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## Grain Quantitative kit

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### 1. INTENDED USE

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

### 2. INTRODUCTION

Pasta is normally made from 100% durum wheat (*Triticum turgidum*) 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 (*T. aestivum*) 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 and allowing its exact quantification in relation to the quantity of wheat belonging to the genus *Triticum*.

### 3. PRINCIPLE OF THE ASSAY

*Grain quantitative kit* allows the detection and relative quantification of the *T. aestivum* genomic DNA by the amplification of a gene common for all species of *Triticum* genus and of a specific gene for the *T. aestivum* cultivar by Real-Time PCR, based on TaqMan probe technology (Table 1). The relative quantification of the *T. aestivum* grain is obtained using two calibration curves, one for each target gene.

The kit provides a ready to use amplification mix. 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 includes 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.

**Table 1: Target and fluorophores**

Target	Fluorophores
<i>Triticum aestivum</i>	FAM
<i>Triticum turgidum</i>	VIC/Cal Fluor Orange 560

### 4. KIT CONTENT

The kit provides all the reagents required for the analysis (Table 2).

**Table 2: Kit content**

Reagent	No vial x Volume	Cap colour
<b>Multiplex PCR Master Mix</b>	3 x 750 µl	BLUE
<b>ROX Solution</b>	1 x 10 µl	BLACK
<b>3% DNA Solution</b>	2 x 30 µl	PURPLE
<b>Negative PCR Control</b>	1 x 100 µl	GREEN
<b>Dilution Buffer</b>	1 x 1000 µl	TRANSPARENT
<b>Standard DNA</b>	5 x 30 µl	RED

## 5. REQUIRED MATERIAL NOT SUPPLIED

- Disposable powder-free gloves
- DNA isolation kit
- Pipettes (adjustable)
- Sterile pipette tips with aerosol-preventive filter
- Vortex mixer
- Bench microcentrifuge
- Real-time PCR instrument
- Consumables compatible with the real-time PCR instrument. White and clear plates, clear 8-well tubes strips, optical sealing foils and optical 8 caps tubes
- Sterile 1.5 mL tubes
- Laboratory freezers from -30°C to -10°C

The kit is validated and can be used with the follow extraction systems and thermalcyclers:

### Thermalcyclers

- CFX96, Biorad
- ABI7500, Applied Biosystems
- Rotor gene Q, Qiagen
- Quant Studio 3, Applied Biosystems

## 6. ASSAY LIMITATIONS

- Samples must be collected, transported and stored using appropriate procedures and conditions. Improper sample collection, transfer, storage and processing may cause erroneous test results.
- Contamination during sample collection, handling or preparation can cause unreliable results
- Contamination during product handling can cause unreliable results

## 7. STORAGE CONDITIONS AND STABILITY OF THE REAGENTS

Store the kit at -20° C, protected from light. If stored at the recommended temperature all reagents are stable until the expiration date.

The performance of kit components is not affected for up to 2-3 cycles of freezing and thawing. If the reagents are to be used only intermittently, they should be stored in aliquots.

## 8. WARNING AND PRECAUTIONS

Before using the kit read the Product Information carefully and completely.

The operator should always pay attention to:

- Set up pre- and post-PCR areas. Do not share instruments or consumables (pipettes, tips, tubes, etc) between those areas;
- Store positive material separately from all other reagents and, if possible, add it to the reaction mix in a separated space;
- Do not use any reagent after the expiration date indicated on the label;
- Wear powder-free gloves during all procedures;
- Thaw all kit components and protect from light before starting the assay. After thawing, mix the components and centrifuge briefly;
- Do not substitute or mix reagents from different batches in order to maintain optimal performances;
- Include in each run at least 1 PCR Negative control and 1 3% DNA Solution;
- Minimize sample handling;
- Change gloves frequently;
- Wash the bench surfaces with 5% sodium hypochlorite;
- Use sterile disposable laboratory materials and do not re-use the tubes and tips;
- Store the reagents at the recommended temperature;
- Use calibrated micropipettes;
- The quality of the sample preparation may influence the quality of the PCR test.

