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# Inverse PCR and quantitative PCR as alternative methods to Southern blotting analysis to assess transgene copy number and characterize

Questa è la Versione finale referata (Post print/Accepted manuscript) della seguente pubblicazione:

*Original Citation:*

Inverse PCR and quantitative PCR as alternative methods to Southern blotting analysis to assess transgene copy number and characterize the integration site in transgenic woody plants / Biricolti Stefano; Bogani Patrizia; Cerboneschi Matteo; Gori Massimo. - In: BIOCHEMICAL GENETICS. - ISSN 0006-2928. - STAMPA. - 54:(2016), pp. 291-305. [10.1007/s10528-016-9719-z]

*Availability:*

This version is available at: 2158/1023751 since: 2018-04-13T16:51:30Z

*Published version:*

DOI: 10.1007/s10528-016-9719-z

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03 May 2024

Biochemical Genetics

# Inverse PCR and quantitative PCR as alternative methods to Southern blotting analysis to assess transgene copy number and characterize the integration site in transgenic woody plants

--Manuscript Draft--

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|---|---|
| **Manuscript Number:** | BIGI-D-15-00256R1 |
| **Full Title:** | Inverse PCR and quantitative PCR as alternative methods to Southern blotting analysis to assess transgene copy number and characterize the integration site in transgenic woody plants |
| **Article Type:** | Original Article |
| **Keywords:** | Southern blot; inverse PCR; quantitative PCR; transgene copy number; transgenic fruit trees |
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| **Order of Authors Secondary Information:** | |
| **Funding Information:** | |
| **Abstract:** | Abstract<br>One of the major unanswered questions with respect to the commercial use of genetic transformation in woody plants is the stability of the transgene expression over several decades within the same individual. Gene expression is strongly affected by the copy number which has been integrated into the plant genome and by the local DNA features close to the integration sites. Because woody plants cannot be subjected to selfing or backcrossing to modify the transgenic allelic structure without affecting the valuable traits of the cultivar, molecular characterization of the transformation event is therefore crucial. After assessing the transgene copy number of a set of apple transgenic clones with Southern blotting, we describe two alternative methods: the first is based on inverse PCR (i-PCR) and the second on the quantitative PCR (q-PCR). The methods produced comparable results with the exception of the data regarding a high copy number clone, but while the q-PCR based system is rapid and easily adaptable to high throughput systems, the i-PCR based method can provide information regarding the transformation event and the characteristics of the sequences flanking the transgenic construct. |

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Dear Editor

The manuscript "**Inverse-PCR and quantitative-PCR as an alternative method to Southern blotting analysis to assess transgene copy number and characterize the integration site in transgenic woody plants" (**BIGI-S-12-00311) has been widely revised. All the modifications requested by the referee has been done and a thorough revision to correct typos and errors in style in the manuscript has been carried out.

As far as I can see all the referee's requirements have been fulfilled. For any further adjustment, I remain. Thank you for all.

Best regards

Stefano Biricolti

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# Inverse PCR and quantitative PCR as alternative methods to Southern blotting analysis to assess transgene copy number and characterize the integration site in transgenic woody plants

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## Introduction

Genetic modification has been exploited to study the physiology and development of plants as well as to improve commercial crops with fine agronomic traits (Mason et al. 2002). Regardless of the aim of the transformation, its application requires that transgenes be stably integrated and expressed in the plant genome. The reason for analyzing many transformants resides in the mechanism of integration itself: since the exogenous DNA is inserted at random into the plant genome and only one of the two allelic chromosomes harbors the transgene, plants with one to several integrated copies are obtained, and the multiple copies can be hosted in one or more chromosome locations. The copy number assessment is particularly important with the use of direct DNA delivery transformation methods such as biolistic technologies which result in very high copy number of the introduced gene and also in fragmented copies integrated into the genome (Walter et al. 2005). Transgenic plants with a single transgene copy are usually preferred because multiple copies often bring on undesirable effects such as gene silencing (Matzke and Matzke 1995; Kumpatla et al. 1997). The issue of gene silencing and gene arrangement based on multiple and fragmented copies appears of great importance to genetic engineering in trees, since in these organisms, transgene is harbored by only one of the two allelic chromosomes and is expected to be expressed correctly and to remain unmodified over a period of 20 years or more (Walter et al. 2005). This condition becomes permanent, given that no further manipulation such as crossing or self-fertilisation are viable approaches for obtaining homozygous single-copy clones (the genotypes usually selected for commercialisation) due to long juvenile period, high level of heterozygosity leading to inbreeding depression and to self-incompatibility and sterility phenomena. Also, backcrossing can not be used to produce large quantities of transgenic woody plants due to the long time needed to produce and test large numbers of transgenic progenies. Therefore, T0 transgenic plants must be vegetatively propagated on a large scale for field plantations. Vegetative propagation, while ensuring genetic stability of the transgene, could make the clonal population prone to large scale transgene silencing (Minocha and Wallace 2000). Few studies have followed the expression of a transgene over years in the same plant or its clonal progeny (Kumar and Fladung 2002). To determine whether the insertion of the transgene is simple (one copy) or complex (multiple and truncated copies), scientists have traditionally relied on nucleic acid blotting techniques (Southern blots) (Southern 1975) which is costly in terms of reagents,

labour and time. In addition, the method response is not always unambiguous. Some investigators have attempted to simplify the method for copy number determination using alternative methods such as real-time polymerase chain reaction (PCR) in animal cells (Lipiński et al. 2012; He et al. 2012) and in plants (Ferradini et al. 2011; Yang et al. 2005; Yi et al. 2008) which is the method of choice when high number of genomes or low quantities of DNA available need to be analysed. Inverse PCR (i-PCR), is an alternative method based on the selective amplification of junction fragments, i.e. plant genomic sequences flanking any known DNA sequence (Ochman et al. 1988; Yu et al. 2012; Chen et al. 2013). Does et al. (1991) proposed a protocol in which the left border sequence of the T-DNA construct have been used to estimate the copy number of the transgenic line by i-PCR, which has been extensively used in our laboratory (Pasquali et al. 2008). In our study, the strategy i-PCR has been implemented by analyzing both T-DNA flanking sequences, in order to obtain more solid data.

The copy number assessment using the real-time PCR was based on the selective amplification of a marker (resistance to hygromycin: *hpt*) and a reporter (*gusA*) gene, both present in the construct T-DNA used for transformation. Both transgenes were analyzed in comparison with a reference gene present in the apple genome in single copy. In our study, we have applied both methods to a species of high commercial value which, as far as we know, has never been subjected to such a thoroughly characterization of the transgene integration. The results of these methods were compared to each other and with the results obtained with Southern blotting as a reference. The methods are very different but, within the limits of the analytical procedure, results are comparable.

## MATERIALS AND METHODS

Transgenic apple plants (clones 1, 2, 5, 6, 10, 13, 22, 25 and 28) were obtained from *Agrobacterium* mediated transformation of apple cv Greensleeves, clone 92 leaf discs (James and Dandekar 1997). The plasmid for transformation (pDR5-*gus*) was kindly provided by Dr John Gittins (University of Southampton) and obtained by inserting a 2.2 kb EcoRI/Klenow/SalI DR5p-*gusA*-nos cassette (from Prof. Guilfoyle, University of Missouri) into a SalI/PmlI 1381 pCambia plasmid (Fig. 1).

