ACKNOWLEDGEMENTS

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K. N. Rai (pear millet), K. N. Rai (pear millet),
Kiran Sharma (cowpea), Athilochan Sarker (gram),
S. Goel (spinach) and Anil Bhagat (gram),
Shrinath Chande (green gram), and Simon Breeze (corn).

For their contribution, Abid Khan (mung beans)
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Abebe Menkir (maize), Stefania Grando (barley),
Stephen Beebe (bean), Kiran Sharma (cowpea),
Anil Bhagat (gram), and Simon Breeze (corn).

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PRECAUTIONARY NOTES ON AVOIDING CONTAMINATION

It is well known that plant breeding is a numbers game, and that screening genotypes requires a substantial effort in both the field and the laboratory. Collecting a representative sample and minimizing soil and dust contamination from harvesting or postharvest processing equipment poses a considerable analytical challenge when breeding iron- and zinc-dense crops.

Before using equipment—especially processing equipment—for sample preparation, determine what material the equipment is made of and whether it could be a source of contamination. For example, many grinding mills have stainless steel components, which generally are thought not to cause iron contamination. However, some stainless steel comes in various levels of hardness and quality and can be a source of contamination. Another example is black rubber products, commonly found in various threshing equipment, which can introduce exogenous iron and zinc.

The following general sampling protocols were developed by Harvestplus plant breeders and their collaborators. These protocols are a work in progress and may be refined over time.

HANDLING HARVESTED MATERIALS

The staff who collect and handle samples must understand the importance of avoiding contamination. Common contaminant sources when sampling or handling crops in the field or the laboratory include:

- soil or dust on hands or equipment,
- skin-care products (such as hand lotions) on bare hands,
- dirty or rusty equipment (such as sampling containers or threshing blades),
- lodging of crop plants (in the case of cereals) that result in the inflorescence lying in soil or water,
- contact or accidental mixing with other samples.

MAINTAIN POSTHARVEST EQUIPMENT

Do not use any equipment that has rusty parts. If you are unsure whether your equipment is causing contamination, equipment should be tested. To test, prepare replicates of two to three varieties and analyze for the mineral content of the samples. A high level of iron with a correspondingly high level of aluminum or titanium may indicate contamination. For example, if a milled rice sample has a higher iron content than its corresponding brown rice sample, then the iron in the milled rice is most likely a contaminant.
Before and after using equipment, use a clean brush, a clean cloth, or compressed air to remove dust and plant material. Clean the equipment after each sample to avoid cross-contamination. When equipment is not in use, keep it covered to protect it from dust and water or moisture (to avoid rusting).

COLLECTING A REPRESENTATIVE SAMPLE

Inherent genotypic variability for iron and zinc has been observed among plants of the same genotype and even within a single plant. For example, iron content varies spatially within the inflorescence of wheat; hence the need for a representative sample that truly reflects the real iron and zinc values of the genotype.

For grains, there are two ways to collect a representative sample:

* Pile the grains evenly on a clean acid-washed tray, flatten the pile, and spread the grains in a circle (see Figure 1). Divide the circle into four roughly equal parts. Discard two diametrically opposite quarters, and remix the remaining two parts. Repeat the quartering procedure until the amount of grains is reduced to the amount of sample required for the experiment.

* Use stainless steel automatic dividers (commonly called sample splitters) to randomly divide grains into two, four, or more streams, any one of which can be taken to represent the gross sample. Repeat the sample-splitting procedure until the amount is reduced to the desired sample amount.

For roots and tubers, the method illustrated in Figure 1 also can be applied. Divide each root or tuber into four roughly equal parts, and discard two diametrically opposite quarters; combine the two remaining quarters as the representative sample. To account for variation between roots or tubers on the same plant, combine diametrically opposite quarters from several tubers or roots from the same plant.

STORING SAMPLES

At any stage of the crop-sampling process when sample material must be transported, packaged, or stored, the importance of a clear labeling and clean, contaminant-free, pest-free environment cannot be overemphasized. Seed and samples must be stored properly to avoid contamination and ensure their integrity for analysis.

First and foremost, always label the sample vessel with the name of the sampled crop, variety name, location, and date.

