



Manual for Allin 2.0

Technical Manual

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I. Description

Allin 2.0 is a **multiplex PCR system** for **detection of GMOs** that contain the **35S-promoter** and / or the **NOS-terminator** derived from the cauliflower mosaic virus and the agrobacterium tumefaciens, which are the most common promoter and terminator utilized in commercially grown transgenic crops. In addition to transgenic markers (35S and NOS), this multiplexed system contains **soya** (lectin) and **maize** (zein) specific primers used to demonstrate the presence of these two species in a sample. An **internal control** determines inhibitors in the reaction.

Picture Allin 2.0

Figure 1 (see quality control sheet)

Table 1. PCR Amplification Product Profile

	Organism	Target Sequence	Size of Product (bp)
	Maize	Zein	278
	GMO	35S-Promoter	227
	Soybean	Lectin	163
Internal Control			132
	GMO	NOS-Terminator	104

biosmart gmbh weihergasse 11
 ch-3005 bern switzerland
 telefon: ++41 (0)31 318 28 08
 telefax: ++41 (0)31 318 28 09
 email: mail@biosmart.ch
 www.biosmart.ch
 post-conto: 46-323024-2
 mwst-nr: 427 357
 akkreditiert nach ISO/IEC 17025, STS 261



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II. Product Components

Product	Size	Cat.#
Biosmart Allin 2.0 GMO Screening System	3x20 Assays	17001 (Biosmart)
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Please contact your nearest AdiaGene or Promega branch by visiting www.adiagene.com or www.promega.com.

Each multiplex reaction requires one PCR reaction. The **Allin 2.0** multiplexes are supplied as master mixes containing primers, buffer, and dNTPs. The user adds sample genomic DNA (160ng), internal control DNA (which is supplied with the kit) and HotStar-Taq polymerase (which must be purchased separately). **We recommend HotStar-Taq polymerase and buffer** from Qiagen (*HotStar-Taq* Polymerases containing Triton -X100 in their buffer system should **not be used** for Allin 2.0 !).

Allin 2.0 includes:

MasterMix Allin 2.0 (red tube) for the 1. PCR

Positive Control Allin 2.0 (yellow tube) 0.5% RoundupReady soybean DNA and Bt-176 maize DNA

Internal Control Allin 2.0 (blue tube)

Storage Conditions

Store all components at +4°C (undissolved). Refreeze the kit only once, avoid multiple freeze-thaw cycles.

III. General Information

A. DNA Isolation

Genomic DNA can be isolated by any protocol that can produce DNA of „high quality“. The Wizard resin protocol published by the Joint Research Centre of the European Commission highlighted in AN071 (<http://www.promega.com/qualitymonitoring/appnotes/gmonew.pdf>) is one of the recommended methods of purification. It is important to add 160ng of genomic sample DNA to the reaction. Excess DNA can overwhelm the system and too little DNA can compromise the sensitivity of the Allin 2.0 kit. If a customer wants to try lower amounts of DNA, he will need to validate the system under his conditions. **RNase treatment** is required prior to nucleic acid quantitation as RNA can cause overestimation of DNA concentrations.

B. Internal Control

The **internal control DNA** utilize the same primer binding sequences as Lectin and will cause competition.

C. Sensitivity and Specificity

Using an optimized cycling-protocol, the following specification can be reached:

Sensitivity

Zein: 6ng

Lectin: 6ng without internal control, 25ng with internal control

35S-promoter: 0.01% by an input of 160ng (cutoff > 95%, validated by Biosmart GmbH)

NOS-terminator: 0.01% by an input of 160ng (cutoff > 95%, validated by Biosmart GmbH)

Specificity: between soya and maize 100% (standards from Fluka).



IV. Reaction protocol

1. Preparation, dilution steps

Positive Control

The positive Control is lyophilized and has to be diluted:

add 50µl PCR-grade-water. This will lead to a concentration of 160µg/µl

(vortex gently about 5sec., and wait until pellet has solved completely, wait about 20min. at room-temp. and centrifuge shortly before use)

store at 4°C for 7 days, store at -20°C for 2 months

Allin Mix 2.0

Allin Mix 2.0 is lyophilized and has to be diluted:

add 626µl PCR-grade-water

(vortex gently about 5sec., and wait until pellet has solved completely, wait about 20min. at room-temp. and centrifuge shortly before use: wait until all particles have dissolved!!!)