Transgenic plants and wild type cultivar were micropropagated according to James and Dandekar (1997). Genomic DNA was isolated from fresh leaves of *in vitro* cultivated plantlets using a method after Dellaporta et al. (1983).

1.Southern blotting

Southern experiments were performed following standard procedures (Sambrook and Russell 2001). Eight µg of each DNA sample were digested with HindIII (Stratagene, La Jolla, CA, USA) which has two cleavage sites within the T-DNA both located upstream the probe. The probe (888 bp) spanned about 50% of the *gus* gene and was fluorescently labeled with digoxigenin (Roche Diagnostics Gmbh, Mannheim, Germany). Restriction fragments were resolved by electrophoresis in a 0.8% agarose gel overnight at 25 volts, blotted using standard methods, and covalently bound to a Nylon+ membrane (Boehringer Mannheim, Indianapolis, IN, USA) by UV exposure in an automated crosslinker Spectroline XL-1000 (Spectronics Corporation, New York, USA). The membrane was hybridized with the *gus* probe and exposed using standard procedures according to the manufacturer's guidelines.

2.Inverse PCR

i-PCR has been carried out according to Does et al. (1991) and Pasquali et al. (2008) adjusting restriction endonucleases and primers suitable for the construct used for transformation. Two different strategies have been devised in order to selectively amplify genomic fragments flanking both sides of the T-DNA. About 1 µg genomic DNA has been completely digested with BfaI or BshFI (4 nucleotide cutters), religated and again digested with three different sets of restriction enzymes (6 nucleotides cutters: BamHI, EcoRI and PstI for the left border and NheI, NruI and MspCI for the right border) according to Does et al. (1991) . 2 µl of the digested DNAs were amplified with two primers oriented in the reverse direction to the usual (copycount1 CAGCGTTGAACTGCGTGATG and copycount2 TGGACCGATGGCTGTGTAGA for the left border e copycount3 TGAATCCTGTTGCCGGTCTT and copycount 4 GTGTACATTGAGTGCAGCCC for the right border) as shown in figure 1. The amplified DNAs were run on agarose gel, or, if necessary, on polyacrylamide gels (6%), to achieve a better resolution.

3.Quantitative PCR

3.1.Primers for q-PCR

The data obtained using i-PCR were compared with quantitative PCR (q-PCR). For this purpose the DNA samples have been diluted to 0.6 ng/µl with ultrapure autoclaved water. Two strategies were developed in order to amplify both marker and reporter genes present in the T-DNA construct. A highly conserved endogenous single copy gene (*SBE*: starch branching enzyme I) was used as a reference to quantitate Malus DNA (Han et al. 2007). Primers were designed on the basis of the accession sequences n. DQ115404 for *SBE*, n. U12639 for *gusA* and n. AY818364 for hygromycin resistance (*hpt*) genes, available in the GenBank® database. The primers have been designed in order to achieve the same amplification efficiency. The sequence and size of amplicons are detailed in Table 1. All primers were synthesized by Sigma Aldrich Co., Milan, Italy.

3.2.Quantitative PCR procedure

PCR analyses were performed with a Rotor-Gene 6000 (Corbett Life Science, Mortlake, Australia) in a 15-µl volume containing 3 ng total DNA, 7.5 µl of 2X SsoAdvanced SYBR Green Supermix (Bio-Rad laboratories srl, Hercules, CA, USA) and 250 nM of each primer. PCR conditions were 98°C for 5 min to activate the DNA polymerase, then 40 cycles at 98°C for 5 seconds and 65°C for 40 s. The melting curves of the PCR products were acquired by a stepwise increase in the temperature from 50°C to 96°C after PCR amplification, which is a built-in program of the Rotor-Gene® system. Each DNA sample was analyzed three times in separate reactions. Prior to the assays, serial dilutions (30, 3, 0.3, and 0.03 ng) of the apple Greensleeves clone 1 genomic DNA (with one copy of the transgene as previously assessed by inverse PCR and further confirmed by blotting) were amplified to evaluate the amplification efficiency by the comparison of the slope of the standard curves of both transgene regions (*gusA* and *hpt*) and the reference (SBE I). In fact , the use of the $2^{-\Delta\Delta Ct}$ method for relative quantification, a comparative technique in which a target gene is normalized to an endogenous control and relative to a calibrator, requires the PCR efficiencies of target and control genes to be approximately equal.

3.3.Relative quantification by the comparative Ct ($2^{-\Delta\Delta Ct}$) method

The most robust method for copy number determination by real-time PCR (q-PCR) is the comparative Ct ($2^{-\Delta\Delta Ct}$) method. For estimating copy number, any absolute quantification of the amount of transgene copies is needed. The method we used has been described previously (Livak and Schmittgen 2001). If all

amplicons amplify with the same efficiency, the difference ΔCt between the Ct for the transgene ($Ct_t$: *gusA* or *hpt*) and the Ct for the endogenous control ($Ct_r$: *SBE*) is constant, provided that, independently from the amount, chromosomal DNA is exactly the same for both amplification reactions (transgene and endogenous control):

$\Delta Ct = Ct_t - Ct_r$

In our study, we adopted apple Greensleeves clone 1 as a calibrator, being a hemizygous one-copy genotype (as assessed with i-PCR and blotting) in which, after transformation, only one of the two allelic chromosomes harbors the transgene. Thus, all samples with the same ΔCt as the calibrator contain one copy of the transgene. More generally, the ratio of the initial amount of transgene in the sample (*Xs*) to the initial amount of transgene in the calibrator (Xcal) can be calculated as follows:

$$\frac{Xs}{Xcal} = (1 + \mathrm{E})^{-\Delta\Delta Ct}$$

where ΔΔCt = ΔCts - ΔCtcal, ΔCts=ΔCt sample, ΔCtcal=ΔCt calibrator and E=amplification efficiency (varying from 0 to 1).

For copy number calculation ΔΔCt will be zero (one-copy plants) or negative (multi-copy plants) (Bubner and Baldwin 2004).

RESULTS

1.Southern blot

The apple cv. Greensleeves transgenic clones were analyzed by Southern blot hybridization (Fig. 2). The size of the detected bands was bigger than 3.0 kbp as expected from the probe-restriction enzyme combination used. Hybridization with the *gus* probe indicated that three plants (clones 10, 13 and 28) had two T-DNA copies, even if clone 10 showed a very faint band (Fig.2). Five plants (clones 1, 2, 6, 22, 25) showed the integration of one T-DNA copy, whereas the clone 5 carried four copies. Control plant did not show any hybridization signal as expected.

