CPVO · OCVV
Community Plant Variety Office
Office Communautaire des Variétés Végétales

PROTOCOL FOR DISTINCTNESS, UNIFORMITY AND STABILITY TESTS

Triticum aestivum L. emend. Fiori et Paol.

WHEAT

UPOV Species Code: TRITI_AES

Adopted on 19/03/2019

Entered into force on 01/08/2019

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CPVO/TP-003/5

1. SUBJECT OF THE PROTOCOL AND REPORTING

1.1 Scope of the technical protocol

This Technical Protocol applies to all varieties of ***Triticum aestivum*** **L. emend. Fiori et Paol.**

The protocol describes the technical procedures to be followed in order to meet the requirements of Council Regulation 2100/94 on Community Plant Variety Rights. The technical procedures have been agreed by the Administrative Council and are based on documents agreed by the International Union for the Protection of New Varieties of Plants (UPOV), such as the General Introduction to DUS (UPOV Document TG/1/3 http://www.upov.int/export/sites/upov/resource/en/tg_1_3.pdf), its associated TGP documents (http://www.upov.int/tgp/en/) and the relevant UPOV Test Guideline TG/3/12 dated 05.04.2017 (http://www.upov.int/edocs/tgdocs/en/tg003.pdf) for the conduct of tests for Distinctness, Uniformity and Stability.

1.2 Entry into Force

The present protocol enters into force on **01.08.2019**. Any on-going DUS examination of candidate varieties started before the aforesaid date will not be affected by the approval of the Technical Protocol. Technical examinations of candidate varieties are carried out according to the TP in force when the DUS test starts. The starting date of a DUS examination is considered to be the due date for submitting of plant material for the first test period.

In cases where the Office requests to take-over a DUS report for which the technical examination has either been finalized or which is in the process to be carried out at the moment of this request, such report can only be accepted if the technical examination has been carried out according to the CPVO TP which was in force at the moment when the technical examination started.

1.3 Reporting between Examination Office and CPVO and Liaison with Applicant

1.3.1 Reporting between Examination Office and CPVO

The Examination Office shall deliver to the CPVO a preliminary report ("the preliminary report") no later than two weeks after the date of the request for technical examination by the CPVO.

The Examination Office shall also deliver to the CPVO a report relating to each growing period ("the interim report") and, when the Examination Office considers the results of the technical examination to be adequate to evaluate the variety or the CPVO so requests, a report relating to the examination ("the final report").

The final report shall state the opinion of the Examination Office on the distinctness, uniformity and stability of the variety. Where it considers those criteria to be satisfied, or where the CPVO so requests, a description of the variety shall be added to the report. If a report is negative the Examination Office shall set out the detailed reasons for its findings.

The interim and the final reports shall be delivered to the CPVO as soon as possible and no later than on the deadlines as laid down in the designation agreement.

1.3.2 Informing on problems in the DUS test

If problems arise during the course of the test the CPVO should be informed immediately so that the information can be passed on to the applicant. Subject to prior permanent agreement, the applicant may be directly informed at the same time as the CPVO particularly if a visit to the trial is advisable.

1.3.3 Sample keeping in case of problems

If the technical examination has resulted in a negative report, the CPVO shall inform the Examination Office as soon as possible in case that a representative sample of any relevant testing material shall be kept.

2. MATERIAL REQUIRED

2.1 Plant material requirements

Information with respect to the agreed closing dates and submission requirements of plant material for the technical examination of varieties can be found on http://cpvo.europa.eu/applications-and-examinations/technical-examinations/submission-of-plant-material-s2-publication in the special issue S2 of the Official Gazette of the Office. General requirements on submission of samples are also to be found following the same link.

2.2 Informing the applicant of plant material requirements

The CPVO informs the applicant that

- he is responsible for ensuring compliance with any customs and plant health requirements.
- the plant material supplied should be visibly healthy, not lacking in vigour, nor affected by any important pest or disease.
- the plant material should not have undergone any treatment which would affect the expression of the characteristics of the variety, unless the competent authorities allow or request such treatment. If it has been treated, full details of the treatment must be given.

2.3 Informing about problems on the submission of material

The Examination Office shall report to the CPVO immediately in cases where the test material of the candidate variety has not arrived in time or in cases where the material submitted does not fulfil the conditions laid down in the request for material issued by the CPVO.

In cases where the examination office encounters difficulties to obtain plant material of reference varieties the CPVO should be informed.

3. METHOD OF EXAMINATION

3.1 Number of growing cycles

The minimum duration of tests should normally be two independent growing cycles.

3.2 Testing Place

Tests are normally conducted at one place. In the case of tests conducted at more than one place, guidance is provided in TGP/9 "Examining Distinctness" http://www.upov.int/edocs/tgpdocs/en/tgp_9.pdf.

3.3 Conditions for Conducting the Examination

The tests should be carried out under conditions ensuring satisfactory growth for the expression of the relevant characteristics of the variety and for the conduct of the examination.

The optimum stage of development for the assessment of each characteristic is indicated by a number in the third column of the Table of Characteristics. The stages of development denoted by each number are described in Chapter 8.3

3.4 Test design

Each test should be designed to result in a total of at least 2000 plants, which should be divided between at least two replicates.

The assessment of the characteristic "Seasonal type" should be carried out on at least 300 plants.

If ear rows are used, the test should be conducted on at least 100 ear rows.

In case of hybrids, the parent lines have to be included in the test and should be tested and assessed as any other self-pollinating variety. The observations on the hybrid variety itself should be made on at least 200 plants.

The design of the tests should be such that plants or parts of plants may be removed for measurement or counting without prejudice to the observations which must be made up to the end of the growing cycle.

3.5 Additional tests

In accordance with Article 83(3) of Council Regulation No. 2100/94 an applicant may claim either in the Technical Questionnaire or during the test that a candidate has a characteristic which would be helpful in establishing distinctness. If such a claim is made and is supported by reliable technical data, an additional test may be undertaken providing that a technically acceptable test procedure can be devised.

Additional tests will be undertaken, with the agreement of the President of CPVO, where distinctness is unlikely to be shown using the characters listed in the protocol.

