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Extraction of DNA from Choline Chloride Feed Additive (CC) and from derived Pre-Mixes (PMCC)

and

Screening of CC and PMCC for (a) presence of rice and (b) presence of BT63

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Abstract

Choline Chloride 60% (CC) is a feed additive that is imported in significant quantities from China. In a number of cases GM rice, harbouring the event Bt63, has been found in imported CC batches but not in derived pre-mixes.

The Member States were asked to provide positive CC samples and derived pre-mixes to the EU-RL GMFF in order to allow the EU-RL GMFF establishing practical approaches to DNA extraction and the subsequent testing for presence of (a) rice and (b) the Bt63 rice event.

This technical note has been derived based on data provided from National Reference Laboratories (NRLs) and experience made by the EU-RL GMFF when re-testing eleven feed additive samples and eight pre-mixes samples (as of 16 May 2014).

It was found that the extraction of DNA from the feed additive Choline Chloride (CC) does not normally pose specific problems. It can be carried out following the available protocols or using available standard DNA extraction kits.

The extraction of DNA from derived pre-mixes (PMCC) is posing problems because of observed strong inhibition and the presence of DNA from additional sources than those present in the CC. The EU-RL GMFF has tried a number of protocols and extraction kits on PMCC samples. On this basis it is concluded that it is advisable to carefully purify the DNA, to verify the possible presence of inhibition effects and eventually to try to reduce any inhibition observed.

Concerning testing the extracted DNA for rice and Bt63 rice event presence, this does not pose a problem in case of the CC while for PMCC rice was not anymore detectable or was detected in trace amounts. A possible explanation could be that much of the extracted DNA is from other plants than rice and hence the concentration of rice DNA is below the LOD of the rice-taxon specific method. The EU-RL GMFF has analysed the available rice (taxon-) specific methods by means of bio-informatics and (partly) in the laboratory and concludes that they all should be suitable for specifically and reliably detecting rice from the *Oryza sativa* species.

The re-testing by the EU-RL GMFF of CC and PMCC samples has in all cases, except one, confirmed the results of the initial tests.



EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit



**EU-RL GMFF Technical Note
concerning**

**Extraction of DNA from Choline Chloride Feed Additive
(CC) and from derived Pre-Mixes (PMCC)
and
Screening of CC and PMCC for (a) presence of rice and (b)
presence of Bt63**

28 May 2014

European Union Reference Laboratory for GM Food and Feed

Executive Summary

Choline Chloride 60% (CC) is a feed additive that is imported in significant quantities from China. In a number of cases GM rice, harbouring the event Bt63, has been found in imported CC batches but not in derived pre-mixes.

The Member States were asked to provide positive CC samples and derived pre-mixes to the EU-RL GMFF in order to allow the EU-RL GMFF establishing practical approaches to DNA extraction and the subsequent testing for presence of (a) rice and (b) the Bt63 rice event.

This technical note has been derived based on data provided from National Control Laboratories and experience made by the EU-RL GMFF when re-testing nine feed additive samples and ten pre-mixes samples.

The EU-RL GMFF found that the extraction of DNA from the feed additive Choline Chloride (CC) does not normally pose specific problems. It can be carried out following the available protocols or using available standard DNA extraction kits.

The extraction of DNA from derived pre-mixes (PMCC) is, however, posing problems because of observed strong inhibition and the presence of DNA from additional sources than those present in the CC. The EU-RL GMFF has tried a number of protocols and extraction kits on PMCC samples. On this basis it is concluded that it is advisable to carefully purify the DNA, to verify the possible presence of inhibition effects and eventually to try to reduce any inhibition observed.

Concerning testing the extracted DNA for rice and Bt63 rice event presence, this does not pose a problem in case of the CC while for PMCC rice was not anymore detectable or was detected in trace amounts. In any case testing for Bt63 presence was impossible. A possible explanation could be that much of the extracted DNA is from other plants than rice and hence the concentration of rice DNA is below the LOD of the rice-taxon specific method. In order to verify this, the EU-RL GMFF has analysed the available rice (taxon-) specific methods by means of bio-informatics and (partly) in the laboratory and concludes that they all should be suitable for specifically and reliably detecting rice from the *Oryza sativa* species.