store at 4°C for 7 days, store at -20°C for 2 months

Internal Control

Internal Control is lyophilized and has to be diluted:

add 105 µl PCR-grade-water

(vortex gently about 5sec., and wait until pellet has solved completely, wait about 20min. at room-temp. and centrifuge shortly before use)

store at 4°C for 7 days, store at -20°C for 2 months

2. Amplification Reaction

PCR Reaction Protocol

- 1) Dilute the RNase treated template to **16ng/10µl**. (DNA concentration was measured spectrophotometrically).
- 2) Mix the prepared **MasterMix Allin 2.0** with the following components according to the table:
 PCR-buffer 10x
 HotStar-Taq polymerase (5 units/µl)
 and Internal Control

Reaction Setup for Small and Larger Sample Numbers

PCR

Component	Single Reactions	Twenty Reactions +5%
Add dissolved MasterMix Allin 2.0 (red tube)	29.8µl	626µl
PCR buffer 10x (PCR Buffer from Qiagen Cat. No. 203203 or 203205)	5µl	105µl
HotStar-Taq polymerase (5 units/µl) (HotStarTaq DNA Polymerase from Qiagen Cat. No. 203203 or 203205)	0.2µl	4.2µl
Internal Control (blue tube)	5µl	105µl
Dissolve gently to mix and wait until pellet has solved completely (about 5-10min)		
make aliquots of 40µl		
add sample template-DNA 16ng/µl (160ng/PCR assay)	10µl per sample	



- 3) Vortex gently to mix, till the pellet is dissolved and give 40µl prepared mastermix into the PCR-tube per assay.
- 4) At the template place, add **10µl template** (total 160ng) to the PCR tube.
- 5) Performe first PCR (see thermocycler-programmes shown in **Appendix**).

2) Internal Control:

- a. Soya samples containing 160ng DNA per sample: for 20 assays add 105µl internal control to the mastermix.
- b. Soya samples containing less DNA (below 80ng/10µl e.g. processed samples): dilute the internal control 1:10 with water. This leads to a higher sensitivity for Lectin.
- c. Soya samples containing less DNA (below 20ng/10µl e.g. Lecithin): You can substitute the internal control by water. This leads to highest sensitivity for Lectin.
- d. Maize samples containing 160ng DNA per sample: for 20 assays add 105µl internal control to the mastermix.
- e. The internal control can also be added individually: 5µl/sample

3. Data Interpretation

A. Analysis-Controls

Determine that the control reactions performed as expected before analyzing your samples.
The assay is valid when:

Negative no-DNA Control: shows only the band for the Internal Control (if applied).

There may be some low molecular weight bands or smearing that are the result of primer interactions.

Positive Control 0.5 (0.5% RoundupReady soya and 0.5% Bt-176 maize) must show bands for Lectin (soya), zein (maize) the 35S-promoter and the NOS-terminator. The sizes of the amplification products are indicated in Table 1 above and on the Qualitycontrol-sheet.

B. Interpretation of the results

The sizes of the amplification products are indicated in Table 1. above or on the Qualitycontrol-sheet.

Symptoms	Comments
Zein band is visible	The sample contains maize.
Lectin band is visible	The sample contains soya.
35S-promoter band is visible	The sample contains the 35S-promoter.
NOS-terminator band is visible	The sample contains the NOS-terminator.
only the internal control shows up	The sample does not contain DNA (or not amplifiable DNA) from maize, soya and 35S-promoter and NOS-terminator (relative to the detection limit). The reaction was not inhibited.
no band (Internal Control was added)	The sample contains inhibitors, and the template has to be purified better.