2.i-PCR

In the i-PCR we estimate the copy number of the transgenic clones by the comparison of the number of amplicons we obtained from the selective amplification of the right and the left borders flanking sequences. PCR amplification of clones 1, 2, 6, 10, 22 and 25 resulted in a single fragment (Fig 3 a-e). The sequencing analysis of clone 2 right border flanking sequence, after excision of the amplified band,

showed that the transformation event also integrated a fragment of the originary pCAMBIA 1381 binary vector (Table 2), as already observed by other authors (Forsbach et al. 2003). As far as clone 28 is concerned, two bands have been observed, even if a third faint band appears in the pattern when the fragment flanking the left border of the T-DNA was amplified. When excised, the faint band did not give any amplification, revealing to be an artifact. The clone 13 showed two amplified fragments on the gel, while a third faint band between the two main fragments was detected in the right border analysis (Fig.3 c). All the bands were excised from a polyacrylamide gel, re-amplified, and sequenced. The sequencing data showed full homology of the faint band with the shorter brighter band, but with an additional 82 bp fragment inserted between two BshFI site (Table 3). Therefore, during ligation, recombinant molecules through insertion of foreign fragments amenable to amplification sometimes occur. Clone 5 contained multiple inserts according to the pattern of the gel. The number of bands obtained amplifying the left border flanking sequences was 3-4 in the agarose gel but a better resolution could be achieved in polyacrylamide gel (Fig.4a). On the right side of the T-DNA the amplification pattern is a bit more difficult to interpret but still discernible (Fig.4b).

3.q-PCR

The use of q-PCR to estimate copy number of transgenic plants requires preliminary additional tests prior to performing the copy number assay. Evaluation of PCR efficiency can be calculated by plotting the Ct as a function of log10 concentration of template (Biosystems 2001); as the slope of the resulting trend line is a function of the PCR efficiency, a slope of -3.32 indicates that the PCR is 100% efficient. To ensure that these requirements were met, we generated standard curves for the *gusA* and *hpt* transgenes and for the endogenous control *SBE* gene amplicons, using the 4 point dilutions 30-3-0.3-0.03 ng. In the standard curves a very efficient amplification was achieved, as indicated by the slope of the linear regression, and good correlation coefficients were observed (Fig.5). Slopes of -3.43, -3.31 and -3.35 for *hpt* and *gusA* and for *SBE*, and correlation coefficients of 0.999, 0.998 and 0.999 were respectively obtained. Moreover, a sensitive method for assessing if two amplicons have the same efficiency is to look at how ΔCt varies with template dilution: absolute slopes of 0.044 (<0.1) for *gusA* and of 0.077 (<0.1) for *hpt* transgenic targets were obtained when plotting the log input amount versus ΔCt ($Ct_{GUS}$-$Ct_{SBE}$ and $Ct_{hygromycin}$-$Ct_{SBE}$), permitting the use of the $2^{-\Delta\Delta Ct}$ method (Biosystems 2001).

Moreover, to assess reaction specificity and to verify product identity, melting curve analysis was performed following amplification. Fig. 6-7 shows the melting curves in triplicate for all the samples of the transgenes (*gusA* and *hpt*) and endogenous gene (*SBE*). The negative first derivative profile of the melting curves from 50°C to 96°C shows a single peak for the amplified genes in all the samples, which represents the melting point (Tm) of the amplicons. Nonspecific products are not detectable.
Copy number determination was performed by the $2^{-\Delta\Delta Ct}$ method using the SBE as an endogenous control and clone n.1 as a calibrator. In the transgenic samples, evaluation of the $2^{-\Delta\Delta Ct}$ indicates the fold change in copy number of the T-DNA relative to the clone chosen as a calibrator. The estimation of transgene copy number by the q-PCR was similar to the results obtained by i-PCR, apart from clone 5 (Table 4), the clone containing multiple copies of T-DNA as estimated with Southern blotting hybridization and i-PCR. The number of copies detected with q-PCR is much higher than those obtained with blotting and i-PCR, to this aim we must point out that when the copy number is high the Ct difference diminishes logarithmically as the copy number increases, thus reducing the accuracy of the quantification. The results obtained were confirmed by the analysis of the copy number estimated using the *hpt* primers, in fact, the copy number of the clones obtained with the amplification of the *hpt* matches those obtained with the amplification of the *gusA* sequences (Table 4).

## DISCUSSION

Estimating transgene copy number is critical to the selection of candidate transgenic plants and for the identification of multiple-copy transformants that may exhibit gene silencing. Such screening can be very difficult to handle, especially when the number of independent transformed events is large. No transformation method can completely control the transgene integrations into the plant genome (Omar et al. 2008; Latham et al. 2006) where the new DNA is randomly inserted and only one of the two allelic chromosomes harbors the transgene. To maintain the traits of the original untransformed cultivar and the additional features conferred by the transgene, most fruit trees must be vegetatively propagated for field plantations. The molecular characterization of the transformation event is therefore of utmost importance because the set of integrated transgene(s) is expected to remain unchanged during the whole transgenic clone's lifetime.

Southern blotting is the method traditionally accepted to estimate transgene copy number, but besides the fact that it is costly in terms of reagents, labor, skill and time, it requires a considerable amount of high quality DNA from fresh or frozen material which is not available at earlier stages of transgenic plant development. Furthermore, the results are sometimes difficult to interpret due to incomplete DNA digestion, unspecific hybridization generating false positives, multiple copies tandemly located. Sothern blot analysis does not allow to discriminate the hemizygous from homozygous individuals and a subsequent segregation analysis is needed.

Finally no additional information about the integration site can be drawn from the identified bands in Southern blots.

i-PCR is a method which can allow not only the quantification of the copy number, but also the investigation, through sequencing, of the flanking genomic regions of the integrated T-DNA, allowing in-depth investigation of the integration site. The accurate examination of transgene insertion sites is a crucial requirement for the improvement of genetic analysis and selection of transgenic plants, and to prevent unintended mutations (Latham et al. 2006).

i-PCR is characterized by a multi-step procedure, and the actual results are not always unambiguous, particularly when the copy number is high. To this end, production of recombinant molecules during ligation amenable to PCR amplification have been demonstrated in this study, but doubtful results could be removed by sequencing or restriction analysis.

Like most of the other methods of copy number estimation, the accuracy of the quantification diminishes as the number of integrated number of copies increases, but usually the selection of candidate transgenic clones request the identification of low copy number lines, and the i-PCR method has been shown to be highly reliable for the identification of single copy plants.

q-PCR also provides a fast and reliable method for the identification of transgenic tissue and selecting low copy number transgenic genotypes. The benefit of early analysis of regenerating plants would expedite plant transformation projects, especially for woody species or fruit trees which show a slow and low shoot regeneration capacity. Thus, it is important to screen the transformants at an early stage to distinguish transformants with low copy number from those with high copy number.

The q-PCR method provides robust values as long as the amplification efficiencies for transgene and endogenous control are the same and calculations with efficiencies lower than 1 are also possible.

The $2^{-\Delta\Delta Ct}$ method is simple to apply, because DNA concentrations do not have to be measured and dilution series are not required. Its utility has been demonstrated for plant copy number determination (Ingham et al. 2001) and zygosity analysis in animals (Tesson et al. 2002) and plants (German et al. 2003).