3.6 Constitution and maintenance of a variety collection

The process for the constitution and the maintenance of a variety collection can be summarized as follows:

Step 1: Making an inventory of the varieties of common knowledge

Step 2: Establishing a collection ("variety collection") of varieties of common knowledge which are relevant for the examination of distinctness of candidate varieties

Step 3: Selecting the varieties from the variety collection which need to be included in the growing trial or other tests for the examination of distinctness of a particular candidate variety.

3.6.1 Forms of variety collection
The variety collection shall comprise variety descriptions and living plant material, thus a living reference collection. The variety description shall be produced by the EO unless special cooperation exists between EOs and the CPVO. The descriptive and pictorial information produced by the EO shall be held and maintained in a form of a database

3.6.2 Living Plant Material
The EO shall collect and maintain living plant material of varieties of the species concerned in the variety collection.

3.6.3 Range of the variety collection
The living variety collection shall cover at least those varieties that are suitable to climatic conditions of a respective EO.

3.6.4 Making an inventory of varieties of common knowledge for inclusion in the variety collection
The inventory shall take into account the list of protected varieties and the official, or other, registers of varieties, in particular:
The inventory shall include varieties protected under National PBR (UPOV contracting parties) and Community PBR, varieties registered in the Common Catalogue, the OECD list, the Conservation variety list and varieties in trade or in commercial registers for those species not covered by a National or the Common Catalogue.

3.6.5 Maintenance and renewal/update of a living variety collection
The EO shall maintain seeds in conditions which will ensure germination and viability, periodical checks, and renewal as required. For the renewal of existing living material the identity of replacement living plant material shall be verified by conducting side-by-side plot comparisons between the material in the collection and the new material.

4. ASSESSMENT OF DISTINCTNESS, UNIFORMITY AND STABILITY

The prescribed procedure is to assess distinctness, uniformity and stability in a growing trial.

4.1 Distinctness

4.1.1 General recommendations
It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 9 'Examining Distinctness' (http://www.upov.int/edocs/tgpdocs/en/tgp_9.pdf) prior to making decisions regarding distinctness. However, the following points are provided for elaboration or emphasis in this Technical Protocol.

To assess distinctness of hybrids, a pre-screening system on the basis of the parental lines and the formula may be established according to the following recommendations:

(i) description of parental lines according to the Technical Protocols;

(ii) check of the distinctness of the parental lines in comparison with the reference collection, based on the characteristics in the table of characteristics in order to screen the closest inbred lines;

(iii) check of the distinctness of the hybrid formula in comparison with those of the hybrids in common knowledge, taking into account the closest inbred lines;

(iv) assessment of the distinctness at the hybrid level of varieties with a similar formula.

Further guidance is provided in document TGP/8 "Trial Design and Techniques Used in the Examination of Distinctness, Uniformity and Stability".

4.1.2 Consistent differences

The differences observed between varieties may be so clear that more than one growing cycle is not necessary. In addition, in some circumstances, the influence of the environment is not such that more than a single growing cycle is required to provide assurance that the differences observed between varieties are sufficiently consistent. One means of ensuring that a difference in a characteristic, observed in a growing trial, is sufficiently consistent is to examine the characteristic in at least two independent growing cycles.

4.1.3 Clear differences

Determining whether a difference between two varieties is clear depends on many factors, and should consider, in particular, the type of expression of the characteristic being examined, i.e. whether it is expressed in a qualitative, quantitative, or pseudo-qualitative manner. Therefore, it is important that users of these Technical Protocols are familiar with the recommendations contained in the UPOV-General Introduction to DUS prior to making decisions regarding distinctness.

If distinctness is assessed using the t-test least significant difference the difference between two varieties is clear if it occurs with the same sign at the 1% significance level or less ($p<0.01$) in two consecutive or two out of three growing cycles.

If distinctness is assessed by the combined over years distinctness analysis (COYD) the difference between two varieties is clear if the respective characteristics are different at the 1% significance level or less ($p<0.01$) in a test over either two or three years.

If the significance level or statistical methods proposed are not appropriate the method used should be clearly described.

4.1.4 Number of plants/parts of plants to be examined

Unless otherwise indicated, for the purposes of distinctness, all observations on single plants should be made on 10 plants or parts taken from each of 10 plants and any other observations made on all plants in the test, disregarding any off-type plants.

In the case of observations of parts taken from single plants, the number of parts to be taken from each of the plants should be 1.

4.1.5 Method of observation

The recommended method of observing the characteristic for the purposes of distinctness is indicated by the following key in the third column of the Table of Characteristics (see document TGP/9 "Examining Distinctness", Section 4 "Observation of characteristics"):

MG: single measurement of a group of plants or parts of plants
MS: measurement of a number of individual plants or parts of plants
VG: visual assessment by a single observation of a group of plants or parts of plants
VS: visual assessment by observation of individual plants or parts of plants

Type of observation: visual (V) or measurement (M)
"Visual" observation (V) is an observation made on the basis of the expert's judgment. For the purposes of this document, "visual" observation refers to the sensory observations of the experts and, therefore, also includes smell, taste and touch. Visual observation includes observations where the expert uses reference points (e.g. diagrams, example varieties, side-by-side comparison) or non-linear charts (e.g. colour charts). Measurement (M) is an objective observation against a calibrated, linear scale e.g. using a ruler, weighing scales, colorimeter, dates, counts, etc.

Type of record: for a group of plants (G) or for single, individual plants (S)
For the purposes of distinctness, observations may be recorded as a single record for a group of plants or parts of plants (G), or may be recorded as records for a number of single, individual plants or parts of plants (S). In most cases, "G" provides a single record per variety and it is not possible or necessary to apply statistical methods in a plant-by-plant analysis for the assessment of distinctness.

In cases where more than one method of observing the characteristic is indicated in the Table of Characteristics (e.g. VG/MG), guidance on selecting an appropriate method is provided in document TGP/9, Section 4.2.

With respect to the use of protein electrophoresis, the Office follows the actual UPOV approach as laid down under point 10 of this protocol. If electrophoresis is used for testing distinctness, the same population standard and the same acceptance probability as for other characteristics should be applied. However, a sequential analysis approach could be applied to reduce the workload.

Electrophoretic characteristics with a lack of uniformity shall not be taken into account for the assessment of distinctness.

