CIMMYT
International Maize and Wheat Improvement Center

CGIAR RESEARCH PROGRAM ON Maize

Quality Assurance/ Quality Control (QA/QC) in Maize Breeding and Seed Production:

Theory and Practice

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Natalia Palacios-Rojas, Gordon Huestis, and B.M. Prasanna

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CIMMYT MR
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Abstract

This technical manual introduces the theory and practice of Quality Assurance/ Quality Control (QA/QC) of seed quality in maize breeding and seed production. It focuses mainly on the genetic quality related issues of the improved maize seed. The seed quality program typically involves Quality Assurance (QA) which focuses on prevention of low-quality or genetically impure seed to be mixed with high-quality seed, while Quality Control (QC) in seed production chain ensures identification and correction of possible errors or mixtures that might have slipped through QA protocols.

The manual outlines in detail the advantages of undertaking QA/QC in maize breeding and seed production by complementing the morphology-based testing (grow-out test) with the molecular markers-based analysis, especially in terms of testing for genetic purity and identity, parent-offspring test, and trait-specific testing using breeder-ready molecular markers. It also highlights the procedure for effectively monitoring the kernel-based nutritional quality traits (e.g., QPM and provitamin A) in maize breeding and seed production.

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Acknowledgments

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1. Introduction

The success of a maize variety in the farmer's fields as well as the long-term economic viability of seed companies is largely dependent on the quality of seed. Production of high quality parental inbred lines of hybrid maize is the combined effort of many trained scientists and field technicians who grow, inspect, rogue, detassel, and care seed production fields throughout the growing season and harvest the crop when it attains physiological maturity. Quality seed of hybrid maize is marketed when genetically pure breeder seed, basic seed and certified seeds are produced.

Quality Assurance/Quality Control (QA/QC) is defined as "the combination of quality assurance, the process or set of processes used to measure and assure the quality of a product, and quality control, the process of ensuring products and services meet consumer expectations." (https://en.wikipedia.org/wiki/QA/QC).

QA and QC are key in deploying high quality seeds, an important factor for increasing crop yields per unit area. The efficiency of other agricultural inputs depends largely on the quality of seeds planted. High quality seeds are genetically and physically pure, properly mature, are free from insect-pests and pathogens, uniform in size, have high germination potential, optimum moisture content, viable, and vigorous.

This manual mainly focuses on the genetic quality related issues of the improved maize seed and how to undertake QA and QC. "Genetic purity" refers to genuineness to originality, or the degree of contamination of seeds caused by undesired genetic varieties from same species. The success of hybrid seed production is dependent on the genetic purity of parental inbred lines. Both outcrossing and the unintentional mixing of seed can compromise seed quality.

Achieving high level of genetic purity in commercial hybrids and elite inbred lines and maintain the original performance for desired agronomic traits is important in order to serve the farmers/customers with high quality seeds. Conventionally, morphological comparisons are used as a basis for genetic purity evaluations. However, replicated field observations are time-consuming, expensive and environment dependent.

Morphology cannot provide information on the purity of specific genetic attributes that relate to grain quality or disease or herbicide resistance etc. Biochemical assays, including isozymes, cannot effectively distinguish closely related inbred lines or hybrids. Isozymes have been routinely used in checking seed-lot purity in maize since 1990s.

Newer DNA-based technologies, especially those based on single nucleotide polymorphisms (SNPs), will enable better discrimination and faster determination of genetic purity of the maize inbred lines or hybrids.

Seed quality features can be broadly divided into three components (Fig 1):

- Physical purity and physiological qualities of the seed in the specific seed lot;
- Genetic quality which relates to genetic purity and identity of the seed lot; and
- Seed health which refers to the freedom from pathogens or insect-pests within the seed lot

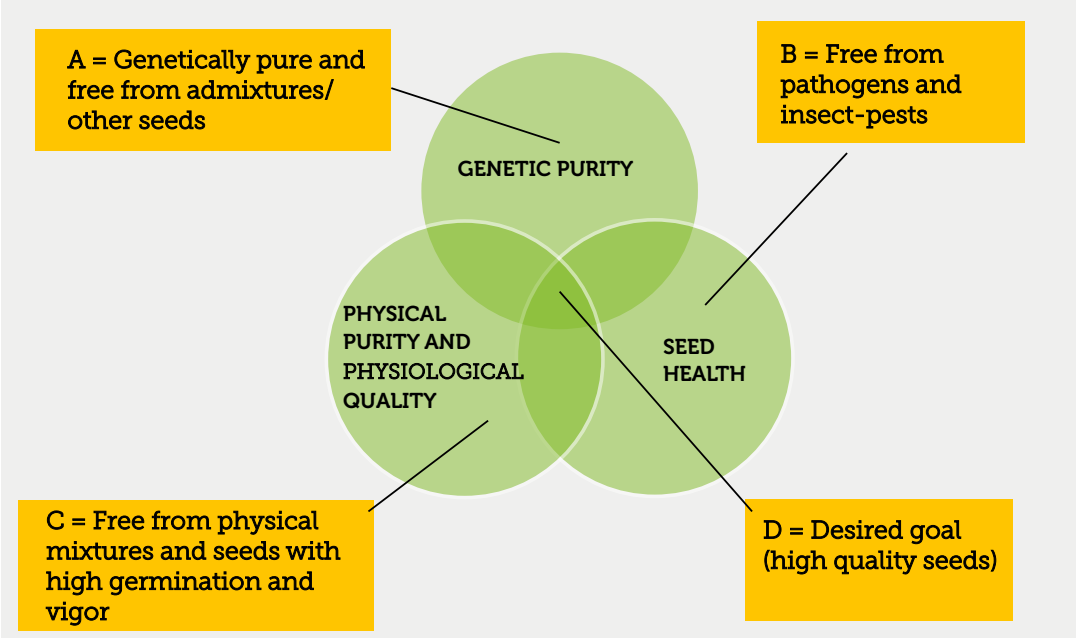


Fig. 1. Quality components of the seed (Source: Yonas Sahilu, Ethiopian Seed Enterprise).

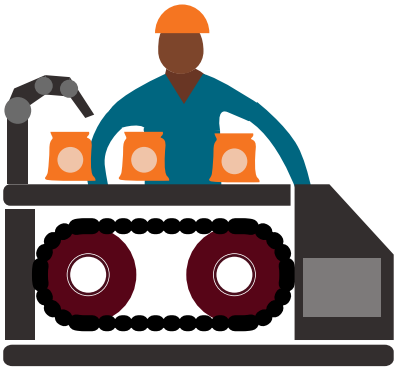
2. Quality Assurance and Quality Control (QA and QC)

The purpose of QA in seed value chain is to provide proper service and support for seed production at various stages, and ensure that the sales and marketing team have high quality products. Quality assurance is, thus, process oriented and focuses on prevention of low-quality or genetically impure seed to be mixed with high-quality seed. A good QA program always starts with established protocols on functions, objectives, action plans, including a set of quality standards. QA is important for proactively preventing the mistakes in the process of creating or maintaining new finished inbred lines, or hybrids.

Product development and commercial seed production may involve diverse environments, locations and staff with different skill levels. For example, in the process of seed production, it is essential to make sure that all staff involved in seed production from different locations have parental lines from appropriate source(s), and use the same protocols for maintaining these lines, drying and storing the seed in proper environments, consistent seed package labeling, etc.

Setting up of QA protocols is important not only to prevent the errors but also to cross-check for mistakes that might have happened inadvertently in the process of seed production. It is better to have protocols in place to prevent common problems, such as a seed mix-up, mislabeled seed packets, improper pollinations, etc. Standard operating procedures (SOPs) should be put in place for all the steps in the seed value chain, including checklists and protocols that staff should follow consistently and rigorously. Periodic inspections can help to identify the gaps in implementation of standardized procedures, and which steps are more prone to errors etc. These inspections can be done either internally by a QA Inspector, or by a third party which can help to consistently improve the standards of quality seed production.
QA needs to be a fully integrated program in the seed value chain, with interface among the breeders, seed production personnel and product sales personnel. Working with the breeders in product development stages can give advance information about the inherent quality of specific parental inbred lines or hybrids. For example, information sourced on the seed performance potential of a parental inbred line can be helpful in realizing the potential of obtaining high-quality seed in a cost efficient manner. QA personnel also need to work closely with the sales team to keep them informed of the seed quality status and also to get feedback on the performance of the product from the customers.

The purpose of QC in seed production chain is to identify and correct the errors or mixtures that might have slipped through QA protocols. QC involves routine testing of the seed materials throughout the process of seed production or breeding using all available seed testing methods, including germination, seed viability, seed vigor, phenotypic and morphological traits based grow-out tests, and implementation of biochemical and/or molecular marker based testing. A well-organized QC program can be a powerful management tool in any seed certification organization, breeding institution, or commercial seed company. This involves a systematic process that controls the validity of analytical results by measuring the accuracy and precision of each method, and by developing expected control limits. By using these limits one can detect the mistakes that might have happened in the field or out-of-control events, and those requiring curative actions to prevent or minimize the repetition of such events. The QC program aims to check whether the currently used inbred/parental lines are genetically identical to the original source, whether the seed has required genetic purity, whether the hybrids are indeed derived from the specified parents, and whether the seed lots are meeting all the seed quality standards etc.



