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(71) Applicant (*for all designated States except US*):
**VLAAMS INTERUNIVERSITAIR INSTITUUT
VOOR BIOTECHNOLOGIE VZW** [BE/BE]; Rijnvis-
schestraat 120, B-9052 Zwijnaarde (BE).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **ANGENON, Geert**
[BE/BE]; Sint-Denijslaan 257, B-9000 Gent (BE). **DE
JAEGER, Geert** [BE/BE]; Kramershoeck 35, B-9940
Evergem (BE). **GOOSSENS, Alain** [BE/BE]; Markt 36,
B-9160 Lokeren (BE). **DEPICKER, Anna** [BE/BE];
Molenstraat 61, B-9820 Merelbeke (BE).

(74) Common Representative: **VLAAMS INTERUNIVER-
SITAIR INSTITUUT VOOR BIOTECHNOLOGIE
VZW**; Rijnvischestraat 120, B-9052 Zwijnaarde (BE).

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(54) Title: HETEROLOGOUS GENE EXPRESSION IN PLANTS

(57) Abstract: The present invention relates to heterologous gene expression in plants. More specifically, the invention relates to high expression of heterologous proteins in seeds, by incorporating the gene between seed specific sequences. Preferably, said heterologous protein is a single-chain antibody variable fragment (scFv).



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HETEROLOGOUS GENE EXPRESSION IN PLANTS

The present invention relates to heterologous gene expression in plants. More specifically, the invention relates to high expression of heterologous proteins in seeds, by incorporating the gene between seed specific sequences. Preferably, said heterologous protein is a single-chain antibody variable fragment (scFv).

The ability to clone and produce a wide range of proteins from diverse sources became feasible with the advent of recombinant technology. The selection of expression hosts for commercial production of heterologous proteins is based on the economics of the production technique, such as the fermentation cost, on the cost of the purification and on the ability of the host to accomplish the post-translational modifications needed for full biological activity of the recombinant protein.

Although in many cases, prokaryotic cells such as *Escherichia coli* or simple eukaryotic cells such as the yeast *Saccharomyces cerevisiae* are the host cells of choice, these systems are not sufficient in all cases and problems can be encountered both in yield and activity of the protein produced. Alternative systems such as plant cells, mammalian cells and insect cells may solve the problem of biological activity, but suffer from a high fermentation cost and a low yield.

Transgenic plants can produce several types of heterologous polypeptides, comprising antibodies (ab's) and antibody fragments (Whitelam *et al.*, 1993; Goddijn and Pen, 1995; Hemming, 1995). Antibodies and antibody fragments are interesting from an industrial point of view: they can be produced against nearly every type of organic molecule and are binding this antigen in a very specific way. However, the major drawback is the production cost. Plants and plant cells are an interesting alternative for the production of these molecules, and other polypeptides that are difficult to produce in other prokaryotic or eukaryotic cells.

US5804694 describes the commercial production of β -glucuronidase in plants, by placing the β -glucuronidase gene after an ubiquitin promoter. With this construction, 0.1% of the total extracted protein is β -glucuronidase. By targeting the heterologous protein to the endoplasmic reticulum, the accumulation can be improved, especially for ab's and ab fragments. Using the cauliflower mosaic virus 35S promoter resulted in expression level of single chain Fv (scFv) antibodies up to 4 - 6.8% of total soluble protein in leaves (Fiedler *et al.*, 1997).

A lot of the efforts for production of proteins in plants have been focused on seeds. Indeed, seeds, especially those of legumes and cereals, contain large quantities of protein; these are mainly storage proteins, which can form up to 7-15% of the dry weight for cereals, and up to 20-40% for legumes. Moreover, those storage proteins are limited in number, and some of them can be responsible for up to 20% of the total protein content in seed (Vitale & Bollini, 1995), which may have important advantages for the purification process. In that respect, the promoters coding for the storage proteins have been considered as ideal tools to obtain high expression levels of proteins in seed (Fiedler *et al.*, 1997)

US5504200 discloses the use of the phaseolin promoter for the expression of heterologous genes in plants and plant cells. WO9113993 describes the expression of animal genes, or the gene from brazil nut 2S storage protein, using a promoter selected from the group consisting of the phaseolin promoter, the α' -subunit of β -conglycinin promoter and the β -zein promoter. The gene is linked to a poly-A signal selected from the group consisting of phaseolin poly-A signal and animal poly-A signal. However, none of these systems leads to high heterologous protein expression. WO9729200 describes a seed specific expression level of 1.9% of heterologous protein on total soluble protein, using the specific legumin B4 promoter. Further improvement of the expression cassettes lead to an expression of 3-4% of scFv antibodies in ripe tobacco seeds (Fiedler *et al.*, 1997)

Recently, the arcelin 5I gene (*arc5I*) of *Phaseolus vulgaris* was isolated and cloned. This gene is responsible for the production of the seed storage protein Arcelin 5a (ARC5a) that accumulates in wild type plants up to 24-32% of the total protein content of the seed (Goossens *et al.*, 1994; Goossens *et al.*, 1995). Expression of the gene in *Arabidopsis thaliana* and *Phaseolus acutifolius* indicated that the seed storage protein ARC5a could be expressed up to 15%, respectively 25% of the total soluble protein (Goossens *et al.*, 1999), when the own promoter and expression signals were used. However, no evidence was shown that the promoter could give efficient expression with other proteins. Surprisingly, we found that the use of the arcelin 5I promoter or the phaseolin promoter, in combination with the arcelin 5I leader sequence or the Tobacco Mosaic Virus (TMV) omega leader and the arcelin 5I 3' sequence could result in an expression level of heterologous protein as high as 12% of the total seed protein, which is far higher than known in the prior art.

It is a first aspect of the invention to provide a seed preferred expression cassette having gene regulatory elements comprising

a) the arcelin promoter comprising the sequence shown in SEQ ID 1 or a phaseolin promoter comprising SEQ ID N° 5.

5 b) the arcelin 5' leader shown in SEQ ID 2 or a TMV omega leader

c) the arcelin 5' 3' end comprising the sequence shown in SEQ ID 3.

Preferably, said seed preferred expression cassette comprises said arcelin promoter, said arcelin 5' leader and said arcelin 5' 3' end.

Said seed preferred expression cassette may also comprise the sequence shown in
10 SEQ ID N° 4, encoding the 2S2 storage albumin signal peptide of *Arabidopsis thaliana* (Krebbbers *et al.*, 1988). In order to obtain seed specific expression of a gene of interest, said gene is placed between said leader sequence and said arcelin 3' end sequence. Preferably, said gene of interest is fused to the sequence encoding the 2S2 storage albumin signal peptide. In one preferred embodiment, the gene of interest is a
15 gene encoding a scFv antibody.

It is another aspect of the invention to provide a seed preferred expression cassette, which is not prone to silencing. When using the expression cassette according to the invention to express a gene of interest, more than 40% of the transformed lines are not silenced and do show a high expression, preferably more than 50% of the lines are not
20 silenced, even more preferably more than 75% are not silenced.

It is another aspect of the invention to provide a method to obtain seed preferred expression of a heterologous protein at a level of at least 10%, preferably a level of at least 15%, 20%, 25%, 30%, 35% or 40 % of the total soluble seed protein, with the proviso that said heterologous protein is not an unmodified seed storage protein such
25 as Arcelin 5a. Preferably, said heterologous protein is not an unmodified Arcelin, Phaseolin or Zein. In a preferred embodiment, a seed preferred expression cassette according to the invention is used. Another preferred embodiment is a method according to the invention, whereby said heterologous protein is a scFv.

Still another aspect of the invention is a plant cell, transformed with an expression
30 cassette according to the invention, or a transgenic plant comprising an expression cassette according to the invention. Indeed, the expression cassette can be incorporated and transformed to a plant cell or plant, using methods known to the person skilled in the art. Said methods include, but are not limited to *Agrobacterium* T-

DNA mediated transformation, particle bombardment, electroporation and direct DNA uptake.

Definitions

- 5 The following definitions are set forth to illustrate and define the meaning and scope of various terms used to describe the invention herein.

Seed preferred expression means that the expression preferably takes place in the seed, but does not exclude expression in other organs of the plant.

- 10 *Gene of interest* as used here means the coding sequence of a gene of which one wants to obtain seed preferred expression.

Leader as used here means the 5' end untranslated sequence.

Signal peptide indicates the initial function of the peptide in the 2S2 albumin storage protein but does not necessarily imply that the peptide has the same function and is processed in the same way when it is fused to the gene of interest.

