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Grain quantitative kit

MBK0062 150 reactions

INTEDED USE

Grain quantitative kit allows the quantification of *Triticum aestivum* in *Triticum* spp. by quantitative Real-Time PCR.

INTRODUCTION

Pasta is normally made from 100% durum wheat due to its special features to obtain a good final product. The production and sale of pasta is strictly regulated by the current Italian law: only a maximum of 3% soft wheat can be tolerated in dry pasta to account for cross contamination that may occur during growing, harvesting and handling practices.
Grain quantitative kit is based on DNA detection that is more stable than other components even when subjected to technological processes used in the food industry. The DNA can be amplified and detected with high sensitivity and specificity, constituting an excellent molecular marker of the presence of soft wheat in the product.

PRODUCT DESCRIPTION

Grain quantitative kit could be used to determine the relative DNA copy number ratio of *Triticum aestivum* in *Triticum* spp. by comparing the amplification of a specific gene of *Triticum aestivum* (soft grain) with normaliser gene common of all species of *Triticum* genus.
The relative quantification is obtained by the use of two calibration curves, one for each target gene.
The kit contains a Standard DNA for the preparation of the calibration curves, containing DNA of *Triticum turgidum* and *aestivum*. The material provided allows to perform five separately quantification experiments.

The Standard DNA supplied with the kit is derived from *Triticum aestivum* and *Triticum durum* (*Triticum turgidum*) certified seeds.

The kit include also the 3% DNA solution, a ready-to-use DNA solution that contains 3±0.9% of *Triticum aestivum/Triticum* spp. prepared from the same certified seeds used for the preparation of the Standard curve.

NOTE: this sample shall be processed in each PCR run to check the accuracy of the qPCR.

SENSITIVITY

Limit of detection (LOD): 0.15% of *T. aestivum* in *Triticum* spp.
Lower limit of quantification (LLOQ): 0.2% of *T. aestivum* in *Triticum* spp.
Upper limit of quantification (ULOQ): 15% of *T. aestivum* in *Triticum* spp.

SPECIFICITY

The specificity of the system was verified using DNA extracts from different species of genus *Triticum* and specific cultivar of *Triticum aestivum*.
The species that have been tested for the amplification of the normaliser gene are: spelt (Triticum turgidum *dicoccum*), kamut (*Triticum turgidum polonicum*) durum wheat (*Triticum turgidum)* and soft wheat (*Triticum aestivum*).
The species that have been tested for selective amplification of the target gene of *Triticum aestivum* are: rice (*Oryza sativa*), barley (*Hordeum vulgare*), maize (*Zea mays*), millet (*Panicum miliaceum*), oat (*Avena sativa*), spelt (*Triticum turgidum dicoccum*), kamut (*Triticum turgidum polonicum*), durum wheat (*Triticum turgidum*).

KIT CONTENTS

Multiplex PCR Master Mix: 4 x 750 µl
Hot rescue DNA Polymerase (5U/µl): 30 µl (175U)
ROX Solution: 4 x 5 µl
DNAse free water: 1 x 1000 µl
Dilution Buffer: 1 x 1000 µl
Standard DNA: 5 x 30 µl
3% DNA solution: 2 x 30 µl

ADDITIONAL EQUIPMENT

- Gloves
- Sterile filters and micropipettes
- 1.5 ml microcentrifuge tubes and 0.2 ml tubes
- Vortexer
- Microcentrifuge

STORAGE

Store the kit at -20°C, protect from light. Avoid repeated freeze-thawing cycles. For discontinued use, storage of mixes in working aliquots is recommended. If properly stored, see the expiration date for the stability of the kit.

PRECAUTIONS

- USE calibrated pipettes;
- To prevent the risk of contaminations, keep two separate working areas, one for extraction/preparation of samples and Standard DNA, and one for PCR set-up;
- Use pipette tips with filters;
- Store positive material (speciments, controls and amplicons) separately from all other reagents and if possible, add it to the reaction mix in a separated space;
- Thaw all components (except Hot rescue DNA Polymerase) and samples at room temperature and then thawed, mix the components and centrifuges briefly.

PROCEDURE

1.1 SAMPLE PREPARATION

For the isolation of DNA use Grains DNA extraction kit (Diatheva, MBK0064) or Mericon food kit (Qiagen) according the manufacturer's instructions. Alternatively use an extraction system capable of providing DNA suitable for the subsequent applications in terms of yield and purity.

1.2 QUANTIFICATION OF DNA

Determine the concentration of the total DNA using a spectrophotometer or other system available in your laboratory. For obtaining a good quantification is recommended the use of 250-150 ng/5 µl of DNA extracted.