The purpose of QC in seed production chain is to identify and correct the errors or mixtures that might have slipped through QA protocols.

3. Seed testing

Seed quality program involves various stages, including production, harvesting, drying, processing, treating, and storage (Fig. 2). Seed testing services are required from time to time to gain information on the planting value of the seed lots. Seed testing determines the standards of a seed lot, including physical and genetic purity, seed health, moisture content, germination, thereby enabling the farming community to get quality seeds.

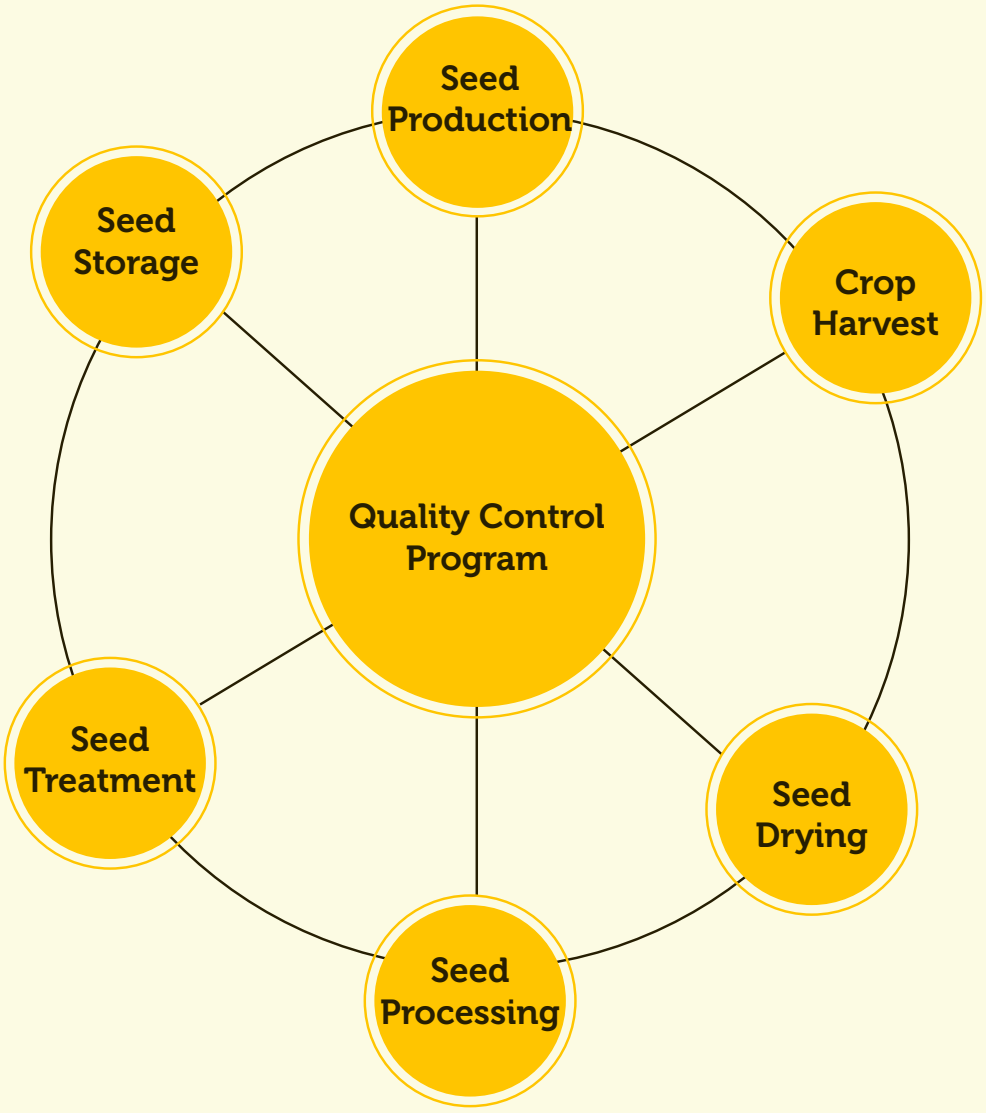


Fig. 2. Quality control laboratory is a hub of seed quality control program

4. Grow-out test

The purity of hybrid seed is conventionally assayed by conducting a Grow-out-Test (GOT) involving a representative sample of the seed to be marketed. A set of qualitative and quantitative characteristics known as "descriptors" stringently related to seed quality are used for hybrid or inbred lines identification. GOT is the official measure in many countries for controlling the genetic purity of the seed lot. It serves as a pre-control as well as a 'post-control' test for avoiding genetic contamination. Generally it is a requirement for seed certification of maize hybrids. The test is also used to validate the seed producers label with respect to genetic purity status of the seed lot as well as to measure the efficacy of the certification agency. Around 1000 grams of maize seed is used as submitted sample for test. The working sample can be obtained through subsequent mixing and dividing of the submitted sample. A minimum of 400 plants is required for taking the observations. The number of seeds required for GOT to obtain the required number of plants depends on the germination percentage of the seed sample and hence seed rate should be adjusted accordingly.

The GOT is conducted in specified areas by either the seed certification agency or by the seed company. The standard sample must not differ significantly in any character from the original source samples. The field plots should be grown in replicates to protect against germination failure and to reduce environmental and soil fertility variations. GOT plots must be examined throughout the growing season with emphasis on the period from the flowering to harvesting. All plants must be examined, keeping in view the distinguishing characters (descriptors) described for the test crop as well as the control. While taking the observations, the plants showing deviations in descriptors against the standard check(s) should be tagged and examined carefully at a later stage to confirm whether they are off-types or not. The number of the total plants and the off-type plants found should be recorded. All morphological variations specific to, and any differences from expected observations, should be recorded for proper identification (Fig. 3).

The results of the grow-out test must be reported as percentage of other off-type plants. If off-type plants are more than 15 per cent, the report should state that the sample consists of mixture of different genotypes, depending on the regulation of each country. Nevertheless, most of quantitative traits included in the descriptors list vary in their expression due to their interaction with the environment, which could be sometimes misleading in terms of identification of an appropriate hybrid or inbred lines. Moreover, GOT is time consuming (takes one full crop season for recording of data), tedious and highly vulnerable to environmental influence and level of skills of manpower recording the data. Molecular markers, in contrast, being based on DNA sequence variation, provide an unbiased means of analyzing the genetic purity and identity of specific inbred lines or hybrids.

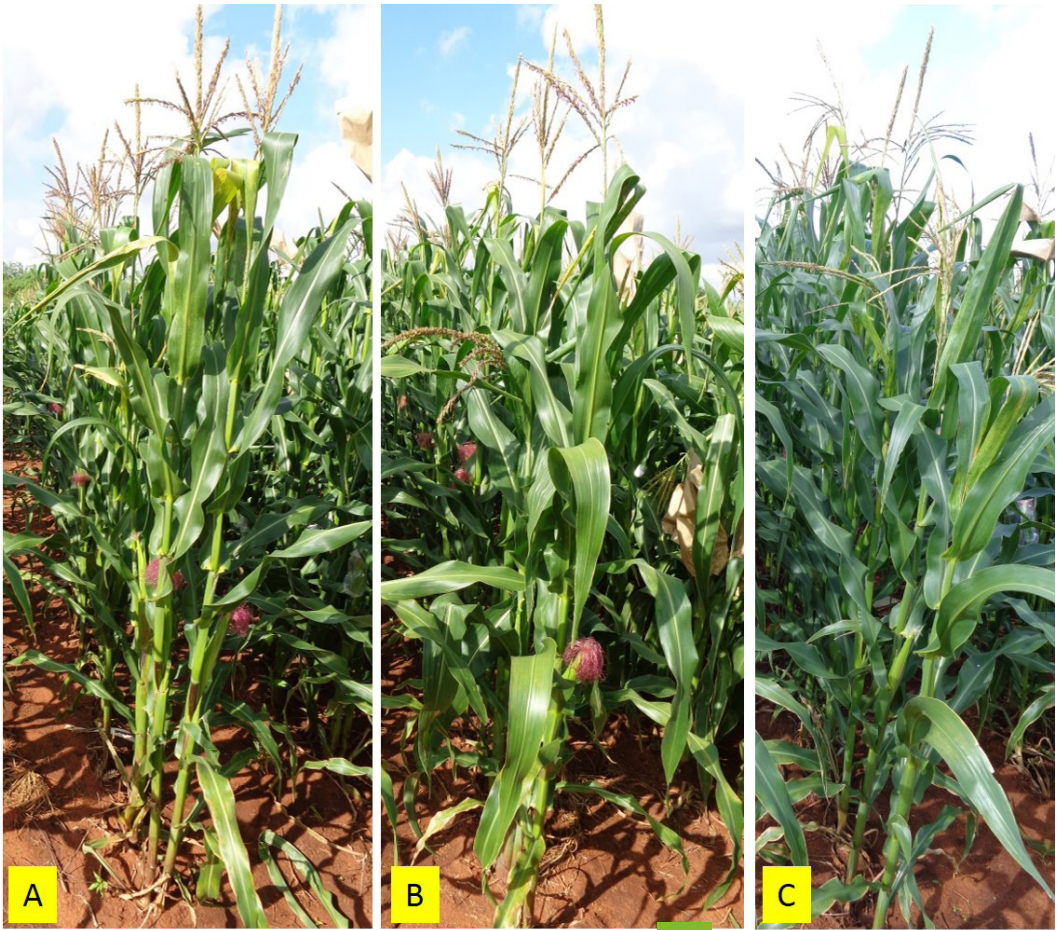


Fig. 3. Morphological comparison, through GOT at Kiboko, Kenya (2016), of a CIMMYT inbred line from three different seed sources (A, B and C). While A (seed from a reference sample) and B were similar in phenotype, C was not true-to-type.