4.2 Uniformity

4.2.1 It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 10 'Examining Uniformity' (http://www.upov.int/edocs/tgpdocs/en/tgp_10.pdf) prior to making decisions regarding uniformity. However, the following points are provided for elaboration or emphasis in this Technical Protocol:

4.2.2 The assessment of uniformity for hybrid varieties depends on the type of hybrid and should be according to the recommendations for hybrid varieties in the General Introduction.

4.2.3 In addition to the examination of the uniformity of the hybrid variety itself, uniformity of the parent lines should also be assessed.

Uniformity is assessed by visual observation and the detection of off-types.

4.2.4 The recommended sample size for the assessment of uniformity is indicated by the following key in the table of characteristics:

A sample size of 100 plants/parts of plants/ear rows
B sample size of 2000 plants or, in case of hybrids, 200 plants

4.2.5 For the assessment of uniformity in a sample of 2000 plants, a population standard of 0.3 % and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 2000 plants, 10 off-types are allowed.

4.26 For the assessment of uniformity in a sample of 100 ear-rows, plants or parts of plants, a population standard of 1 % and an acceptance probability of at least 95 % should be applied. In the case of a sample size of 100 ear-rows, plants or parts of plants, 3 off-types are allowed. An ear-row is considered to be an off-type ear-row if there is more than 1 off-type plant within that ear-row.

4.2.7 For "A" characteristics, with the exception of characteristics 2 and 3, the assessment of uniformity can be done in 2 steps. In a first step, 20 plants or parts of plants are observed. If no off-types are observed, the variety is declared to be uniform. If more than 3 off-types are observed, the variety is declared not to be uniform. If 1 to 3 off-types are observed, an additional sample of 80 plants or parts of plants must be observed.

4.2.8 For the assessment of uniformity of hybrid varieties, a population standard of 10% and an acceptance probability of at least 95% should be applied. In case of characteristics indicated by "B", the sample size for the assessment of uniformity may be reduced to 200 plants. In case of a sample size of 200 plants, 27 off-types are allowed. In case of a sample size of 100 ear-rows, plants or parts of plants, 15 off-types are allowed.

4.2.9 For all varieties except hybrid varieties, a re-submission of plant material may be allowed for the second growing cycle if in the first growing cycle the number of off-types did not exceed 18 plants in a sample size of 2000 plants (Population standard of 0.6% with an acceptance probability of ≥ 95%) or 9 plants, parts of plants or ear rows in a sample size of 100 (Population standard of 5% with an acceptance probability of ≥ 95%).

4.3 Stability

4.3.1 It is of particular importance for users of this Technical Protocol to consult the UPOV-General Introduction to DUS (link in chapter 1 of this document) and TGP 11 'Examining Stability' (http://www.upov.int/edocs/tgpdocs/en/tgp_11.pdf).

In practice, it is not usual to perform tests of stability that produce results as certain as those of the testing of distinctness and uniformity. However, experience has demonstrated that, for many types of variety, when a variety has been shown to be uniform, it can also be considered to be stable.

4.3.2 Where appropriate, or in cases of doubt, stability may be further examined by testing a new seed stock to ensure that it exhibits the same characteristics as those shown by the initial material supplied.

5. GROUPING OF VARIETIES AND ORGANIZATION OF THE GROWING TRIAL

5.1 The selection of varieties of common knowledge to be grown in the trial with the candidate varieties and the way in which these varieties are divided into groups to facilitate the assessment of distinctness are aided by the use of grouping characteristics.

5.2 Grouping characteristics are those in which the documented states of expression, even where produced at different locations, can be used, either individually or in combination with other such characteristics: (a) to select varieties of common knowledge that can be excluded from the growing trial used for examination of distinctness; and (b) to organize the growing trial so that similar varieties are grouped together.

5.3 The following have been agreed as useful grouping characteristics:

(a) Lower glume: hairiness on external surface (characteristic 12)

(b) Ear: scurs or awns (characteristic 17)

(c) Ear: colour (characteristic 19)

(d) Seasonal type (characteristic 27)

5.4 If other characteristics than those from the TP are used for the selection of varieties to be included into the growing trial, the EO shall inform the CPVO and seek the prior consent of the CPVO before using these characteristics.

6. INTRODUCTION TO THE TABLE OF CHARACTERISTICS

6.1 Characteristics to be used

The characteristics to be used in DUS tests and preparation of descriptions shall be those referred to in the table of characteristics. All the characteristics shall be used, providing that observation of a characteristic is not rendered impossible by the expression of any other characteristic, or the expression of a characteristic is prevented by the environmental conditions under which the test is conducted or by specific legislation on plant health. In the latter case, the CPVO should be informed.
The Administrative Council empowers the President, in accordance with Article 23 of Commission Regulation N°874/2009, to insert additional characteristics and their expressions in respect of a variety.

States of expression and corresponding notes
In the case of qualitative and pseudo-qualitative characteristics, all relevant states of expression are presented in the characteristic. However, in the case of quantitative characteristics with 5 or more states, an abbreviated scale may be used to minimize the size of the Table of Characteristics. For example, in the case of a quantitative characteristic with 9 states, the presentation of states of expression in the Test Guidelines may be abbreviated as follows:

State	Note
small	3
medium	5
large	7

However, it should be noted that all of the following 9 states of expression exist to describe varieties and should be used as appropriate:

State	Note
very small	1
very small to small	2
small	3
small to medium	4
medium	5
medium to large	6
large	7
large to very large	8
very large	9

6.2 Example Varieties

Where appropriate, example varieties are provided to clarify the states of expression of each characteristic.

6.3 Legend

For the CPVO N° column:

G	Grouping characteristic	– see Chapter 5
QL	Qualitative characteristic	
QN	Quantitative characteristic	
PQ	Pseudo-qualitative characteristic	
(+)	See Explanations on the Table of Characteristics in Chapter 8.2	

For the UPOV N° column:

The numbering of the characteristics is provided as a reference to the UPOV guideline.
(*) UPOV Asterisked characteristic – Characteristics that are important for the international harmonization of variety descriptions.