## 9. INSTRUCTION FOR USE

### 9.1. SETUP REAL TIME PCR INSTRUMENT

Before preparing the reaction mix, program the real-time PCR instrument with the following thermal profile (Table 3):

**NOTE: In the Rotor gene Q instrument, NO SET THE GAIN OPTIMIZATION**

**Table 3: Thermal profile setting**

Phase	Temperature and time	Cycles
Initial denaturation	95°C 3 min	1 X
Denaturation	95°C 15 sec	40 X**
<b>Annealing-extension*</b>	62°C 40 sec	

\*fluorescence of different fluorophores is detected during the **annealing-extension** step in the channels specified in the following table

\*\* set **38** cycles when using the Rotor gene Q instrument

**Table 4: Fluorescence channels setting**

Multiplex	Fluorescence reading channels	
	FAM (Green Channel)	VIC/ Cal Fluor Orange 560 (Yellow Channel)
Target	<i>Triticum aestivum</i>	<i>Triticum spp.</i>

- The final reaction volume is **20 µL**
- **For the instruments that provide it, in the respective "Quencher" box of each target, select "NFQ-MGB" es: ABI 7500, QS3**
- **For the instruments that provide it, select "ROX" at the item "Select the dye to use as passive reference" es. ABI 7500, QS3**
- To set the standard curves, enter the following total ng values respectively for both targets as shown in table 5

**Table 5: Quantity (ng total) for the standard curves**

Standard	ng <i>Triticum</i> total/5 µl	ng <i>Triticum aestivum</i> /5 µl
<b>STD1</b>	828.6	33.9
<b>STD2</b>	414.3	16.9
<b>STD3</b>	207.1	8.4
<b>STD4</b>	103.5	4.2
<b>STD5</b>	51.7	2.1

## 9.2. DNA PREPARATION

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

## 9.3. 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 250ng/5 µl of DNA extracted (this quantity can be re-evaluated based on the extraction kit in use in the laboratory). If the extraction yield does not make it possible to load total 250ng, it is possible to load a minimum of 150 ng total.

## 9.4. PREPARATION OF STANDARD CURVE

The kit contains Standard DNA and the Dilution Buffer for the preparation of the standard curve.

To prepare the standard curve:

- 1) Thaw the Dilution Buffer to use for the standard preparation and prepare 4 tubes (1.5 mL) in series from STD2 to STD5 tube;
- 2) Pipette 15 µl of Dilution buffer in the prepared 4 tubes;
- 3) Thaw, mix by vortex for 10" and centrifuge briefly the Standard DNA, this is the STD1;
- 4) Pipette 15 µl of STD1 into the tube containing 15 µl of the Dilution Buffer named STD2;
- 5) Mix by vortex for 10" and centrifuge briefly;
- 6) Change tip and pipette 15 µl from tube STD2 to STD3;
- 7) Mix by vortex for 10" and centrifuge briefly;
- 8) Repeat steps 6 and 7 to complete the STD4 and STD5 dilutions.

**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).

## 9.5. REACTION SETUP

All detection experiments should include a PCR negative control (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% DNA Solution to verify the qPCR accuracy.

- Thaw and protect from light the components.

The Multiplex PCR Master Mix reagent is generally ready to use for all Real-Time PCR instruments except for instruments that require a higher concentration of ROX (High ROX) such as: ABI Prism® 7000, 7300, 7700, 7900, 7900HT, StepOne, StepOne Plus.

**Notes: ONLY for instruments that require a ROX higher concentration, at the first use of the Multiplex PCR Master Mix add the ROX Solution reagent as follows: 2.3 µl of ROX Solution to 1 vial of Multiplex PCR Master Mix containing 750 µl.**

- Vortex gently the **Multiplex PCR Master Mix** for 10 sec and centrifuge briefly;
- For each sample to test, PCR Negative Control and positive controls, aliquot **15 µL of the Mastermix** into the PCR tubes or into the wells of the plate prepared for the experiment;
- Add **5 µL** of PCR Negative Control into the corresponding tube;
- In a separate area, add **5 µL** of DNA sample to analyse, **5 µL** of each standard prepared as in the 9.4 section and double loaded and **5 µL** of 3% DNA Solution into the respective tube in which the Mastermix was previously aliquoted;
- Hermetically seal the tubes and load them into the real-time PCR instrument, following the manufacturer's instructions

**NOTE: It is recommended to centrifuge the tubes or the PCR plate before inserting them into the thermalcycler to eliminate air bubbles and collect all the reagents at the bottom of each well. Verify that the liquid is at the bottom of each well, otherwise centrifuge again at higher rpm for a longer time**

## 9.6. ANALYSIS OF RESULTS

The analysis of the results must be performed using the PCR software of each specific instrument and referring to the manual for detailed information. Some software set baseline automatically, in this case it is advisable to check these settings. Analyze standard curves and samples in both acquisition channels. Using the instruments CFX96, Rotorgene Q, ABI 7500 and Quant Studio 3 refer respectively to **appendices A, B, C and D** for the set-up of the analysis parameters. Once the parameters for each instrument have been set, before proceeding with the analysis of the samples, check that the standard curves respect the following values:

**Table 6: Validity criteria of standard curves**

	Channel	Efficienza	R <sup>2</sup>
<i>Triticum aestivum</i> Standard curve	FAM	85-115%	≥0.98
<i>Triticum</i> spp. Standard curve	VIC/CF0560	85-115%	≥0.98

- **Negative PCR Control:** Amplification should not be observed within the 35 cycle (peltier block instrumentation) or within the 33 cycle with the Rotor-gene Q instrument. If NTC Ct values lower than those indicated above are observed, check that the Ct obtained is higher than the intercept value ( $y = \text{intercept}$ ). If the value is higher, proceed with the analysis of the results, if the value is lower, contamination may be present, therefore clean the work area and proceed with a new test. Any amplification signals after the indicated cycles are signals due to the formation of primers dimers that do not interfere with the analysis.
- **3% DNA Solution:** the quantity of *Triticum aestivum* in the sample must be  $3 \pm 0.9\%$ .