- 15 *Heterologous protein* refers to any protein that can be expressed in seed but which is not an unmodified seed storage protein. In contrast, modified seed storage proteins (specific mutants, fusion proteins, improved seed storage proteins...) are part of the present invention and are thus included in said definition.

20 Brief description of the figures

Figure 1: Overview of the T-DNA vectors for the evaluation of scFv production in seeds of *Arabidopsis thaliana*.

LB and RB = left- en right border of the T-DNA

- 25 pVS1 = plasmid insertion of *Pseudomonas aeruginosa* for vector stability and replication in *Agrobacterium tumefaciens*.

pBR = ori of replication in *Escherichia coli*.

NptII = selection marker *neomycine phosphotransferase II* under control of the *nos*-promoter and the *ocs* 3'-termination and poly-adenylation-signals.

Sm/SpR = bacterial resistance gene for spectinomycin and streptomycin

- 30 **Figure 2:** Construction of pBluescript (2S2-G4)

Figure 3: Construction of patag5 (3'-arc5I)

Figure 4: Construction of pSP72 (Parc5I/ARC5a^{CS})

Figure 5: Amplification of DNA fragments for vector construction

Figure 6: Construction of pParc5I-G4. This vector has been, in accordance with the Budapest Treaty, deposited with the Belgian Coordinated Collections of Microorganisms-BCCMTM Laboratorium voor Moleculaire Biologie-Plasmidencollectie (LMBP), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium by Dr. Ann Depicker, Molenstraat 61, 9820 Merelbeke, Belgium (work: K.L. Ledeganckstraat 35, 9000 Gent, Belgium) and has accession number: LMBP 4128.

Figure 7: Construction of pParc5I-Ω-G4

Figure 8: Construction of pP35S-G4

Figure 9: Construction of pPβ-phas-G4

Figure 10: Results of scFv-G4 quantification in seed extracts by quantitative Western blot. 500 ng protein of A-, P-, and Ω-seed extracts and 2,5 µg protein of 35S- and Col O-extracts were loaded on a 10 % SDS-PAGE gel. G4 proteins were detected by monoclonal anti-*c-myc* antibody and anti-mouse antibody coupled to alkaline phosphatase. ColO = negative control.

Figure 11: scFv-G4 quantification in seed extracts from floral dip transformants by quantitative Western blot. Results are shown from 10 segregating T2 seed stocks transformed with pParc5I-G4 (upper blot), and 4 segregating T2 seed stocks transformed with pPβ-phas-G4 (lower blot). We loaded 1 microgram seed protein for A1, A7, F28, F31, F38, and F39, 1.5 microgram for A3, A5, A8, A15, A22, and A42; and 2 microgram for A14 and A16 on a 10% SDS-PAGE gel. G4 proteins were detected by monoclonal anti-*c-myc* antibody and anti-mouse antibody coupled to alkaline phosphatase. M = molecular weight marker.

Figure 12: Coomassie blue stained SDS/page gel showing separated *Arabidopsis* seed proteins from transgenics F28 (lanes 3 and 7), F31 (lanes 4 and 8), F38 (lanes 5 and 9), F39 (lanes 6 and 10), transformed with pPβ-phas-G4 and from an untransformed control plant (lane 2). Lanes 3, 4, 5, and 6 contain 30 microgram protein and lanes 7, 8, 9, and 10 contain 20 microgram protein. The arrow indicates the recombinant scFv protein band. Lane 1 contains the molecular weight marker. On basis of the coomassie stained protein bands in separate lanes, Image master VDS software measured following G4 contents for each line: 9.9% for F28 (11.3% by Western blot), 15.4% for F31 (20.0% by Western blot), 16.0% for F38 (19.0% by Western blot), and 9.6% for F39 (12.0 % by Western blot).

Figuur 13: Schematic representation of the ELISA-test used to analyse the antigen binding activity of *ex planta*-extracted and *E. coli*-extracted scFv-proteins. (1) Coating

of microtiter well with monoclonal antibody 9E10 binding to the *cmyc*-tag of the scFv;
 (2) co-incubation of several amounts of scFv from seed or *E. coli* with an excess of the
 antigen dihydroflavonole-4-reductase (DFR) from *Petunia hybrida*; detection of bound
 DFR with (3) polyclonal antiserum against DFR from rabbit and (4) polyclonal anti-
 5 rabbit serum coupled to alkaline phosphatase (AP).

Figure 14: Results of the analysis of antigen-binding activity of seed- and *E.coli*-
 extracted scFv proteins by ELISA. The ELISA test (figure 12) was performed with
 different G4-concentrations (X-axis) in presence or absence (controls) of DFR-antigen.
 Y-axis represents the ELISA-signal (Δ FU/min).

Figure 15: Construction of patag6 (3'-arc5l).

Figure 16: Construction of pParc5l-G4bis.

Figure 17: scFv-G4 quantification in different seeds (3/2, 3/3, 3/4, and 3/5) from a
 single transgenic *Phaseolus acutifolius* plant. From each seed extract, we loaded 3
 (lanes a) or 4 (lanes b) microgram seed protein on a 10% SDS-PAGE gel. G4 proteins
 15 were detected by monoclonal anti-c-myc antibody and anti-mouse antibody coupled to
 alkaline phosphatase. M = molecular weight marker.

Examples

**Example 1: Cloning of the T-DNA vectors for the evaluation of scFv production
 20 in *Arabidopsis thaliana* under control of the *arc5l* expression signals of
Phaseolus vulgaris.**

Four T-DNA vectors were constructed to evaluate and compare scFv production under
 control of the *arc5l* expression signals (vectors pParc5l-G4 and pParc5l- Ω -G4), the
 35S promoter of the cauliflower mosaic virus (vector pP35S-G4), and the promoter of
 25 the β -phaseolin gene of *Phaseolus vulgaris* (vector pP β -phas-G4) (figure 1). A first
 step in the cloning procedure consisted of the construction of three pilot vectors:
 pBluescript (2S2-G4) (figure 2), patag5 (3'-arc5l) (figure 3) en pSP72 (Parc5l/ARC5a^{CS})
 (figure 4).

Construction of pBluescript (2S2-G4) (figure 2):

30 The coding sequence of the scFv fragment G4, fused at its 3'-end to the coding
 sequence of the *c-myc* tag (Evan *et al.*, 1985) and the ER-retention signal KDEL
 (Denecke *et al.*, 1992), was cut from the T-DNA vector pG4ER (De Jaeger *et al.*, 1999)
 by the restriction sites *NcoI* and *XbaI*. Besides, an oligonucleotide was made that
 encodes the signal sequence of the seed storage protein 2S2 from *Arabidopsis*

thaliana (Krebbers *et al.*, 1988). This oligonucleotide was flanked at its 3'-end by the 'sticky' end of the restriction site *NcoI* and was flanked at its 5'-end by a 'sticky' end that complements the restriction site *XbaI* but destroys it after ligation, followed by the restriction sites *EcoRI*, *BglII* en *HindIII*. The G4 fragment and the oligonucleotide were
5 cloned together in the vector pBluescriptKS (Stratagene, La Jolla, CA), after cutting with *HindIII* en *XbaI*. The sequence of the insert was checked and a clone containing a correct insert was selected for further cloning steps. As such, we obtained the pilot vector pBluescript (2S2-G4), which contained the G4-encoding sequence, preceded by a series of unique restriction sites that could be used for the insertion of the different
10 promoters in combination with a mRNA-leader sequence.

Construction of patag5 (3'-arc5I) (figure 3):

An oligonucleotide was made to insert a few unique restriction sites in the T-DNA-vector patag4 (Goossens *et al.*, 1999). The oligonucleotide contained the following
15 restriction sites from its 5'-end to its 3'-end: the 5'-'sticky' end of *EcoRI*, the restriction sites *XbaI*, *XhoI*, and *BglII* and a 3'-'sticky' end that complements *XbaI* but destroys it after ligation. The oligonucleotide was ligated in patag4, after cutting with *EcoRI* en *XbaI*. This resulted in the vector patag5. The sequence of the inserted oligonucleotide was checked and a clone with correct insert was selected for further cloning steps. From the vector pBluescript (*arc5I*) (Goossens *et al.*, 1995), which contains the
20 genomic sequence of the *arc5I*-gene, we cut the 3'-expression signals of *arc5I* (3'-arc5I) by using *XbaI* en *EcoRI*. The 3'-arc5I-fragment was ligated in patag5, after cutting with *XbaI* en *EcoRI*. This resulted in the T-DNA-vector patag5 (3'-arc5I).

