GCMTI RD-1:2022

GCMTI method publications

Identification of Bulbus Fritillariae Ussuriensis – a common adulterant found in Bulbus Fritillariae Cirrhosae by qualitative real-time polymerase chain reaction method

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Safety precautions: *This method involves the use of hazardous materials. It is the user's responsibility to apply appropriate precaution when handling such materials. Use eye and hand protection and where necessary carry out the work in a fume cupboard or biological safety cabinet.*

1 Introduction

1.1 This method describes the identification of Bulbus Fritillariae Ussuriensis (Ping bei mu) in the presence of Bulbus Fritillariae Cirrhosae (Chuan bei mu) by qualitative real-time PCR assays. Bulbus Fritillariae Ussuriensis is a common adulterant found in Fritillariae Cirrhosae Bulbus. According to the Chinese Pharmacopoeia (edition 2020), the source of Bulbus Fritillariae Ussuriensis is the dried bulb of *Fritillaria ussuriensis* Maxim. The source of Bulbus Fritillariae Cirrhosae is the dried bulb of 6 *Fritillaria* species *Fritillaria cirrhosa* D. Don, *Fritillaria unibracteata* Hsiao et K.C. Hsia, *Fritillaria przewalskii* Maxim, *Fritillaria delavayi* Franch, *Fritillaria taipaiensis* P. Y. Li and *Fritillaria unibracteata* Hsiao et K. C. Hsia var. *wabuensis* (S. Y. Tang et S. C. Yue) Z. D. Liu, S. Wang et S. C. Chen.

1.2 A test protocol is included in this method and the annexes as reference for ease of operation. The analytical processes of this method include the following steps: 1. DNA extraction; 2. Amplification and detection of target DNA region for differentiation by real-time PCR; and 3. data analysis.

2 Reagent and materials

All chemicals or reagents involved shall be of molecular biology or PCR grade and suitable for DNA analysis as a rule. The water used for the analysis shall be double distilled or PCR grade water free from nuclease and nucleic acids. Chemicals, reagents and water shall be autoclaved prior to use, if feasible. Operator shall wear powder-free gloves for all operations. The use of

aerosol-protected pipette tips as protection against cross contamination is recommended.

Test reagents and materials for the method are listed for reference in the Supplementary Information.

3 Apparatus

General laboratory equipment and apparatus for this method are listed in the Supplementary Information. Critical equipment in the entire analytical processes are as follows:

3.1 **Laminar flow hood**, to provide unidirectional flow of HEPA-filtered air over the work area to prevent contamination.

3.2 **Temperature controlled incubator**, to provide experimental condition for nucleic acid extraction.

3.3 **Bench top microcentrifuge**, to spin sample at high speed for nucleic acid extraction.

3.4 **Spectrophotometer**, to estimate the purity and yield of double-stranded DNA.

3.5 **Real-time PCR system**, for PCR amplification of DNA and detection of amplified PCR product.

4 General procedure

The test protocol described in Annex A and the Supplementary Information is for reference purpose only. The selection and suitability of method is an experience-based choice of the operator, taking into account the scope and given matrices. It is recommended that the operator shall make reference to relevant international/ national standards if available. To guarantee the validity of test results, controls shall be run in parallel with test samples as described in Clause 5, where relevant, in order to detect DNA contamination of the analytical reagents or sample-to-sample contamination.

4.1 Preparation of test portion

Operator responsible for preparation of test samples, such as reducing particle size, homogenization, and taking test portion, shall have good laboratory practices and cross-contamination avoidance to preclude invalid test results.

4.2 DNA extraction

4.2.1 Acquisition of amplifiable DNA is a prerequisite for DNA method. The process in general can either be carried out by using a commercial DNA isolation kit or reagents prepared in laboratory. Extraction methods validated by GCMTI are stipulated in Annex A for reference. Using commercial kits are much easier for quality control of extraction process and save time for troubleshooting as the quality of the products is well controlled and guaranteed by manufacturers. However, the use of other kits or any procedural modification from procedures stipulated in Annex A shall be subjected to adequate validation by the user. The user shall bear the responsibility to assess the suitability of the testing items adopted for the test.

