Real Time PCR Chloroplast Amplification Control Kit

**PCR Real Time test for the evaluation of the plant DNA amplifiability**

Code:  
- IC-02-0092  50 tests
- IC-02-0093  100 tests

*In vitro test*
Store at –20°C
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PCR Real Time test for the evaluation of the plant DNA amplifiability

**Brief description**

♦ By means of PCR Real Time technology, the kit is able to detect a chloroplastic marker universally present in the majority of flowering food plants (*Magnoliophyta*); in particular this marker is genetically retained within *Liliopsida* (monocotyledon plants) and within *Eudicotyledons* (some dicotyledon), both representative of the majority of food plants. For specifics details, please refer to the NCBI taxonomy [http://www.ncbi.nlm.nih.gov/Taxonomy](http://www.ncbi.nlm.nih.gov/Taxonomy)

♦ The kit contains all reagents for the DNA amplification, including a positive and negative control reagents.

♦ Detection: probe labelled with fluorescent dyes (Taqman®).

♦ This kit contains sufficient reagents for 50 (or 100) reactions.

Sample preparation: DNA extraction

Time requirement: test execution (10 samples) .......... approx 2 hours
results analysis…………………………… approx 15 minutes

Functionality: through a duplex PCR reaction, *PLANT DNA AMPLIFICATION CONTROL KIT* is capable of revealing the presence of plant DNA in the sample (thus confirming the success of the extraction steps) as well as the presence of possible masked inhibition effects (by the amplification of an internal Inhibition Positive Control).
1. Introduction

1.1 Intended use

*PLANT DNA AMPLIFICATION CONTROL KIT* is designed for the qualitative detection of an universal chloroplastic marker in DNA obtained from the sample under study. The use of the kit is particularly suggested in Real Time PCR analysis based on products for human and animal applications (detection of GMOs, allergens, identification of species or food frauds).

1.2 General

The advance of genetic engineering and recombinant DNA techniques has meant an increase on the number and variety of DNA purification techniques whose use depends on the original sample from which DNA is purified, as well as on the application to which it is to be used. In order to avoid false results and prevent failure of downstream applications, it is of great relevance to ensure the quantity and quality of the DNA obtained from the sample, which could inhibit downstream applications.

To support the researchers, InCura Srl has developed a test capable of revealing the presence of plant DNA by Real Time PCR analysis. Through the observation of both the DNA amplification profile and the internal Inhibition Positive Control, it is able to give information about the quality of DNA extracted as well as the eventual presence of inhibition contaminants.

1.3 Test principle

*PLANT DNA AMPLIFICATION CONTROL KIT* allows to verify the plant DNA presence within the sample. By Real Time PCR, the reaction detects an universal chloroplastic marker present in the majority of flowering food plants (*Magnoliophyta*); in particular this marker is genetically preserved within the biology class of *Liliopsida* (monocotyledon) and the biology group of *Eudicotyledons* (some dicotyledons), both representative of the majority of food plants.


The amplified DNA segment (126bp) is detected by a probe labelled with fluorescent dyes (Taqman®). The increase in fluorescence is continuously measured in a PCR real-time detection instrument (see Fig.1). The kit functionality within the listed taxonomic groups was verified by experimental and by bioinformatic alignment of the sequences ¹.


Species by which the *PLANT DNA AMPLIFICATION CONTROL KIT Real Time PCR* functionality has been verified through bioinformatics tools (sequence comparison analysis): Acorus calamus, Arabidopsis thaliana, Atropa belladonna, Avena sativa, Bertholletia excelsa, Brassica napus, Brassica nigra, Brassica oleracea var. borytris, Brassica oleracea var. italicca, Carastium beeringianum, Carex subcernua, Cichorium intybus, Cucumis melo, Cucumis sativus, Cucurbita pepo, Cuspidia cernua, Eucalyptus globulus, Fragaria x ananassa, Gossypium hirsutum, Helianthus anum, Lupinus luteus, Medicago truncatula, Morus alba, Nicotiana tabacum, Phalaris arundinacea, Robinia hispida, Rosa canina, Salix rotundifolia.
Additional informations: through the bioinformatic alignement it is possible to deduce that species within Coniferophyta, Cycadophyta, Ginkgophyta (Magnoliophyta) groups (species which can not be detected by Real Time PCR) can be detected by electrophoresis on agarose gel using the kit as "classic PCR".

2. Reagents

2.1 Kit content

The kit contains sufficient reagents for 50 (or 100) reactions with a final reaction volume of 25 µl each.