Construction of pSP72 (Parc5I/ARC5a^{CS}) (figure 4):

The *arc5I* promoter and the coding sequence of the ARC5a-protein (ARC5a^{CS}) were
25 cut from pBluescript (*arc5I*). This fragment was ligated in the cloning vector pSP72 (Promega, Madison, WI) after cutting with *EcoRI* en *XbaI*. This resulted in the vector pSP72 (Parc5I/ARC5a^{CS}), containing the *arc5I*-promoter preceded by the restriction sites *EcoRI* and *BglII*.

Besides these three pilot-vectors, four DNA fragments were amplified by PCR. These
30 fragments were called PCR1, PCR2, PCR3 en PCR4 (figure 5). Fragments PCR1 en PCR2 were amplified from the 3'-end of the *arc5I*-promoter (Parc5I) in pBluescript (*arc5I*). PCR1 contained the 3'-end of Parc5I followed by the *arc5I*-'leader' and the 5'-end of the 2S2 signal sequence. PCR2 contained the same 3'-end of Parc5I, but followed by the Ω -'leader' and the 5'-end of the 2S2 signal sequence. Both fragments

were flanked by the restriction sites *SacI* en *HindIII*. PCR3 was obtained by amplifying the 35S promoter of the cauliflower mosaic virus from the vector pGEJAE1 (De Jaeger *et al.*, 1999). At the 3'-end of the 35S promoter, we built in the *arc5l*-leader', followed by the 5'-end of the 2S2 signal sequence. PCR3 was flanked by the restriction sites *BglII* and *HindIII*. PCR4 contained the promoter of the β -phaseolin gene of *Phaseolus vulgaris*, amplified from the vector pBluescript (P β -phas) (van der Geest *et al.*, 1994). At the 3'-end of PCR4, we built in the *arc5l*-leader' followed by the 5'-end of the 2S2-signal sequence. This fragment was flanked by the restriction sites *XhoI* and *HindIII*. The pilot vectors and the four PCR fragments were used to clone the four T-DNA vectors from figure 1.

Construction of pParc5l-G4 (figure 6):

First, the 5'-end of the *arc5l* promoter was cut from the vector pSP72 (Parc5l/ARC5a^{CS}) by using *BglII* and *SacI* enzymes. This promoter fragment, together with the PCR1 fragment, was ligated in de vector pBluescript (2S2-G4), after restriction digest with *BglII* en *HindIII*. This resulted in the vector pBluescript (Parc5l-*arc5l*'leader'-2S2-G4). The sequence of the inserted PCR1 fragment was checked and a clone with correct insert was selected for further cloning steps. Finally, the *arc5l*-promoter with the *arc5l*-leader' and the G4-coding sequence was cut from the former construct by the restriction sites *BglII* en *XbaI* and ligated in the vector patag5(3'-*arc5l*), after digestion with *BglII* en *XbaI*. This resulted in the T-DNA vector pParc5l-G4. This plasmid, transformed in *E. coli* MC1061 is deposited at BCCM under deposit number LMBP 4128. The plasmid contains the full *arc5l*-promoter and the full 3'end of *arc5l*, as used in the expression cassettes comprising the *arc5l*-promoter and the 3'end of *arc5l*.

Construction of pParc5l- Ω -G4 (figure 7):

First, the 5'-end of the *arc5l* promoter was cut from the vector pSP72 (Parc5l/ARC5a^{CS}) by restriction digest with *BglII* and *SacI*. This promoter fragment, together with the PCR2 fragment, was ligated in de vector pBluescript (2S2-G4), after restriction digest with *BglII* en *HindIII*. This resulted in the vector pBluescript (Parc5l- Ω 'leader'-2S2-G4). The sequence of the inserted PCR2 fragment was checked and a clone with correct insert was selected for further cloning steps. Finally, the *arc5l*-promoter with the Ω -leader' and the G4-coding sequence was cut from the former construct by the restriction sites *BglII* en *XbaI* and ligated in the vector patag5 (3'-*arc5l*), after digestion with *BglII* en *XbaI*. This resulted in the T-DNA vector pParc5l- Ω -G4.

Construction of pP35S-G4 (figure 8):

The PCR3 fragment was ligated in the vector pBluescript (2S2-G4), after restriction digest with *Bgl*II and *Hind*III. This resulted in the vector pBluescript (P35S-arc5l'leader'-2S2-G4). The sequence of the inserted PCR3 fragment was checked and a clone with correct insert was selected for further cloning steps. Finally, the 35S-promoter with the arc5l-'leader' and the G4-coding sequence was cut from the former construct by the restriction sites *Bgl*II en *Xba*I and ligated in the vector patag5 (3'-arc5l), after digestion with *Bgl*II en *Xba*I. This resulted in the T-DNA vector pP35S-G4.

Construction of pP β -phas-G4 (figure 9):

The PCR4 fragment was ligated in the vector pBluescript (2S2-G4), after restriction digest with *Xho*I en *Hind*III. This resulted in the vector pBluescript (P β phas-arc5l'leader'-2S2-G4). After checking the DNA-sequence of the inserted PCR4-fragment, we found in the *β -phaseolin* promoter a few basepairs that differed from the original sequence (Bustos *et al*, 1991; Genbank accession number J01263). However, the 3'-end of the cloned promoter sequence, starting from the *Nde*I-site, was completely the same as the given sequence. Therefore, this piece, together with the coding sequence of scFv G4, was cut from the vector pBluescript (P β phas-arc5l'leader'-2S2-G4) by using the restriction sites *Nde*I en *Xba*I. Besides, the 5'-end of the *β -phaseoline*-promoter was cut from pBluescript (P β -phas) by the restriction sites *Xho*I en *Nde*I. Both DNA-fragments were ligated in patag5 (3'-arc5l), after restriction digest with *Xho*I en *Xba*I. This resulted in the final vector pP β -phas-G4. Again we checked the sequence of the *β -phaseoline*-promoter and the same differences were found with the original sequence. The sequence between the *Xho*I site and the *Nde*I site, as used in the construct is depicted in SEQ ID N° 5.

The four T-DNA vectors were purified from *Escherichia coli* and electroporated in *Agrobacterium tumefaciens* C58C1Rif^R (pMP90). After colony purification, plasmids were purified from *Agrobacterium* and checked. The *Agrobacterium*-strains were used in *Arabidopsis* transformation.

Example 2: Transformation of *Arabidopsis thaliana* and regeneration of transgenic plants

Arabidopsis thaliana (Columbia genotype O) was transformed by root-transformation (Valvekens et al., 1988) with the constructs pParc5I-G4, pParc5I- Ω -G4, pP35S-G4 en pP β -phas-G4. After selection of transformed calli on kanamycin-selective medium, 150 calli were transferred to shoot inducing medium. Finally, shoots were transferred to root inducing medium. After root formation plants were transferred to the greenhouse and seeds were collected from the following numbers of transgenic *Arabidopsis* plants: 36 for pParc5I-G4, 4 for pP35S-G4, 18 for pP β -phas-G4, and 13 for pParc5I- Ω -G4.

In parallel, the same constructs were used for *Arabidopsis* transformation by 'floral dip' (Clough & Bent, 1998). Transformed T1-plants were selected on kanamycin-containing selective medium, transferred to the greenhouse, and seeds were collected.

Example 3: scFv accumulation in transgenic *Arabidopsis* seeds**Seed extraction and protein quantification**

Crude seed protein extracts were obtained following a modification of the extraction protocol of van der Klei *et al.* (1995) (Goossens *et al.*, 1999). Ground seeds were extracted twice with hexane to remove lipids. The residue was lyophilized and subsequently extracted twice with 50 mM Tris/HCl, 200 mM NaCl, 5 mM EDTA, 0,1% Tween 20, pH 8 (Fiedler *et al.*, 1997) for 15 min at room temperature under continuous shaking. To prevent protein degradation, a protease inhibitor mix (2x CØmplete™, Roche Molecular Biochemicals, Germany) was added to the extraction buffer. The pellet was removed by centrifugation at 20000xg and supernatants were pooled. Total protein quantity in the crude extracts was determined by the Lowry method using the DC Protein Assay (BioRad, Hercules, USA) with BSA as a standard (Table 1). The reliability of *Arabidopsis* seed protein quantification by this method was proven by Goossens *et al.*, 1999.