4.2.2 DNA extraction kits or protocols should be selected depending on the chemical/ cellular composition of herbal materials. It is advisable, where relevant, to remove the following compositions in herbal materials in order to obtain sufficient quality and yield of DNA extracts for subsequent PCR amplification process:

4.2.2.1 polysaccharides such as pectin, cellulose, hemi-cellulose, and starch, which may be removed by the treatments with appropriate enzymes, e.g. pectinase, cellulose, α-amylase.

4.2.2.2 RNA and /or proteins, which may be removed by enzymatic treatment using RNase and proteinase, respectively.

4.2.2.3 Lipid fractions, which may be removed by enzymatic treatments or solvent extraction such as n-hexane.

4.2.2.4 Pigments such as phenolics compounds, tannins and other secondary metabolites, which may be removed using polyvinylpyrrolidone (PVP) and/or beta-mercaptoethanol.

4.2.3 Estimation of the quality of DNA extracts by means of

4.2.3.1 spectrophotometric analysis, and

4.2.3.2 amplification of endogenous gene of plant taxon.

4.3 Amplification by real-time PCR

4.3.1 This method provides 2 assays with different primer/probe for the following purposes, deem appropriate:

4.3.1.1 Specific amplification assay (SA) uses specific primer/probe set for detection of characteristic DNA region of *F. ussuriensis* in this method. SA recognizes and binds to a chloroplast DNA region of *F. ussuriensis* and shows positive amplification. On the other hand, there were no amplification observed in *F. cirrhosa, F. unibracteata, F. przewalskii, F. delavayi, F. taipaiensis* or *F. unibracteata* var. *wabuensis* and other plant materials tested.

4.3.1.2 Internal positive amplification control assay (IPAC) is the quality control primer/probe set for providing assurance that the genomic DNA of tested sample is amplifiable. IPAC recognizes and binds to a partial chloroplast DNA region of plants tested including *F. ussuriensis, F. cirrhosa, F. unibracteata, F. przewalskii, F. delavayi, F. taipaiensis* or *F. unibracteata* var. *wabuensis* showed positive amplification.

4.3.1.3 Every test sample shall be analyzed by the two assays simultaneously. The information of all primer/probe sets used in the method is available in Table B1 of Annex B.

4.3.2 It is recommended to perform duplicate analysis for each test sample and each of them at two template DNA levels, which are prepared according to the concentration measured under Clause 4.2.3.1:

4.3.2.1 Normalized DNA extract — the DNA extract is diluted to 10 ng/μL using molecular grade water.

4.3.2.2 Diluted DNA extract — the normalized DNA extract is further diluted 5-fold using molecular grade water.

4.3.3 Normalized DNA extract and diluted DNA extract together with appropriate controls stated in Clause 5.1 shall be analyzed by real-time PCR in parallel.

4.3.4 Prepare PCR reaction mix and perform PCR analysis according to Table B2 and Table B3 of Annex B, respectively.

4.4 Data analysis

4.4.1 Analyze the data with the following setting:

4.4.1.1 Manual threshold: set 0.05 for the SA and 0.04 for IPAC analysis.

4.4.1.2 Manual baseline: from cycles 3-15.

Note:

a) Data evaluation shall be according to the usual analysis program recommended by the real-time PCR instrument manufacturer. This setting is applicable to GCMTI in-house validated protocol and the specified real-time system.

b) By using this test protocol to analyze dual-target plasmid, containing a single copy sequence of SA and IPAC, an input

of 450,000 copies plasmid DNA for real-time PCR analysis resulted in the mean Ct value of 20.4 ± 0.18 and 19.0 ± 0.27 in SA and IPAC assay respectively. The threshold shall be set in the linear phase of the amplification plot. The plasmid map and real-time PCR performance are given in Annex C.