The re-testing by the EU-RL GMFF of CC and PMCC samples has confirmed the results of the initial tests.

EU-RL GMFF Technical Note concerning extraction of DNA from Choline Chloride Feed Additive (CC) and from derived Pre-Mixes (PMCC) and Screening of CC and PMCC for (a) presence of rice and (b) presence of Bt63

1. Introduction and Background

This technical note has been prepared taking account of the data received from official control laboratories that reported detection of Bt63 in Chlorine Chloride 60% but not in derived pre-mixes, and data generated by the EU-RL GMFF when re-testing those samples of Chlorine Chloride 60% and derived pre-mixes that were provided.

1.1. Choline Chloride alerts

Since early 2014, companies and national control laboratories have found traces of Bt63 rice in a significant number of choline chloride 60% corn cob feed additive consignments from China. While numerous findings in the feed additive were notified to RASFF by different Member States of the EU, Bt63 rice was never reported in pre-mixes prepared with positive Choline Chloride batches.

1.2. Legal situation

Bt63 rice is un-authorized in the EU and in China.

1.3. Detection

As a first step, taxon specific methods should be used to detect rice in the feed additive Choline Chloride 60% (CC) and, if detected, in pre-mixes derived from positive-tested choline chloride batches (PMCC). In case of rice being present, the samples from the CC and the PMCC should also be tested for Bt63.

2. DNA extraction

Using the available PCR-based detection methods does require DNA of suitable quality and quantity to be extracted from the test samples (CC or PMCC).

2.1. General considerations – existing ENGL guidance

ENGL guidance “Definition of minimum performance requirements for analytical methods of GMO testing” (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>) describes acceptance criteria for DNA extracted from food and feed matrices. The forthcoming ENGL guidance on sample preparation (expected to be published on the EU-RL GMFF website <http://gmo-crl.jrc.ec.europa.eu/default.htm> in June 2014) explains how to derive representative analytical samples from a laboratory sample provided by the inspection service.

2.2. DNA extraction from Choline Chloride Feed Additive

The fact that an increasing number of control laboratories have been able to identify Bt63 in CC samples proves that it is possible to extract DNA of sufficient quantity and quality from that matrix. The EU-RL GMFF has in the meantime re-tested a number of samples provided by national reference laboratories (NRLs) and found that for extracting DNA from CC samples, the CTAB-based protocol, with the slight modification described in Annex 1, works well.

The performance of the DNA extraction method should be controlled as provided for in the ENGL document “Definition of minimum performance requirements for analytical methods of GMO testing” (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

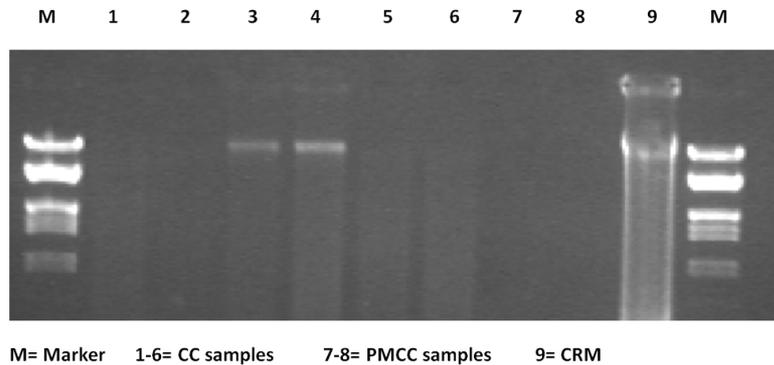
According to this guidance, DNA extracts should be assessed, if necessary, to determine DNA concentration, fragmentation state and absence of PCR inhibitors.

Given the nature of the CC samples and the experience gained by the EU-RL GMFF, the following should be taken into consideration by the testing laboratories:

2.2.1. DNA fragmentation state

Given the low concentration of CC extracts, the analysis of fragmentation state is not always meaningful. Depending on the DNA extraction method and the CC sample characteristics, DNA can appear intact or fragmented on agarose gel (Figure 1).

Figure 1. Gel electrophoresis of CC and PMCC samples



2.2.2. DNA concentration

Two approaches for determining concentrations of DNA extracts are routinely applied in molecular biology and GMO testing laboratories: UV readings of DNA extracts (based on absorbance properties of nucleic acids) and fluorometric measurement (based on light emission of dye intercalating in the DNA double strand via a dedicated calibration curve, e.g. PicoGreen[®] test).