Table 1: Total protein concentration in transgenic seed extracts (500 microliter) from 10 mg transgenic <i>Arabidopsis</i> seeds				
A 1	A 105	A 140	A 143	A 165A
3,665 µg/µl	3,395 µg/µl	3,042 µg/µl	3,708 µg/µl	3,675 µg/µl
P 3A	P 5	P 15	P 22A	P 102B
2,852 µg/µl	4,028 µg/µl	3,623 µg/µl	3,151 µg/µl	3,339 µg/µl
Ω 7A	Ω 33	Ω 65C	Ω 98A	Ω 130
3,873 µg/µl	3,527 µg/µl	3,184 µg/µl	3,754 µg/µl	3,517 µg/µl
35S 93	35S 101	35S 116	35S 131A	Col O
3,945 µg/µl	3,837 µg/µl	3,879 µg/µl	3,906 µg/µl	3,215 µg/µl

Col O = non-transformed *Columbia* genotype O used as a control

A = pParc5l-G4 transformed *Arabidopsis* line

P = pPβ-phas-G4 transformed *Arabidopsis* line

35S = pP35S-G4 transformed *Arabidopsis* line

Ω = pParc5l-Ω-G4 transformed *Arabidopsis* line

3.2. Quantification of scFv-G4 accumulation

Proteins were separated on SDS/PAGE and accumulation levels of scFv-G4 proteins were determined by quantitative Western blot analysis using the anti-c-myc monoclonal antibody 9E10 (Evan *et al.*, 1995) followed by anti-mouse IgG coupled to alkaline phosphatase (Sigma, St Louis, MO, USA), according to De Jaeger *et al.*, 1999 (figure 10). Different amounts of scFv-G4 proteins purified from *Escherichia coli* (De Jaeger *et al.*, 1999) were used as standards. The constructs pParc5l-G4, pParc5l-Ω-G4, and pPβ-phas-G4 give very high scFv-G4 accumulation levels in *Arabidopsis* seeds, in the range of 10% of total soluble seed protein. These are the highest levels ever reported for scFv proteins produced in plants. Moreover, lines with such high levels were easily found, as only 5 lines were screened for each construct, which is an indication that the expression cassettes are not very sensitive to silencing.

Table 2: ScFv-G4 accumulation levels in transgenic *Arabidopsis* seeds.

PParc5I-G4		pP β -phas-G4		pParc5I- Ω -G4		pP35S-G4	
Line	G4-level (*)	Line	G4-level (*)	Line	G4-level (*)	Line	G4-level (*)
A 1	< 4%	P 3A	10%	Ω 7A	12%	35S 93	< 0,8%
A 105	10%	P 5	10%	Ω 33	< 4%	35S 101	3%
A 140	6%	P 15	< 4%	Ω 65C	6%	35S 116	< 0,8%
A 143	8%	P 22A	12%	Ω 98A	< 4%	35S 131A	< 0,8%
A 165A	8%	P 102B	12%	Ω 130	< 4%		

(*) accumulation level as % of total soluble protein content in transgenic seeds

Example 4: Transformation of *Arabidopsis thaliana* by 'floral dip' and regeneration of transgenic plants

Arabidopsis thaliana (Columbia genotype O) was transformed by 'floral dip' (Clough & Bent, 1998) with the constructs pParc5I-G4, pParc5I- Ω -G4, pP35S-G4, and pP β -phas-G4. Transformed T1-plants were selected on kanamycin-containing selective medium, transferred to the greenhouse, and T2-segregating seed stocks were collected.

ScFv accumulation in transgenic T2-segregating seed stocks

Seed extraction and protein quantification

Seed extraction and protein quantification was determined as described for Example 3.

Quantification of scFv-G4 accumulation

Proteins were separated on SDS/PAGE and accumulation levels of scFv-G4 proteins were determined by quantitative Western blot analysis using the anti-c-myc monoclonal antibody 9E10 (Evan et al., 1995) followed by anti-mouse IgG coupled to alkaline phosphatase (Sigma, St Louis, MO, USA), according to De Jaeger *et al.*, 1999 (figure 11). Different amounts of scFv-G4KDEL proteins purified from *Escherichia coli* were used as standards. Most seed stocks were at least two times analysed. The constructs pParc5I-G4 and pP β -phas-G4 give very high scFv-G4 accumulation levels in *Arabidopsis* seeds, in the range of 5-10% and 10%-20% of total soluble seed protein, respectively (tabel 3). Use of the *arc5I*-untranslated leader in pParc5I-G4 or

the TMV(omega)-leader in pParc5I-Ω-G4 give similar accumulation levels (tabel 3), showing that both leaders allow efficient translation start in seeds. Besides, inter-transgenic variation is low for all four constructs, which is an indication that the expression cassettes are not very sensitive to silencing. Seed extracts of four pPβ-phas-G4 plant lines with highest G4-accumulation were further analysed by SDS/PAGE and Coomassie-blue staining (figure 12). A clear scFv-protein band could be identified at the expected size, which was absent in the untransformed control line. By using the Imagemaster VDS software (Pharmacia, Uppsala, Sweden), the scFv-percentage of total soluble seed protein was measured in each lane. Similar scFv-accumulation levels were obtained for those lines as with the quantitative Western blot analysis.

Table 3: ScFv-G4 accumulation levels in transgenic *Arabidopsis* segregating T2-seed stocks. 20 independent seed stocks were analysed, scFv levels were ranked from highest to lowest.

pParc5I-G4 (*)	pPβ-phas-G4 (*)	pParc5I-Ω-G4 (*)	pP35S-G4 (*)
8.0 ± 1.4	20.0 ± 0.0	5.8 ± 0.4	1.1 ± 0.1
7.8 ± 0.4	19.0 ± 1.4	4.9 ± 0.2	1.1 ± 0.1
6.7 ± 0.1	12.0 ± 0.0	4.5 ± 0.7	1.1 ± 0.1
6.4 ± 0.5	11.3 ± 1.8	4.0 ± 0.0	1.0 ± 0.0
5.3 ± 1.8	7.2 ± 0.2	3.9 ± 0.2	1.0 ± 0.0
4.7 ± 0.5	5.4 ± 0.9	3.9 ± 0.2	0.8 ± 0.1
4.5 ± 0.7	5.0 ± 0.0	3.5 ± 0.2	0.7 ± 0.1
3.9 ± 0.2	4.5 ± 0.7	3.2 ± 0.2	0.7 ± 0.1
3.8 ± 0.4	4.4 ± 0.5	3.1 ± 0.6	0.7 ± 0.1
3.7 ± 0.5	4.0 ± 1.0	3.0 ± 0.0	0.7 ± 0.0
2.4 ± 0.5	3.9 ± 0.2	3.0 ± 0.0	0.7 ± 0.1
1.7 ± 0.5	3.8 ± 0.3	2.7 ± 0.4	0.7 ± 0.1
1.3 ± 0.4	1.8 ± 0.4	1.6 ± 0.4	0.6 ± 0.0
0.9 ± 0.2	1.8 ± 0.3	1.5	0.5 ± 0.1
0.8 ± 0.0	1.6	1.4 ± 0.1	0.5 ± 0.0
0.5 ± 0.1	1.3	0.7 ± 0.0	0.4 ± 0.0
0.5 ± 0.1	1.2	0.6 ± 0.1	0.3 ± 0.0
0.4 ± 0.1	0.8	0.3	0.2 ± 0.1
<0.3	<0.4	0.2	0.1
<0.3	n.d.	0.1	n.d.

(*) scFv accumulation level as % of total soluble protein content in transgenic seeds, with standard deviation. n.d. = not detectable

ScFv accumulation in transgenic T3-homozygous seed stocks

For each construct, 10 T2-seed stocks containing highest G4-levels were genetically screened by segregation analysis. 72 seeds from each seed stock were germinated on kanamycin-containing selective medium and by statistical analysis we identified plant lines containing a single T-DNA locus. For the constructs pParc5I-G4 and pP β -phas-G4, 4 lines containing a single T-DNA locus were chosen to grow and select homozygous seed stocks. 10 T2-plants per line were grown, T3-seeds were collected and homozygous T3-seed stocks were selected by growing plants on kanamycin-containing selective medium. For one of the four lines containing construct pParc5I-G4, no homozygous seed stocks were found. G4-accumulation was measured by quantitative Western blot in T3-segregating and T3-homozygous seed stocks.

Most pParc5I-G4 and all pP β -phas-G4 homozygous seed stocks gave higher G4-levels than the corresponding T3-segregating stocks (tabel 4). For both constructs pParc5I-G4 and pP β -phas-G4, homozygous seed stocks were obtained containing more than 10% G4 of total soluble seed protein. Homozygous seed stocks, transformed with pP β -phas-G4, contained extraordinary high G4 levels, up to 36.5% of TSP in seeds. This is the highest heterologous protein level ever reported for plants.

Tabel 4: ScFv-G4 accumulation levels in transgenic *Arabidopsis* segregating and homozygous T3-seed stocks.