### Samples:

- If there is no amplification of a sample in both acquiring channels, the sample analyzed does not contain DNA of *Triticum* genus.
- if a sample is amplified in both channels it is possible to quantify the % of *Triticum aestivum* in *Triticum* spp. In each channel the concentrations of the samples are extrapolated starting from the calibration lines obtained from the Ct values at which the standards come out and from the relative quantities. The ng values are automatically calculated by the thermal cycler software. The relative quantification of soft wheat is obtained by calculating the ratio between the ng of *Triticum aestivum* (calculated in the Green channel) and the ng of total *Triticum* (calculated in the Yellow channel) multiplied by 100 (Table 7)

**Table 7: Example of calculation of the *Triticum aestivum* percentage**

SAMPLE	ng <i>Triticum aestivum</i> / ng <i>Triticum turgidum</i>	RESULT
Semolina containing 1% soft wheat	$(4.8/459.4)*100$	1%
Semolina containing 3% soft wheat	$(2.231/74.578)*100$	3%

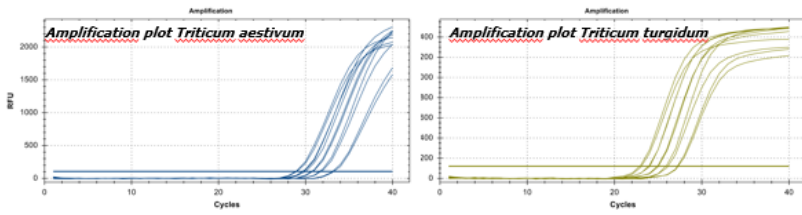
## 9.7. TROUBLESHOOTING

OBSERVATION	PROBABLE CAUSES	SOLUTION
No amplification signal for 3% DNA Solution, Standards and samples	Instrument not correctly set up	Verify the thermal protocol settings and repeat the test with the right settings
	Wrong reagent conservation or use of the reagents beyond the expiration date.	Verify the storage conditions and use a new kit if necessary
The sample is amplified with a ct value lower than Standard 1	High extraction yield, very concentrated sample	Quantify the sample and dilute it until a concentration equal to 50ng/μL is obtained. Repeat the sample amplification test.
The sample is amplified with a ct value higher than the Standard 5	Low extraction yield, poorly concentrated sample	Check that the sample has been well homogenized and has a very fine particle size, repeat the extraction and amplification

**APPENDIX A - CFX96 Analysis parameters**

From the Setting topic select "baseline setting" and set up "baseline subtracted curve fit". To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 1).

Fig 1: Parameter setting and data analysis on CFX96

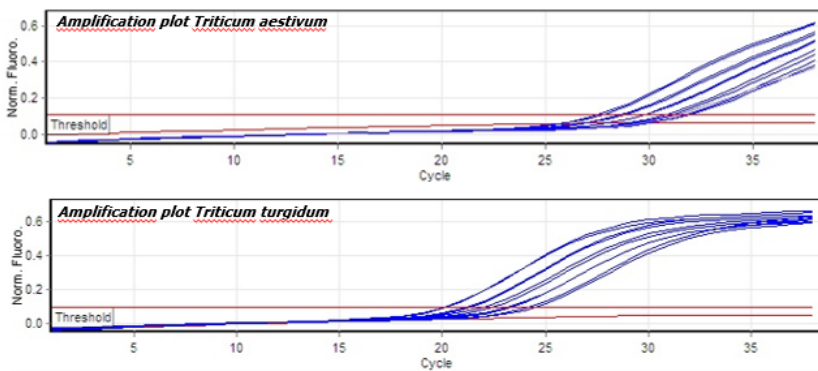


	<u>Triticum aestivum</u>	<u>Triticum turgidum</u>
STD1	28.93	22.98
STD2	29.86	23.97
STD3	30.74	24.96
STD4	31.68	26.00
STD5	33.16	27.24
Efficienza	96.4%	93%
R(2)	0.983	0.994

**APPENDIX B - Rotor gene Q Analysis parameters**

For each fluorophore proceed with the analysis as follows: Select the "Dinamic Tube" function. To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 2).