1.3 PREPARATION OF STANDARD CURVE

The kit contains Standard DNA and the Dilution Buffer for the preparation of calibration curves. In a separate area proceed with the preparation of serial dilutions to create the standard curve as described below:

1) Thaw the Dilution Buffer and prepare 4 tubes in series from tube STD2 to STD5;
2) Pipette 15 µl of Dilution Buffer into the 4 tubes;
3) Thaw, vortex 10" and centrifuge briefly the Standard DNA, this is the first level of the calibration curve (STD1);
4) Pipette 15 µl of STD1 into the tube containing 15 µl of Dilution Buffer named STD 2;
5) Vortex 10" and centrifuge briefly;
6) Change tip and pipette 15 µl from tube STD2 to STD3;
7) Vortex 10" and centrifuge briefly;
8) Repeat steps 6 and 7 to complete the dilution series for STD4 and STD5.

Standard	**ng *Triticum* spp./5 µl**	**ng *Triticum aestivum*/5 µl**
STD1	828.6	33.9
STD2	414.3	16.9
STD3	207.1	8.4
STD4	103.5	4.2
STD5	51.7	2.1

Note: the standard solution is not long-term stable and will lose sensitivity under prolonged storage. Use only fresh prepared solutions as quantification references. Older solutions can be used as a qualitative standard (positive control).

1.4 PCR SET-UP

All detection experiments should include an NTC (No-Template Control), containing all the components of the reaction except for the template. This enables detection of potential contamination. Optionally you should include in the PCR run also the 3% Control sample.

1. Thaw the components protect from light. Vortex Multiplex PCR Master Mix and ROX solution for 10" and centrifuge briefly.

2. Upon first use of Multiplex PCR Master Mix, only for instruments that require a passive reference dye (see below), it is necessary to add the ROX solution:
   - Low ROX: ABI Prism® 7500, 7500 Fast.
   - High ROX: ABI Prism® 7000,7300, 7700, 7900, 7900HT, StepOne, StepOne Plus.

3. The ROX solution should be completed immediately before the use by adding 15 µl of DNAse free water to the vial containing the 5 µl ROX solution and vortex for 30". The kit provides 4 vials of ROX (5 µl), one for each Multiplex PCR Master Mix. It is recommended to complete the ROX solution and add to the Multiplex PCR Master Mix only before the use. The ROX solution cannot be stored after the preparation.

4. Proceed by completing Multiplex PCR Master Mix with the addition of ROX solution according to the following scheme.

	MBK0062 (1 vial- 750 µl)
Low Rox	0.85 µl
High Rox	9.25 µl

5. In one sterile 1.5 ml tube prepare amplification reaction mix needed for each sample to be tested following the pipette scheme below:

	1 sample*
PCR mix	19.8 µl mix + 0.2 µl Hot rescue DNA Polymerase

* For the analysis of more than one sample, simply multiply the volumes of mix and Hot rescue DNA polymerase for the number of samples to be tested considering the NTC and calibration curve in duplicate (optionally test also the 3% DNA solution in duplicated) plus one or two additional reactions to cover pipetting losses.

6. Vortex for 15" the vial containing the prepared master mix and centrifuge briefly;
7. Aliquot 20 µl of master mix in 0.2 ml tubes or alternatively in the plate prepared for the experiment;
8. Add 5 µl of DNAse free water into NTC;
9. In a separate area, add 5 µl of DNA samples to be tested (prepared as indicated in section 1.1), into the corresponding PCR tubes or wells containing amplification mixes;
10. Add 5 µl of each standard dilutions prepared as indicated in section 1.3 into the corresponding PCR tubes or wells containing amplification mixes. The final volumes of each reaction is 25 µl.

Note: it is recommended amplify standard curve in duplicate.

1.5 THERMAL PROFILE

The kit has been optimized to be used with ABI 7500 and Rotor-Gene Q thermal cyclers. Otherwise it could be used also with other instruments, in this case please contact technical service of Diatheva s.r.l.
Program the Real-Time PCR instruments with the following thermal profile:

Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95°C	10 min	1X
Denaturation	95°C	20 sec	40X
Annealing*	62°C	30 sec	
Extension	72°C	30 sec	

*Fluorescence is detected during annealing step on Green channel (FAM; ex 495 nm-em 520 nm) and Yellow channel (VIC; 538 nm-em 554 nm). For instruments that allow the gain optimization on the acquiring channels, set the gain optimization on NTC sample (tube position 1). Select ROX as passive reference dye for instruments that require it.

Target	**Reporter dye**	**Quencher Dye**
Triticum aestivum	Green (FAM dye)	BHQ-NFQ
Triticum spp.	Yellow (VIC dye)	BHQ-NFQ

1.6 ANALYSIS OF RESULTS

The analysis of results should be done with the program included in the recommendations provided by the manufacturer of the instrument. In some cases it is possible that the program will go automatically setting the baseline. In this case it is advisable to check these settings. For a correct definition of the thresholds it is necessary to select a value distinction from the background after the linear phase growth.
Analyze the standard curves and samples in each acquisition channel. Before proceeding with the analysis of the samples, check the validity standard curves:

	Channel	**Efficiency**	**R^2**	**Ct STD 1**
Standard curve of *Triticum aestivum*	Green - FAM	90-110%	≥0.985	26-28*
Standard curve of *Triticum* spp.	Yellow -VIC	90-110%	≥0.985	20-23*
NOTE: the difference between the PCR efficiencies obtained for each target of the duplex assay should not exceed 10%				

* These values may differ slightly between different thermal cyclers.