Plant line	T2-segregating seed stocks (*)	T3-segregating seed stocks (*)	T3-homozygous seed stocks (*)
A1	8.0 $\pm$ 1.4	8.2 $\pm$ 1.0 6.7 $\pm$ 0.7	4.4 $\pm$ 0.8 12.5 $\pm$ 1.9
A15	4.7 $\pm$ 0.5	5.6 $\pm$ 1.0 4.7 $\pm$ 0.0	6.4 $\pm$ 1.4 7.7 $\pm$ 1.4
A16	4.5 $\pm$ 0.7	3.8 $\pm$ 0.7 5.1 $\pm$ 1.4	6.0 $\pm$ 1.0 3.5 $\pm$ 0.4
F3	4.5 $\pm$ 0.7	5.8	18.0
F24	5.0 $\pm$ 0.0	7.0 $\pm$ 1.4 4.9 $\pm$ 0.2	13.5 $\pm$ 2.1 15.0 $\pm$ 1.4
F28	11.3 $\pm$ 1.8	13.0 $\pm$ 1.4 10.5 $\pm$ 0.7	21.0 $\pm$ 4.2 18.5 $\pm$ 0.7

F38	19.0 ± 1.4	17.5 ± 0.7 17.5 ± 3.5	36.5 ± 3.4
(*) scFv accumulation level as % of total soluble protein content in transgenic seeds, with standard deviation. n.d. = not detectable. For most lines more than one T3-segregating and homozygous seed stocks were analysed. A1, A15, and A16 are plant lines transformed with construct pParc5I-G4. Lines F3, F24, F28, and F38 were transformed with pPβ-phas-G4.			

Analysis of scFv-quality in seed extracts

Antigen-binding activity of seed extracted G4-proteins was measured and compared with *E. coli*-extracted G4 by ELISA. We used seed extract of the 'phas'-seed stock containing 36.5% G4 (tabel 4). Different amounts of scFv..were incubated with the antigen dihydroflavonole-4-reductase in excess and bound antigen was measured by sandwich-ELISA (figure 13). ELISA-signal curves were set up for both the bacterial and plant scFv and compared. The curves overlap each other (figure 14), indicating that the plant-produced scFv has similar antigen-binding activity as the bacterial scFv.

Example 5: Transformation of *Phaseolus acutifolius* and scFv accumulation in transgenic segregating bean seeds

Phaseolus acutifolius TB1 was transformed with pParc5I-G4bis (figure 16). pParc5I-G4bis contains the same T-DNA as pParc5I-G4, except that it contains an additional P35S-GUS-construct for segregation analysis of transgenic plants.

For the construction of this T-DNA vector, we first made the pilot construct patag6(3'-arc5I) (figure 15) according to the cloning step procedure for patag5(3'-arc5I) (figure3). An oligonucleotide was made to insert a few unique restriction sites in the T-DNA-vector patag3 (Goossens *et al.*, 1999). The oligonucleotide contained the following restriction sites from its 5'-end to its 3'-end: the 5'-'sticky' end of *EcoRI*, the restriction sites *XbaI*, *XhoI*, and *BglII* and a 3'-'sticky' end that complements *XbaI* but destroys it after ligation. The oligonucleotide was ligated in patag3, after cutting with *EcoRI* en *XbaI*. This resulted in the vector patag6. The sequence of the inserted oligonucleotide was checked and a clone with correct insert was selected for further cloning steps.

From the vector pBluescript (*arc5I*) (Goossens *et al.*, 1995), which contains the genomic sequence of the *arc5I*-gene, we cut the 3'-expression signals of *arc5I* (3'-arc5I) by using *XbaI* en *EcoRI*. The 3'-arc5I-fragment was ligated in patag6, after

cutting with *Xba*I en *Eco*RI. This resulted in the vector patag6 (3'-arc5I). This pilot construct was used to make pParc5I-G4bis (figure 16). The *arc5I*-leader' and the G4-coding sequence was cut from the vector pBluescript (Parc5I-arc5I'leader'-2S2-G4) (figure 6) by the restriction sites *Bgl*II en *Xba*I and ligated in the vector patag6 (3'-arc5I), after digestion with *Bgl*II en *Xba*I. This resulted in the T-DNA vector pParc5I-G4bis.

Three transgenic plants were obtained by using the protocol of Dillen *et al.* (1997). As the three plants were regenerated from the same callus, it was expected that they were clones from the same transformation event. Seeds were collected and protein extracts were made from separate seeds in the same buffer as the *Arabidopsis* seed extraction, and according to Goossens *et al.* (1999). Total soluble protein concentration was measured spectrophotometrically at 280nm and G4 accumulation was determined by quantitative Western blot.

G4 was detected as a single protein band (figure 17) in, on average, 3 of 4 seeds for all three segregating seed stocks. Therefore, these transformants most probably contain a single T-DNA-locus. All analysed G4-accumulating seeds contained 2-2,5% G4 of total soluble protein or 2-2,5 milligram scFv per gram fresh weight seed. So far, only one paper reported scFv production in leguminous species. Perrin *et al.* (2000) obtained 9 microgram scFv per gram fresh weight in pea seeds with the *legA* promoter. The accumulation with the *arc5I* promoter construct is thus 4 to 5 hundred times higher than the reported levels with the *legA* promoter. As we only obtained one transgenic plant line, most probably plant lines with even higher scFv levels can be obtained. Goossens *et al.* (1999) already obtained 4 times higher levels of ARC5I protein in homozygous seed stocks compared to segregating seed stocks and this under control of the same *arc5I* promoter. As such, after obtaining more transgenic lines and selection of homozygous lines, we expect to reach 10% scFv levels in *Phaseolus acutifolius*, by using this *arc5I* promoter construct.

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Claims

1. A seed preferred expression cassette having gene regulatory elements comprising:

- the arcelin promoter comprising the sequence shown in SEQ ID N° 1,
- the arcelin 5I leader shown in SEQ ID N° 2, and
- the arcelin 5I 3'end comprising the sequence shown in SEQ ID N° 3.

2. A seed preferred expression cassette according to claim 1 wherein said arcelin promotor is replaced by a phaseolin promoter comprising the sequence shown in SEQ ID N° 5.

3. A seed preferred expression cassette according to claim 1 wherein a TMV omega leader replaces said arcelin 5I leader.

4. A seed preferred expression cassette according to any of claims 1-3 further comprising the sequence shown in SEQ ID N° 4 encoding the 2S2 storage albumin signal peptide.

5. A seed preferred expression cassette according to any of claims 1-4 whereby a gene of interest is placed between said leader sequence and said arcelin 5I 3' sequence.

6. A seed preferred expression cassette according to claim 5, whereby said gene of interest is fused the sequence encoding the 2S2 storage albumin signal peptide.

7. A seed preferred expression cassette according to claim 5 or 6, whereby the gene of interest encodes a single chain Fv fragment.

8. A method to obtain seed preferred expression of a heterologous protein at a level of at least 10% of the total soluble seed protein, with the proviso that said heterologous protein is not an unmodified seed storage protein.