Based on the experience with extracting DNA from CC samples, the EURL GMFF concludes that the UV-based readings and fluorometric estimates of DNA concentration in extracts from CC samples can differ significantly. There seems to be a trend for UV readings to indicate higher concentrations than fluorometric estimates. That difference - which can be up to 3-8 times the fluorometric based - mainly depends on the principles of the two methodologies, the degree of DNA fragmentation, and the presence of co-extracted compounds.

For example, in one case fluorometric estimation of the DNA concentration achieved with the above indicated modified CTAB method was 1-10 ng/ul while the estimation via UV readings (Nanodrop) was 3-36 ng/ul.

Therefore, given the degree of uncertainty provided by differing UV and fluorometric estimates when applied to DNA from CC samples, the appropriate amount of DNA extract (expressed in microliters) required by the downstream PCR application should always be used to test for the presence of the target-rice sequence and the target Bt63 sequence, regardless the estimation of the concentration of total DNA extracted.

An alternative solution is the estimation of the target DNA via calibration curve with a reference material using a validated real-time PCR method. The latter test can be of appreciable interest to determine the practical LOD of the CC sample.

2.2.3. Absence of PCR inhibitors

This test should be conducted according to the indications provided in the ENGL guidance document “Verification of analytical methods for GMO testing when implementing inter-laboratory validated methods” (<http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm>).

If the estimated DNA yield is very low (e.g. below 5-10 ng/μl), a simplified procedure for testing presence/absence of inhibitors can be applied, consisting in testing the DNA extract in qPCR with the rice-specific reference system of choice on a dilution series of the DNA extract by loading the sample undiluted (1:1) and diluted (e.g. 1:4 and 1:16). A ΔC_q of 2 should be measured from one dilution point to the next. Given that there is no agreed acceptance criteria, this approach should be limited to cases where very little amount of DNA is extracted and proved insufficient to test in accordance to the ENGL guidance document.

2.3. DNA extraction from pre-mixes

For the extraction of DNA from pre-mixes the same finding and recommendation prevail but the situation is further aggravated by the fact that the total rice DNA (GM and non-GM) becomes an even smaller fraction of the total DNA of a given sample because the pre-mix frequently contains other plant material. Due to the presence of too much non-rice DNA, it is likely that the practical LOD of a rice detection method in general and of the Bt63 construct specific method in particular (absolute LOD of the latter is around 5 copies per experiment) is significantly higher than assumed.

2.4. Result of DNA extraction experiments of the EU-RL GMFF

2.4.1. Choline Chloride Feed Additive

DNA could be extracted but the standard quality control (PicoGreen[®], Nanodrop) seemed to indicate low yields and low DNA quality and gave sometimes very different results (see above). However, when five microliters of such extracts were analysed with the rice-specific reference system (PLD), C_q values of 24-27 were measured, clearly indicating the presence of rice.

Therefore, it was found that the extracted DNA was still sufficient for running the required rice-specific assay and, if rice was found, the Bt63 specific one. Whenever this was the case, the findings of the control laboratories that initially tested the samples were confirmed.

2.4.2. Pre-mixes

DNA extraction from pre-mixes was found to be prone to the same quality and quantity issues as extraction from the feed additive, aggravated, as expected, by the fact that DNA extracted from PMCC samples was also from other species (e.g. soybean, maize). Therefore, also the EU-RL GMFF could not detect any rice DNA, and subsequently Bt63, in the PMCC samples it re-analysed.

2.5. Conclusion concerning DNA extraction from CC and PMCC

Testing laboratories should use the DNA extraction procedures they normally use for matrices similar to the Choline Chloride feed additive (CC) or the derived pre-mixes (PMCC) but the EU-RL GMFF found that the slightly modified CTAB protocol attached as Annex 1 worked properly. Standard DNA quality control methods should be applied but might provide very different estimates and indicate very low yields and quality of the extracted DNA, apparently due to confounding factors linked to the nature of the samples. It is therefore advised in any case to use the extracted DNA for the intended PCR experiments, together with a positive control, if available. However, for Bt63 GM-rice the EU-RL GMFF is currently not in the position to provide any positive control, unless new material is provided by the Chinese Authorities.