In woody plants, estimating transgene zygosity is a minor priority given that selfing or backcrossing are often not employable.

We do not agree with Bubner and Baldwin (2004) who have stated that SYBR green is not suitable for copy number estimation. The amplification of primer-dimers or falsely amplified by-products contributing to total fluorescence can be ruled out by the melting curve analysis which establishes the recorded signals originating from the transgene amplicon alone. Even if we can't obtain the sequence-specificity of a primer-fluorescent probe system, using two different transgene amplicons (*gusA* and *hpt*) in the quantitative PCR analysis of a single sample, we can greatly increase the reliability of the analysis. The limit for the application of this method is the availability of an internal single-copy reference gene and a single copy transgene calibrator, but this last can be easily provided by i-PCR or blotting.

The use of a combination of the two methods could be implemented by establishing a single-copy number calibrator with i-PCR and subsequent mass screening with q-PCR to identify single copy candidate transgenic clones.

The molecular characterization of the selected clones can be completed by i-PCR to investigate the T-DNA flanking sequences. A few studies have already examined T-DNA insertions, chromosomal rearrangements, and deletion of host DNA (Gheysen et al. 1987; Kim et al. 2003; Kumar and Fladung 2002). Only one large-scale study has investigated the chromosomal mutations created by single-copy transgene insertions, the type of event usually selected for commercial purposes (Forsbach et al. 2003). The i-PCR procedure is time consuming and sometimes difficult to interpret but, differently from Southern blotting, additional manipulation of the amplified bands through band excision from gel, reamplification or sub-cloning, followed by sequencing, can provide a thorough information on the characteristics of the genomic flanking sequences. If the procedure is carried on analyzing both T-DNAs flanking regions, a complete characterization of the integration site can be achieved. Once all the information about the T-DNA flanking sequences are available, databases hosting the sequence of whole genomes, such as that of apple, can be queried to locate the chromosome where T-DNA has been

inserted. Otherwise, primers located within the left and right flanking regions could be used to recover the target sites from the untransformed plants (Fladung 1999). All this information can be obtained at an early stage after the transgenic plant regeneration, as a few mg of fresh weight are sufficient to carry out the analysis.

Keeping into account that the accuracy of the quantification diminishes when the transgene copy number is high, the combination of i-PCR and q-PCR can be considered an alternative to the blotting techniques to determine transgene copy number and provide additional information on the molecular characterization of the integration event.

References


**Biosystems A (2001) Relative Quantitation of Gene Expression User Bulletin #2 ABI PRISM 7700 Sequence Detection System**

**Bubner B, Baldwin IT (2004) Use of real-time PCR for determining copy number and zygosity in transgenic plants Plant Cell Rep 23:263-271 doi:10.1007/s00299-004-0859-y**

**Chen X, Zhang Z, Visser RG, Broekgaarden C, Vosman B (2013) Overexpression of IRM1 enhances resistance to aphids in Arabidopsis thaliana PLoS One 8:e70914 doi:10.1371/journal.pone.0070914**

**Dellaporta S, Wood J, Hicks J (1983) A plant DNA minipreparation: Version II Plant Mol Biol Rep 1:19-21 doi:10.1007/BF02712670**

**Does MP, Dekker BM, de Groot MJ, Offringa R (1991) A quick method to estimate the T-DNA copy number in transgenic plants at an early stage after transformation, using inverse PCR Plant Mol Biol 17:151-153**

**Ferradini N, Nicolia A, Capomaccio S, Veronesi F, Rosellini D (2011) Assessment of simple marker-free genetic transformation techniques in alfalfa Plant Cell Reports 30:1991-2000 doi:10.1007/s00299-011-1107-x**

**Fladung M (1999) Gene stability in transgenic aspen (Populus). I. Flanking DNA sequences and T-DNA structure Mol Gen Genet 260:574-581**

**Forsbach A, Schubert D, Lechtenberg B, Gils M, Schmidt R (2003) A comprehensive characterization of single-copy T-DNA insertions in the Arabidopsis thaliana genome Plant Mol Biol 52:161-176**

**German MA, Kandel-Kfir M, Swarzberg D, Matsevitz T, Granot D (2003) A rapid method for the analysis of zygosity in transgenic plants Plant Science 164:183-187 doi:http://dx.doi.org/10.1016/S0168-9452(02)00381-3**

**Gheysen G, Montagu MV, Zambryski P (1987) Integration of Agrobacterium tumefaciens Transfer DNA (T-DNA) Involves Rearrangements of Target Plant DNA Sequences Proceedings of the National Academy of Sciences of the United States of America 84:6169-6173 doi:10.2307/29794**

**Han Y, Gasic K, Sun F, Xu M, Korban SS (2007) A gene encoding starch branching enzyme I (SBEI) in apple (Malusxdomestica, Rosaceae) and its phylogenetic relationship to Sbe genes from other angiosperms Mol Phylogenet Evol 43:852-863 doi:10.1016/j.ympev.2006.09.001**

**He L, Winterrowd C, Kadura I, Frye C (2012) Transgene copy number distribution profiles in recombinant CHO cell lines revealed by single cell analyses Biotechnology and Bioengineering 109:1713-1722 doi:10.1002/bit.24428**

**Ingham DJ, Beer S, Money S, Hansen G (2001) Quantitative real-time PCR assay for determining transgene copy number in transformed plants BioTechniques 31:132-134, 136-140**

**James D, Dandekar A (1997) Regeneration and Transformation of Apple (Malus pumila Mill.). In: Lindsey K (ed) Plant Tissue Culture Manual. Springer Netherlands, pp 335-352. doi:10.1007/978-94-009-0103-2_19**

**Kim S-R, Lee J, Jun S-H, Park S, Kang H-G, Kwon S, An G (2003) Transgene structures in T-DNA-inserted rice plants Plant Mol Biol 52:761-773 doi:10.1023/A:1025093101021**

**Kumar S, Fladung M (2002) Transgene integration in aspen: structures of integration sites and mechanism of T-DNA integration The Plant Journal 31:543-551 doi:10.1046/j.1365-313X.2002.01368.x**

**Kumpatla SP, Teng W, Buchholz WG, Hall TC (1997) Epigenetic Transcriptional Silencing and 5-Azacytidine-Mediated Reactivation of a Complex Transgene in Rice Plant Physiology 115:361-373 doi:10.1104/pp.115.2.361**

**Latham JR, Wilson AK, Steinbrecher RA (2006) The Mutational Consequences of Plant Transformation Journal of Biomedicine and Biotechnology 2006:25376 doi:10.1155/JBB/2006/25376**

**Lipiński D, Zeyland J, Pławski A, Słomski R (2012) Determination of the Absolute Number of Transgene Copies in CMVFUT Transgenic Pigs vol 12. doi:10.2478/v10220-012-0029-z**

**Livak KJ, Schmittgen TD (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method Methods 25:402-408 doi:http://dx.doi.org/10.1006/meth.2001.1262**

**Mason G, Provero P, Vaira AM, Accotto GP (2002) Estimating the number of integrations in transformed plants by quantitative real-time PCR BMC Biotechnology 2:20**