9. A method according to claim 8 wherein said level is at least 15% of the total soluble seed protein.

10. A method according to any of claims 8-9, using an expression cassette according to any of claims 1-7.

11. A plant cell transformed with an expression cassette according to any of the claims 1-7.

12. A transgenic plant comprising an expression cassette according to any of the claims 1-7.

Figure 1

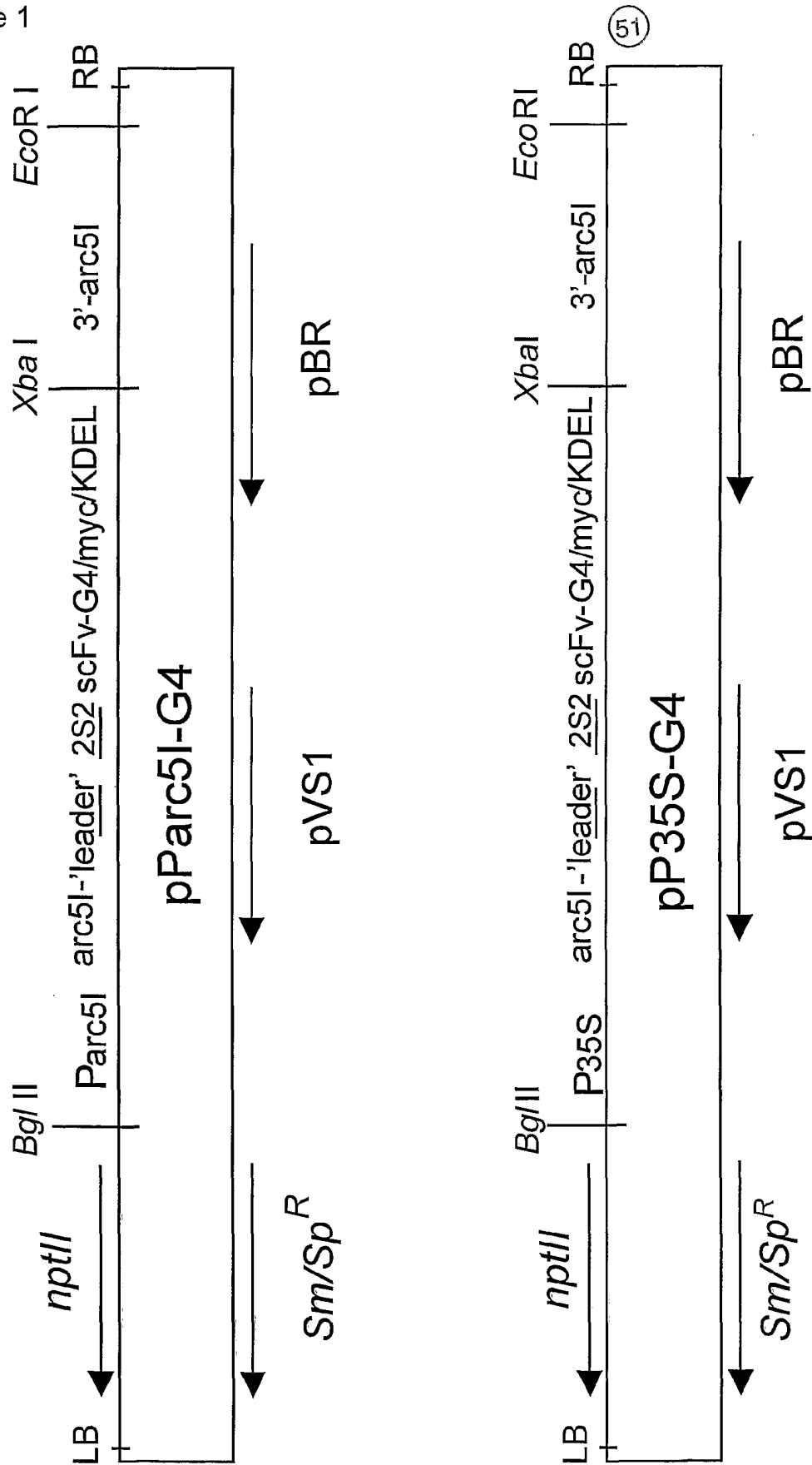
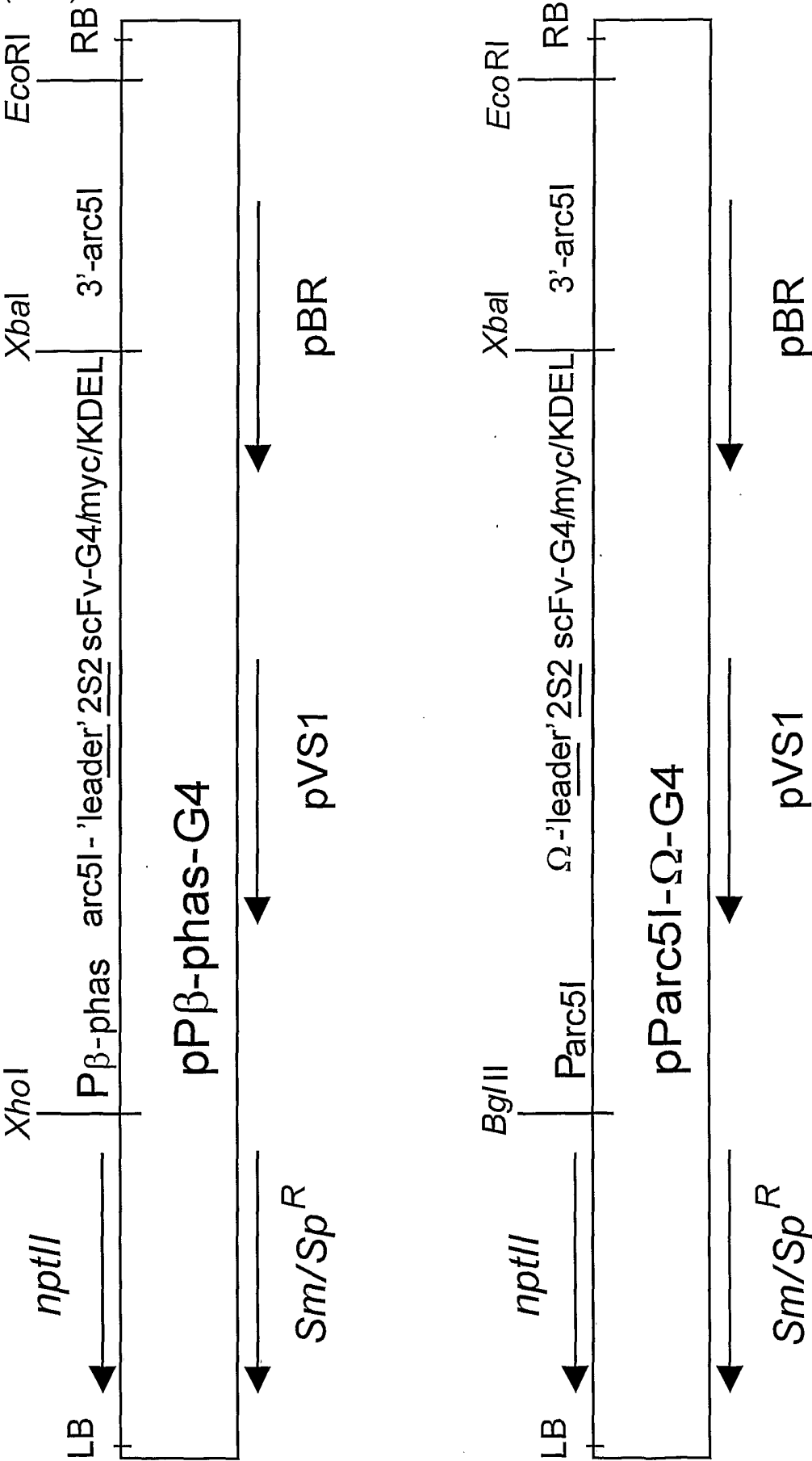
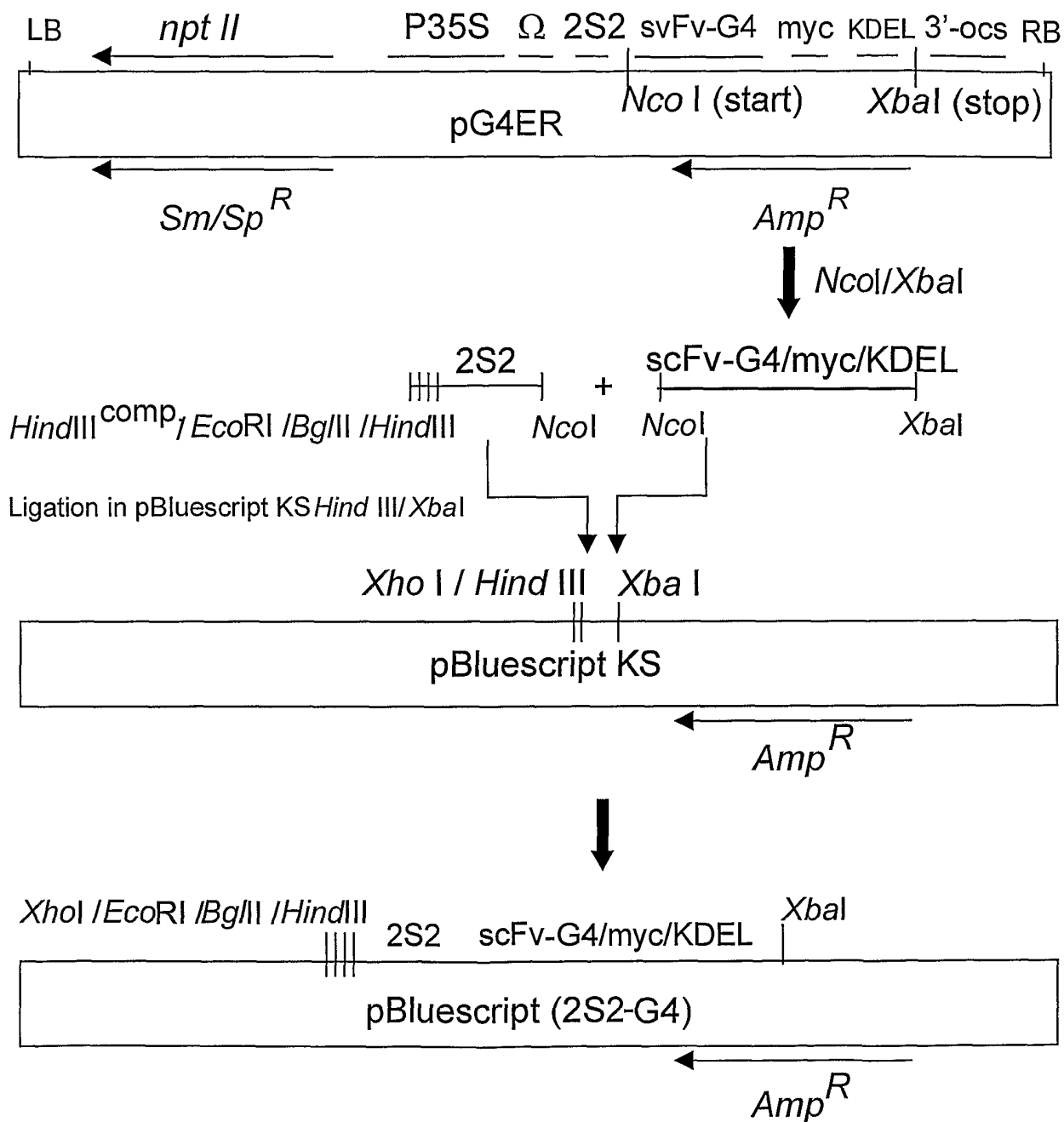