Whenever a sampling protocol calls for storage, the vessel—like any postharvest processing equipment—must be clean and uncontaminated. Paper bags and envelopes should be new and unused, preferably taken from a sealed package that ensures that they are free of soil, dust, and other contaminants; for most dry samples to be stored between preparation and analysis, #1 coin envelopes are the perfect size.
Plastic containers, plastic trays, and Petri dishes should be acid-washed to ensure their cleanliness. Wash the vessels with soapy (i.e. Pyroneg) water, then rinse in reverse osmosis (RO) water. Place the vessels in a 10% nitric acid bath for 24 hours, then rinse with high-purity water [preferably Milli-Q water (>18-milliohm resistivity)]. If acid washing is not possible, then wash vessels with soapy water and rinse with RO water; however, there is a slight chance of contamination in the absence of acid washing.

For long-term storage, naphthalene mothballs can be used to avoid insect infestation. In such a case, place sealed sample bags in a carton or other box, and place the mothballs around the sample bags (not inside the sample bags—not touching the crop samples).

**GRINDING**

Grinding often is a prerequisite to sample digestion and analysis, and a contaminant-free grinding mill must be used. The following HarvestPlus crop-sampling protocols mention using a Retsch grinding mill with Teflon chambers and zirconium balls and the IKA A10 grinder (used by many HarvestPlus collaborators). However, these mills are not the only suitable ones on the market. To obtain a list of mills that HarvestPlus has tested so far (with assessments of their performance), contact Dr. James Stangoulis.

**Figure 1: Collecting a representative sample from roots and tubers.**

*For more information, contact:*

Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
BARLEY SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. After the crop has reached maturity, choose 10–20 mature heads at random. Break off heads by hand, or cut with a pair of stainless steel scissors.

3. Place heads in clean, properly labeled paper bags and seal.

IN THE LABORATORY

4. Thresh the spikes and bulk seed from the heads.

5. Pearl the grain using a noncontaminating pearler (such as the Satake TM-05).

6. Package the pearled grain in clean, new, properly labeled, paper #1 coin envelopes, and store the samples in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

Dr. Stefania Grando, HarvestPlus Barley Crop Leader (s.grando@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@adelaide.edu.au)
BEAN SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Before the main harvest, randomly collect approximately 10 well-filled pods. Place pods in clean new paper envelopes (to avoid contamination with dust and dirt while uprooting plants and threshing in bulk).

IN THE LABORATORY

3. Thresh the pods by hand, and collect 5 grams of seed. Clean the seeds using a cloth dampened with high-purity water.

4. Dry seeds to 7–8% moisture content (typically 10–12% in fresh seeds) in a clean, contaminant-free (uncorroded) oven at 60ºC.

5. Grind the 5 gram sample with a noncontaminating grinding mill (such as a Retsch mill with Teflon chambers and zirconium balls or an IKA A10) to avoid iron and zinc contamination. The required sample amount for atomic absorption spectroscopic (AAS) or inductively coupled plasma–optical emission spectroscopic (ICP-OES) analysis is 0.5–0.8 grams of ground sample.

6. Package the ground samples in clean, new, properly labeled, paper #1 coin envelopes or in plastic screw-top tubes, and store them in a clean, dry, insect-free location until ready for analysis.

For more information contact:

Dr. Stephen Beebe, HarvestPlus Bean Crop Leader (s.beebe@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
COWPEA SAMPLING PROTOCOL

FIELD PROCEDURE
1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.
2. Collect 1 kilogram of mature pods from 5-10 plants in two middle rows, and place the samples in clean, properly labeled paper bags.

IN THE LABORATORY
3. Dry samples at 60ºC for 3 days in a clean, contaminant-free (uncorroded) oven.
4. Thresh the pods by gentle twisting over a clean, dust-free plastic tray.
5. Winnow the seeds, and collect approximately 10 grams of seed. Store the samples in clean dry paper bags.
6. Grind up seeds using a non-contaminating mill such as a Retsch mill with Teflon chambers and zirconium balls, or an IKA A10.
7. Package the milled samples in clean, new, properly labeled, paper #1 coin envelopes, and store them in a clean, dry, insect-free environment until ready for analysis.