3. Detection of Rice DNA and Bt63 in CC and PMCC samples

Note: the following is based on the assumption that DNA is extracted (see above) from the CC or the PMCC that is of sufficient quality to run the required PCR experiment, e.g. it indicates presence of rice DNA at Cq values below 30.

Note: Neither sampling nor the processing of the laboratory samples into the analytical samples is addressed in this technical note. It is assumed that the analytical sample is representative of the sampled consignment.

3.1. Available methods

3.1.1. Taxon specific methods for detection of Rice DNA

The EU-RL GMFF carried out a bio-informatics analysis by establishing the expected performance and specificity of 3 taxon-specific methods that are available in the GMOMethods database, run by the EU-RL GMFF (<http://gmo-crl.jrc.ec.europa.eu/gmomethods/>), by carrying out *in silico* PCR against all available rice genomes (Annex 2). It was concluded that all methods should detect the *Oryza sativa* varieties for which genome sequences were available, and also most of the other available *Oryza* genomes, with the remarkable exception of *Oryza brachyantha*, that would not be recognised by any of the methods.

Experimental test verification of a PLD based method, carried out by the EU-RL GMFF in the context of another project, confirms that this target gene is indeed very reliable and should detect most, if not all, of the commercialised *Oryza sativa* rice varieties. The PLD system that was used by the EU-RL GMFF to test the CC and PMCC samples provided, was validated by the EU-RL GMFF¹.

3.1.2. Construct specific method for detection of the GM-event Bt63

A construct-specific method for detection of Bt63 rice was fully validated by the German Federal Office of Consumer Protection and Food Safety (BVL), and verified by the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/summaries/Bt63_Rice_verification_report_final.pdf). It is also annexed to the ISO standard ISO 21569:2011². The absolute LOD is 5 copies, i.e. it gives a positive signal whenever 5 or more copies of the target amplicon are in the reaction. This might be problematic to achieve if the dilution, e.g. in a PMCC by other DNA, is too strong, which would explain why no Bt63 GM-rice can be detected in a pre-mix prepared with a CC known to contain (traces of) Bt63 GM rice. It is therefore important to load into each reaction the maximum amount of DNA possible.

3.1.3. Results achieved by the EU-RL GMFF

The EU-RL GMFF re-tested a number of CC and PMCC samples, received from National Control Laboratories, and established that the available taxon specific methods and the construct specific method for Bt63 work sufficiently well for DNA extracted from samples taken from the Choline Chloride feed additive (CC). For PMCC the DNA extraction issues (see above) create the expected problems and Bt63 could not be found in any of the samples tested so far, even if it was known that the pre-mixes were prepared from CC batches that had been tested positive for Bt63 GM-Rice.

3.2. Conclusion concerning detection of rice and Bt63 in CC or PMCC

Once adequate DNA is extracted for the sample taken from a batch of CC or PMCC, the available taxon-specific methods will work and rice DNA can be detected if present. The SOP for the 3 methods listed in the GMOMethods database of the EU-RL GMFF can be found there.

The same holds true for detecting the Bt63 GM-event. If at least 5 copies are in the reaction, the available method (QL-CON-00-009) will give a positive signal, if correctly carried out (<http://gmo-crl.jrc.ec.europa.eu/gmomethods/entry?db=gmometh&id=ql-con-00-009&q=bt63>).

¹ QT-TAX-OS-017. EU Database of Reference Methods for GMO Analysis, <http://gmo-crl.jrc.ec.europa.eu/gmomethods/>

² ISO 21569:2011. C7

The re-testing by the EU-RL GMFF of CC and PMCC samples has confirmed the results of the initial tests.