**Matzke MA, Matzke AJM (1995) How and Why Do Plants Inactivate Homologous (Trans)genes? Plant Physiology 107:679-685 doi:10.2307/4276379**

**Minocha S, Wallace J (2000) Gene Transfer Techniques and their relevance to Woody Plants. In: Jain SM, Minocha S (eds) Molecular Biology of Woody Plants, vol 66. Forestry Sciences. Springer Netherlands, pp 1-24. doi:10.1007/978-94-017-2313-8_1**

**Ochman H, Gerber AS, Hartl DL (1988) Genetic Applications of an Inverse Polymerase Chain Reaction Genetics 120:621-623**

**Omar AA, Dekkers MGH, Graham JH, Grosser JW (2008) Estimation of transgene copy number in transformed citrus plants by quantitative multiplex real-time PCR Biotechnology Progress 24:1241-1248 doi:10.1002/btpr.62**

**Pasquali G, Biricolti S, Locatelli F, Baldoni E, Mattana M (2008) Osmyb4 expression improves adaptive responses to drought and cold stress in transgenic apples Plant Cell Reports 27:1677-1686 doi:10.1007/s00299-008-0587-9**

**Sambrook J, Russell D (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. doi:citeulike-article-id:144016**

**Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis Journal of Molecular Biology 98:503-517 doi:http://dx.doi.org/10.1016/S0022-2836(75)80083-0**

**Tesson L, Heslan J-M, Ménoret S, Anegon I (2002) Rapid and accurate determination of zygosity in transgenic animals by real-time quantitative PCR Transgenic Research 11:43-48 doi:10.1023/A:1013928600442**

**Walter C, Find J, Grace L (2005) Somatic Embryogenesis and Genetic Transformation in Pinus radiata. In: Jain SM, Gupta P (eds) Protocol for Somatic Embryogenesis in Woody Plants, vol 77. Forestry Sciences. Springer Netherlands, pp 11-24. doi:10.1007/1-4020-2985-3_2**

**Yang L, Ding J, Zhang C, Jia J, Weng H, Liu W, Zhang D (2005) Estimating the copy number of transgenes in transformed rice by real-time quantitative PCR Plant Cell Reports 23:759-763 doi:10.1007/s00299-004-0881-0**

**Yi CX, Zhang J, Chan KM, Liu XK, Hong Y (2008) Quantitative real-time PCR assay to detect transgene copy number in cotton (Gossypium hirsutum) Analytical Biochemistry 375:150-152 doi:http://dx.doi.org/10.1016/j.ab.2007.11.022**

**Yu C, Han F, Zhang J, Birchler J, Peterson T (2012) A transgenic system for generation of transposon Ac/Ds-induced chromosome rearrangements in rice Theor Appl Genet 125:1449-1462 doi:10.1007/s00122-012-1925-4**

| Gene | Primer name | Size amplicon | Sequence (5'->3') | Tm° |
|---|---|---|---|---|
| *hpt* | qIgro2 Forward | 117 | GCGAAGAATCTCGTGCTTTCAG | 63,1 |
| | qIgro2 Reverse | | CCGATGCAAAGTGCCGATAAAC | 63,7 |
| *SBE* | qSBE1 Forward | 119 | GGTTTGCGGGTATTGATGGATG | 63,2 |
| | qSBE1 Reverse | | CTATCTCCTGTGTGGAAGTAGGAC | 62,8 |
| *gusA* | qDR52Forward | 116 | AGTGTGATATCTACCCGCTTCG | 63 |
| | qDR5S2 Reverse | | CGCATCTTCATGACGACCAAAG | 63,2 |

**Table 1** Sequences and temperature of melting (Tm°) of the primers producing amplicons of the reported size within the regions of the *hpt*, starch branching enzyme I (*SBE*) and *gus* genes, used in q-PCR for assessing copy number of transgenic clones

**GTAAACCTAAGAGAAAAGAGCGTTTA**TTAGAATAATCGGATATTTAAAAGGGCGTGAAAA
GGTTTATCCGTTCGTCCATTTGTATGTGCATGCCAACCACAGGGTTCCCCTCGGGATCAAAGT
ACTTTGATCCAACCCCTCCGCTGCTATAGTGCAGTCGGCTTCTGACGTTCAGTGCAGCCGTCT
TCTGAAAACGACATGTCGCACAAGTCCTAAGTTACGCGACAGGCTGCCGCCCTGCCCTTTTC
CTGGCGTTTTCTTGTCGCGTGTTTTAGTCGCATAAAGTAGAATACTTGCGACTAGAACCGGA
GACATTACGCCATGAACAAGAGCGCCGCCGCTGGCC

**Table 2** Sequence of the clone 2 T-DNA right border flanking sequence: the fragment of the pCAMBIA 1381 binary vector between the T-DNA right border (in bold) and the first BshFI (GGCC) site present in the vector downstream to the right border is underlined

CATGTAATAATTAACATGTAATGGCATGNACGTTATTTATGAGTATGGGTTTTTATGATTAGA
GTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATA
AATTATCGCGCGCGGTGTCACCTCATTCCCTCTTAGAATCAACAACTTTTTCCTTCCTCTGTT
GGAATAATTTTTGGATCGTCGGGCCCACCCCACTCTAACGACATCGATATTGTCCCCAACTT
AACCACCTGCCCAATCCGTCAGGTGTGGGGTTTTAGCACAAAAGGCCTGGCAGGAGAAACT
GCATCAGCCGATTATCATCACCGAATACGGCGTGGATACGTTAGCC**GGGCTGCACTCATGG**
**TACAC**

CGTTGGATTACGTTAAGNCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGNAT
GGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGATAGAAAACAAAATATAG
CGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCACCTCATTCCCTCTTAGAATCAACAAC
TTTTTCCTTCCTCTGTTGGAATAATTTTTGGATCGTCGGG-
CCTGGCAGGAGAAACTGCATCAGCCGATTATCATCACCGAATACGGCGTGGATACGTTAGCC
**GGGCTGCACTCAATGTACAC**