For the column "stage, method":

00 – 99	See Explanations on the Table of Characteristics in Chapter 8.3	
MG, MS, VG, VS	Method of observation	– see Chapter 4.1.5
A	Sample size 100	– see Chapter 4.2
B	Sample size 2000 (200 for hybrids)	– see Chapter 4.2
(a)	See explanations on the Table of Characteristics in chapter 8.1	

7. TABLE OF CHARACTERISTICS

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
1.	**1.**	**00**	**Seed: colour**			
(+)		**VG/ A**	white	Blini	Heroldo	1
PQ			reddish	Granary	Solehio	2
			purple		Indigo	3
			bluish		Skorpion	4
2.	**2.**	**00**	**Seed: coloration with phenol**			
(+)		**VG/A**	absent or very light		Bitop	1
QN			light	Lavett	Courtot	3
			medium	Sensas	SY Moisson	5
			dark	Granary	Antonius	7
			very dark	Lennox	Callobre	9
3.	**3.**	**09-11**	**Coleoptile: anthocyanin coloration**			
(+)		**VG/A**	absent or very weak	Cornetto	Rubisko	1
QN			weak	FD 1 24	Antonius	3
			medium	Specifik	Maxwell	5
			strong	Sensas	Zephyr	7
			very strong		Cellule	9
4.	**4.**	**25-29**	**Plant: growth habit**			
(+)	**(*)**	**VG/B**	erect			1
QN			semi erect	CH Campala	Callobre	3
			intermediate	Sensas	Apache	5
			semi prostrate	Feeling	Solehio	7
			prostrate		Stelarka	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
5.	**5.**	**47-51**	**Plant: frequency of plants with recurved flag leaves**			
(+)		**VG/B**	absent or very low		Genius	1
QN			low	Triso	Solehio	3
			medium	Specifik	Callobre	5
			high	Blini	Antonius	7
			very high	FD 1 24	Boryana	9
6.	**6.**	**49-60**	**Flag leaf: anthocyanin coloration of auricles**			
(+)		**VG/B**	absent or very weak	Triso	Soissons	1
QN			medium	Antille		2
			strong	LCS Star	Astardo	3
7.	**7.**		**Time of ear emergence**			
(+)	**(*)**	**MG/B**	very early	Badiel	Accor	1
QN			early	Sensas	Solehio	3
			medium	Granary	Genius	5
			late	Triso	Maxence	7
			very late		Luxaro	9
8.	**8.**	**60-65**	**Flag leaf: glaucosity of sheath**			
	(*)	**VG/B**	absent or very weak		Basilio	1
QN			weak	CH Campala	Saturnus	3
			medium	Faiza	Maxwell	5
			strong	Triso	Solehio	7
			very strong		Waximum	9
9.	**9.**	**60-65**	**Flag leaf: glaucosity of blade**			
(+)		**VG/B**	absent or very weak		Courtot	1
QN			weak	FD 1 24	Saturnus	3
			medium	Blini	SY Moisson	5
			strong	Lennox	Accor	7
			very strong		Waximum	9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
10.	**10.**	**60-69**	**Ear: glaucosity**			
	(*)	**VG/B**	absent or very weak		Soissons	1
QN			weak	KWS Charing	Callobre	3
			medium	Granary	Solehio	5
			strong	Specifik	Edgar	7
			very strong		Waximum	9
11.	**11.**	**60-69**	**Culm: glaucosity of neck**			
QN		**VG/B**	absent or very weak		Basilio	1
			weak	CH Campala	Soissons	3
			medium	Granary	Ronsard	5
			strong	Lennox	SY Moisson	7
			very strong		Waximum	9
12.	**12.**	**69-92**	**Lower glume: hairiness on external surface**			
	(*)	**VG/B**	absent	Triso	Soissons	1
QL G		**(a)**	present	Galera	Franz	9
13.	**13.**	**75-92**	**Plant: length**			
(+)	**(*)**	**MG/B**	very short		Courtot	1
QN			short	Lennox	Apache	3
			medium	FD 1 24	Solehio	5
			long		Lavett	7
			very long		Capo	9
14.	**14.**	**80/92**	**Straw: pith in cross sections**			
(+)	**(*)**	**VG/A**	thin	FD 1 24	SY Moisson	1
QN			medium	Granary	Vulcanus	2
			thick or filled	Olivart	Synchro	3

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
15.	**15.**	**80-92**	**Ear: density**			
(+)	**(*)**	**MS/B VG/B**	very lax			1
QN			lax	Lennox	Kranich	3
			medium	Granary	Solehio	5
			dense	Virgile	Cellule	7
			very dense			9
16.	**16.**	**80-92**	**Ear: length**			
(+)		**MS/B VG/B**	very short			1
QN			short	Granary	GK Berény	3
			medium	Sensas	Rubisko	5
			long	Specifik	Antonius	7
			very long		Edgar	9
17.	**17.**	**80-92**	**Ear: scurs or awns**			
(+)	**(*)**	**VG/B**	both absent	Gorda		1
QL			scurs present	Granary	Apache	2
G			awns present	Sensas	Solehio	3
18.	**18.**	**80-92**	**Ear: length of scurs or awns**			
(+)	**(*)**	**MS/B VG/B**	very short		Fidelius	1
QN			short	Tybalt	Apache	3
			medium	Triso	Boryana	5
			long	Granary	Courtot	7
			very long	FD 1 24	SY Moisson	9
19.	**19.**	**80-92**	**Ear: colour**			
(+)	**(*)**	**VG/B**	white	Granary	Solehio	1
QL G			coloured	Bastian	Sertori	2

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
20.	**20.**	**80-92**	**Ear: shape in profile**			
(+)		**VG/B**	tapering	Tybalt	Solveig	1
PQ			parallel sided	Granary	Solehio	2
			slightly clavate		Pierrot	3
			strongly clavate		Vulcanus	4
			fusiform	FD 1 24	Soissons	5
21.	**21.**	**80-92**	**Apical rachis segment: area of hairiness on convex surface**			
(+)		**VG/A**	absent or very small		Soissons	1
QN		**(a)**	small	Specifik	Solehio	3
			medium	Granary	Fidelius	5
			large	KWS Bittern	Kranich	7
			very large		Mv Bodri	9
22.	**22.**	**80-92**	**Lower glume: shoulder width**			
(+)		**VG/A**	absent or very narrow		Courtot	1
QN		**(a)**	narrow	Tybalt	Soissons	3
			medium	Sensas	Solehio	5
			broad	KWS Collada	Sosthene	7
			very broad			9
23.	**23.**	**80-92**	**Lower glume: shoulder shape**			
(+)		**VG/A**	strongly sloping	Amulett	Courtot	1
QN		**(a)**	slightly sloping	Tybalt	Solehio	3
			horizontal	Lennox	Solveig	5
			slightly elevated	Virgile	Sosthene	7
			strongly elevated			9