5. Biochemical markers-based QA/QC

This biochemical test for cultivar identification/differentiation involves analyses of protein/isoenzyme profiles. Here single seeds are defatted and extracted for storage protein(s). The extracted proteins are separated by polyacrylamide gel electrophoresis (PAGE). Based on the banding pattern of protein(s), the inbred lines or cultivars are differentiated or identified.

Seeds, seedlings or mature leaves etc. of a crop plant have a specific mix of proteins which are not only crop-specific but are often also variety-specific (genotype-specific). The electrophoresis in a suitable medium separates the mixture of proteins extracted from seeds, seedlings or mature leaves into distinct bands. Each variety (or an inbred line or hybrid) could have a specific "banding pattern" on the basis of which admixtures of other varieties, differing in "banding pattern" could be detected. This is done by comparing the banding pattern of an analyzed sample with the standard banding pattern of that variety. Isozymes are used for determining the genetic purity of seed samples. Some of isozymes used in maize seed genetic purity tests are listed in Table 1.

However, wide use of isozymes for genetic purity tests in seed production is limited due to their limited numbers and low polymorphism (= low discriminative capacity) for effectively distinguishing closely related genotypes, and high environmental influence.

Table 1. List of commonly used isozymes in maize for genetic purity test. (Source: ISTA Seed Congress, 2010)

Enzyme	Abbreviation	Structure	Chromosomal poistion(s)	Locus
Malate dehydrogenase	MDH	Dimeric	8	Mdh1
			6L	Mdh2
			3L	Mdh3
			1L	Mdh4
			5S	Mdh5
Isocitrate dehydrogenase	IDH	Dimeric	8	Idh1
			6	Idh2
6-phoshogluconate dehy-rogenase	PGD	Dimeric	6	Pgd1
			3L	Pgd2
Phosphogucomutase	PGM	Monomeric	1L	Pgm1
			5S	Pgm2
Phosphoglucoisomerase	PGI	Dimeric	1L	Pgi1
Acid phosphatase	ACP	Dimeric	9L	Acp1
Diaphorase	DIA	Monomeric	2	Dia1
		Dimeric	1L	Dia2
Alcohol dehydrogenase	ADH	Dimeric	1L	Adh1

6. Molecular markers-based QA/QC

Many of the modern maize inbred lines or hybrids could be morphologically or biochemically difficult to distinguish. Molecular markers are innumerable, environmentally insensitive and have the discriminative power to effectively perform the task of QA/QC in crops like maize. The DNA based markers are ideal for genetic testing because (i) they detect variation directly at DNA level, (ii) codominant (=can differentiate a genetically homozygous vs a genetically heterozygous genotype), (iii) highly abundant and polymorphic, (iv) independent from environment and reproducible, (v) expressed at all the developmental stages, (vi) have known positions in the genome, (vii) often also linked with trait(s) of interest, and (viii) possible to automate, so that one can handle thousands of samples in quick time. The Biochemical and Molecular Techniques Group of the International Union for the Protection of New Varieties of Plants (UPOV) is using different DNA marker based tests in establishing distinctness, uniformity and stability (DUS) of plant varieties (Jones et al., 2010).

6.1. What is a SNP?

DNA is the genetic material that codes for expression of phenotypic traits. Among different types of DNA markers available, single nucleotide polymorphism (SNP) markers are commonly used because of their low genotyping cost per data point, high genomic abundance, locus-specificity, codominance, simple documentation, and potential for high throughput analysis. Therefore, SNPs are markers of choice for QA/QC applications in crops like maize.

SNP is a variation at a single position in a DNA sequence among a group of genotypes. DNA sequence is formed from a chain of four nucleotide bases: A, C, G, and T. If more than 1% of a population does not carry the same nucleotide at a specific position in the DNA sequence, then this variation can be classified as a SNP. Widely used SNP genotyping platforms, ranging from single SNP methods such as KASP™ from LGC Genomics (http://www.lgcgroup.com) and TaqMan™ from Applied Biosystems (http://www.lifetechnologies.com), to larger fixed array based systems, including Bead Xpress™ and Golden Gate™ from Illumina (http://www.illumina.com) and Axiom1 Genotyping Array from Affymetrix (http://www.affymetrix.com), and finally to the next-generation sequence based genotyping platforms, such as KeyGene1 SNPSelect (http://www.keygene.com) and DArTSeq, developed by Diversity Arrays Technology (http://www.diversityarrays.com) offer an array of SNP-based genotyping services worldwide to ensure high seed quality.

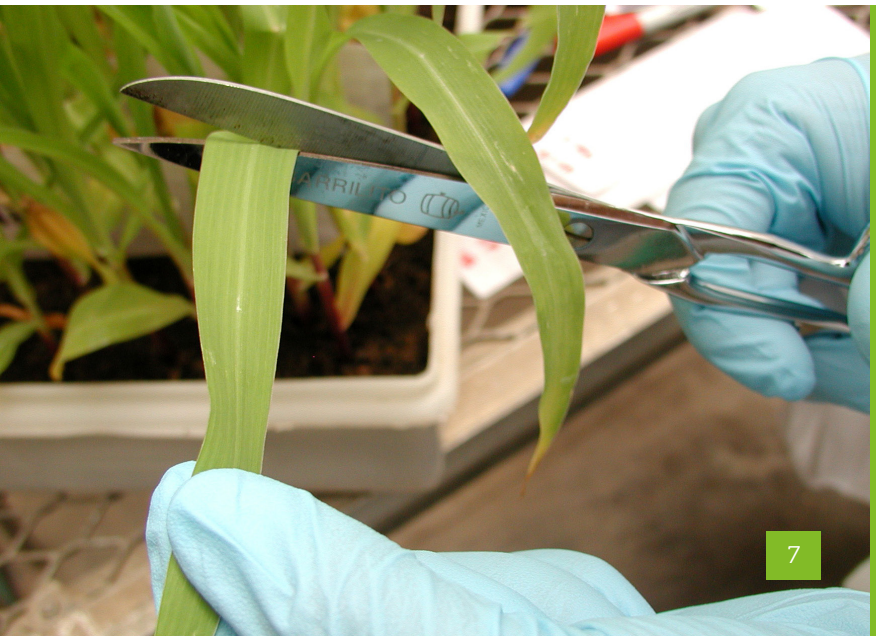
Based on several data sets obtained from a wide range of sample sizes and diverse genetic backgrounds (inbred lines), CIMMYT Global Maize Program recommended a subset of 50–100 SNPs for routine and low-cost QA/QC genotyping; these SNPs have been validated in an array of tropical/subtropical maize inbred lines developed by CIMMYT and widely used by breeding programs across sub-Saharan Africa, Latin America and Asia. Based on this, Semagn et al. (2012) outlined a QA/QC protocol that could be used to minimize errors in genetic analyses and QA/QC related tests.

These SNPs have been selected for their high polymorphic information content (PIC) and uniform coverage of the maize genome.

6.2. Procedure for DNA Fingerprinting

For DNA fingerprinting of inbred lines or hybrids, depending on the objective of the test(s), 50–100 seeds are planted for each entry in the field or in greenhouse. When the seedlings are about 2–4 weeks old, one leaf is harvested from at least 30 different plants. DNA is extracted either from each plant or bulking leaves from 5-10 plants, so that each sample is represented by two to three bulks. The extracted DNA is checked for quality and quantity, and the required working concentration is prepared for polymerase chain reaction (PCR). PCR analysis of the samples is performed using a specific set of SNPs that have been optimized for QC genotyping.

Broadly there are two types of QC approaches – "broad QC" and "rapid QC" (Chen et al. 2016). "Broad QC" focuses particularly on analysis of genetic identity of a sample, using 50–100 carefully selected SNPs (Table 2). This type of QC can help ensure the identity and purity of the founding parents and to evaluate the levels of residual heterozygosity within a particular parental inbred line. "Rapid QC" uses a smaller subset of 10-15 selected markers, and can help quickly assess with high accuracy the possibility of mislabeling of entries across a panel of inbred lines or seed lots. For example, if the objective is to know just whether two maize inbred lines are different or the same, both samples may initially be genotyped using a rapid QC marker set. If the test shows significant difference in the molecular marker profiles of the two inbred lines, the laboratory can conclusively say that the two inbred lines are indeed genetically different. However, if one wants to know how much or up to what extent the specific inbred lines are genetically different, then the number of markers needs to be increased. If no differences is found after genotyping with even 50-100 markers, it can be concluded that the two inbred lines or hybrids are only as different as random fluctuations caused by sampling or laboratory error would look; in essence, the two inbred lines are genetically indistinguishable from one another. The marker based laboratory tests results could be available within 4–6 weeks after sample submission.