**Table 3** Sequence of the faint band in comparison to the lighter and brighter band present in the pattern of clone n.13. The underlined 84 bp sequence represent a recombinant fragment which has been inserted in the BshFI site during ligation. Primer copycount 4 (reverse complement) in bold

| Sample | Ct of *SBE* gene (Ctr.) | Ct of *gusA* gene (Ctt) | ΔCt = Ctt – Ctr | ΔΔCt | Copy number |
|---|---|---|---|---|---|
| **1** | 20,3 | 21,57 | 1,27 | Calibrator | 1 |
| **2** | 20,26 | 21,74 | 1,48 | 0,21 | 1 (1,15) |
| **5** | 20,3 | 18,69 | -1,54 | 2,81 | 7 (7,01) |
| **6** | 20,41 | 21,86 | 1,45 | 0,18 | 1 (1,13) |
| **10** | 20,52 | 22,23 | 1,71 | -0,44 | 1 (0,73) |
| **13** | 21,29 | 21,35 | 0,06 | 1,21 | 2 (2,31) |
| **22** | 20,66 | 21,99 | 1,33 | 0,06 | 1 (1,04) |
| **25** | 20,35 | 21,8 | 1,45 | 0,19 | 1(1,12) |
| **28** | 20,30 | 20,32 | 0,02 | 1,25 | 2 (2,37) |

| Sample | Ct of *SBE* gene (Ctr) | Ct of *hpt* gene (Ctt) | ΔCt = Ctt - Ctr | ΔΔCt | Copy number |
|---|---|---|---|---|---|
| **1** | 24,44 | 25,53 | 1,09 | Calibrator | 1 |
| **2** | 23,97 | 25.17 | 1,2 | -0,11 | 1 (0.93) |
| **5** | 23,95 | 22,24 | -1,71 | 2,8 | 7 (6,96) |
| **6** | 23,49 | 24,74 | 1,25 | 0,16 | 1 (1,12) |
| **10** | 24,71 | 25,73 | 1,02 | 0,07 | 1 (1,05) |
| **13** | 25,27 | 25,15 | -0,12 | 1,21 | 2 (2,31) |
| **22** | 24,33 | 25,75 | 1,42 | 0,33 | 1 (1,24) |
| **25** | 24,25 | 25,50 | 1,25 | 0,15 | 1(1,10) |
| **28** | 24,08 | 23,97 | -0,11 | 1,2 | 2 (2,30) |

**Table 4** Calculation of the copy numbers of the transgenic clones applying the $2^{-\Delta\Delta Ct}$ method to the q-PCR data obtained with the amplification of the *gusA* and the *hpt* transgenes and *SBE* as a reference gene

**Fig.1** Diagram of T-DNA and flanking sequences: dashed lines indicate genomic plant DNA sequences with a putative BfaI site (left border) or a BshFI site (right border). Two primers are directed in the reverse direction in order to amplify a template which is a restriction fragment that has been ligated upon itself to form a circle composed by: the fragment between the reverse primer (copycount 2 or 4) and the border; the flanking genomic DNA up to the first BfaI (or BshFI) site; the fragment between the BfaI (or BshFI) site internal to the T-DNA and the forward primer (copycount1 or 3). The fragments amplified with the two couples of primers are indicated by the black solid lines. Dotted lines connect the restriction sites (BfaI or BshFI) to be re-ligated. The back solid bar indicates the probe used in Southern blotting hybridization. Upstream the probe one of the two HindIII cleavage sites is reported. DR5 prom: DR5 promoter, CaMV35SP: CaMV35S promoter, CaMV polyA: CaMV terminator, nos polyA: nos terminator, LB: left border, RB: right border, OD: overdrive

**Fig. 2** Southern analysis with the *gus* probe on genomic DNA extracted from the apple transgenic clones. DNA molecular weight Marker II (Roche Diagnostics Gmbh, Mannheim, Germany), clone 1, 2, 5, 6, 10, 13, 22, 25, 28, Wt non-transgenic apple cv. Greensleeves, DNA molecular weight Marker II. An arrow indicates a faint band detected in clone 10

**Fig.3** Agarose gel of the selective amplification of the left (a and b) and right (c, d and e) border flanking sequences digested with the three restriction endonucleases (BamHi, EcoRI and Pst for the left border and NheI, NruI and MspCI for the right border. The number of bands visible in each run should correspond to the number of copies of DNA integrated into the respective clone genome. PCR on the EcoRI (a) and BamHI (b) digested fragment of clone 6, 22 and 1 did not show any amplification probably due to the presence of an EcoRI and BamHI restriction site between the genomic BfaI site and the integrated T-DNA. The arrows indicate the faint bands observed in clone 13 and 28. In figure a, b and e a 100 bp ladder has been used, in c and d 1 kb ladder (Invitrogen Carlsbad, CA, USA)

**Fig.4** Polyacrylamide gel electrophoresis of the selective amplification of the left (a) and right (b) border flanking sequences of the clones n.5. Polyacrylamide gel permit to resolve banding patterns which sometimes cannot be resolved with agarose gel

**Fig.5** Standard curve comparison. Standard curves for the starch branching enzyme (*SBE*) gene, *hpt* and *gusA* transgene in serially diluted (1000-fold) PCR amplifications. Very efficient amplification coefficients were obtained, as indicated by the slopes of the standard curves. Ct, cycle threshold

**Fig.6** The first derivative of raw fluorescence plotted against an increase in temperature from 50°C to 96°C. The single melt peak indicates a single PCR product for *SBE* and for *gusA* is being amplified (from triplicate measurements for all the sample)

**Fig.7** The first derivative of raw fluorescence plotted against an increase in temperature from 50°C to 96°C. The single melt peak (one for *SBE* and one for *hpt*) indicates a single PCR product is being amplified (from triplicate measurements for all the sample)

Figure 1

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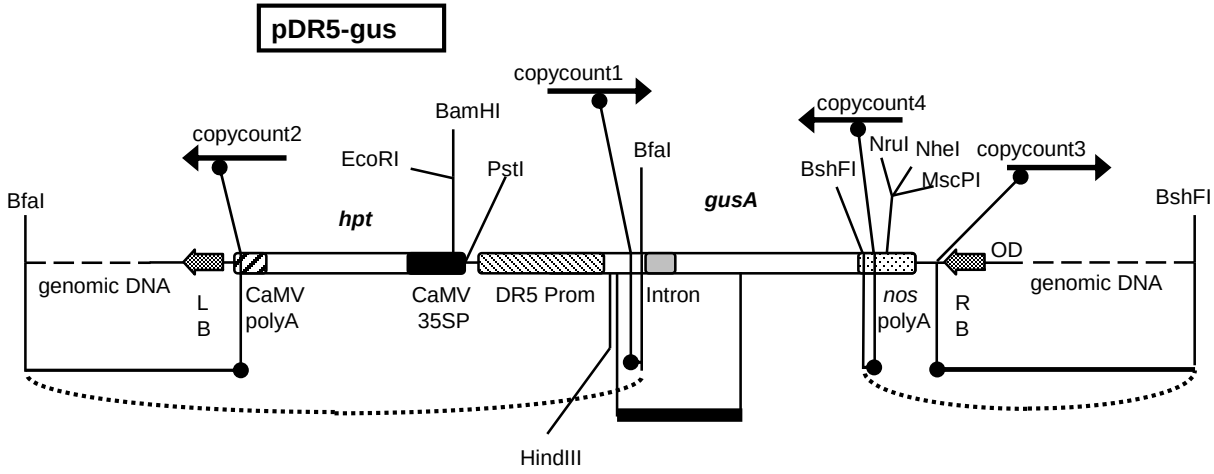