CPVO N°	UPOV N°	Stage, Method	Characteristics	Example Varieties Spring type	Example Varieties Winter type	Note
24.	**24.**	**80-92**	**Lower glume: length of beak**			
(+)		**MG/A MS/A VG/A**	very short		Solveig	1
QN		**(a)**	short	Tybalt	Kranich	3
			medium	Blini	Zephyr	5
			long	Sensas	Soissons	7
			very long	FD 1 24	Rubisko	9
25.	**25.**	**80-92**	**Lower glume: shape of beak**			
(+)	**(*)**	**VG/A**	straight	FD 1 24	Solveig	1
QN		**(a)**	slightly curved	Granary	Cellule	3
			moderately curved	Harenda	Edgar	5
			strongly curved		Sertori	7
			geniculate		Velocity	9
26.	**26.**	**80-92**	**Lower glume: area of hairiness on internal surface**			
(+)		**VG/A**	very small		Lupus	1
QN		**(a)**	medium	KWS Scirocco	Solehio	3
			very large	Lennox	Apache	5
27.	**27.**		**Seasonal type**			
(+)	**(*)**	**VG**	winter type		Solehio	1
PQ			alternative type		Buteo	2
G			spring type	Lennox		3

8. EXPLANATIONS ON THE TABLE OF CHARACTERISTICS

8.1 Explanations covering several characteristics

Characteristics containing the following key in the first column of the Table of Characteristics should be examined as indicated below:

(a) Characteristics on the lower glume must be observed on spikelets in the mid-third of the ear.

8.2 Explanations for individual characteristics

Ad. 1: Seed: colour

The seed colour should be observed on dry seeds or by using NaOH solution (seeds soaked for 10 minutes at 60°C or 60 minutes at room temperature in a 5M NaOH solution).

Ad. 2: Seed: coloration with phenol

The seed coloration with phenol cannot be observed on purple nor bluish seeds.

Method for Determination of Phenol Reaction:
Number of seeds per test:.....100 seeds. The seeds should not have been treated chemically.
Preparation of seeds:.....Soak in tap water for 16 to 20 hours, drain and remove surface water, place the seeds with crease downwards, cover dish with lid
Concentration of solution: .....1 per cent Phenol-solution (freshly made up)
Amount of solution: .....The seeds should be about 3/4 covered
Place: .....Laboratory
Light: Daylight .....- out of direct sunshine
Temperature: .....18 to 20°C
Time of recording: .....4 hours (after adding solution)
Note: .....At least two example varieties should be included as a control.
Any alternative method may be used if it gives the same results.

Ad. 3: Coleoptile: anthocyanin coloration

Method for the Determination of Anthocyanin Coloration
Number of seeds per test .....100 seeds
Preparation of seeds .....Set up non-dormant seeds on moistened filter paper covered with a Petri dish lid during germination.
Place .....Laboratory or greenhouse.
Light.....After the coleoptiles have reached a length of about 1 cm in the dark, they are placed in artificial light (daylight equivalent), at 13000 to 15000 lux continuously for 3-4 days.
Temperature .....15 to 20°C.
Time of recording .....Coleoptiles fully developed (about 1 week) at stage 09-11.
Note .....At least two of the example varieties should be included as a control.
Any alternative method may be used if it gives the same results.

Ad. 4: Plant: growth habit

The growth habit should be assessed visually from the attitude of the leaves and tillers. The angle formed by the outer leaves and the tillers with an imaginary vertical axis should be used.

1	3	5	7	9
erect	semi-erect	intermediate	semi-prostrate	prostrate

Ad. 5: Plant: frequency of plants with recurved flag leaves

1. (absent or very low): all flag leaves are rectilinear
3. (low):about 1/4 of the plants with recurved flag leaves
5. (medium): about 1/2 of the plants with recurved flag leaves
7. (high): about 3/4 of the plants with recurved flag leaves
9. (very high): all flag leaves are recurved

Ad. 6: Flag leaf: anthocyanin coloration of auricles

The appropriate scoring time between stages 49 and 60 should be determined depending on the location. All varieties should be assessed at the same stage.

Ad. 7: Time of ear emergence

Time of ear emergence is reached when the first spikelet is visible on 50% of ears.

Ad. 9: Flag leaf: glaucosity of blade

Observations should be made on the lower side of the blade.

Ad. 13: Plant: length

The length of plant includes stem, ear, awns and scurs.

Ad. 14: Straw: pith in cross section

Pith in cross section should be observed half way between base of ear and uppermost node. All stems of the plant should be checked and the highest score per plant recorded.

1
thin

2
medium

3
thick or filled

Ad. 15: Ear: density

The density is the ratio of the number of spikelets per ear length.

Ad. 16: Ear: length

Length of ear should be observed excluding awns and scurs.

Ad. 17: Ear: scurs or awns

Observations should be made at the tip of the ear.

1
both absent

2
scurs present

3
awns present

Ad. 18: Ear: length of scurs or awns

Cannot be observed on varieties with no scurs nor awns.
Observations should be made at the tip of the ear.

1
very short

3
short

5
medium

7
long

9
very long

Ad. 19: Ear: colour

White ear varieties may be slightly coloured due to environmental conditions.

Ad. 20: Ear: shape in profile

1
tapering

2
parallel sided

3
slightly clavate

4
strongly clavate

5
fusiform

Ad. 21: Apical rachis segment: area of hairiness of convex surface

1	3	5	7	9
absent or very small	small	medium	large	very large

Ad. 22: Lower glume: shoulder width

1	3	5	7	9
absent or very narrow	narrow	medium	broad	very broad

Ad. 23: Lower glume: shoulder shape

1	3	5	7	9
strongly sloping	slightly sloping	horizontal	slightly elevated	strongly elevated