For the EU-RL GMFF



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Date: 28 May 2014

ANNEX 1

SOP

DNA Extraction - CTAB

Note

The present SOP was applied for extraction of DNA from CC and PMCC samples with the following modifications for increased DNA yield:

- a) At Step 1 of the DNA extraction procedure 150 mg were processed instead of 100 mg**
- b) At Step 18, the upper layers from two samples were pooled in a reaction tube**
- c) At Step 24, the pellet was dissolved in 50 µl 0.2x TE buffer**

European Commission - Directorate General Joint Research Centre

Institute for Health and Consumer Protection
Molecular Biology and Genomics Unit

Standard Operating Procedure

DNA Extraction - CTAB [RNase A + Proteinase K]

Revision	Author	Reviewer	QS Agreement	Approver
3	A. Bogni	A. Bogni	S. Cordeil	M. Mazzara
Document history				
Revision	Date	Drafted by	Comments	
3	31/01/2012	A. Bogni	<i>Changes in Paragraph 6, point 14)15 minutes. In Sub-paragraph 7.3 the record sheet number to be used for preparation of buffers is added.</i>	
2	14/04/2011	D. Charels	<i>Annual Check: addition of title page to document, update in sections 2, 3 and 7, numbering of protocol in section 6, reference to RU120B4 introduced.</i>	
a	30/01/2009	M. Mazzara	Document put in the system at MBG Level	
{a}	09/01/2006	M. Mazzara	{S40B6 in the CRL-GMFF quality system}	

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1. Scope and Field of Application

This method is used to extract and purify genomic DNA from a wide variety of matrices, including plant seed and grain materials. Application of the method to complex matrices may require adaptation.

2. Terms and definitions:

SO = Scientific officer; STSO = Scientific and technical support officer; QM = Quality Manager; CGL = Competence Group Leader EURL-GMFF

3. Abbreviations:

CTAB: Cetyltrimethylammonium Bromide.
 DNA: Desoxiribonucleic acid
 EDTA: Ethylenediaminetetraacetic acid disodic salt
 Tris: Tris[hydroxymethyl] aminomethane

4. Equipment

Water bath or heating block
 Microcentrifuge (Eppendorf or equivalent):
 Micropipettes
 Vortexer
 Microcentrifuge tubes
 Rack for reaction tubes
 Vinyl or latex gloves
 Vacuum device to dry DNA pellets
 Mortar and pestle

NOTE:

Plastic ware has to be sterile and free of DNAses, RNases and nucleic acids. Filter pipette tips protected against aerosol should be used.

5. Reagents

2 % CTAB-buffer
 0.5 % CTAB-precipitation solution
 NaCl 1.2 M
 Ethanol-solution 70% (v/v)
 Chloroform (99% at least)
 Isopropanol (99,7% at least)
 Sterile distilled water
 RNase A solution 10 mg/ml
 Proteinase K solution 20 mg/ml
 Liquid nitrogen

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6. Procedure

PRELIMINARY REMARKS:

The procedure requires sterile conditions. Contamination may be avoided during sample preparation by using single-use equipment, decontamination solutions and by avoiding the formation of dust.

During all steps of DNA preparation, wear gloves and change gloves regularly. Whenever contamination of the gloves could have occurred, use a new pair of gloves.

Never vortex genomic DNA. Vortexing of genomic DNA can result in degradation (shearing) of the DNA.

Fresh samples (e.g. leaf tissue) should be finely ground with mortar and pestle in liquid nitrogen before extraction; dry samples (e.g. flour, biscuits, corn flakes) are moistened with the threefold amount of water (w/v).

Fill out RU120B4 when applying the procedure described below.

- 1) Transfer 100 mg of a homogeneous sample into a sterile 1.5 ml reaction tube.
- 2) Add 300 µl of sterile deionised water; mix with a loop if necessary.
- 3) Add 500 µl of CTAB-buffer; mix with a loop.
- 4) Add 20 µl of Proteinase K solution (20 mg/ml), mix and incubate at 65°C for 30-60 min
- 5) Add 20 µl of RNase solution (10 mg/ml), mix and incubate at 65° C for 5-10 min.
- 6) Centrifuge for 10 min at 13000 rpm
- 7) Transfer supernatant to a tube containing 500 µl chloroform; mix completely by inverting the tubes several times for 30 sec.
- 8) Centrifuge for 10 min at 13000 rpm until phase separation occurs.
- 9) Transfer 500 µl of upper layer into a new tube containing 500 µl chloroform; mix completely by inverting the tubes several times for 30 sec.
- 10) Centrifuge for 5 min at 13000 rpm.
- 11) Transfer upper layer to a new tube.
- 12) Add 2 volumes of CTAB precipitation solution, mix completely by pipetting.
- 13) Incubate for 60 min at room temperature.
- 14) Centrifuge for 15 min at 13000 rpm. Discard the supernatant.
- 15) Dissolve precipitate in 350 µl NaCl (1.2 M).
- 16) Add 350 µl chloroform; mix completely by inverting the tubes several times for 30 sec.
- 17) Centrifuge for 10 min at 13000 rpm until phase separation occurs.
- 18) Transfer upper layer to a new reaction tube.
- 19) Add 0.6 volumes of isopropanol, mix gently and completely by inverting the tube.