For more information, contact:
Dr. Christian Fatokun, HarvestPlus Cowpea Crop Leader (c.fatokun@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
LEN TILE SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. When plants begin to turn yellow and the lower pods turn brown to yellow–brown but still contain sufficient moisture to toughen the pods, randomly select 10 lentil plants in the field for sampling. (Because pods can easily shatter, closely monitor the lentil plants as harvest time approaches). Harvest lentils by cutting or swathing. (Note: Swathing should not be done during a hot, dry time of day).

IN THE LABORATORY

3. Store samples in a dry location to reduce the moisture content to around 14% before threshing. If necessary, dry lentils in air dryers heated to no more than 43°C, to minimize the cracking of seed coats. Natural air drying has advantages over heated air, but a proper system design is necessary. Irrespective of the drying method, good airflow is required through the seed, which usually means that thinner layers of the seed must be used in this process.

4. Thresh the lentils. If using a commercial thresher, to prevent cracking, use a slower cylinder speed and set the concaves wider than for harvesting. Initial wind and sieve settings for wheat may be used. To avoid contamination, ensure that the thresher parts that may come into contact with the sample are rust-free.

5. Clean seed of foreign matter. Collect a representative sample of approximately 5 grams (refer to Collecting a Representative Sample and Figure 1 in Precautionary Notes on Avoiding Contamination). Package the samples in clean, new, properly labeled, paper #1 coin envelopes, and store them in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

Dr. Ashutosh Sarker, HarvestPlus Lentil Crop Leader (a.sarker@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
MAIZE SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Before harvesting, cover the ears with clean paper bags to avoid iron contamination from dust or soil (for example, if the plant lodges during a severe storm).

3. After the crop has reached physiological maturity (80–120 days after planting), harvest enough cobs to give a representative sample. For open-pollinated varieties, harvest 100 randomly selected ears and thresh ear-to-row to make a balanced bulk; for fixed inbred lines, harvest 3–10 good ears and thresh in bulk.

IN THE LABORATORY

4. Remove the husk from each ear manually, using a clean instrument such as a high-quality stainless steel knife or a plastic stick.

5. Store the husked ears in a clean basket with a loose cover (or a clean woven-plastic bag used solely for this purpose).

6. Place the husked ears on clean plastic drying trays. (If wooden trays are used, prevent contamination by placing the husked ears in clean, unused brown paper bags before placing them on the trays). Dry samples at 40°C for 5 days in a clean, contaminant-free and uncorroded oven. (Ovens used to dry samples for iron analysis should NOT be used to dry other samples, such as soil and root tissues, which may leave contaminant residues in the oven).

7. Shell the husked ears with clean, bare hands or wearing contaminant-free gloves. Place kernels onto a clean plastic tray and thoroughly mix kernels. Collect a representative sample of approximately 250 grams (refer to Collecting a Representative Sample and Figure 1 in Precautionary Notes on Avoiding Contamination).

8. Grind kernels to fineness (grains must pass through a 30-mesh sieve) using a noncontaminating mill (such as a Retsch mill with Teflon chambers and zirconium balls or an IKA A10).

9. Collect a subsample of 25 grams for analysis. Package the samples in clean, new, properly labeled brown paper bags, and store them in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

Dr. Kevin Pixley, HarvestPlus Maize Crop Leader (k.pixley@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
PLANTAIN AND BANANA SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Harvest three mature fruit from three hands situated top, middle and bottom of the bunch 3–4 months after flowering (when the distal end of the fruit or leaves is dry or upon full, robust fruit formation).

IN THE LABORATORY

3. Peel the plantain or banana using a clean, well-made stainless steel knife.

4. Cut the plantain or banana longitudinally and then cross-wise, and discard two diametrically opposite quarters. Combine the two remaining parts as the representative sample. (Refer to Collecting a Representative Sample and Figure 1 in Precautionary Notes on Avoiding Contamination for an example of how this is done). Combine the samples from each bunch (six quarters in total).

6. Place the collected pieces of the fruit in clean, acid-washed, properly labeled plastic Petri dishes, and dry the sample in a clean, contaminant-free (uncorroded) oven at 60ºC for 3 days.

