavis.edu), Forage Specialist, Department of Plant Sciences, MS#1, University of California, One Shields Ave., Davis, CA 95616. **In:** Proceedings, 2014 California Alfalfa, Forage, and Grain Symposium, Long Beach, CA, 10-12 December, 2014. UC Cooperative Extension, Plant Sciences Department, University of California, Davis, CA 95616. (See <http://alfalfa.ucdavis.edu> for this and other Alfalfa Symposium Proceedings.)

However, sampling for detection of a genetically-engineered (GE) trait may require different considerations. In cases where 100% of the hay mass may contain a trait, sampling is a trivial matter, since the trait is likely to be present in every portion of the hay mass. However, if a trait is present in very small amounts (e.g. less than 1% of the mass), sampling becomes critical.

**Need for Testing.** In recent years it has become necessary to sample alfalfa hay to determine the presence or absence of a Genetically-Engineered (GE) trait in alfalfa hay, which may be present as an unwanted presence in otherwise non-GE crop. This is known as Adventitious Presence (AP) or Low Level Presence (LLP). The Roundup-Ready (RR) trait is currently the only GE trait commercialized in alfalfa hay – but others may follow. Why may this be necessary? Organic markets do not allow GE crops. In addition, some countries which import alfalfa hay have not approved the Roundup-Ready (RR). Although some countries (e.g. Japan) have approved importation of the trait, some export buyers do not want a GE trait for commercial purposes. It has been estimated that less than 10% of the US market is sensitive to the GE trait, but the primary sensitive markets are likely to be export.

**What are the thresholds?** It's important to emphasize that thresholds for tolerance of LLP are not well defined, but may range from non-detect by laboratory analysis to commercial assurances of non-GE crop (written or verbal). For human food markets, GE presence (of approved traits) up to 1% is tolerated in products imported into the EU, and above that amount, the product must be labelled as containing a GE product. The level for labelling in Japan is approximately 5%. However, in countries where the trait is not approved, any detection may result in regulatory action. This is the case currently with China and several other countries – although testing has been inconsistent.

## **SOURCES OF LOW LEVEL PRESENCE IN ALFALFA HAY**

There are several possible sources of low level presence of a GE trait in an otherwise non-GE alfalfa hay lot. A lot here is defined as a single group of bales (in a container or group of containers), typically from the same field and cutting. It is important to understand these sources since it impacts the validity of the sampling method.

- **Traces transferred from Seed at planting to the Hay Crop:** Small amounts of Adventitious Presence (AP) in hay may originate in the seed, if that seed has not been tested to be non-detect. This is the most important potential source of AP in alfalfa hay. The non-detect protocols for seed companies require a protocol which tests seed so that the level of AP is below 0.1%. Due to the 'environmental barriers' to further gene flow, it is highly likely that this hay will continue to be non-detect at that level, if other sources of gene movement into hay do not occur.
- **Partial Bales transferred during Harvest.** If balers move between fields from a GE field to an otherwise non-GE field, partial bales of RRA may be included in the hay lot. This is a source of contamination for partial bales, but would not be randomly distributed across the hay lot.
- **Mix-up of bales during handling.** It is possible for a few bales, or entire stacks to be mislabeled, and included in an otherwise non-GE hay lot. Again, this source is not likely to be distributed uniformly over the mass of hay.

- **Gene Flow during hay production.** Hay that originates from non-detect seed can occasionally have gene flow from neighboring fields. However, this is a low probability event, and is very likely to be orders of magnitude below the 0.1% level in fields that have been tested at planting to be non-GE.

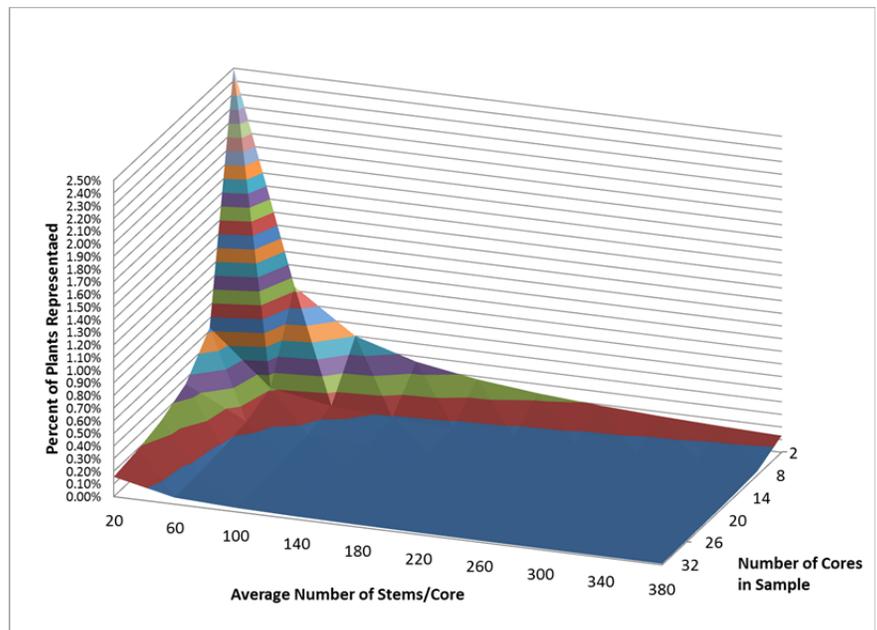
These are listed in approximate level of risk, with seed being the most important risk, followed by transfer of partial bales, mixing up of hay lots, and then gene flow in hay fields.