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- 20) Centrifuge for 10 min at 13000 rpm. Discard the supernatant.
- 21) Add 500 µl of 70% ethanol solution, mix gently and completely by inverting the tube.
- 22) Centrifuge for 10 min at 13000 rpm. Discard the supernatant.
- 23) Dry pellets in vacuum for 10 min at 30 °C
- 24) Dissolve dry pellet in 100 µl 0.2x TE buffer.

The DNA solution may be stored in a refrigerator for a maximum of two weeks, or in the freezer at -20°C for longer periods.

7. Procedure Buffer preparation and required reagents

These buffers/solutions are required the CTAB DNA extraction procedure.

7.1 Equipment

Weighting equipment
 pH meter
 Heating stirrer.
 Autoclave
 Pipets and micropipets.

7.2 Reagents.

CTAB: Cetyltrimethylammonium Bromide (Ultrapure grade)
 Tris: Tris[hydroxymethyl] aminomethane (Molecular Biology grade) or 1 M Tris-HCl pH 8.0 (e.g. Sigma)
 EDTA: Ethylenediaminetetraacetic acid (titration 99.9%)
 Ethanol (96% at least)
 NaCl (99% at least)
 NaOH (98% at least, anhydrous)
 Distilled sterile water
 RNase A (Molecular Biology grade)
 Proteinase K (Molecular Biology grade)

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7.3 Formulations of buffers and solutions

- **2 % CTAB-buffer, 200ml (record to be used: RU300B4)**

20 g/l CTAB	4 g
1.4 M NaCl	16.4 g
0.1 M Tris*	3.15 g
20 mM Na ₂ EDTA	1.5 g

Alternatively 20 ml of a 1 M ready-to-use solution of Tris-HCl pH 8.0 can be used.

- add 100 ml of distilled water.
- adjust pH to a value of 8.0 with 1M NaOH.
- fill up to 200 ml and filter sterilise.
- store buffer at room for 12 months.

- **0.5 % CTAB-precipitation solution (RU301B4)**

5 g/l CTAB	1 g
0.04 M NaCl	0.5 g

- add 100 ml of distilled water.
- adjust pH to a value of 8.0 with 0.1 M NaOH.
- fill up to 200 ml and filter sterilise.
- store solution at 4° C for max. 6 months.

- **NaCl 1.2 M (RU302B4)**

- dissolve 7.01 g of NaCl in 100 ml distilled water.
- autoclave and store at room temperature.

- **Ethanol-solution 70 % (v/v) (RU303B4)**

70 ml of pure ethanol are mixed with 30 ml of distilled sterile water.

- **NaOH 1M (RU403B4)**

Dissolve 2 g of NaOH in 50 ml of sterile water.

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- *NaOH 0.1M (RU350B4)*

Dilute 10 ml of NaOH 1M in 90 ml of sterile water.

- *RNase A 10 mg/ml (RU315B4)*

a. Dissolve the RNase A at a final concentration of 10 mg/ml in sterile water.

b. If required from RNase A preparation supplier: boil the RNase A solution at 95°C for 15' in order to remove any residual nuclease activity.

- *Proteinase K 20 mg/ml (RU321B4)*

Dissolve the Proteinase K at a final concentration of 20 mg/ml in sterile distilled water according to the supplier's specifications.

- *Ready-to-use 1x Tris-EDTA buffer solution pH 8.0*

A ready-to-use 1x TE solution BioUltra, for molecular biology, pH 8.0 can for example be purchased from Fluka.

- *0.2x TE buffer (RU350B4)*

TE 0.2x buffer is obtained through five-fold dilution of the 1x TE buffer.