DNA is extracted either from each plant or bulking leaves from 5-10 plants

Table 2. List of 100 selected SNP markers recommended by CIMMYT for QA/QC in maize.

SNP name	Chr.	Physical position (bp)	SNP name	Chr.	Physical position (bp)
PZA00175_2	1	8,510,027	PHM662_27	5	135,569,668
PZB01062_3	1	56,846,728	ae1_7	5	167,873,309
PZE-101093951	1	87,301,345	PZA00352_23	5	191,075,557
PZE0186065237	1	87,301,459	PZA02480_1	5	214,953,055
PZE0186365075	1	87,601,397	PZA00440_1	6	22,404,308
PHM10621_29	1	101,421,468	PZA03120_1	6	57,774,238
PZA02741_1	1	161,072,169	PZA00355_2	6	78,756,133
PHM1968_22	1	183,647,544	PZA00214_1	6	91,704,092
PHM12706_14	1	212,356,401	PZB01658_1	6	102,953,833
PZA00664_3	1	227,542,649	lac1_3	6	120,230,802
PZA02269_3	1	252,722,026	PZA02187_1	6	139,106,115
PZB01403_1	1	285,273,845	PZA02436_1	6	149,251,173
PHM4752_14	1	298,874,066	PZA01462_1	6	155,546,716
PHM13440_13	2	2,527,344	PHM3466_69	6	167,148,384
PHM6111_5	2	21,990,814	PHM3078_12	7	5,963,009
PZA02378_7	2	35,040,818	PHM4080_15	7	20,240,404
PHM3457_6	2	62,804,122	PZA00084_2	7	43,948,264
PHM13360_13	2	107,146,579	PZA01607_1	7	68,051,112
PHM3626_3	2	125,642,617	PZA03645_1	7	73,892,322
PZA03211_6	2	148,837,605	PZA01933_3	7	98,070,498
PZA00495_5	2	170,377,814	PZD00054_1	7	130,488,434
PHM3668_12	2	195,555,350	PZA01533_2	7	162,381,818
PZA00527_10	2	216,833,071	PZA02174_2	8	4,101,256
PZD00022_5	2	233,128,511	PHM2350_17	8	23,985,819
PZA02090_1	3	4,138,512	PZA00498_5	8	48,775,713
PHM2343_25	3	27,981,649	PZA00793_2	8	64,421,988
PZA01447_1	3	53,549,251	PHM11114_7	8	70,899,841
PHM5502_31	3	67,284,067	PHM4134_8	8	105,795,742
PZA02742_1	3	97,441,783	PHM5805_19	8	120,875,248
PZA00413_20	3	125,192,432	PZA00770_1	8	134,140,609
PZA00667_2	3	161,516,227	PZA03182_5	8	152,155,087
PHM17210_5	3	178,229,653	PHM2749_10	8	171,703,522
PZB01109_1	3	194,643,731	sh1_12	9	11,340,882
PHM15964_16	3	221,986,592	PHM5181_10	9	15,582,065
PZA02358_1	4	11,329,241	PHM229_15	9	30,003,189
PHM5572_19	4	35,384,118	PZA01791_2	9	77,467,426
PZA00726_10	4	60,768,063	PZA01062_1	9	88,057,320
PZA00218_1	4	78,946,415	PZB01899_1	9	98,502,843
PZA03536_1	4	107,751,353	PZA02325_4	9	117,870,773
PZA03409_1	4	128,632,208	PHM7916_4	9	132,762,904
PZA01477_3	4	172,301,064	PZA01715_2	9	142,948,449
PZA02779_1	4	207,114,208	PHM1752_36	10	9,746,552
PZA00399_11	4	229,644,826	PHM3922_32	10	17,722,938
PZA03322_5	4	242,019,440	PHM4066_11	10	41,187,565
PZA02462_1	5	6,820,571	PHM1155_14	10	62,063,210
PZA01427_1	5	23,135,578	PHM2770_19	10	72,565,410
PZA00981_3	5	37,030,384	PZA00814_1	10	87,194,491
PHM4165_14	5	65,741,535	PZA01919_2	10	111,260,278
PZA00643_13	5	91,096,945	PZA00866_2	10	124,203,565
PZA02164_16	5	112,179,855	PZA03605_1	10	141,830,532

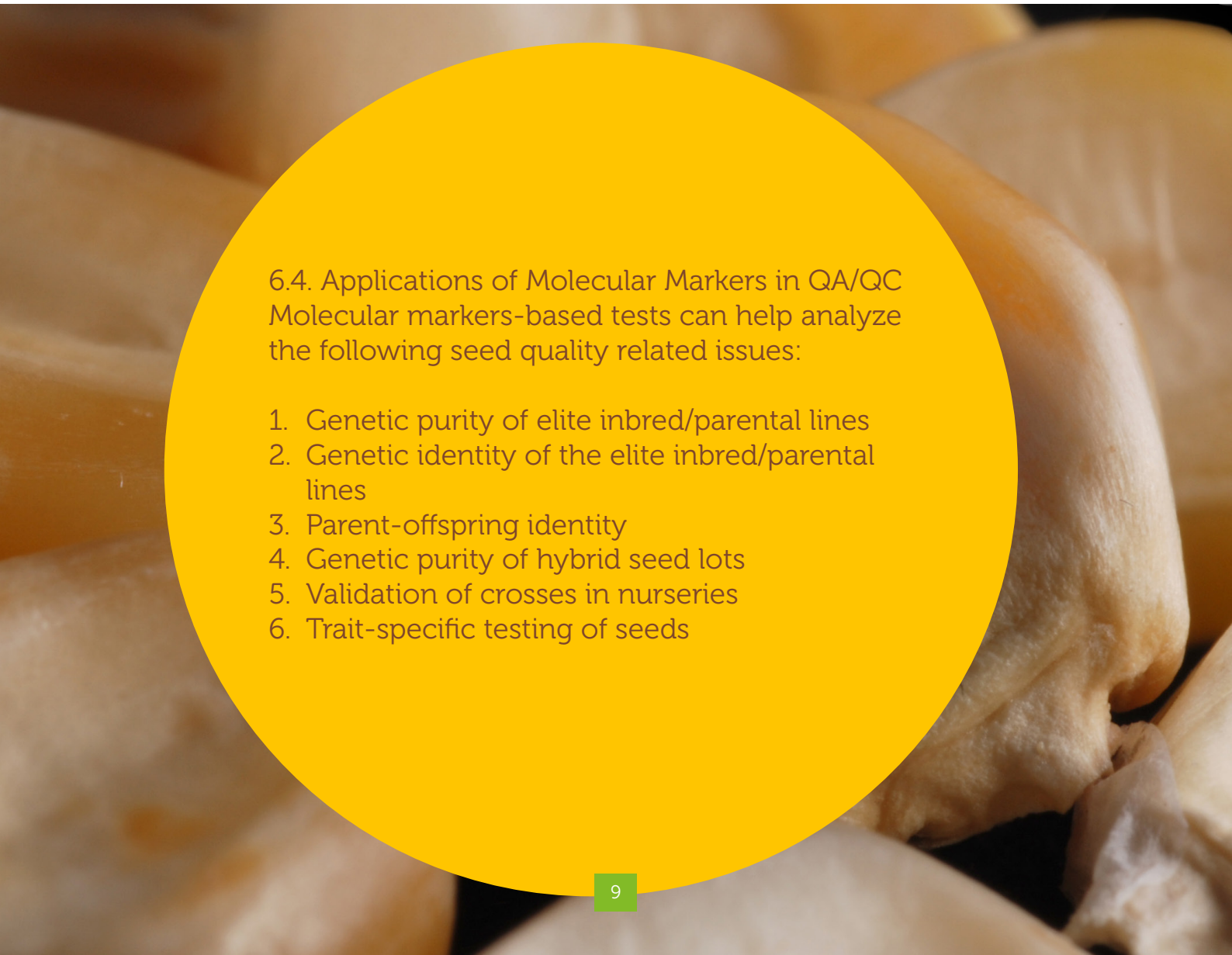
6.3. Sampling for Molecular Marker Analysis

Since it is not practical to test every seed within a lot, sampling is necessary. Each sample taken from a seed lot must be genotyped individually, including DNA extraction. The number of samples submitted is an important factor for QA/QC genotyping since it directly influences the cost, time, and accuracy of off-type detection. These tests will only reflect the sample that was delivered to the laboratory. Therefore, when submitting a sample, ensure that the seeds are sent from a pure and known source. A sample of about 100 seeds can be sent to the laboratory for analysis. Up to 1000 grams should be collected at the time the sample is taken from the seed lot, and the remainder of the seed can be kept under cold storage (as reference sample) until the tests are complete. If there are doubts regarding the results of the tests, a second sample can be drawn from the reference sample and sent to the laboratory again for testing. In CIMMYT, based on several studies, we follow the following two types of sampling for DNA extraction.