V. Troubleshooting

Symptoms	Possible Causes	Comments
Low yield or no amplification product, missing bands	Annealing-temperature and annealing-time are not optimized, maybe too high	Optimize annealing-temperature and annealing-time by changing.
	Thermal cycler programmed incorrectly	Verify that the times and temperatures are correct.
	Temperature too low in some positions of thermal cycler	Perform a set of control reactions to determine if certain positions in the thermal cycler yield little or no product.
	Top of thermal cycler open	The top must be closed for correct heating and cooling.
	Degraded reagent	Store the Multiplex Master Mixes at -20°C and keep on ice once thawed. Avoid multiple freeze-thaw cycles.
	Incorrect tubes	Thin-walled microcentrifuge tubes are recommended
	Too little DNA	Too little DNA can compromise the sensitivity.
	Missing reaction component	Check the reaction components and repeat the reaction.
	Inhibitory agents in the DNA sample	Repurify the DNA sample to remove inhibitors.
Multiple, nonspecific amplification products, smear effect	Annealing temperature too low	Check the accuracy of the thermal cycler. Increase the annealing temperature.
	Too much genomic DNA	We recommend using not more than 160ng per assay (total volume 50µl). Excess DNA can overwhelm the system.
	Too much <i>HotStar-Taq</i> DNA polymerase	Do not use more than 1 unit of <i>HotStar-Taq</i> DNA Polymerase per assay (total volume 50µl).
	Thermal cycler programmed incorrectly	Verify that the times and temperatures are correctly programmed.
Contamination of negative controls	Contamination by amplicons	Vigorous cleaning is recommended. Kit of lots 260203 or later, contain UTP . Enzymes like e.g. Amperease, UTPase may be effective to avoid contamination by amplicons.

VI. References

Meyer, R. et al. (1996) Polymerase chain reaction (PCR) in the quality and safety assurance of food: detection of soya in processed meat products. *Z Lebensm Unters Forsch* **203**, 339-344

Studer, E. et al. (1997) Nachweis des gentechnisch veränderten „Maximizer“-Mais mittels der Polymerase-Kettenreaktion (PCR). *Mitt. Gebiete Lebensm. Hyg.* **88**, 515-524

Zimmermann, A. et al. (1998) Quantitative and qualitative evaluation of nine different extraction methods for nucleic acids on soya bean food samples. *Z Lebensm Unters Forsch A* **207**, 81-90

biosmart gmbh weihergasse 11
 ch-3005 bern switzerland
 telefon: ++41 (0)31 318 28 08
 telefax: ++41 (0)31 318 28 09
 email: mail@biosmart.ch
 www.biosmart.ch
 post-conto: 46-323024-2
 mwst-nr: 427 357
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VII. Appendix

Thermal Cycling Programme Protocols

These cycling-protocols are important clues. Individual adaptations may be necessary.

Thermocycler	Hybaid Omn-E Crocodile II (Modus: Tubecontrol, 0.5ml tubes thin-walled)	Perkin Elmer 9600 (Modus: Blockcontrol, 0.2ml tubes thin-wall):	ABI PRISM 7000
Pre-Denaturation	15min/95°C	15min/95°C	15min/95°C
No. of Cycles Denaturation Annealing	5x 10sec/95°C 20sec/66°C	5x 20sec/95°C 60sec/66°C	5x 20sec/95°C 60sec/66°C
No. of Cycles Denaturation Annealing Extension	5x 10sec/95°C 20sec/62°C 20sec/72°C	5x 20sec/95°C 60sec/62°C 60sec/72°C	5x 20sec/95°C 60sec/62°C 60sec/72°C
No. of Cycles Denaturation Annealing Extension	5x 10sec/95°C 20sec/58°C 20sec /72°C	5x 20sec/95°C 60sec/58°C 60sec /72°C	5x 20sec/95°C 60sec/58°C 60sec /72°C
No. of Cycles Denaturation Annealing Extension	10x 10sec/95°C 20sec/54°C 20sec /72°C	10x 20sec/95°C 60sec/54°C 60sec /72°C	10x 20sec/95°C 60sec/54°C 60sec /72°C
No. of Cycles Denaturation Annealing Extension	10x 10sec/95°C 20sec/50°C 20sec /72°C	15x 20sec/95°C 60sec/50°C 60sec /72°C	15x 20sec/95°C 60sec/50°C 60sec /72°C
No. of Cycles Denaturation Annealing Extension	15x 10sec/95°C 20sec/45°C 20sec /72°C	15x 20sec/95°C 60sec/45°C 20sec /72°C	15x 20sec/95°C 60sec/45°C 20sec /72°C
Endestension			35sec/72°C

a) For Research Use Only. Not for use in diagnostic procedures.

b) Biosmart GmbH, Switzerland developed the screening system Allin 2.0. Modifications are reserved.

c) The PCR-method stays under US-Patent owned by Roche Inc. The application of PCR requires a license. Nothing of this publication may be understood as a warrant or as a licence for applying PCR method.

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Product claims are subject to change. Please contact Promega Technical Services or Promega online catalog for the most up-to-date information.