Fig 2: Parameter setting and data analysis on Rotor gene Q

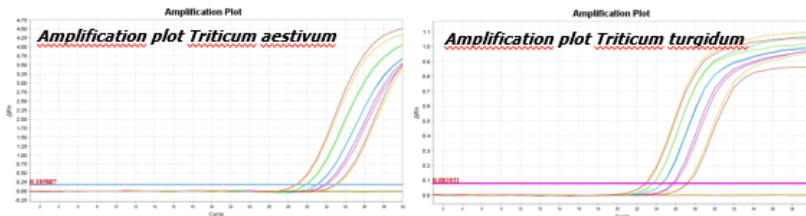


	<u>Triticum aestivum</u>	<u>Triticum turgidum</u>
STD1	27.65	20.17
STD2	28.74	21.25
STD3	29.80	22.23
STD4	31.04	23.24
STD5	31.92	24.39
Efficienza	90%	94%
R(2)	0.994	0.991

**APPENDIX C - ABI 7500 Analysis parameters**

For analysis, in the "Amplification plot" screen and "Options" section, deselect "Auto" respectively at the threshold and select "Auto Baseline". To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 3).

Fig 3: Parameter setting and data analysis on ABI 7500

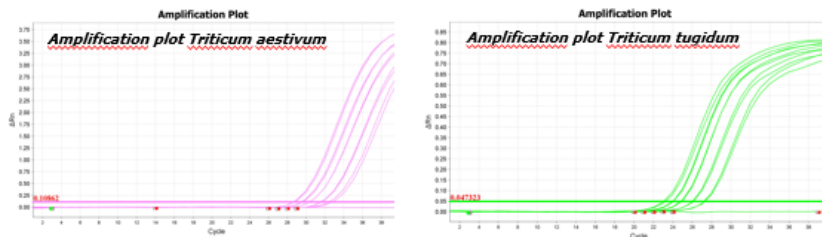


	<u>Triticum aestivum</u>	<u>Triticum turgidum</u>
STD1	28.96	22.72
STD2	29.91	23.49
STD3	31.05	24.41
STD4	31.94	25.58
STD5	33.08	26.97
Efficienza	96.74%	92.45%
R(2)	0.996	0.984

**APPENDIX D – Quant Studio 3 Analysis parameters**

For analysis, in the "Amplification plot" screen and "Options" section, deselect "Auto" respectively at the threshold and select "Auto Baseline". To set the threshold, manually move the line in the exponential phase of the fluorescence curves (Fig. 4).

Fig 4: Parameter setting and data analysis on Quant Studio 3



	<u>Triticum aestivum</u>	<u>Triticum turgidum</u>
STD1	28.72	23.11
STD2	29.58	23.97
STD3	30.39	24.83
STD4	31.65	25.98
STD5	32.64	27.21
Efficienza	101.7%	97.4%
R(2)	0.983	0.994

## APPENDIX E – PRODUCT VALIDATION

**Specificity:** All cereals belonging to the specific genus *Triticum* spp. are amplified in the VIC / CFO560 channel by the kit. While it does not amplify samples of cereals belonging to different genera. The FAM channel is selective only for soft wheat, *Triticum aestivum* (Table 8).

**Table 8: Specificity**

Cereal	VIC channel amplification ( <i>Triticum</i> spp.)	FAM channel amplification ( <i>Triticum aestivum</i> )
Durum wheat ( <i>Triticum turgidum</i> )	✓	-
Soft wheat ( <i>Triticum aestivum</i> )	✓	✓
Kamut ( <i>Triticum turgidum polonicum</i> )	✓	-
Emmer ( <i>Triticum turgidum dicoccum</i> )	✓	-
Rice ( <i>Oryza sativa</i> )	-	-
Maize ( <i>Zea mays</i> )	-	-
Mile ( <i>Panicum miliaceum</i> )	-	-
Oat ( <i>Avena sativa</i> )	-	-

**Limit of detection (LOD):** LOD value of the test, determined on both DNA and semolina samples, corresponds to 0.1% of *Triticum aestivum* in *Triticum turgidum*.

**Limit of detection (LOQ):** LOQ value of the test, determined on both DNA and semolina samples, corresponds to 0.2% of *Triticum aestivum* in *Triticum turgidum*.

**Reproducibility:** The reproducibility of the test has been evaluated on artificially created semolina mixtures containing 1, 3 and 5% of *Triticum aestivum* in *Triticum turgidum*. The relative standard deviation value for all levels tested was  $\leq 25\%$ .

**Repeatability:** The repeatability of the test has been evaluated on artificially created semolina mixtures containing 3 and 5% of *Triticum aestivum* in *Triticum turgidum*. The relative standard deviation value for all levels tested was  $\leq 25\%$ .