8. References

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ANNEX 2

**Bioinformatics analysis of reactivities of rice-reference systems
against *Oryza* species and other taxa**

		QT-TAX-OS-17 (PLD)	QL-TAX-OS-003 (GOS9)	QT-TAX-OS-003 (SPS)
<i>Oryza sativa</i>	<i>O. sativa indica</i> 9311	1	3	1
	<i>O. sativa indica</i> Pa64	2	2	1
	<i>O. sativa japonica</i> Nipponbare IRGSP5	1	2	1
	<i>O. sativa japonica</i> Syngenta	1	1	1
Other <i>oryza</i> species	<i>O. barthii</i> (AA genome) Africa	1	1	1
	<i>O. brachyantha</i> (AA genome)	0	0	0
	<i>O. glaberrima</i> (African rice) Africa	1	1	1
	<i>O. glumaepatula</i> (AA genome)	1	2	1
	<i>O. meridionalis</i> (AA genome) Australia	1	1	0
	<i>O. nivara</i> (AA genome) India	1	1	1
	<i>O. punctata</i> (BB genome)	1	0	0
Other plants	<i>Aegilops tauschii</i>	0	1*	0
	<i>Triticum aestivum</i>	0	1*	0
	<i>Panicum vulgare</i>	0	0	1*
		* >=3 mismatches and/or gaps		

Green cells represent *in silico* predicted amplifications; figures within cells indicate the expected number of target amplicons per species.

Orange cells represent possible *in silico* amplifications with predicted number of mismatches.

Red cells represent absence of *in silico* predicted amplification.

Bioinformatics analysis conducted at MBG Unit do not predict amplification for: *Arabidopsis thaliana*, *Amborella trichopoda*, *Brachypodium distachyon*, *Brassica rapa*, *Chlamydomonas reinhardtii*, *Cyanidioschyzon merolae*, *Elaeis oleifera*, *Glycine max*, *Hordeum vulgare*, *Medicago truncatula*, *Physcomitrella patens*, *Populus trichocarpa*, *Prunus persica*, *Selaginella moellendorffii*, *Setaria italic*, *Solanum lycopersicum*, *Solanum tuberosum*, *Sorghum bicolor*, *Triticum urartu*, *Vitis vinifera*, *Zea mays*, *Beta vulgaris*, *Betula nana*, *Cajanus cajan* Asha, *Cannabis sativa*, *Citrullus lanatus*, *Cucumis mela*, *Cucumis sativus*, *Gossypium raimondii* BGI-CGP, *Hevea brasiliensis*, *Lotus japonicum*, *Panicum virgatum*, *Phoenix dactylifera*, *Phyllostachys heterocycla*, *Prunus mume*, *Pyrus bretschneideri*

Other *Oryza* species not yet sequenced: *O. alta*, *O. australiensis*, *O. coarctata*, *O. eichingeri*, *O. grandiglumis*, *O. granulata*, *O. latifolia*, *O. longiglumis*, *O.*

longistaminata (long-staminate rice), *O. malampuzhaensis*, *O. meyeriana*, *O. minuta*, *O. neocaledonica*, *O. officinalis*, *O. perennis*, *O. rhizomatis*, *O. ridleyi*, *O. rufipogon* (common wild rice), *O. schlechteri* + some crosses of them (i.e. *O. nivara* x *O. sativa*, considered as amphidiploids genomes)

QT-TAX-OS-017	Quantitative PCR method for detection of rice Phospholipase D alpha 2 gene (Mazzara et al., 2006). Targets:PLD<--->PLD
QL-TAX-OS-003	Qualitative PCR method for detection of rice root-specific GOS9 gene Targets:GOS9<--->GOS9
QT-TAX-OS-003	Quantitative PCR method for detection of rice sucrose-phosphate synthase gene (Jiang et al., 2009). Targets:SPS<--->SPS

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Title:

Extraction of DNA from Choline Chloride Feed Additive (CC) and from derived Pre-Mixes (PMCC)

and

Screening of CC and PMCC for (a) presence of rice and (b) presence of BT63

Author(s): Maria Grazia Sacco, Francesco Gatto, Valentina Paracchini, Elena Scaravelli, Elena Nardini, Cristian Savini,
Mauro Petrillo, Alexandre Angers, Marco Mazzara, Joachim Kreysa

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