Single plant genotyping: This requires sampling of 10-30 individuals for each entry, and each entry should be represented from at least three different sources. This provides the possibility to trace the possible cause if there are any issues on genetic identity and purity. It is possible to do this type of testing for inbred lines at an advanced stage in the seed production pipeline. However, this is 10-30 times more expensive than genotyping one bulk sample per entry.

A bulk of 5-15 plants per entry: Molecular marker analysis of a bulked sample of 5-15 plants per entry will provide a quick answer whether there are any issues in terms of genetic identity and purity, while being significantly more economical compared to single plant genotyping. However, analysis of the bulked samples does not provide clear information on the extent of genetic contamination, if there is any.

Note: An Import Permit and necessary compliance with phytosanitary rules and regulations is a must if seed samples are sent for QA/QC from one country to another. Once the import permit has been issued from the country where the seed will be sent, a phytosanitary certificate must be obtained from the country where the seed or leaf sample comes from. A phytosanitary certificate declares that the seed is free from quarantined pests and pathogens.



6.4. Applications of Molecular Markers in QA/QC

Molecular markers-based tests can help analyze the following seed quality related issues:

1. Genetic purity of elite inbred/parental lines
2. Genetic identity of the elite inbred/parental lines
3. Parent-offspring identity
4. Genetic purity of hybrid seed lots
5. Validation of crosses in nurseries
6. Trait-specific testing of seeds

6.4.1. Testing for Genetic Purity:

Maize inbred lines are expected to be highly genetically pure or homogeneous. Theoretically, inbred lines should be at least 95% identical at every locus for every SNP analyzed, while in case of a doubled haploid (DH) line, the homozygosity is expected to be 100%. We recommend to use at least 50-100 SNPs to minimize the possible genotyping or sampling errors. An inbred line may be considered genetically pure or homogeneous if the proportion of heterozygous loci does not exceed 5%. Any inbred line with more than 5% but less than 15% genetically heterozygous loci requires purification by performing ear-to-row selection while one with >15% heterozygous loci is likely to be contaminated with unrelated genetic material and requires to be either discarded or extensive reselected for the original genotype. In general, inbred lines with >95% of genetic purity is acceptable in commercial maize seed production.

By applying molecular marker based seed tests, in commercial seed production, one can

i. confirm whether the selected parental inbred line meets genetic purity standards,
ii. find and measure the extent of selfing and outcrossing in hybrid seed lots,
iii. determine the genetic variants, segregation and seed mixes in hybrid or inbred line seed production lots, and
iv. help verify the appropriate hybrids, ensuring that producers and customers receive what they expect.

Further, molecular marker-based genetic tests also help the commercial breeders

i. to identify any outcrossing present in the breeder seed lots,
ii. to ascertain the genetic purity of an inbred line prior to the production of basic seed,
iii. to have assurance about genetically pure and segregation-free high-value inbred selections, and
iv. to determine the level of homozygosity in the selected breeder seed.

To understand better how genetic purity testing using molecular markers works, let us follow an example of verifying two parental inbred lines from three different sources, tested initially using 10 different SNPs (Table 3).

Table 3. Comparison of two parental inbred lines collected from three different sources, genotyped using 10 SNP markers.

SNP	Parental Line 1			Parental Line 2		
	Source_A	Source_B	Source_C	Source_A	Source_B	Source_C
SNP_01	C:C	C:C	C:C	C:C	C:C	C:C
SNP_02	A:G	A:A	A:A	A:A	A:A	A:A
SNP_03	T:T	T:T	A:T	A:A	A:A	A:T
SNP_04	A:A	A:A	A:A	C:C	C:C	C:C
SNP_05	A:G	G:G	G:G	G:G	A:G	G:G
SNP_06	C:C	C:C	C:C	G:G	G:G	A:G
SNP_07	A:A	A:A	A:T	T:T	A:T	T:T
SNP_08	A:A	A:A	A:A	A:A	A:A	A:A
SNP_09	T:T	T:T	T:T	C:C	C:C	C:T
SNP_10	A:T	A:A	A:A	A:A	A:A	A:A
Purity (%)	**70**	**100**	**80**	**100**	**80**	**70**
Results	Reject	Accept	Reject	Accept	Reject	Reject

6.4.2. Testing for Genetic Identity: In the commercial seed production it is important to have assurance that the producer is using the right parental inbred lines. To confirm this we can use the genetic identity test, where we compare the molecular marker profiles of specific parental inbred line(s) with the original source of the line(s). If we do not have a reference profile from the original source then we can use genetic distance based approach. When two or more seed sources of the same inbred line shows >5% of genetic distance or marker mismatch proportion, they were considered as different; otherwise, they are considered identical. For example, if the breeder is looking to create a new OPV or synthetics for marketing, it may not be that important that the parents are exactly what they think they are, but if the breeder is creating a new hybrid specifically from a well-known parental inbred line, he/she needs to ensure that the new line is indeed a derivation of the original parent. Molecular marker based genetic identity test will help to ascertain this (Table 4).

Table 4. Comparison of two parental inbred lines from two sources with their original reference profile, to find their true identity, based on genotyping with 10 SNP markers.

SNP	Original source	Line 1_A	Line 1_B	Original source	Line 2_A	Line 2_B
SNP_11	C:C	C:C	C:C	G:G	G:G	G:G
SNP_12	A:A	A:G	A:A	G:G	G:G	G:G
SNP_13	A:A	T:T	A:A	A:A	A:A	T:T
SNP_14	C:C	C:T	C:C	T:T	T:T	T:T
SNP_15	G:G	A:G	G:G	G:G	G:G	A:A
SNP_16	G:G	C:C	G:G	C:C	C:C	C:G
SNP_17	T:T	A:T	T:T	A:A	A:A	A:A
SNP_18	A:A	A:A	A:A	T:T	T:T	T:T
SNP_19	C:C	C:C	C:C	G:G	G:G	C:C
SNP_20	A:A	A:T	A:A	A:A	A:A	A:A
Identity (%)	100	30	100	100	100	60
Results		Reject	Accept		Accept	Reject

When two or more seed sources of the same inbred line shows >5% of genetic distance or marker mismatch proportion, they were considered as different;

Figure 4 is an example to illustrate genetic identity based on molecular markers-based genetic distance analysis. Simply explained, in this figure, if two samples are placed at the same level on a horizontal line, they are considered identical. If two samples are on different branches, they are genetically different; the length of the branch indicates the degree of genetic difference. The first type of error illustrated in this figure is shown by comparing CML488 with CML489. We know that these two are two different inbred lines, but based on the genetic distance results, they are shown here as the same inbred line! This indicates there was either an error in labeling of the seed or a mix-up that happened during seed harvest and storage. Secondly, one can see that the samples of CML312 are shown as similar but not identical as would be expected, since different sources of CML312 are not on the same horizontal line. This suggests that there has been genetic drift between the two samples, or contamination of one of the samples by stray pollen during seed production. A small amount (<5%) of genetic drift may be acceptable (and could be possibly rectified) but as the genetic distance between two sources of the same inbred line increases, intervention (either by discarding the contaminated seed sample or by reselection) becomes necessary. When samples show up on separate limbs of the tree, such as with sample CZL003, it is suspected that the different samples of each of these inbred lines are not the same. This suggests again a seed mix-up during handling.