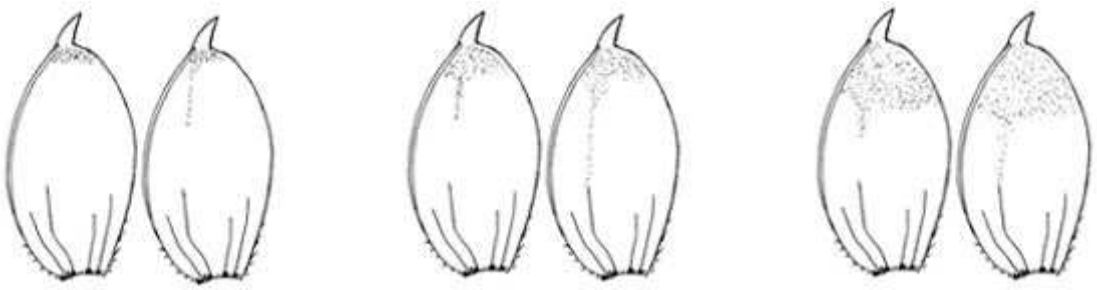
Ad. 24: Lower glume: length of beak

1	3	5	7	9
very short	short	medium	long	very long

Ad. 25: Lower glume: shape of beak

1	3	5	7	9
straight	slightly recurved	moderately curved	strongly curved	geniculate

Ad. 26: Lower glume: area of hairiness on internal surface



1	3	5
very small	medium	very large

Ad. 27: Seasonal type

The seasonal type (need of vernalization) should be assessed on plots sown in springtime. Example varieties should always be included in the trial. When the example varieties behave according to their descriptions, candidate varieties can be described. At the time when the latest spring type variety is fully mature (stage 91/92 of the Zadoks decimal code) growth stage reached by the respective variety should be assessed. The states of expression are defined as follows:

1- Winter type (high need of vernalization): the plants have reached stage 45 of the Zadoks decimal code (boots swollen) at maximum.
2- Alternative type (partial need of vernalization): the plants have exceeded stage 45 of the Zadoks decimal code (they should have normally exceeded stage 75) and have reached stage 90 at maximum.
3- Spring type (no need or very weak need of vernalization): the plants have exceeded stage 90 of the Zadoks decimal code.

8.3 Growth stages

Descriptions of the growth stages of the Zadoks decimal code for cereals (ZADOCKS et al., 1974)

Zadoks Decimal code	Description
00	Dry seed
01	Start of imbibition
03	Imbibition complete
05	Radicle emerged from seed
07	Coleoptile emerged from seed
09	Leaf just at coleoptile tip
10	First leaf through coleoptile
11	First leaf unfolded
12	2 leaves unfolded
13	3 leaves unfolded
14	4 leaves unfolded
15	5 leaves unfolded
16	6 leaves unfolded
17	7 leaves unfolded
18	8 leaves unfolded
19	9 or more leaves unfolded
20	Main shoot only
21	Main shoot only and 1 tiller
22	Main shoot only and 2 tillers
23	Main shoot only and 3 tillers
24	Main shoot only and 4 tillers
25	Main shoot only and 5 tillers
26	Main shoot only and 6 tillers
27	Main shoot only and 7 tillers
28	Main shoot only and 8 tillers
29	Main shoot only and 9 or more tillers
30	Pseudo stem erection
31	1st node detectable
32	2nd node detectable
33	3rd node detectable
34	4th node detectable
35	5th node detectable
36	6th node detectable
37	Flag leaf just visible
39	Flag leaf ligule/collar just visible
40	-
41	Flag leaf sheath extending
45	Boots just swollen
47	Flag leaf sheath opening
49	3First awn visible
50	First spikelet of inflorescence visible
53	¼ of inflorescence emerged
55	½ of inflorescence emerged
57	¾ of inflorescence emerged
59	Emergence of inflorescence completed
60	Beginning of anthesis
65	Anthesis half-way
69	Anthesis completed
70	-
71	Kernel watery ripe
73	Early milk
75	Medium milk
77	Late milk
80	-
83	Early dough
85	Soft dough
87	Hard dough
90	-
91	Kernel hard (difficult to divide with thumbnail)
92	Kernel hard (no longer dented with thumbnail)
93	Kernel loosening in daytime
94	Overripe, straw dead and collapsing

95	Seed dormant
96	Viable seed giving 50% germination
97	Seed not dormant
98	Secondary dormancy induced
99	Secondary dormancy lost

9. LITERATURE

Payne, P.I., and Lawrence, G.J., 1983: Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1 and Glu-D1 which code for the high-molecular-weight subunits of the glutenin in hexaploid wheat. Cereal Res. Commun., 11: pp. 29 to 35.

Zadoks, J. C., Chang, T. T. and Konzak, C. F., 1974: A decimal code for the growth stages of cereals. Weed Research, 14: pp. 415 to 421.

10. ELECTROPHORESIS

10.1 Introduction

The following Annex contains a list of characteristics based on storage proteins revealed by electrophoresis and a description of the method to be used. UPOV decided to place these characteristics in an Annex to the Test Guidelines, thereby creating a special category of characteristic, because the majority of the UPOV members is of the view that it is not possible to establish distinctness solely on the basis of a difference found in a characteristic based on storage protein markers revealed by electrophoresis. Such characteristics should therefore only be used as a complement to other differences in morphological or physiological characteristics. UPOV reconfirms that these characteristics are considered useful but that they might not be sufficient on their own to establish distinctness. They should not be used as a routine characteristic but at the request or with the agreement of the applicant of the candidate variety.

For the analysis of high molecular weight (HMW) glutenins, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS PAGE) should be used. Any alternative method may be used if it gives the same results. Glutenins are encoded by three compound loci, known as Glu-A1, Glu-B1 and Glu-D1 on the long arms of the group 1 chromosomes (Payne, 1987). There are a number of alleles at each locus and the analysis of HMW glutenins is based on the recognition of these alleles from proteins, which appear on gels as a series of well-defined bands or patterns of bands. The alleles are described by band numbers according to the definition given to them by Payne and Lawrence, 1983 (see Chapter IX, Literature). The corresponding letters and apparent molecular weights are reproduced in the description of the method used.