4.4.2 Export the data, including but not limited to Ct values, into a spreadsheet program for further analysis.

4.5 Result interpretation

4.5.1 The determination of test result was based on the quantification cycle, here called cycle threshold (Ct). The cycle at the fluorescence from the reaction crosses a specified threshold level at which the signal can be distinguished from background levels. The determination of SA and IPAC are as below:

Ct values	**Determination**
Undetermined	Negative
≤37	Positive
>37 (High Ct value)	Weak positive

When a positive result with a high Ct value is obtained, it indicates a low amount of target in the reaction which is close to but lower than the detection limit. The entire analysis shall be repeated to affirm positive finding.

4.5.2 System controls shall exhibit the expected performance as below. Each control shall has a valid value and, if the observed result for any control is different from the valid value, the analysis shall be repeated.

System controls	**Assay**	**Valid Ct values**
Environment control (EC)	SA	Undetermined
	IPAC	Undetermined
Extraction positive control (EPC)	SA	Ct value ≤37
	IPAC	Ct value ≤37

System controls	Assay	Valid Ct values
Extraction negative control (ENC)	SA	Undetermined
	IPAC	Undetermined
Positive DNA target control (P)	SA	Ct value ≤37
	IPAC	Ct value ≤37
Negative DNA target control (N)*	SA	Undetermined
	IPAC	Ct value ≤37
No template control (NTC)	SA	Undetermined
	IPAC	Undetermined

*The negative DNA target control is recommended. It demonstrates the ability of the nucleic acid amplification procedure and avoids false positive amplification in the absence of the nucleic acid representative of the target. Negative DNA target control may be the DNA of Bulbus Fritillariae Cirrhosae.

5 Quality control parameters

The analytical performance of each run is checked for conformance with the acceptance criteria of quality controls, which determines if the results of analyses are acceptable and able to meet the objective of the method. In consideration of the compliance with quality control (QC) plan, user should determine the number of samples in a batch to give a reasonable handling capacity for operator to conduct DNA analysis.

5.1 Use of system controls

The system controls of the method are tabulated as below. The arrows indicated that this control should be applied in the indicated analytical steps.

Analytical steps	Environment control [a]	Extraction negative control [b]	Extraction positive control [c]	Sample duplicate control [d]	Positive DNA target control [e]	Negative DNA target control [f]	PCR negative control [g]
Homogenization	↓						
DNA extraction	↓	↓	↓	↓			
Nucleic acid	↓	↓	↓	↓	↓	↓	↓

amplification and detection							

Remarks:

a) The use of environment controls enables the laboratory to identify sources of contamination at an early stage and work area for which contamination is present. The control is a tube containing a suitable volume of nucleic acid free water that is left open to the air throughout the entire grinding process.

b) Extraction negative control is the negative control of DNA extraction process which does not contain any added sample. This control is used to demonstrate the absence of contaminating nucleic acid during extraction and shall always be placed after the last DNA extraction sample. At least one extraction negative control shall be included each time when DNA is extracted from one or more samples.

c) Extraction positive control is used to demonstrate that the DNA extraction procedure has been performed in a way that will allow extraction of a target DNA. The control shall be a certified reference material or known positive sample that contains the sequence under study. This control is preferably analyzed in duplicate.

d) Test sample shall be run in duplicate for entire analysis to demonstrate the consistence of test results.

e) Positive DNA target control can be the reference DNA, or DNA extracted from a certified reference material or known positive sample, which act as the representative of the sequence or organism under study. This control is used to demonstrate the unique performance of the PCR assay. This condition can also be fulfilled by an appropriate positive extraction control.

f) Negative DNA target control is the DNA containing no sequence under study, which is extracted from a certified reference material or known negative sample. This control demonstrates that the results of analyses of test samples not containing the target sequence will be negative.

g) PCR negative control is the negative control to reveal any defect and problems in the reagents used during the PCR or in the operation of such process. This control can demonstrate that the PCR reagents used are free from any contaminating nucleic acids. This control shall be analyzed in duplicates.