### IMPLICATIONS FOR SAMPLING METHOD

The validity of the sampling method will depend upon both the level of detection desired, and the source of the LLP. Low level Presence can be endemic (widely distributed throughout the hay mass, as per LLP originating from seed or from gene flow during production), or it could be random and occasional (specific to a specific portion of the hay lot, as per identification mixup or partial bales during baling). Sampling methods suggested below are considered to be valid for endemic traits (where a trait is distributed throughout the stack), but not for a small amount of a partial bale in a hay stack which may originate from a randomly-present bale or portion of a bale. It is doubtful that any sampling protocol will reliably succeed in finding these minor random sources of LLP. However, traditional cored hay sampling methods are likely to be successful at detecting an endemic trait (where the trait is randomly spread throughout the hay mass), depending upon limits to detection. A hay lot which is 100% GE (in this case Roundup-Ready Alfalfa) is quite easy to sample and test – small handfuls will typically represent the entire hay lot – and therefore simple strip tests will suffice. However, low level presence (below 1%) may require PCR techniques and a more vigorous sampling protocol.

### RECOMMENDED PRINCIPLES FOR PROPER HAY SAMPLING FOR DETECTION OF GE TRAITS IN ALFALFA HAY

**Principles of Sampling.** The principles of sampling for a low level presence (LLP) of a GE trait are similar in many respects to sampling for forage quality (see <http://foragetesting.org> for a description of hay sampling protocols developed for quality analysis). Sampling must attempt to provide a subsample which truly represents the entire mass of a ‘lot’. But there are some important differences. The most important difference is that the objective of GE sampling is to determine a specific (small) concentration of a gene or gene product, not the average characteristic of the hay.



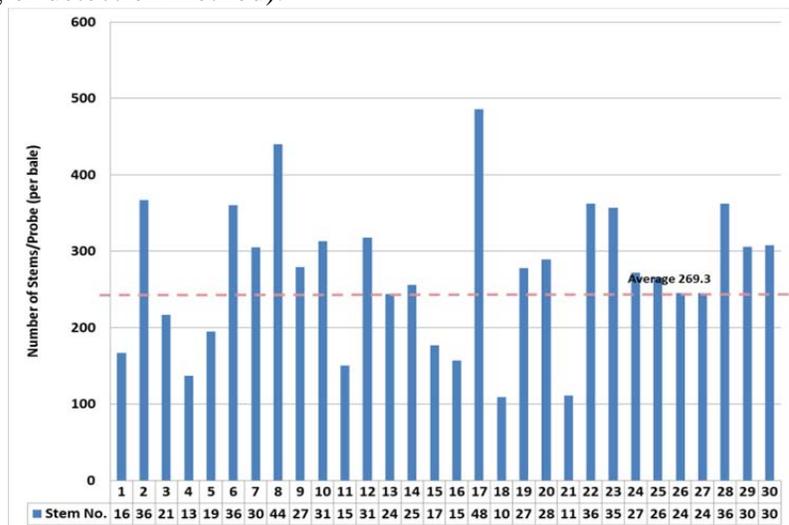
**Figure 1.** Effect of core number and average numbers of stems per core on the detection of a single plant represented in a sample.

While representing the leaf-stem ratio, or the weed-crop mix is an important consideration for quality, this is not important for GE traits. A more important consideration is to represent a large number of plants – since LLP in an alfalfa field will be present only in a few plants in the field. Additionally, the desirable threshold level of detection must be established for GE traits, since a smaller detection threshold will require a greater number of stems for detection.