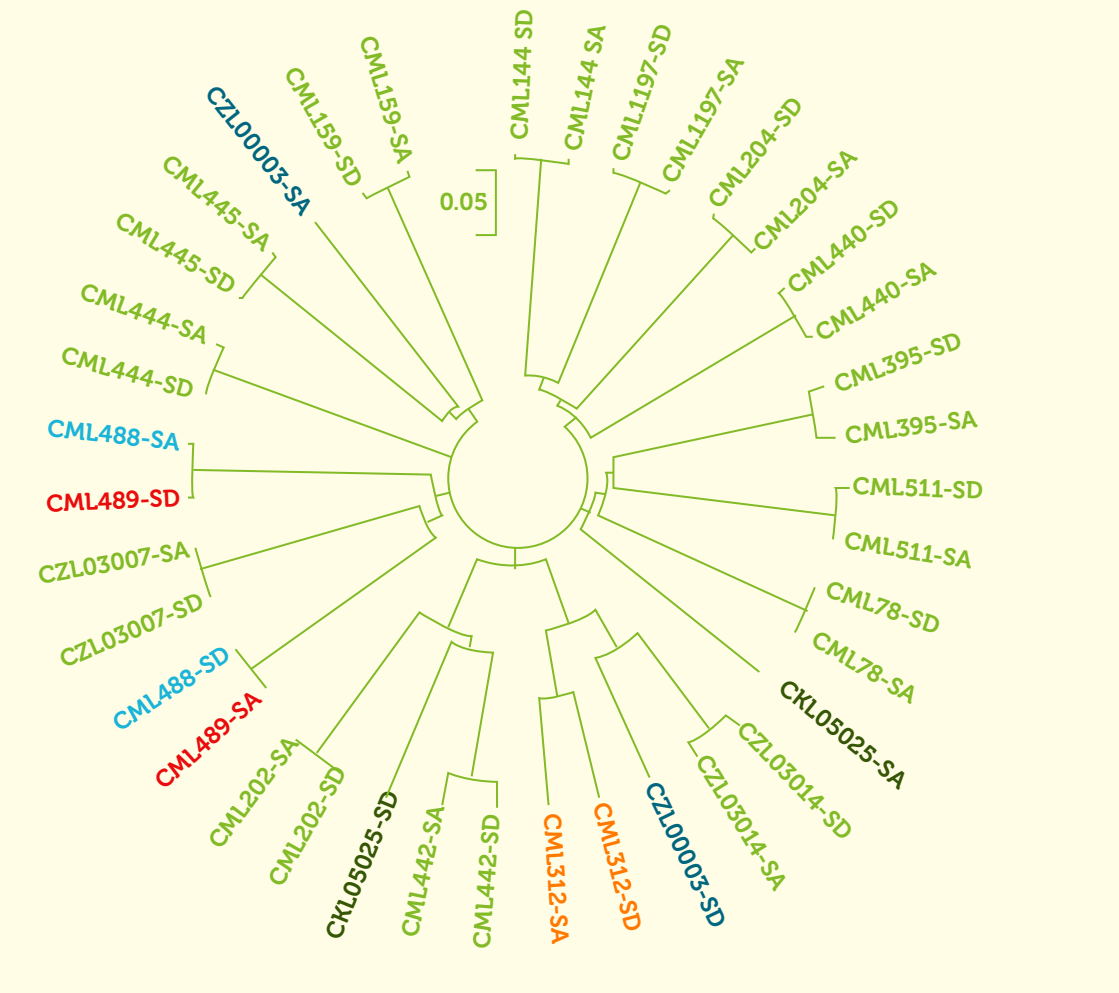


Fig. 4. Neighbor-joining tree for a set of inbred lines based on Roger's genetic distance. Two seed sources of the same line designation that showed >5% genetic distance are indicated with same color in boldface. SA and SD indicate Seed source A and Seed source D, respectively (Semagn et al. 2012)

6.4.3. Parent-offspring Test: The main objective of a parent-offspring test is to confirm whether a specific hybrid (either a single-cross hybrid or a three-way cross hybrid) is indeed derived from the original parental inbred lines (without any foreign pollen contamination). This test helps the commercial seed producer to monitor whether the hybrid was derived by proper crossing at different locations where seed production of that particular hybrid might have happened.

In sub-Saharan Africa, low-cost three-way maize hybrids are widely commercialized as compared to single-cross hybrids. In the process of developing three-way hybrids, and during the formation of single-cross parents of such hybrids, there is a possibility of contamination to happen if the seed production is not properly isolated. Therefore, parent-offspring test is a must to increase the confidence of seed producer on production of genuine high-quality seed (Table 5).

The Pedigree Verification tool in Flapjack (https://ics.hutton.ac.uk/flapjack; Flapjack Documentation, Release 1.16.10.31, 2017) is a very useful tool in pedigree verification of hybrids.

Table 5. Comparison of parental inbred lines versus single-cross hybrid and three-way hybrid to confirm parent-offspring relationship and proper pollination in seed production plots. Here we used data from 20 SNP markers.

SNP	Parent1	Parent2	SCH	SCH?	Parent3	TWCH	TWCH?
SNP_21	C:C	A:A	C:A	YES	A:A	A:A	Not a perfect marker for TWCH identification
SNP_22	G:G	A:A	A:G	YES	G:G	A:G	Not a perfect marker for TWCH identification
SNP_23	T:T	A:A	A:T	YES	T:T	T:T	Not a perfect marker for TWCH identification
SNP_24	A:A	C:C	A:C	YES	C:C	A:C	Not a perfect marker for TWCH identification
SNP_25	A:A	G:G	A:G	YES	A:A	A:G	Not a perfect marker for TWCH identification
SNP_26	C:C	G:G	C:G	YES	G:G	G:G	Not a perfect marker for TWCH identification
SNP_27	A:A	T:T	A:T	YES	T:T	T:T	Not a perfect marker for TWCH identification
SNP_28	C:C	A:A	C:C	NO	A:A	A:C	NO
SNP_29	T:T	C:C	T:T	NO	C:C	C:T	NO
SNP_30	G:G	A:A	A:A	NO	A:A	A:A	NO
SNP_31	A:A	A:A	A:A	Monomorphic	C:C	A:C	YES
SNP_32	C:C	C:C	C:C	Monomorphic	G:G	G:C	YES
SNP_33	G:G	G:G	G:G	Monomorphic	T:T	G:T	YES
SNP_34	T:T	T:T	T:T	Monomorphic	G:G	G:T	YES
SNP_35	G:G	G:G	G:G	Monomorphic	A:A	A:G	YES
SNP_36	T:T	T:T	T:T	Monomorphic	C:C	C:T	YES
SNP_37	C:C	C:C	C:C	Monomorphic	T:T	C:T	YES
SNP_38	A:A	A:A	A:A	Monomorphic	G:G	A:G	YES
SNP_39	G:G	G:G	G:G	Monomorphic	A:A	A:G	YES
SNP_40	A:A	A:A	A:A	Monomorphic	C:C	A:C	YES

6.4.4. Trait-specific Testing using Breeder-ready Markers: Molecular markers also give added advantage over normal QA/QC process by including trait-specific markers. CIMMYT has an ongoing molecular marker discovery-validation-deployment program for prioritized traits in sub-Saharan Africa, Asia and Latin America. For several traits, we have identified and validated specific QTL (Quantitative Trait Loci) or genomic regions, and developed breeder-ready markers or SNP marker assays to select for such high-value QTL or genomic regions. CIMMYT Global Maize Program has identified and validated and is deploying molecular markers for resistance to Maize Streak Virus or MSV (Nair et al., 2015; Table 6), Maize Lethal Necrosis or MLN (Gowda et al., 2015), provitamin A (Babu et al., 2013; Table 6), and Quality Protein Maize or QPM (Babu and Prasanna., 2014; Jompuk et al., 2006; Vivek et al., 2008). Molecular markers for some other important traits, such as Grey leaf spot (GLS), Turcicum leaf blight (TLB), and Tar spot complex (TSC), are also in pipeline for validation and deployment.

Table 6. Selection of inbred lines for favorable alleles for resistance to MSV and for provitamin A (PVA) by using specific SNPs.

MSV_SNP	Resistance	Susceptible	Line1	Line2	Line3	Line4
PZE0186365075	C:C	A:A	C:C	A:A	A:A	C:C
PZE-101093951	A:A	G:G	A:A	G:G	G:G	A:A
PZE0186065237	C:C	T:T	C:C	T:T	T:T	C:C
MSV_Selection			Select	Reject	Reject	Select

PVA_SNP	Favorable	Unfavorable	Line1	Line2	Line3	Line4
S10_134583972	G:G	C:C	G:G	G:G	C:C	C:C
S10_134655704	C:C	T:T	C:C	C:C	T:T	T:T
SYN11355	A:A	G:G	A:A	A:A	G:G	G:G
PZE-110083653	G:G	A:A	G:G	G:G	A:A	A:A
S10_136072513	G:G	T:T	T:T	T:T	G:G	G:G
S10_136840485	C:C	T:T	C:C	C:C	T:T	T:T
S10_137904716	C:C	T:T	C:C	C:C	T:T	T:T
PVA_Selection			Select	Select	Reject	Reject

Trait-specific SNPs can be incorporated into the QA/QC SNP set, and any inbred lines submitted for testing could be assessed for these SNPs for the presence/absence of those high-value genomic regions. It is important to note, however, that QTL are genotype-specific with background effects playing sometimes an important role. So if you are breeding for a trait with a different trait donor source than that was used in the QTL discovery program or if you are introgressing a QTL into a different genetic background, the markers may not necessarily confirm that the trait is present in your inbred line. QTL are also subject to environment-by-genotype interactions; therefore, the stability of any QTL across environments need to be assessed.

7. Maize breeding and seed production stages for QA/QC

In the course of maize breeding, application of molecular markers could start as early as selection of appropriate parents (Fig .5) in breeding programs. In the absence of clear information about heterotic groups, divergent inbred lines identified based on genetic distance estimates using molecular markers could be useful. In the nursery, when breeders have large number of F1 plants from different parental combinations but in the same field, it is appropriate to follow QA/QC to confirm whether all the F1s are purely derived from specific parents (and to rule out potential cross-contamination). In maize breeding, GOT and/or molecular marker-based QA/QC can also be applied on the final finished inbred lines to confirm their genetic purity and identity. Only fixed inbred lines must be entered into commercial hybrid seed production pipeline as breeder's seed. Multiplication and maintenance of breeder's seed of inbred line(s) is an important task, and the basic foundation for the successful commercialization of hybrid seed. It is essential to have a reference sample of seed of each finished inbred line, along with a reference DNA fingerprint on such seed by using at least 50-100 SNPs from QA/QC set.