5.2 The system control is evaluated by real-time PCR. The following parameters shall be met:

	Parameter	**Acceptance criteria**
A	Environment control	Negative results in IPAC and SA
B	Extraction negative control	Negative results in IPAC and SA
C	Extraction positive control	Positive results in IPAC and SA
D	Sample duplicate control	Consistent results in duplicate analysis of test sample
E	Positive DNA target control	Positive results in IPAC and SA

	Parameter	**Acceptance criteria**
F	Negative DNA target control	Positive results in IPAC Negative results in SA
G	PCR negative control	Negative results in IPAC and SA

6 References

6.1 Chinese Pharmacopoeia Commission (n.d.). Pharmacopoeia of the People's Republic of China (2020 ed.) Beijing: China Medical Science Press.

6.2 Generating DNA Barcodes for Plant-derived Chinese Materia Medica (CMM) (GCMTI RD-5:2020). Available online: https://www.cmro.gov.hk/html/eng/useful_information/gcmti/research/testing_methods/plant_derived.html (accessed on 13 April 2021)

Annex A

(Normative)

Spin Column DNA Extraction Protocol

This protocol has been in-housed validated by GCMTI for the identification of Bulbus Fritillariae Ussuriensis, a common adulterant found in Bulbus Fritillariae Cirrhosae, by qualitative real-time PCR method. Any procedural modification from the method stipulated in Annex A shall be subjected to adequate validation by the user, who has the responsibility to assess the suitability of the testing items when adopted for the test.

A1. Spin down briefly the powdered sample material contained in grinding vial or microtube before open.

A2. Freshly prepare ProK-Lysis Buffer (PLB) by mixing Lysis Buffer (LB) with Proteinase K (20 mg/mL) at 10:1 ratio. For example, add 2 mL Proteinase K into 20 mL of LB and mix.

A3. Add 1 mL of PLB, 25 μL of α-amylase and 5 μL of RNase A into grinding vial or microtube.

A4. Close the cap and then mix the sample by vortexing.

Note: It should ensure that tissue particles are able to move freely in the lysis mix and not stuck to the bottom of the tube. Additional volume of buffer may be required.

A5. If deem appropriate, put the microtube or grinding vial into the adaptor of Tissue Lyser and set frequency of 28 Hz for 30 sec. Then, disassemble adapters and rotate 180°, and apply 28 Hz for another 30 sec.

A6. Before incubation, shake each sample by hand.

A7. Incubate at 56°C for 30 min and follow by 65°C for 1 h incubation in thermomixer.

A8. After incubation, centrifuge at maximum speed for 1 min to pellet the debris. Extension of centrifugation may be required for separation of supernatant from loose cell debris.

A9. Transfer 350 μL of lysate into a clean 1.5-mL tube and add 350 μL of Extraction Binding Buffer (EBB) to each sample.

Note: If any re-crystallization occurs in EBB, pre-warm at 56°C to dissolve and mix well before use. On the other hand, if necessary, recovery of more volume of lysate for low DNA yield sample is feasible but EBB should be added in proportion.

A10. Mix the lysate with EBB thoroughly before loading to column.

A11. Transfer all lysate mixed with EBB from the tubes into the spin column placed on top of the collection tube.

A12. Centrifuge at 5000–6000 g for 2 min to bind DNA to the membrane of spin column. After centrifuge, replace with a new collection tube for each sample.

A13. First wash step: Add 180 µL of Extraction Wash Buffer (EWB) to column. Centrifuge at 5000–6000 g for 2 min.

A14. Second wash step: Add 600 µL of EWB to column. Centrifuge at 5000–6000 g for 4 min. Replace a new collection tube for each sample and repeat once of this step.

Note: If necessary, an additional wash may be needed for sample rich in pigment or an extra volume of lysate has been recovered in Clause A9.

A15. After centrifuge, replace with a new collection tube for each sample and centrifuge at maximum speed for 4 min to spin dry the column membrane.

A16. Replace collection tube with a new 1.5-mL microtube with lid opened. Open the column lid and incubate at 56°C for 30 min to remove residual ethanol in the column membrane.

A17. Add 80 µL pre-warmed water directly onto membrane of the column and incubate at room temperature for 1 min.