10.2 Characteristics derived by using electrophoresis

Number	Characteristic	Example Varieties	Note
28.	**Glutenin composition: allele expression at locus Glu-A1**		
	band 1	Meister	1
	band 2*	Sonett, Spontan	2
	no band	JB Asano	3
29.	**Glutenin composition: allele expression at locus Glu-B1**		
	bands 6 + 8	Meister	1
	bands 7 + 8	KWS Loft	2
	bands 7 + 9	Tobak	3
	band 7 (or 7 + 9 in the presence of bands 5 + 10 of char. Glu-D1)	JB Asano	4
	bands 13 + 16	Fanion, Ronsard	5
	bands 14 + 15	Atomic	6
	bands 17 + 18	Tabasco	7
	band 20	Ilias	8
	bands 6.1 + 22	Zollernspelz, Schwabenkorn	9
30.	**Glutenin composition: allele expression at locus Glu-D1**		
	bands 2 + 12	Tobak	1
	bands 3 + 12	Matrix	2
	bands 4 + 12	-	3
	bands 5 + 10	JB Asano	4

10.3 Description of methods used

Glutenin composition: allele expression at loci Glu-A1, Glu-B1 and Glu-D1

SDS PAGE Method for Analysis of HMW Glutenins from T. aestivum

1. Apparatus and equipment

Any suitable vertical electrophoresis system can be used, provided that the gels can be kept at a constant temperature. A gel thickness of no more than 1.5 mm is recommended. The power supply used should be capable of delivering both constant current and constant voltage output.

2. Chemicals

All chemicals should be of 'Analytical Reagent' grade or better.

Acrylamide (specially purified for electrophoresis)
Bisacrylamide (specially purified for electrophoresis)
Tris (hydroxymethyl) methylamine (TRIS)
Sodium dodecyl sulphate (SDS)
Ammonium persulphate (APS)
2-mercaptoethanol
TEMED (NNN'N'-tetramethylethylenediamine)
Trichloroacetic acid (TCA)
Hydrochloric acid
Glacial acetic acid
Glycine
n-Butanol
Pyronin Y (or G)
Glycerol (d = 1.256)
Methanol or ethanol
Coomassie Brilliant Blue R-250 (or equivalent)
Coomassie Brilliant Blue G-250 (or equivalent)

3. Solutions

3.1 Extraction solution

3.1.1 Extraction of glutenins only

Stock solution:

6.25 ml 1M TRIS HCl buffer, PH 6.8 (see 3.3.2)
12.05 ml distilled water
2g SDS
10 mg Pyronin Y (or G)
10 ml glycerol
This solution can be stored for two months at 4°C.

Immediately before use, extraction solution is prepared as follows:

4.25 ml stock solution (above) plus 0.75 ml 2-mercaptoethanol made up to 10.0 ml with distilled water. This solution must be prepared immediately prior to use and cannot be stored.

3.1.2 Extraction of glutenins following gliadins

Solution A - 25 ml 2 - chloroethanol + 50 mg Pyronin Y/G, made up to 100 ml with distilled water.
Solution B - 27.0 g urea, 3.0 ml 2 - mercaptoethanol + 10.0 g SDS, made up to 100 ml with distilled water.

3.2 Electrophoresis (running) buffer

Stock solution:

141.1 g glycine
30.0 g TRIS
10.0 g SDS
made up to 1 l with distilled water.
Immediately before use, the stock solution is diluted 1:10 with distilled water.

The stock buffer solution can be stored for 2 months at room temperature. Do not store the diluted buffer more than one week. The pH of the buffer must be close to 8.3.

3.3 Gel preparation solutions

3.3.1 Stock resolving gel buffer (1M TRIS HCl, pH 8.8)

121.14 g TRIS plus approximately 20 ml HCl (d = 1.19) made up to 1 l with distilled water. This buffer can be stored at 4°C for 2 months.

3.3.2 Stock stacking gel buffer (1M TRIS HCl, pH 6.8)

121.14 g TRIS plus approximately 78 ml HC1 (d = 1.19) made up to 1 l with distilled water. This buffer can be stored at 4°C for 2 months.

3.3.3 10% (w/v) SDS solution

10g of SDS dissolved in distilled water and made up to 100 ml. This solution can be stored at 4°C for 2 months. Prior to use, stir and heat gently to re-dissolve the SDS, if it comes out of solution.

3.3.4 1% (w/v) ammonium persulphate solution

1g of APS dissolved in distilled water and made up to 100 ml. This solution must be prepared immediately prior to use.

3.3.5 Stock acrylamide solution

40.02g acrylamide made up to 100 ml with distilled water.

3.3.6 Stock bisacrylamide solution

0.5198g bisacrylamide made up to 130 ml with distilled water.

3.4 Staining solutions

3.4.1 0.25g Coomassie Brilliant Blue G-250 plus 0.75g Coomassie Brilliant Blue R-250, made up to 100 ml with water.

3.4.2 55g TCA, 65 ml glacial acetic acid, 180 ml methanol or ethanol plus 25 ml solution 3.4.1, made up to 11 with distilled water.

4. Procedure

4.1 Protein extraction

4.1.1 Glutenins only

Individual seeds are ground using a hammer (or other device). Ground seed meal is mixed with diluted sample extraction buffer (3.1.1) in a 3 ml polypropylene hemolyse or similar tube with a screw-on or fitted cap. The ratio of meal/extraction buffer is 50 mg/0.75 ml. The samples are extracted for 2 hours at room temperature, mixed several times using a vortex mixer, heated in a boiling water bath for 10 minutes and then allowed to cool. The tubes are centrifuged at 18000g for 5 minutes.

4.1.2 Glutenins following gliadins

If desired, glutenins and gliadins can be analyzed from the same grain. Gliadins are extracted first by adding 0.25 ml of Solution A (3.1.2) to a crushed grain (or half-grain) in a microtiter plate or micro-centrifuge tube and incubating overnight at room temperature. Following this, glutenins are extracted by adding 0.5 ml of Solution B (3.1.2) to the crushed grain and incubating overnight at room temperature.

According to the gel thickness and the size of the wells, the volume of extract loaded can vary. Between 10 and 25 μl is usually sufficient.

4.2 Preparation of the gel

Clean and dry gel cassettes are assembled, according to the design of the equipment used. If tape is used to seal the cassettes, it is advisable to assemble them at least one day in advance of use, to enable the tape to 'age' and adhere better.

4.2.1 Resolving (main) gel (10% acrylamide, pH 8.8)

To make two slab gels of 180 x 160 x 1.5 mm, the following is required:

20 ml stock acrylamide solution (3.3.5)
26 ml stock bisacrylamide solution (3.3.6),
30 ml stock gel buffer (3.3.1).