Breeder's stock is the source, normally kept in ear-to-row (ETR) that is maintained by the breeder. Breeder's seed is the increase (by selfing) of the Breeder's stock in ear-to-row and then bulked to produce the Breeder's Seed Generation 1 that can be used as foundation to make new increases or make-up hybrids (parental or commercial). In order to maintain the breeder's seed in its original form, breeder's stock has to be tested in specific intervals with molecular marker-based tests. In general, breeder chooses 20 ears from the breeders stock and plant these in ETR. Sample each ETR and ship these to lab for DNA fingerprinting. The results before flowering will help discard the seed from specific ETR and the marker-based test would help reveal further about genetic purity and genetic identity, if an original true to type source is provided for comparison. It may happen that there is no old "true to type" source in the lab for comparison; in that case the results will indicate which among the 20 ETR samples have the highest homozygosity and how that sample differs from others in terms of genetic distance and or marker mismatch proportion. Then the breeders have to choose the 2 or 3 best ETR among the ones that the test results indicate as uniform and have <5% of marker mismatch proportion, and advance these further. Further, all the plants in the ETR that were "true-to-type" and are homozygous as indicated by marker based test should be selfed, and only 2-3 best rows harvested that are true-to-type. From these, 20 ears from the 2-3 rows may be selected and shelled individually. This can now form the Breeders' Stock.

Only fixed inbred lines must be entered into commercial hybrid seed production pipeline as breeder's seed.

Inclusion of trait-specific markers (e.g., pro-vitamin A, QPM, MSV resistance or MLN resistance) could be of added value in breeding programs. Basic seed is derived from breeder's/pre-basic seed by increasing the seed of parental inbred lines and single-cross hybrid parent (derived from the first two parents in case of three-way hybrid development). In the process of developing basic seed, there is a possibility of quality failure due to poor detasseling, poor labelling of parents, improper formation of single-cross hybrid parent, displacement of seed packets, poor control on pollination, failure to keep recommended isolation distances, etc. To know how properly one has managed the seed production plots, molecular marker-based test gives quick answer within 4-6 weeks by conducting genetic purity and parentage verification test. Once the seed producer /company is sure of high quality basic seed, similar stringent procedures can be followed to produce high-quality certified seed. By applying appropriate QA/QC tests at different stage of breeding and seed production, the seed producer/company can ensure high quality of seed, derive trust from the clients, and avoid the possibility of huge losses that are possible due to mistakes that may happen at different points in the seed value chain.

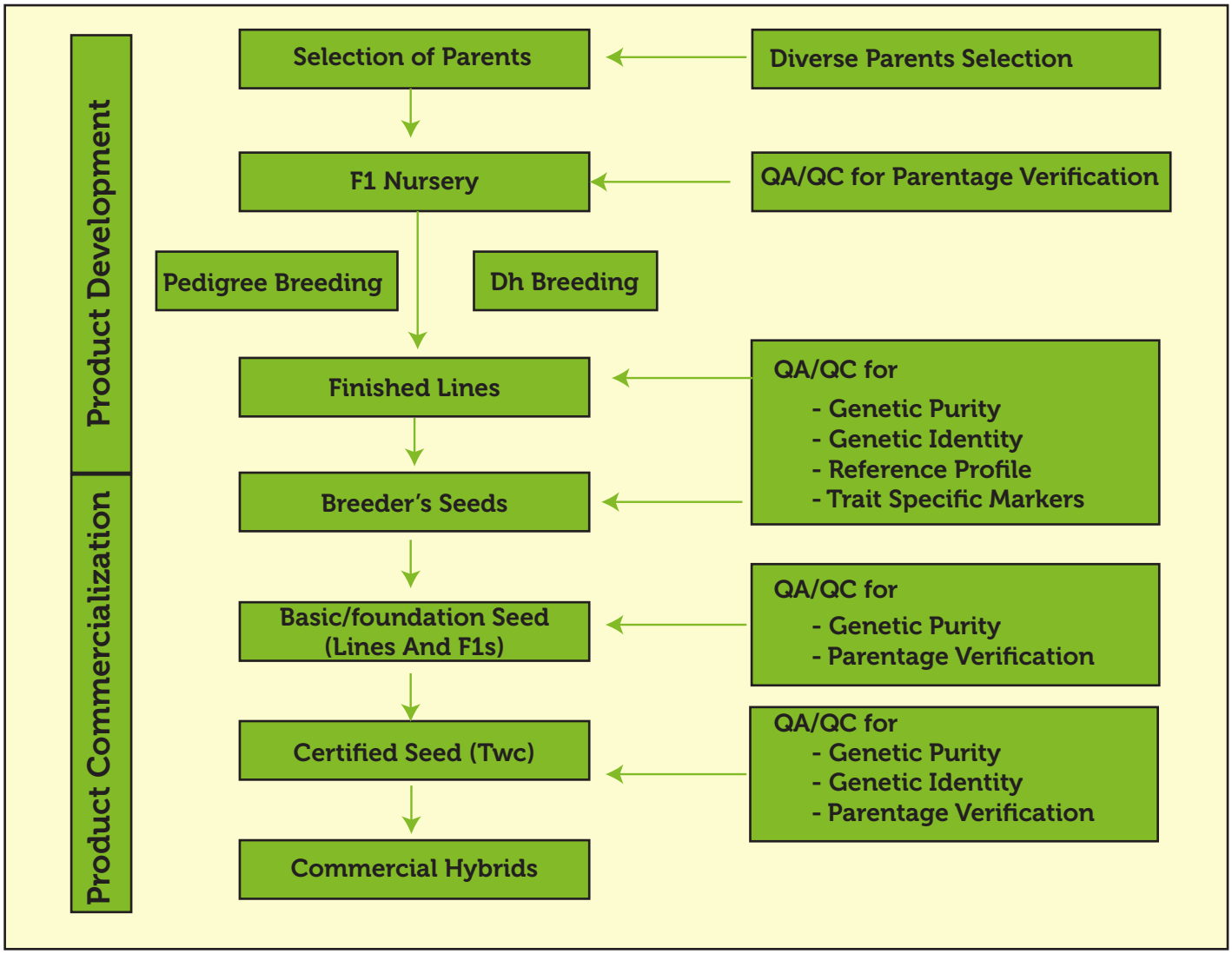


Fig. 5. Flow diagram of maize breeding from parental selection to commercial hybrid seed production and the type of QA/QC required at different stages.

8. QA/QC of nutritional quality traits in the seed

In addition to the quality assurance standards applied during conventional maize seed production, nutritionally enhanced maize, such as QPM (Quality Protein Maize) and provitamin A-enriched maize, require high standards of QA/QC. High-throughput analytical methodologies are critical for integration of biofortification strategies into mainstream plant breeding as well as for assurance of the nutritional quality in the seed that reaches the farmers and consumers (Guild et al., 2017). Given the fact that nutritional traits are not seen by a naked eye, it is extremely important that these are measured in an appropriate laboratory where such methodologies are well-established.

8.1. QPM

Because the QPM trait is conferred mainly by the recessive opaque 2 gene in a homozygous state (o2o2) and endosperm modifiers (that converts the opaque endosperm into a hard, translucent endosperm), the protein quality of QPM seed produced in the seed production fields can be affected mainly through contamination by pollen from conventional maize (if grown in close vicinity, and if the flowering periods overlap). Visually, maize kernels carrying the o2 allele in homozygous state (o2o2) can be distinguished from conventional maize kernels because o2o2 results in an endosperm that is soft, chalky and opaque to light transmission in contrast to conventional maize kernels (O2O2 or O2o2) which are usually hard, vitreous and translucent. When maize kernels are spread on a light table, opaque kernels can easily be separated from the non-opaque (Vivek et al., 2008).

As described by Twumasi-Afriyie et al. (2016), analytical information on quality is vital for successfully selecting and commercializing QPM varieties. The sampling methodology and experimental design used to phenotype many quality traits will depend on the purpose of the study, number of samples, cost of the analysis, etc. Quality traits can be measured through: a) single-phase experiments where varieties are grown without field replicates and one sample is taken for laboratory analysis, or b) through multi-phase experiments in which varieties are first grown in a replicated field trial and then further processed in a laboratory. The common practice is to test a single field replicate for each variety or a single composite sample formed by combining seed/grain samples (Smith et al., 2012). Such an approach may be applied for commercial varieties or advanced breeding materials, where the traits to be analyzed are fixed. For early-generation breeding samples, other approaches can be followed, such as partial replication, where a subset of the varieties is tested using multiple field replicate samples, and then a subset of the selected field plots is split to produce replicate samples for the laboratory analysis. A similar approach can be used in single-phase experiments, where some varieties are tested using individual replicate samples and others are tested using composite samples (Smith et al., 2012). It is therefore very important to discuss the purpose of the study with the laboratory analyst and come to an agreement on a suitable sampling process to reduce possible errors and, also the cost of analysis.

In addition to the above, here are some important general considerations when sending samples for protein quality analysis:

1. Self-pollinate the plants that you are planning to use for quality analysis.
2. If possible, only sample kernels from the central part of the ears.
3. For OPVs, send at least 500g per sample to the lab.
4. For early-generation materials, send at least 100 kernels per sample.
5. For advanced lines and hybrids, send at least 50 kernels per sample.