Figure 2

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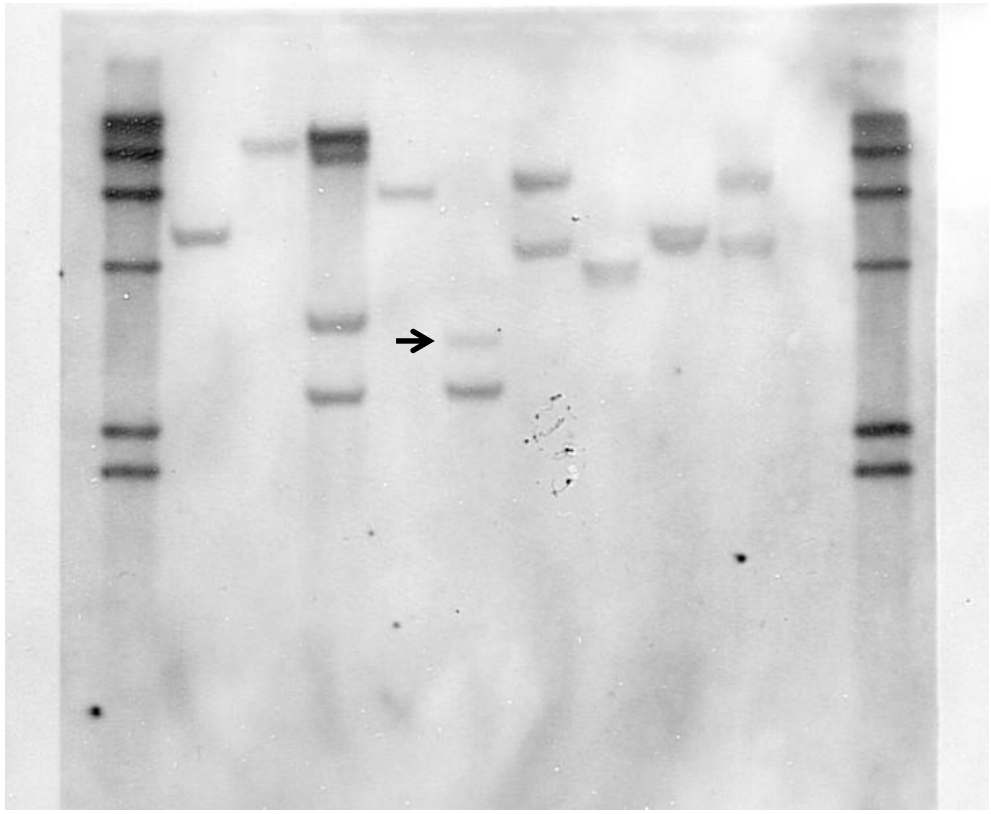

Figure 3

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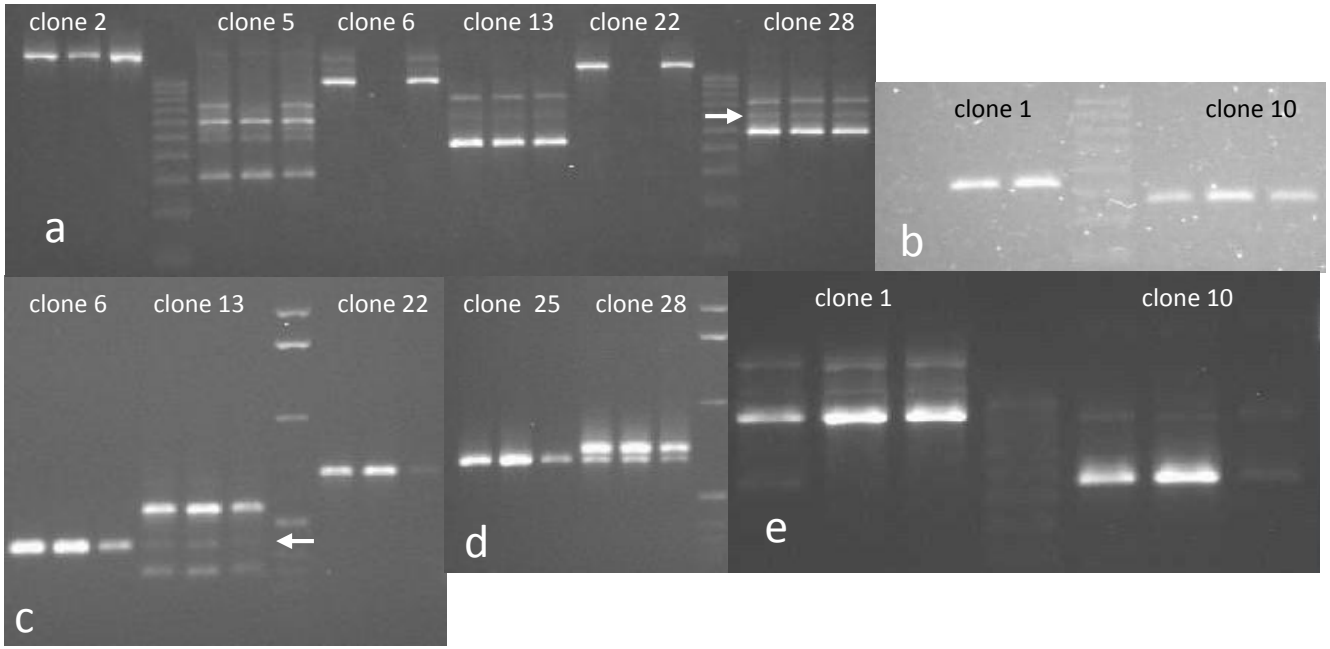

Figure4

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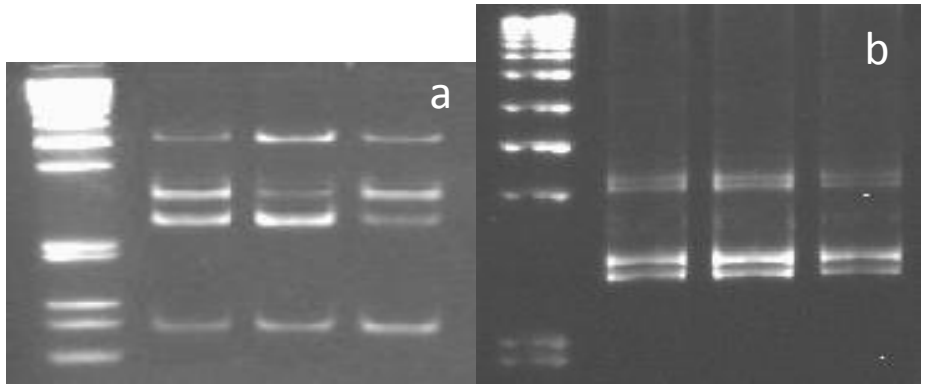