If we assume that each stem represents one plant, then 30 samples will likely needed to detect LLP a single individual plant at less than 0.1% of the mass, if about 250 stems are contained per probe. Fewer samples are needed if the needed threshold is higher (Figure 1). Our research has shown that the numbers of stems present in 14” deep cored samples ranges from a low of about 100 to a high of nearly 500 with an average of 269 (Figure 2). In this example, a single stem would represent approximately 0.013% or greater of the mass collected in 30 probes given these assumptions – if the analytical method is capable of detecting this amount (or greater), then the sampling method is adequate. The observed level of variation is largely due to degree of compaction (more compacted bales have larger numbers of stems) and other factors. In double-compressed bales, compaction is greater, so larger numbers of stems are likely to be represented in 30 cores. If PCR analysis is used, with detection limits at approximately 0.1, this sampling method should be capable of detecting this level.

**The Impossibility of Zero Tolerance in Analysis.** Some governments have not approved some GE traits, and thus have essentially zero tolerance for the importation of that trait in an agricultural product. Likewise, some buyers or consumers wish to have ‘GE-Free’ crops. However, analytically and practically to declare an agricultural product as containing none of a trait or ‘GE Free’ is an absolute impossibility. In order to assure a hay mass ‘GE Free’, every last gram of that mass must be tested, leaving none for its intended use! Furthermore, there is a prescribed limit of detection for any lab method, including PCR. A single stem present in a 200 ton hay crop would constitute ‘contamination’ in a technical sense– but it’s highly unlikely that any sampling or analytical method would detect this (since the LLP is likely to be much lower than the capability of any sampling or detection method).

Thus, declaration of non-detect is made within a definition of the threshold of tolerance, analytical limits of detection, and the sampling method. Here, sampling methods are used to supplement a declaration of “Non-GE” hay which may include other stewardship methods, such as care in labelling, management of inventory, and prevention of contamination in the field.



**Figure 2.** Approximate numbers of plants (stems) encountered per probe core in a single lot to a depth of 14” core in one bale (one lot, Davis, CA)

## PROTOCOL FOR GE HAY SAMPLING

The protocols for sampling hay for exports are nearly the same as for domestic markets for forage quality with some differences. Thus it is highly recommended that those involved with hay analysis should read the hay protocols and take the hay sampling certification offered by the National Forage Testing Association (NFTA) – and become a certified hay sampler. Over 2,000 people have done so, and improved their sampling techniques.

Here are the important principles of hay sampling for detection of a GE trait in alfalfa:

- 1. Identify a single “lot” of hay.** This is a key first step to proper hay sampling, and one frequently ignored. Normally, a hay lot should be identified which is a single cutting, a single field and variety, and generally be less than 200 tons. For exports, a hay lot can be considered a single container or group of containers. However, in principle, the lot should consist of hay originating from a single field, single cutting, and as uniform as possible. For exports, identification of grower(s) and source of hay is important.
- 2. When to Sample?** For detection of GE traits for export, sampling can occur in defined lots before double compression. Care should be taken to keep identification of these lots throughout the re-packaging process, and quality-assurance processes by exporters (including spot-checking compressed bales) may assist confirming the determination of non-GE status in the final double-compressed product. Sampling can also occur after double compression to confirm these results.
- 3. Choose a sharp, well-designed coring device.** We generally recommend a sharp coring device 3/8-3/4” in diameter, approximately 16-24” length, which is capable of penetrating a bale 12-14” (Figure 3). Do not use flakes or grab samples. The probe should be capable of penetration (whether single- or double-compressed), and fairly represent the leaf stem ratio (\*see note below for double-compressed bales). Probes larger than 3/4” are acceptable for GE testing, but may inadvertently create samples which are too large to be handled by a laboratory, or make the sampler stop before the prescribed number of samples is obtained. Thus, smaller diameter probes are preferred, as long as they are capable of obtaining a cross-section of stem and leaf. The principle is to sample as many stems as possible, not maximize the size of the sample – larger diameter probes are not likely to sample a greater number of stems than a smaller diameter probe at 12-14”. A range of probe tip designs have



Figure 3. Probes should be taken perpendicular to the butt-ends of bales, so that stems are arranged perpendicular to the probe. Spiral assists and gas-powered drills may assist in penetrating highly-compressed bales.

been successfully used, from serrated to non-serrated tips. It is probably most important that the tip be sharp (and maintained sharp), and create a clean cut across a cross-section of hay, and not heat during the sampling process. Not all probes meet this criteria. **Note:** only a few probes can be practically used for double compressed hay. Gas-powered drills utilizing a spiral-assist probe (like the Star-Quality Sampler, Edmonton, AB) have been shown to work, as have some punch-type probes which require strength. In both cases, tips must be sharp. We have found serrated-type probes (e.g. Penn State probe) to heat too fast to be practical.