The following components are provided:

<table>
<thead>
<tr>
<th>Component</th>
<th>Reagent</th>
<th>Volume for 50 tests</th>
<th>Volume for 100 tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>Mix Amplification Control</td>
<td>1000 µl</td>
<td>1000 µl x 2</td>
</tr>
<tr>
<td>C2</td>
<td>DNA Positive Control</td>
<td>100 µl</td>
<td>100 µl x 2</td>
</tr>
<tr>
<td>C3</td>
<td>sterile H2O – DNase free</td>
<td>500 µl</td>
<td>500 µl x 2</td>
</tr>
</tbody>
</table>

2.2 General precautions

Only for in vitro diagnostic use

- The test must be performed by specialised, trained, and authorised staff.
- Never pipet reagents with the mouth.
- Do not use reagents after the due date indicated on the label.
- The alteration of a reagent can cause wrong results.
- Nucleic acids are subject to degradation by nucleases present in the atmosphere and on the skin. Use gloves during every procedure.
- Use sterile pipette tips with filters.

Specificity control group tested for cross-reactivity: Anchovy (Engraulis encrasicolus), Bovine (Bos Taurus), giant tiger prawn (Peneaus monodon), Swordfish (Xiphias gladius), Chicken (Gallus gallus), Swine (Sus domesticus), Human (Homo sapiens).
2.3 Storage instructions

The components of PLANT DNA AMPLIFICATION CONTROL KIT should be stored at -20°C and are stable until the expiry date stated on the label. Repeated thawing and freezing of C1 and C2 and light exposure of C1 should be avoided as this may reduce test sensitivity. If the reagents are to be used only sporadically, they should be frozen in aliquots.

3. Material required but not provided

1. PCR Real Time instrument
2. Pipettes
3. Sterile pipette tips with filters
4. Nuclease-free 0.2 ml reaction tubes
5. Standard benchtop mini-centrifuge

4. DNA preparation

In order to efficiently isolate the DNA, it is strongly recommended that the choice of DNA extraction method be suited to the sample matrix under investigation.

5. Control reactions

Internal quality control checks include the following:
- **Positive control:** to verify all reagents work properly.
- **Negative control:** to verify no false positive results are due to DNA contamination (through FAM reporter dye) and to distinguish true target negative results from inhibition reactions (through JOE reporter dye).

6. Execution

Different assay formats are possible. For each sample, either one DNA extraction is analysed in two PCR replicates, or two parallel DNA extractions are analysed (i.e., as recommended by national and international standards). The assay format described in the following section is recommended.

1. Reagents preparation
   Thaw the reagents, mix by vortexing, and centrifuge carefully before using.

2. Sample preparation
   While preparing the reaction mixture, keep the component C2 on ice or use a cooling block. The mixture C1 can be maintained at room temperature since it contains Hot Start Taq DNA Polymerase. **Avoid light exposure and the contact of hands, gloves**

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ISO 22174:2005–Microbiology of food and animal feeding stuffs–Polymerase chain reaction (PCR) for the detection of food-borne pathogens—General requirements and definitions.
and ink with the optical parts involved in the fluorescence lecture as adhesive films and optical caps.

- For a single Sample test, combine:
  1. 20 µl of Mix Amplification Control (C1);
  2a. 5 µl of the DNA to be tested.

For the Negative and the Positive control reactions, add the previous component C1 and substitute the DNA to be tested as following:

- 2b. 5 µl of sterile H2O (C3) to the Negative Control tube.
- 2c. 5 µl of DNA Positive Control (C2) to the Positive control tube.

- 3. gently mix each sample tube and briefly centrifuge the reaction tubes or plate.

<table>
<thead>
<tr>
<th></th>
<th>Negative Control</th>
<th>Positive Control</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix Amplification Control (C1)</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>DNA to be tested</td>
<td>-</td>
<td>-</td>
<td>5 µl</td>
</tr>
<tr>
<td>DNA Positive Control (C2)</td>
<td>-</td>
<td>5 µl</td>
<td>-</td>
</tr>
<tr>
<td>H2O (C3)</td>
<td>5 µl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Final Volume</td>
<td>25 µl</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
</tbody>
</table>

3. PCR Real Time conditions

**PLANT DNA AMPLIFICATION CONTROL KIT** works on several Real Time PCR instruments (ABI PRISM series - 5700, 7700, 7300, Stratagene Mx series, Realplex Eppendorf). The following scheme is related to reaction parameters optimised on the thermalcycler Real Time Biorad iCycler iQ5\textsuperscript{N1}. For other thermal cyclers, the optimisation of the reaction conditions may be required. Please contact InCura if necessary.