Figure5

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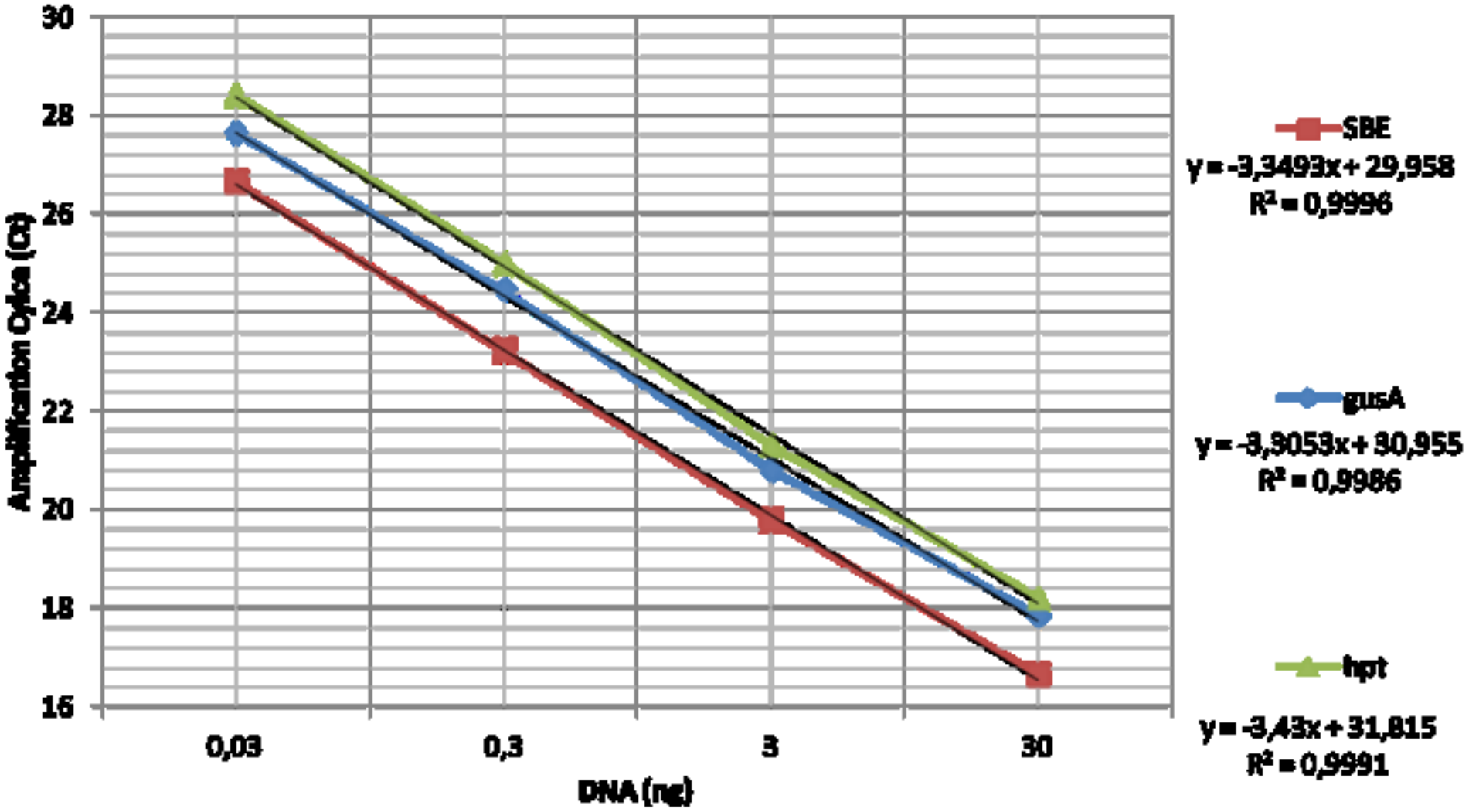

Figure6

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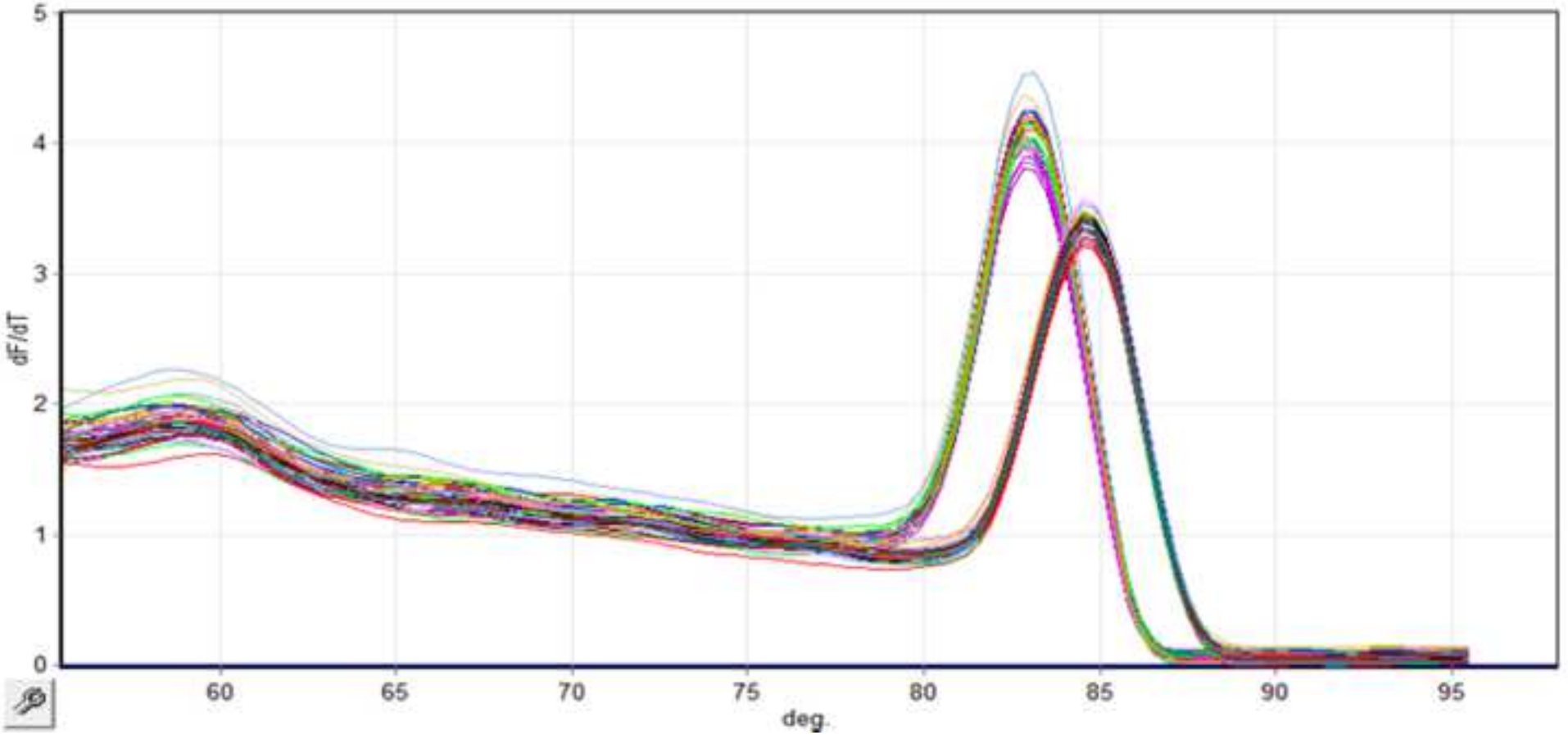

Figure7

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