1.7 INTERPRETATION OF RESULTS

Negative control (NTC): no amplification within 40 cycles;

Samples:

- If there is no amplification of a sample in both acquiring channels, the sample analyzed does not contain DNA of *Triticum* genus.
- If there is amplification of a sample in both acquiring channels, it is possible to determine the relative DNA copy number ratio of *Triticum aestivum* in *Triticum* spp.. DNA quantification of the two targets is extrapolated from the calibration curves which is obtained from Ct values to which exit standards and related quantities. The ng values were automatically calculated by the software of the instrument. The relative quantification of soft wheat in durum wheat is obtained by a ratio between ng of *Triticum aestivum* (calculated in the Green Channel) and ng of *Triticum* spp. (calculated in Yellow channel) multiplied by 100.

Note: Alternatively it is possible to set the thermal cycler's software for the analysis with the two standard curves method. In this way, the software will determine automatically the relative concentration of *Triticum aestivum* in *Triticum* spp. To set the parameters refer to the manual handbook of the software.

3% DNA solution: the amount of *Triticum aestivum* in the sample should be 3±0.9%.

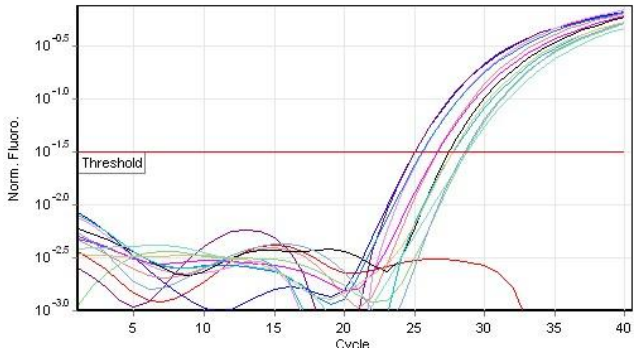
Example of calculation

Green-*Triticum aestivum*		
Sample	**Ct**	**ng**
A	31.09	2.231

Yellow-*Triticum* spp.		
Sample	**Ct**	**ng**
A	24.62	74.578

Sample	**ng soft grain/total grain**	**Result**
A	(2.231/74.578)*100	3%

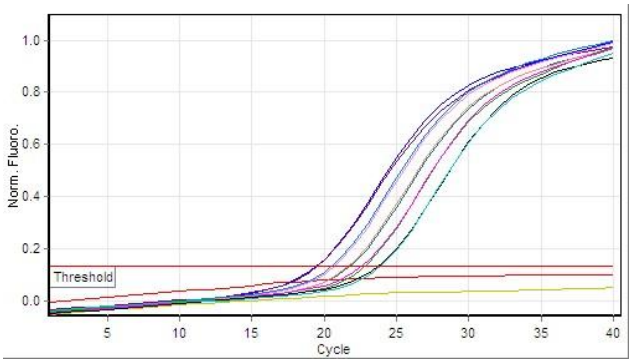
Green/FAM_Triticum aestivum



y=-3,326+38,268
R^2= 0,99
Eff=100%

Standard	**Ct**
STD1	25,76
STD2	26,80
STD3	27,86
STD4	28,92
STD5	29,61

Yellow/VIC_*Triticum* spp.



y=-3,57+29.96
R^2= 0,99
Eff=91%

Standard	Ct
STD1	19,54
STD2	20,75
STD3	21,69
STD4	22,62
STD5	23,77

Figures 1 and 2: amplification plots of the two standard curves, Ct values and efficiencies.

TROUBLESHOOTING

Observation	Possible cause	Suggested solutions
No amplification of standard or DNA samples	Incorrect programming of the thermal cycler	Repeat the PCR with the correct settings.
	Pipetting mistake	Check pipetting and repeat the PCR.
	Degraded reagents	Store PCR reagents at -20 °C protect from light and keep on ice once thawed. Avoid multiple freeze thaw cycles.
	Amplification in NTC is due to contamination of PCR reaction	Vigorous cleaning is recommended before repeat the amplification.
The sample is positive and not inhibited but its Ct is lower than the standard STD1	High concentration of tested samples	Repeat the test by diluting the DNA placed in the reaction.
The Ct of the sample is superior to the standard STD5	Low concentration of tested samples	The sample may contains low quantity of the target gene or alternatively, some problems occurred during extraction step. In this case repeat the extraction.

The certificate of analysis of the corresponding kit batch is available on demand.