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>N. Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95 °C</td>
<td>5 min.</td>
<td>35 cycles\textsuperscript{N2}</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
<td>15 sec.</td>
<td></td>
</tr>
<tr>
<td>Annealing /Extension</td>
<td>60 °C</td>
<td>1 min.</td>
<td></td>
</tr>
<tr>
<td>Ramp Rate</td>
<td>Max</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Detection Parameters: Fluorescence detection
- Reporter dye (chloroplast): FAM
- Reporter dye (Internal Control): JOE
- Quencer dye (chloroplast): BHQ1
- Quencer dye (Internal Control): BHQ1
- Passive reference: none

**The duplex reaction implies the detection of both the reporters dyes for each analysis.**

\textbf{N1:} We recommend setting the function “collect well factors from experimental plate” and reducing the initial denaturation step to 2 minutes and 30 seconds.

\textbf{N2:} It’s possible to extend the reaction beyond this value, as when this system is coupled with another one with similar thermal profile but higher number of cycles (like InCura RoundUp Ready\textsuperscript{®} SoyKit). The results evaluation however must be done only relative to the first 35 cycles.

4. We recommend to initially use approx. 25 ng of the DNA extracted from the matrix under study.
7. Analysis of results

7.1 Setting of parameters

The evaluation has to be conducted according to the data analysis software recommended by the PCR Real-Time instrument manufacturer. If the automatic data analysis is not satisfactory, the baseline and/or threshold values used for the calculation of the Ct should be manually adjusted. As a general guideline, the baseline can be set considering, in linear scale, the range from the cycle with stable detected signal (approximately cycle 4) until 1–2 cycles before the amplification of the sample. To set the threshold, consider, in log scale, fluorescence values clearly higher than the background for which the positive control (FAM reporter dye) has $18 < \text{Ct} < 20$ (the positive test simulates the behaviour of 25ng of DNA extracted from soy flour IRMN standard; the DNA has been quantified by 1% agarose gel).

7.2 Interpretation of results

The control reactions of the analysis run will give the following:

- **Positive Control test (FAM reporter dye)** → amplification / $18 < \text{Ct} < 20$
- **Negative Control test (FAM reporter dye)** → no amplification within the 35 reaction cycles
- **Negative Control test (JOE reporter dye)** → amplification / $27 < \text{Ct} < 30$

Both the Ct value and the curve shape (FAM reporter dye) determine the sample positiveness. As a general rule, the following apply:

- a proper amplification profile shows a curve with low initial fluorescence and a regular sigmoid trend.
- for good DNA extraction process, a sample can be considered positive for the presence of plant DNA when $\text{Ct} < 35$. To be known, higher is the Ct value, lower is the DNA amount in reaction. The positive control mimics the behaviour of 25ng of soy flour extracted DNA; if the DNA sample is used to determine the GMO presence, it is strongly recommended to use sufficient DNA in reaction to obtain a Ct values close to the positive control;
- **no** DNA from plant is present in the reaction with sample DNA if the following apply:
  - no amplification (No Amp/Ct=35, FAM reporter dye)
  - Internal Inhibition Control (JOE reporter dye) amplification profile similar to the negative control one
- in the case the Internal Inhibition Control (JOE reporter dye) shows no amplification or its amplification profile is markedly different compared to the negative control one (when **no** amplification for the chloroplast target with the FAM reporter dye is present), then a strong inhibition of the PCR reaction has occurred for that sample (see point 7.3)
equally, if the amplification profile of the chloroplastic curve (FAM reporter dye) is heavily different from the **positive control** profile (mainly regarding the curve slope), then inhibition effects can be present (see point 7.4).

Scheme:

<table>
<thead>
<tr>
<th>PCR Controls</th>
<th>DNA Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAM</td>
<td>JOE</td>
</tr>
<tr>
<td><strong>√</strong></td>
<td>±</td>
</tr>
<tr>
<td>DNA sample <strong>positive for plant DNA</strong> (DNA target detectable)</td>
<td></td>
</tr>
</tbody>
</table>

| **√**        | ±           | **×** | **√** | **×** | **√** |
| DNA sample **negative for plant DNA** (DNA target not detectable) |

**√** stands for proper amplification profile, **×** stands for amplification absent or inefficient

± JOE reporter dye could give amplification in some case. This result has to be considered valid and this does not modify the final sample evaluation.