7. Grind dried fruit to fineness in a noncontaminating mill.

8. Package the ground samples in clean, new, properly labeled, paper #1 coin envelopes, and store them in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

Dr. Abdou Tenkouano, HarvestPlus Banana Crop Leader (a.tenkouano@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)

This sampling protocol was adapted from the following paper:

PEARL MILLET SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. To obtain a truly representative sample, sib-mate through hand pollination with bulk pollen collected from 50–60 plants and crossed on 20 plants of the same entry. When panicles develop, cover with clean paper bags to reduce exposure to dust.

3. When sib-mated plants have reached physiological maturity (85–90 days after planting), harvest sib-mated panicles (with paper bags on) and place them in clean, new brown paper bags.

IN THE LABORATORY

4. Dry the panicles in their paper bags in a clean, dry location in full sunlight.

5. Remove the grains from the panicles with a thresher.

6. Manually clean the seed of any panicle residue.

7. Collect a representative grain sample of 100 grams for analysis (use a sample splitter, if one is available; if not, refer to Collecting a Representative Sample and Figure 1 in Precautionary Notes on Avoiding Contamination). Package samples in clean, new, properly labeled, paper #1 coin envelopes, and store them in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

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or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
RICE SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Harvest panicles manually (120 days after planting), only from upright and unlodged plants.

IN THE LABORATORY

3. Manually thresh the panicles and place the fresh grains in clean, unused, properly labeled brown paper bags.

4. Dry the rough rice in an oven at 35–45°C for up to 3 days to achieve a moisture content or 12–14%. (To minimize grain breakage or fissures, avoid overdrying; periodically monitor the moisture content of the samples). Allow the dried grains to equilibrate for about 3 days. To obtain optimum milling yields, after drying, store the rough rice in clean sacks or bags for at least 2 months after harvest.

5. Collect a representative sample of 50–120 grams of rough rice (refer to Collecting a Representative Sample and Figure 1 in Precautionary Notes on Avoiding Contamination), depending on which mill will be used.

6. To date, no dehuller has been identified that does not contaminate a sample. Two alternatives can be used for dehulling paddy grains:

   • Manually remove hulls with clean Teflon-covered forceps.
   • Replace the contaminating compound on the Satake THU-35A dehuller with noncontaminating PVC compound (contact Dr. Stangoulis for details on this product) and then dehull the grains.

7. The following mills tested by the International Rice Research Institute (IRRI) can be used to mill large samples (>70 grams) of brown rice:

   • The McGill No. 2 friction-type mill or Grainman No. 60 (Grain Machinery Mfg. Corp, Hialeah, FL, USA) require 120 grams of rough rice and will produce approximately 60 grams of milled rice, depending on the quality of the grains and the moisture content before milling.
   • The Satake TM-05 pearling (abrasive) mill with abrasive mesh # 36 requires at least 50 grams of rough rice to achieve a milling degree and quality similar to that produced by the Grainman mill.
   • The modified Brazilian-made Suzuki rice dehuller and polisher can mill a sample as small as ~10 grams. (Contact Dr. Barry for details on this mill).
   • A modified Kett mill will take a very small (<10 g) sample size. (Contact Dr. Stangoulis for details).
8. Collect 2–5 grams of milled rice. Package samples in clean, new, properly labeled, paper #1 coin envelopes, and store them in a cool (20°C), dry, insect-free location until ready for analysis.

For more information, contact:
Dr. Gerard Barry, HarvestPlus Rice Crop Leader (g.barry@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)

a This sampling protocol was adapted from a manual developed by Professor Bienvinido O. Juliano for the Asian Development Bank’s Micronutrient Project: Breeding Rice for Better Iron Nutrition The manual can be obtained from Dr. Stangoulis or Dr. Barry.

b Avoid any soil, hull, or bran contamination, and avoid touching the brown rice with your bare hands. If required, use powder-free plastic gloves, not rubber gloves.

c Clean the mill thoroughly after each sample.
SORGHUM SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Before the main harvest, cut dried selfed ears (peduncles) with secateurs and place them in a clean, new paper bag.