Note: Lower elution volumes may be used to give a higher DNA concentrations in comparison to the recommended volumes, but at the expense of DNA yield.

A18. Centrifuge at maximum speed for 4 min to eluate the DNA.

A19. DNA extracts are ready for normalization for real-time PCR analysis or keep at -20°C for storage.

Annex B

(Normative)

Real-time PCR Assays

Table B1. Primer/probe sets

Primer/probe sets are tabulated below. Unlabeled PCR primers and TaqMan probes may be ordered from a supplier who specializes in the synthesis of oligonucleotides.

Assay[1]	**Primer/probe**	**Oligonucleotide DNA sequence (5'-3')**	**Final concentration**	**Location of target site**
Specific amplification (SA)	Forward primer	TCCTTAATGTTTACTTCTGC TTTATCCTTGT	900 nM	Relative to location between bp 118239–118357 of *F. ussuriensis* chloroplast genome (NCBI accession no. KY646166.1). Probe recognizes location between bp 118284–118306.
	Reverse primer	GTCGATGAGTTAAACCAG ATAGTTATATGAGT	900 nM	
	Probe[2]	ATGTGTAGTAAAAAGAGA AAATC	250 nM	
Internal positive amplification control (IPAC)	Forward primer	CGGACGAGAATAAAGAGA GAGT	900 nM	Relative to location between bp 45480–45602 of *F. ussuriensis* chloroplast genome (NCBI accession no. KY646166.1). Probe recognizes location between bp 45544–45558.
	Reverse primer	TATTGGGGATAGAGGGACT TGA	900 nM	
	Probe[2]	AAAAGGAAAATCCGT	250 nM	

Remark

1. The assay ID for specific amplification assay (SA) and Internal positive amplification control assay (IPAC) are APGZHAC and APNKWA7 respectively.
2. TaqMan probe is with a dye label (FAM) on the 5' end, and a minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3' end.

Table B2. PCR conditions for amplification of target amplicons

Components	**Specific Amplification assay (SA)**	**Internal Positive Amplification Control assay (IPAC)**
20X assay containing 5 µM TaqMan probe and 18 µM primers	0.5 µL	0.5 µL
2X PCR master mix[3]	5.0 µL	5.0 µL
Template DNA[4]	4.5 µL	4.5 µL
Total volume per reaction	10.0 µL	10.0 µL

3. A ready-to-use optimized 2X PCR master mix containing all of the components, excluding the template DNA, primers and probe, was used. The 2X PCR master mix contains thermostable DNA polymerase, a blend of dNTPs with dUTP and uracil-N-glycosylase (UNG) to minimize carry-over PCR contamination, and ROX dye (passive reference) and optimized buffer components for fast real-time PCR analysis.
4. Normalized DNA extract at concentration of 10 ng/µL and 5-fold diluted DNA extract should be analyzed by real-time PCR in parallel.

Table B3. Real-time PCR cycling conditions for Specific Amplification (SA) and Internal Positive Amplification Control (IPAC)

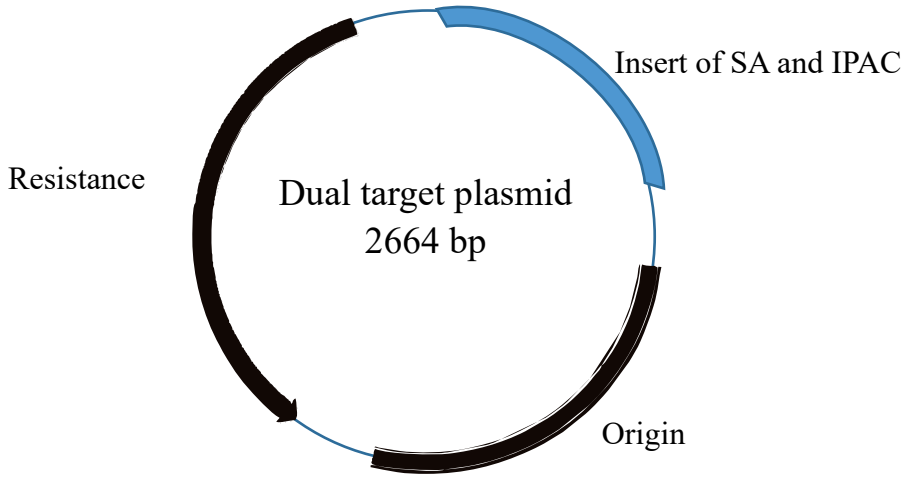
Temperature	**Time**	**No. of cycle(s)**
50°C	120 sec	1
95°C	120 sec	1
95°C 65°C	1 sec 20 sec	40