7.3 “Internal Inhibition Control” profile evaluation

The “Internal Inhibition Control” profile monitoring (JOE reporter dye run) allows to assess if the “negative” samples do not amplify (within the FAM reporter dye run) for the effective absence / scarcity of the DNA in reaction or for strong inhibition phenomena. This "Internal Control" refers to the **Negative Control** reaction: in the absence of inhibition effects their amplification profiles (linear scale) should be similar.

Samples with absent FAM reporter dye amplification (so resulting apparently **negative** to the chloroplast reaction) but showing the "Internal inhibition Control" amplification profile markedly different from the **Negative Control** one are subject to strong inhibition effects and should be considered **false negatives**.

On the other hand, samples which amplify within the FAM reporter dye run could show the "Internal Control" amplification profile markedly different from the **Negative Control** one or even absent: high amounts of the target DNA can compete so as to reduce or completely suppress the internal control amplification. In these cases, to assess the possible inhibition effects the profile evaluation of the chloroplastic target has to be observed (section 7.4).
7.4 Evaluation of inhibition levels (in the case of positive chloroplast amplification)

An example of different inhibition levels in DNA samples extracted from soy flour not adequately purified is reported in Fig.2.

The graphic shows the chloroplastic marker amplification obtained with different dilutions of the DNA sample. For a better interpretation of the amplification lines of the DNA sample different dilutions it is recommended to consider the linear scale (Fig.2a).

The shape of the not-diluted sample amplification lines shows the presence of an inhibition degree (the slope and plateau are markedly different from positive control) that could lead to false negative result in the analysis performed to search GMO or allergens in the DNA sample.

The DNA sample diluted 1:2 shows a small inhibition degree, while the DNA sample diluted 1:10 shows an amplification profile similar to the positive control one (Fig.2b).
8. References


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For clarification regarding the execution of the assay and/or the results analysis, please contact InCura srl.

InCura srl products are warranted to meet the specifications stated in the Manual when stored and used in accordance with instructions and purposes. InCura srl assures only for the quality of materials used in its products. If any materials are defective, InCura srl will provides a replacement products. Further details are provided in General Sales Conditions and in Products & Prices List.
**Introduction**

The advance of genetic engineering and recombinant DNA techniques has meant an increase in the number and variety of DNA purification techniques whose use depends on the original sample from which DNA is purified, as well as on the application to which it is to be used. In order to avoid false results and prevent failure of downstream applications, it is of great relevance to ensure the quantity and quality of the DNA obtained from the sample. Another aspect that must be considered is the presence of contaminants in the sample, which could inhibit downstream applications.

Through the amplifications by PCR Real Time of a universal chloroplastic marker, the new InCura Plant DNA Amplification Control Kit is capable of revealing the presence of plant DNA in the sample thus confirming the success of the extraction steps; the **duplex PCR reaction** allows in addition to verify the presence within the sample of possible masked inhibition effects.

The use of the kit is particularly suggested in Real Time PCR analysis based on products for human and animal applications (detection of GMOs, allergens, identification of species or food fraud).

**Technical Features**

- **Number of Tests:**
  50/100 reactions.

- **Functionality:**
  
  *PLANT DNA AMPLIFICATION CONTROL KIT* allows the detection of plant DNA in products for human and animal nutrition. The kit is based on the amplification by PCR Real Time of a chloroplastic marker, universally present in the majority of flowering food plants (*Magnoliophyta*); particularly this chloroplastic marker is genetically maintained within *Liliopsida* (monocotyledon plants) and *Eudicotyledons* (dicotyledon), both representative of the majority of food plants. The amplified DNA segment is detected by hybridisation with a probe labelled with fluorescent dyes

- **Kit components:**
  
  *PLANT DNA AMPLIFICATION CONTROL KIT* contains all reagents for the DNA amplification (dNTPs mix, PCR buffer, primers, taq polymerase). To guarantee the best result reliability and facilitate troubleshooting, two amplification controls are included.

<table>
<thead>
<tr>
<th>Description</th>
<th>Size</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
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<td>50</td>
<td>IC-02-0092</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>IC-02-0093</td>
</tr>
</tbody>
</table>

**References**

- Etichettatura degli allergeni, linee guida di Federalimentare. (2005) [www.federalimentare.it](http://www.federalimentare.it)