These should be at room temperature. The mixture is degassed in a 100 ml Büchner flask for 2 - 3 minutes. To this is added:

2 ml APS (3.3.4),
0.8 ml SDS (3.3.3),
40 μl TEMED (use straight from bottle).

The gels are then carefully poured, avoiding the formation of air bubbles, and polymerization allowed to take place at room temperature.

The gel cassettes should not be filled entirely, in order to leave room for a 3-4 cm layer of stacking gel. The gel surface is carefully overlaid with n-butanol (or distilled water) using a syringe. When polymerization is finished (about 30 min.), the gel surface is carefully rinsed with distilled water and dried with filter paper.

4.2.2 Resolving (main) gel (7% acrylamide, pH 8.8)

To resolve the sub-units 2 and 2*, it is necessary to use main gels of 7% acrylamide concentration.

To make two slab gels of 180 x 160 x 1.5 mm, the following is required:

14 ml stock acrylamide solution (3.3.5)
6 ml distilled water
26 ml stock bisacrylamide solution (3.3.6),
30 ml stock gel buffer (3.3.1).

These should be at room temperature. The mixture is de-gassed in a 100 ml Büchner flask for 2 - 3 minutes. To this is added:

2 ml APS (3.3.4),
0.8 ml SDS (3.3.3),
40 μ TEMED (use straight from bottle).

The gels are then carefully poured, avoiding the formation of air bubbles, and polymerization allowed to take place at room temperature.

The gel cassettes should not be filled entirely, in order to leave room for a 3-4 cm layer of stacking gel. The gel surface is carefully overlaid with n/butanol (or distilled water) using a syringe. When polymerization is finished (about 30 min.), the gel surface is carefully rinsed with distilled water and dried with filter paper.

4.2.3 Stacking gel (3% acrylamide, pH 6.8)

In a 50 ml Büchner flask, mix:

1.50 ml stock acrylamide solution (3.3.5),
2.15 ml stock bisacrylamide solution (3.3.6)
2.50 ml stock gel buffer (3.3.2) and
13.15 ml distilled water.

Following de-gassing add:

0.75 ml APS (3.3.4),
0.2 ml SDS (3.3.3),
15 µl TEMED (straight from bottle)

Mix carefully and immediately pour the stacking gels to the top of the gel cassettes. Insert the well-forming "comb", avoiding air bubbles. Allow to polymerize for about 2 hours at room temperature. The "combs" are then removed carefully from the gel cassettes and the wells rinsed using diluted electrophoresis running buffer (3.2).

4.3 Electrophoresis

The tank is filled with the appropriate volume of running buffer (3.2), cooled to 15°C. Following sample loading, electrophoresis is carried out at a constant current of 8 mA/cm^2 (cross-sectional area) of gel until the pyronin Y/G has moved through the stacking gel, and then at 16 mA/cm^2 of gel (maximum voltage 300V) until the marker is at the bottom of the gel. The temperature should be maintained at 15°C.

4.4 Fixing and staining

The gel cassettes are removed from the tank, opened and the gels fixed in 250 ml of 15% (w/v) TCA for at least 30 minutes. The gels are rinsed in distilled water and stained overnight in 250 ml of staining solution (3.4.2) at room temperature. Destaining is not usually necessary but gels should be washed in distilled water before being stored in sealed polythene bags.

Other staining procedures can be successfully used (e.g. Coomassie Brilliant Blue G or equivalent in TCA alone). The final quality control criterion, both for gel preparation and gel staining, is to analyze the suggested example varieties on each batch of gels. The separation of the suggested bands, and their relative electrophoretic mobilities (molecular weights) must be clear in order for the procedures to be judged satisfactory.

5 Recognition of Glutenin Alleles

This Table is designed to illustrate the molecular weight of all of the glutenin bands from each locus.

Sub-Units of HMW Glutenins: nomenclature of the individual bands

Band number	**Molecular weight (kDa)**
1	113
2	108
2*	108
3	107
4	106
5	105
6	100
6.1	99
7	98
8	86
9	83
10	83
12	80
13	94
14	94
15	91
16	90
17	89.5
18	89.5
20	94
22	87

Characteristic: **Glu-A1** locus

		Note		
		1	2	3
1	(113)---	1---		
2/2*	(108)---		2*---	no band
3	(107)---			
4	(106)---			
5	(105)---			
6	(100)---			
6.1	(99)---			
7	(98)---			
13/14/20	(94)---			
15	(91)---			
16/17/18	(90/89.5)---			
22	(87)---			
8	(86)---			
9/10	(83)---			
12	(80)---			

Characteristic: **Glu-B1** locus

		Note								
		1	2	3	4	5	6	7	8	9
1	(113)---									
2/2*	(108)---									
3	(107)---									
4	(106)---									
5	(105)---									
6	(100)---	6---								
6.1	(99)---									6.1---
7	(98)---		7---	7---	7---					
13/14/20	(94)---					13---	14---		20---	
15	(91)---						15---			
16/17/18	(90/89.5)---					16---		17/18---		
22	(87)---									22---
8	(86)---	8---	8---							
9/10	(83)---			9---						
12	(80)---									

Characteristic: **Glu-D1** locus

		Note			
		1	2	3	4
1	(113)---				
2/2*	(108)---	2---			
3	(107)---		3---		
4	(106)---			4---	
5	(105)---				5---
6	(100)---				
6.1	(99)---				
7	(98)---				
13/14/20	(94)---				
15	(91)---				
16/17/18	(90/89.5)---				
22	(87)---				
8	(86)---				
9/10	(83)---				10---
12	(80)---	12---	12---	12---	

Note: Certain bands (e.g. bands 9 and 10) have similar molecular weights. This leads to the fact that in the presence of bands 5 + 10 of characteristic Glu-D1 two states of expression of characteristic Glu-B1, band 7 and bands 7 + 9, cannot be differentiated from one another. Therefore, in the presence of bands 5 + 10 of characteristic Glu-D1, note 4 of characteristic Glu-B1 could be either band 7 or bands 7 + 9. Other bands having similar molecular weights can be differentiated from one another by their known association with other bands. For characteristic Glu-B1, band 13 is always associated with band 16 and band 14 with band 15 while band 20 remains alone.

11. TECHNICAL QUESTIONNAIRE

The Technical Questionnaire is available on the CPVO website under the following reference: CPVO/TQ-003/5