When determining nutritional quality of QPM it is important to ensure that the protein content of the sample is within the range of the normal maize (8 to 11%). Then, the protein quality, that is, the levels of kernel tryptophan and lysine need to be monitored. Lysine levels in protein of conventional and QPM maize average 2.0% and 4.0%, respectively, but depending on the genetic background, they may range from 1.5-2.8% and 2.6-5.0%, respectively. Tryptophan levels in kernel protein of conventional maize and QPM usually average 0.5% and 0.8%, respectively (Vivek et al., 2008). The levels of tryptophan and lysine in maize are highly correlated (>0.9) and, since analysis for tryptophan is easier and less expensive, breeders typically use tryptophan levels as an indicator of the nutritive value of a QPM seed (Nurit et al., 2009). Table 7 adapted from Twumasie-Afriyie et al. (2016) provides guidance on how to monitor QPM traits during maize breeding, varietal registration, and seed production chain.

8.2. Provitamin A

Yellow maize contains carotenoids but only three of the 6 carotenoids present in maize have provitamin A activity, α-carotene, β-carotene and β-cryptoxanthin. Therefore only those are considered provitamin A carotenoids (PVAC). An average yellow normal maize seed usually has 2 ppm PVAC (Ortiz-Monasterio et al., 2007). Provitamin A-enriched maize varieties developed by CIMMYT have more than 8-10 ppm PVAC.

Like in any carotenoid analysis there are many sources of errors including different physiological stage of the ears analyzed, improper storage of samples, incomplete extraction, and physical losses during extraction with the organic solvents, faulty measurement and calculation, degradation, and isomerization. The analytical methods used for PVAC analysis in maize are High Performance Liquid Chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC) (Galicia et al., 2009). However, more recently, robust Near Infrared Spectroscopy (NIRS)-based models for PVAC quantification have been developed at the Maize Nutritional Quality Lab at CIMMYT-Mexico.

NIRS methodology can be effectively used for early breeding generations to identify and reject materials that have low values of PVAC. However, for advanced generation materials or commercial samples, it is always recommended to conduct the wet chemistry analysis using UPLC or HPLC (Fig. 6). Given the nature of carotenoids which are chemical molecules prone to degradation due to factors like high temperature, oxygen and light, complete seeds must be submitted to the laboratory (no flour) and samples must be kept at the lowest temperature possible (less than 8°C) until at least before shipments to the lab. Communication with the laboratory analyst is important to agree on details of sample submission to avoid carotenoid degradation and ensure appropriate quantification of the compounds.

Yellow maize contains carotenoids but only three of the 6 carotenoids present in maize have provitamin A activity,

- **α-carotene**
- **β-carotene**
- **β-cryptoxanthin.**

Table 7 - Recommended scheme for monitoring of QPM quality in the seed.

Monitoring Stage	Type of QPM product	Recommended QPM quality monitoring trait					
		Light table endosperm modifica-tion	Kernel protein	Kernel trypto-phan	Kernel lysine	Protein quality Index	Who should do it?
Breeding	F2 seed	Yes	No	No	No	No	Breeder
	F3-F4 seed	Yes	Yes	Yes	No	Yes	Breeder and chemical analyst
Quality recovery following contamination, if any	F2 seed	Yes	No	No	No	No	Breeder and chemical analyst
	F3-F4 seed	Yes	Yes	Yes	No	Yes	Breeder and chemi-cal analyst
Variety registration	Variety - Hybrid/ OPV seed	Yes	Yes	Yes	Yes	Yes	Seed regulatory body
Seed bulking							
Breeder seed	Breeder seed	Yes	Yes	Yes	Yes	Yes	Seed regulatory body & breeder
Pre-basic seed§	Foundation : Pre-basic seed	No	Optional	Optional	No	Optional	Seed producer and chemical analyst
Basic seed§	Foundation: Basic seed	No	Optional	Optional	No	No	Seed producer and chemical analyst
Certified seed	Certified seed	No	Yes	Yes	No	No	Seed regulatory body Seed producer and chemical analyst

Monitoring QPM quality of pre-basic and basic seed is particularly desirable when these classes of seeds are produced by one company and handed over to another.

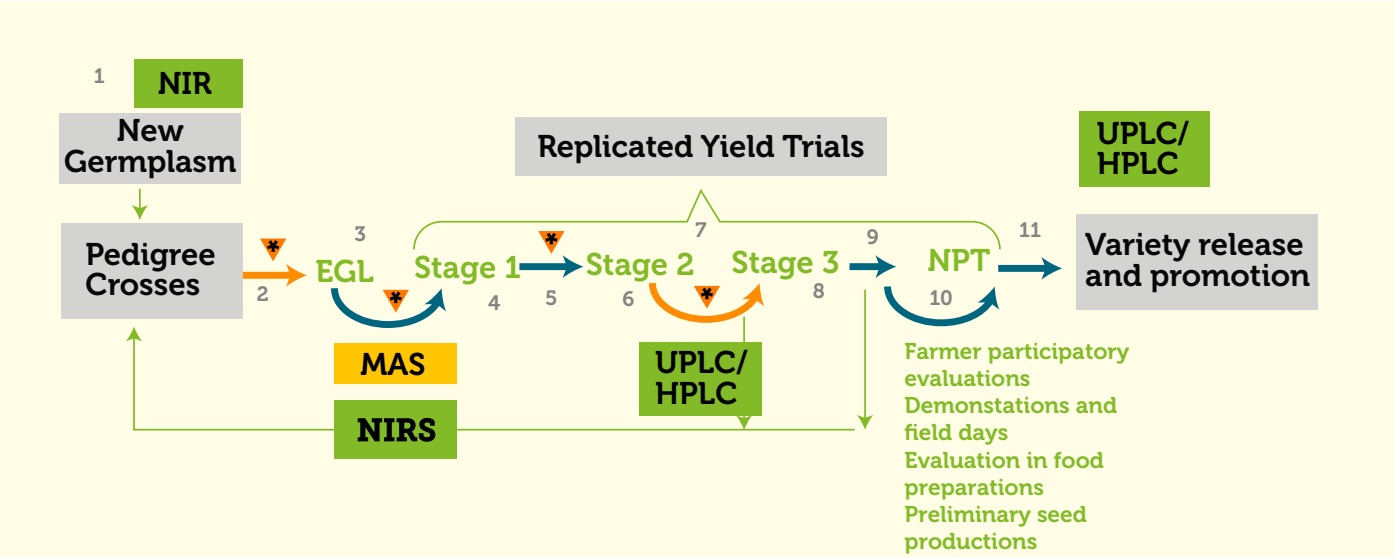


Fig. 6. A schematic strategy for developing provitamin A biofortified maize including critical points where nutritional quality analysis is required (modified based on Pixley et al., 2013).

Table 8. Comparison of UPLC versus NIRS for PVA analysis.

FACTOR	UPLC	NIRS
Accuracy	Quantity and Quality	Quantity
Time of analysis	~3 h	~4 min
Cost/ sample	37.10 US	2.62 US
Risk		
Waste generation		
HR	3 people	1 Person

9. Other relevant seed tests

Seed germination test involves sowing seeds on a uniform substrate (rolled paper towels, sterilized sand, etc.) and incubating the test at an optimum temperature for germination of the seeds. After the prescribed period of incubation, the numbers of normal seedlings, abnormal seedlings, and ungerminated seeds are determined. Seed vigor tests are based on the simulation of environmental stress conditions encountered during storage or during field emergence. Cold test simulates early spring field conditions by providing high soil moistures and low soil temperature. In the accelerated ageing test seeds are stressed at temperatures of 40-45^{0}C and near 100% relative humidity for varying lengths of time prior to a germination test. In the complex stress vigor test seeds are soaked in sodium hypochlorite at a low temperature prior to a germination test. Seedlings are then measured and classified according to length. Seedling vigor classification is similar to the standard germination test but normal seedlings are further classified as 'strong' and 'weak'.

Another commonly used seed viability test is tetrazolium test. In the living cells, dehydrogenase enzymes reduce colorless tetrazolium chloride salt to form the water insoluble red compound formazan. The red-colored living parts of the seeds can then be distinguished from the colorless dead parts. The detailed protocols of these tests are explained in a laboratory guide for seed testing in maize and wheat developed by CIMMYT (Warham et al., 1996; http://repository.cimmyt.org/xmlui/discover).

10. Conclusions

Production and distribution of high quality seed is an integral part of the seed business cycle. Establishing proper QA/QC system is a fundamental requirement for all seed companies – small, medium or large – as well as seed certification agencies. Hybrid maize is an important means for improved productivity, food security and enhanced income and livelihoods of farmers if the seed meets the high standards of genetic and physical purity, besides physiological and seed health standards. Proper seed testing is required to assure genetically pure and disease-free seed to farmers. GOT is used conventionally for seed genetic purity test since beginning of quality seed multiplication chain, but is tedious, time-consuming and sometimes not quite effective. Molecular marker-based seed tests are more efficient, economical, user-friendly, reliable and quick, and can help determine the genetic purity and identity of the seed lots. Markers tightly linked to or present within genes controlling traits of interest are also of great value when testing for genetic quality. Same markers and analytical systems can be used for variety development, management of seed production and QA/QC, and to ensure traceability downstream in the seed value chain. Overall, it is important to effectively use marker-based QA/QC routinely in maize breeding and seed production chain to produce and distribute high quality certified seed to the farmers. Equally important is the effective QA/QC for relevant nutritional quality traits in the biofortified maize both during the breeding program as well as during the seed production to make sure that high-quality biofortified seed reaches the farmers and consumers.

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