Annex C
(Informative)

Information on the dual-target plasmid DNA

C1. Plasmid DNA description and plasmid map

The synthetic dual target plasmid was assembled from synthetic oligonucleotides of SA and IPAC. The fragments were inserted into pUC18-like plasmid, pMA-T. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence identity within the insertion sites was 100%.



Map of the dual target plasmid

C2. Complete sequence of nucleotides (nt) and annotation of the insertion in plasmid

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1      GAGTTTATACTCCTTAATGTTTACTTCTGCTTTATCCTTGTATATAGGGAATGAGATTTT 60

61     CTCTTTTTACTACACATTGATAAGCTGTTTTGTTTTACTCATATAACTATCTGGTTTAAC 120

                            HindIII
                            |
121    TCATCGACCTGAATAACTCTGAAGCTTACTGATTATTCGGACGAGAATAAAGAGAGAGTC 180

181    CCATTCTACGTGTCAATACCGACAACAATGAAATTTATAGTAAAAGGAAAATCCGTCGAC 240

241    TTTATAAGTCGTGAGGGTTCAAGTCCCTCTATCCCCAATAAAAGCCCATT 290
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Key

Single underline nt 1~141 = SA amplicon (141 bp)

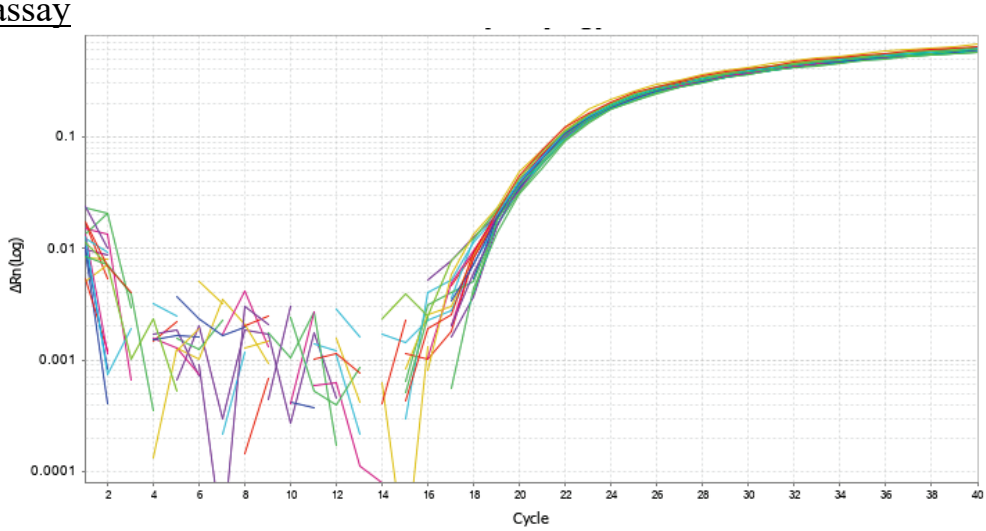
Shaded in grey nt 148 ~ 290 = IPAC amplicon (143 bp)

Two target sequences were separated by HindIII restriction enzyme cutting site nt 142 ~ 147

C3. Amplification plot of dual-target plasmid at 450,000 copies in SA and IPAC assays.

More than twenty replicates from four independent real-time PCR runs were conducted. The mean Ct obtained in SA and ICA assay were 20.4 ± 0.18 and 19.0 ± 0.27, respectively.

SA assay



IPAC assay