- 4. Take enough cores.** For GE sampling, 30 cores, composited to a single sample is likely to provide a sample which represents LLP (if present) at about 0.1%, if the number of stems in each probe is above about 250 per probe (Figure 1). In practice we have found the range of stems to be from about 100-400 per probe. This is greater than the 20 probes recommended for forage quality testing – since in this case we are looking for low level presence, not the average quality of the sample. This is the same recommendation for larger (e.g. 1 ton), or smaller packages, as long as these group of bales fit the definition of a ‘lot’ (see step #1). The key aspect of sampling hay bales is number of samples composited, not necessarily the mass of the sample collected, as long as the mass is sufficient to represent discrete stems in each probed sample.
- 5. Sample at random.** These 30 cores should be taken without bias. Ideally, the sampler should walk around the stack as much as possible, and sample bales at random, and both ends of bales should be sampled. This is sometimes difficult since all of the bales are not available to the sampler (they may be against walls of a barn, present only on one face of a container, or up too high for practical sampling). However, the sampler should make every attempt to sample in a random fashion. This means not to bias either for or against any bales in the stack. For example, the sampler may walk 15 steps, sample, walk 20 steps, sample, walk 5 steps, sample, while walking around stack, trying to represent all areas of the stack. Don’t avoid or choose bales because they look especially bad or good-If 20 cores are taken, they won’t make much difference anyway. Avoiding or choosing bales introduces bias. Note for bales in a container: 25-30 Cores can be taken from the face presented to the sampler, even multiple samples taken from the same bale, since there is often as much variation within bales as between bales. The key is to make sure that 25-30 cores are taken, and that each core is at least 30” (0.7 meter) away from other sample cores to assure that the same plants are not sampled.
- 6. Use proper technique.** Sample butt ends of hay bale, near the center in the compacted area between strings or wires, not near the edge. The butt ends of the bales should have the stems of the product perpendicular to the probe. Probe should be inserted at 90° angle, 12-14” deep. Sample multiple bales if possible, but if sampling the same bales, assure that samples are at least 30” apart. The sides or the top of the bale should not be sampled, since these cores will only represent one flake from a single area of the field, and the stems will not be perpendicular to the probe. With round bales, sample towards middle of bale on an angle directly towards the center of the bale.

7. **Sample amount: not too big, not too small.** Sampling should be done so that about ½ lb (226 g) to 1 lb. (454 g) of sample is produced-taking a minimum of 30 cores. For LLP testing, larger is better – but make sure the laboratory grinds the entire sample. Too-small samples don't fairly represent the full range of variation in the hay lot. Very big samples (common with large length or diameter probes) are excellent at representing the hay but have practical disadvantages. Large samples cannot be easily ground by the labs. The sampler should ensure that the entire sample is ground by the lab. Only work with labs that are willing to grind the entire sample. But you should also assure that you are providing a reasonable weight sample, so that it can be practically handled by the lab.
8. **Handle samples correctly.** Seal Composite 30-core sample in a well-sealed plastic bag and protect from heat. Double bagging is beneficial, especially for DM measurements. Deliver to lab as soon as possible. Do not allow samples to be exposed to excess sun (e.g. in the cab of a pickup truck). Refrigeration of hay samples is helpful, however, dry hay samples (about 90% DM) are considered fairly stable. Note: this is more important for quality analysis than for GE analysis.
9. **Choose an Lab with Established Methods.** For forage quality testing, we recommend labs that participate in the NFTA proficiency certification program ([www.foragetesting.org](http://www.foragetesting.org)). For PCR testing, consult recommendations of the regulatory agency for labs which have been approved.

#### SUMMARY

Sampling for GE-detection requires the following protocol:

1. Identification of a hay lot, consisting of a single cutting, from a single farm and field.
2. Sampling either at the point of sale, before or after compression for transport.
3. Use of a sharp, effective hay coring device, capable of multiple samples to a depth of 12-14" with either non-double compressed or double compressed hay.
4. Take 30 cores composited into a single sample.
5. Random sampling methods – removal of bias.
6. Use of proper technique – 90° probe into butt-ends center of bales, with stems arranged perpendicular to the probe.
7. Obtain approximately ½ to 1 lb (227 to 454 g) of sample.
8. Double seal samples in zip-lock bags, protect from heat, sun.
9. Carefully choose a lab which has met quality control standards and has been approved for GE analysis.