IN THE LABORATORY

3. Thresh the ears manually in a clean location.

4. Winnow the grains.

5. Wipe dust from the grain with a clean cloth, if required.

6. Package the cleaned grain in clean, new, properly labeled, paper #1 coin envelopes, and store them in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

Dr. Belum Reddy, HarvestPlus Sorghum Crop Leader (b.reddy@cgiar.org)
or
Dr. James Stangoulis (james.stangoulis@flinders.edu.au)
TUBER AND ROOT
CROP SAMPLING PROTOCOL
POTATO AND SWEETPOTATO

FIELD PROCEDURE
1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Randomly collect 30 medium-sized tubers per genotype from different plants growing in the same field.

IN THE LABORATORY

Prepare three compound samples per genotype:

3. Wash the tubers with plain tap water and then rinse with deionized water.

4. Dry the tubers with paper towels. Peel and cut each tuber longitudinally into four wedges; each wedge represents one sample. Or, refer to Collecting a Representative Sample and Figure 1 in Precautionary Notes on Avoiding Contamination: Divide each root or tuber into four roughly equal parts, discard two diametrically opposite quarters, and combine the remaining quarters as a representative sample. (To account for variation between roots or tubers on the same plant, combine diametrically opposite quarters from several tubers or roots from the same plant).

5. Slice each wedge with a stainless steel slicer. Mix the slices manually, and collect one 50 gram sample.

6. Cut the slices into smaller pieces, and place the sample in a clean Petri dish. Dry the sample in an oven at 80ºC for at least 24 hours.

7. Weigh the dried samples, then grind them with a non-contaminating mill.

8. Package the ground samples in clean, new, properly labeled paper bags, and store them in a clean, dry, insect-free location until ready for analysis.

For more information, contact:

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CASSAVA AND YAM SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Collect four to five roots from three plants of each variety.

IN THE LABORATORY

3. Wash the roots with plain tap water.

4. Peel the roots, and rinse with deionized water.

5. Cut slices from the distal, central, and proximal sides of each root. Mix the slices manually, and collect one 50 gram sample.

6. Chop the slices into smaller pieces.

7. Place the sample in a clean plastic container or Petri dish. Dry the sample in an oven at 60°C for at least 24 hours.

8. Grind the dry sample in a non-contaminating grinding mill (such as a Retsch mill with Teflon chambers and zirconium balls or the IKA A10).

9. Package the sample in a clean, acid-washed plastic container labeled with the variety name, and store at room temperature.

For more information, contact:

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WHEAT SAMPLING PROTOCOL

FIELD PROCEDURE

1. Familiarize the field team with the Precautionary Notes on Avoiding Contamination.

2. Collect a representative sample of around 250 grams from bulk harvest, or if grains are harvested from a small plot, collect about 10 randomly selected heads before the main harvest. Place the samples in clean, new, properly labeled paper envelopes to avoid contamination from dust and soil.

IN THE LABORATORY

3. Thresh heads by hand (do not use a rubber-coated rubbing board, which can contaminate the sample), and store the seed in clean, new paper envelopes.

4. Pass the grains through an air cleaner to remove all foreign material.

5. Sample split the clean grains to obtain 10 grams of sample, and manually remove any visible soil particles. Place the resulting sample in new, clean brown paper bags.

6. In areas where Karnal bunt is a problem, dry the grains in a clean oven at 75°C for 48 hours to destroy the spores. Wash the seed in high-purity water, then dry again in a clean oven for 24 hours.

7. For ICP analysis, collect 5 grams of whole-grain samples. For atomic absorption spectroscopic (AAS) analysis, grind 5 grams of grains in a noncontaminating grinding mill (such as a Retsch mill with Teflon chambers and zirconium balls or an IKA A10) and collect 0.5 grams of ground wheat flour. Package the samples in clean, new, properly labeled, paper #1 coin envelopes and store in a dry location until ready for analysis.

For more information, contact:

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Breeding Crops for Better Nutrition

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• Bean Sampling Protocol
• Cowpea Sampling Protocol
• Lentil Sampling Protocol
• Maize Sampling Protocol
• Pearl Millet Sampling Protocol
• Plantain and Banana Sampling Protocol
• Rice Sampling Protocol
• Sorghum Sampling Protocol
• Tuber and Root Crop Sampling Protocol
• Cassava and Yam Sampling Protocol
• Wheat Sampling Protocol