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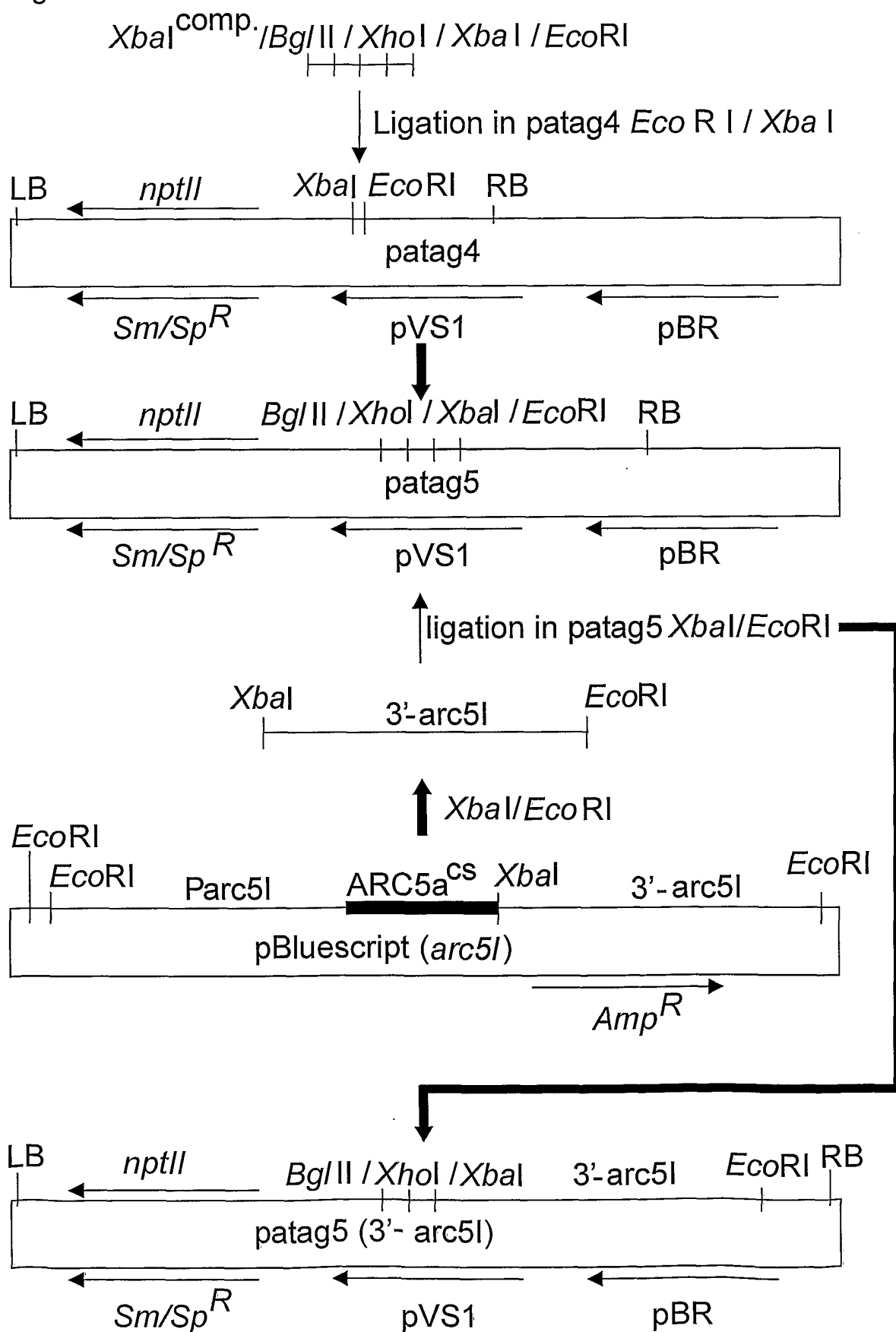
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Figure 2



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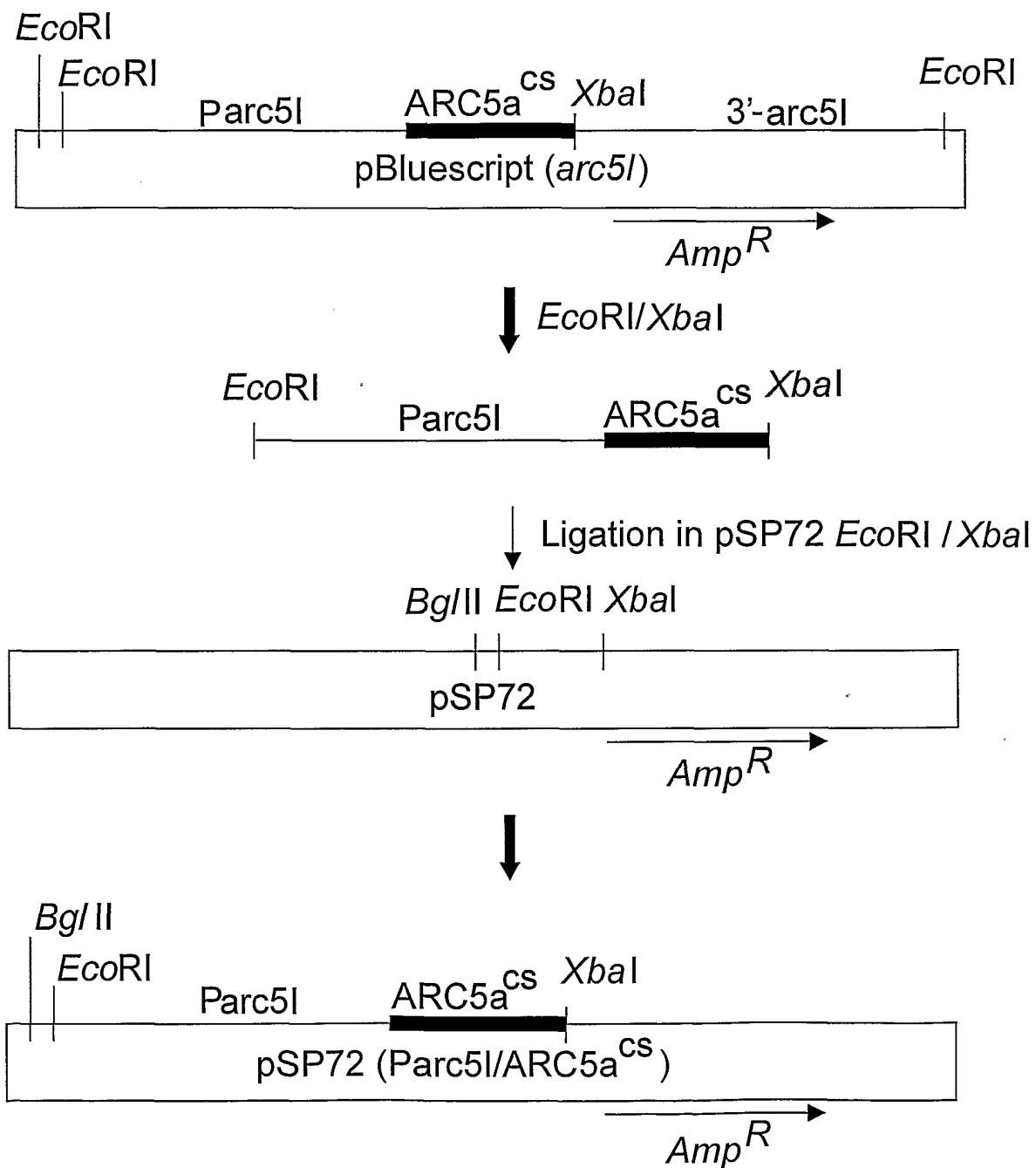
Figure 3



ARC5a^{CS} = coding sequence of the ARC5a - protein

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Figure 4



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Figure 5

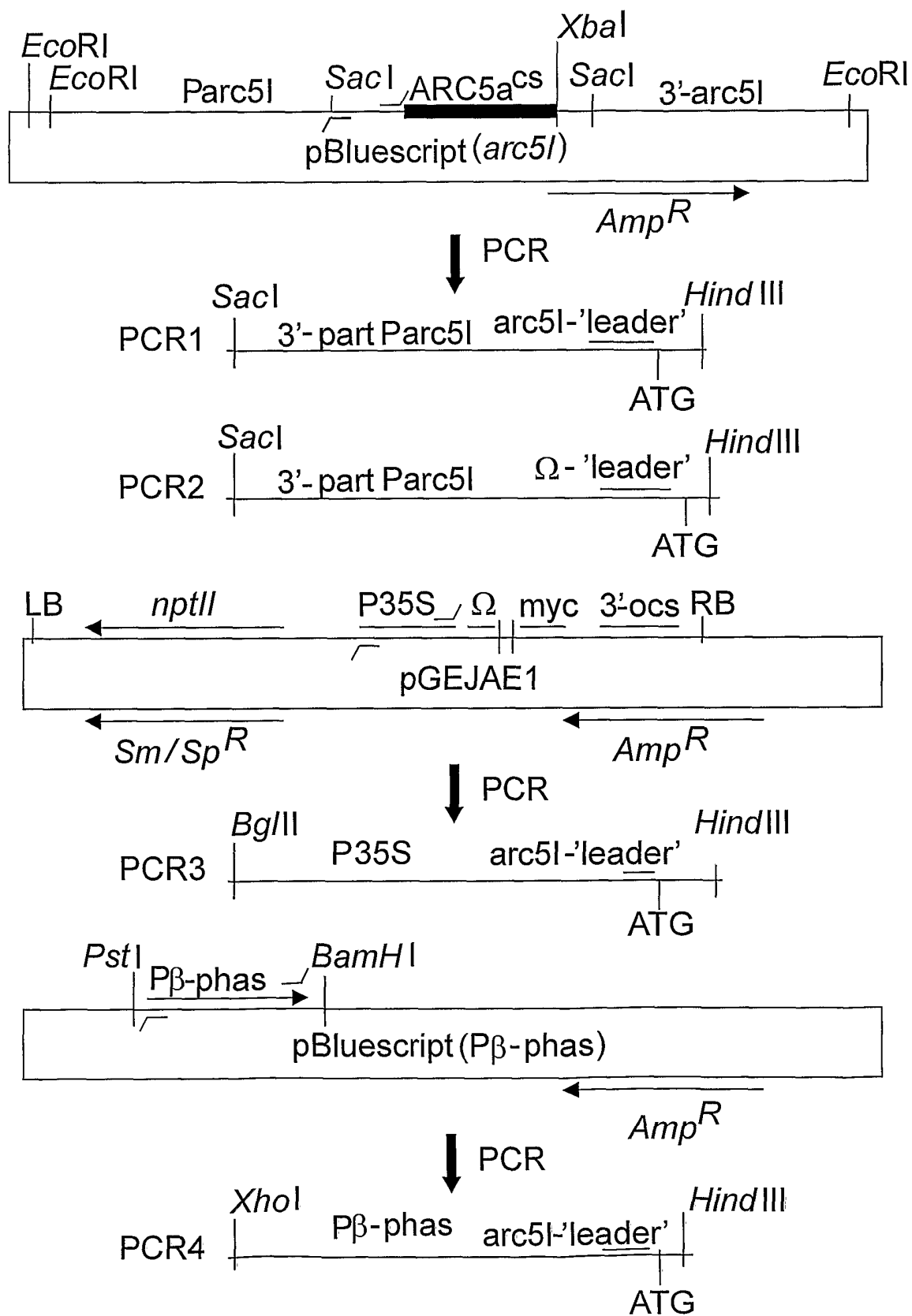
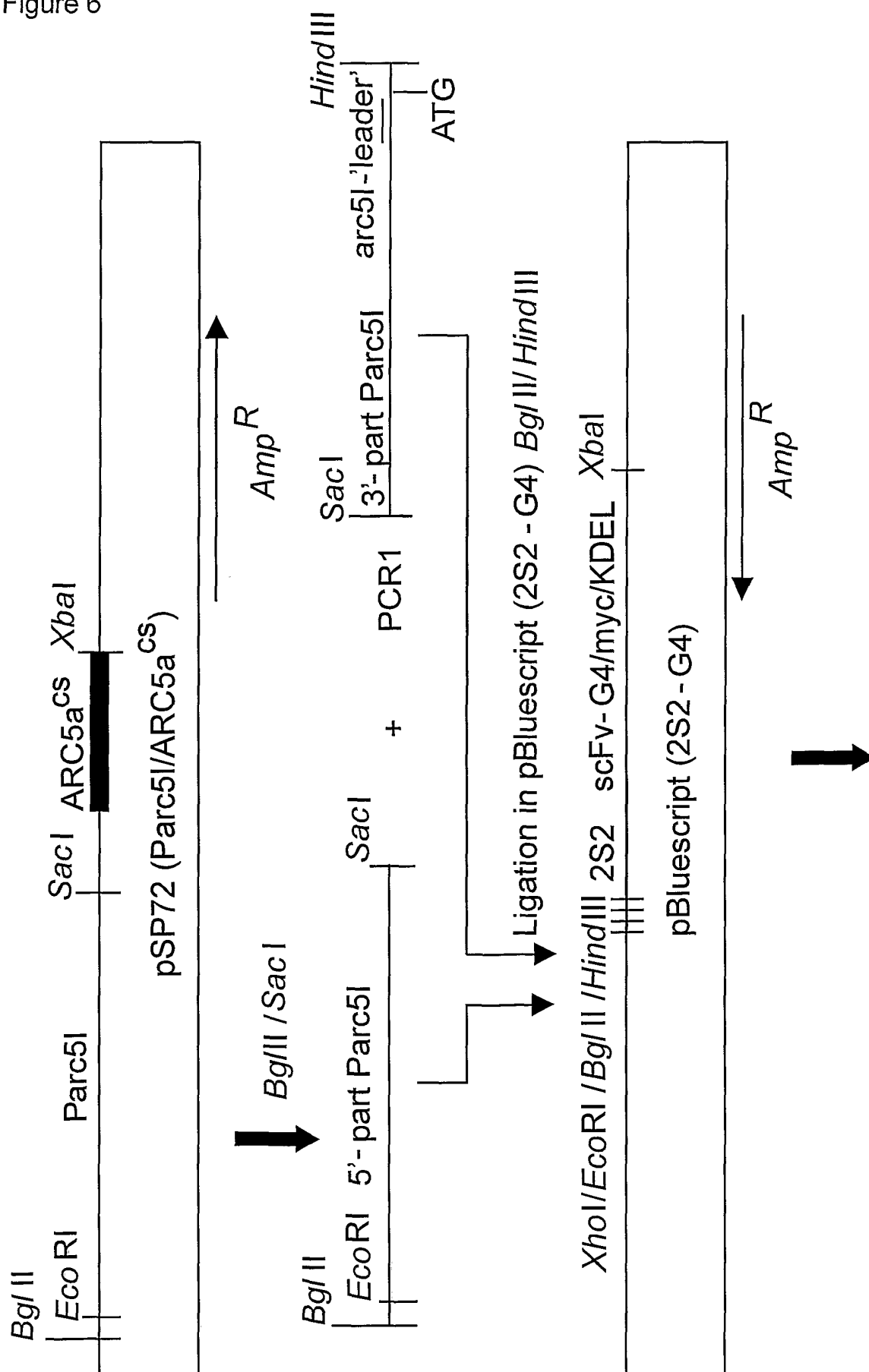
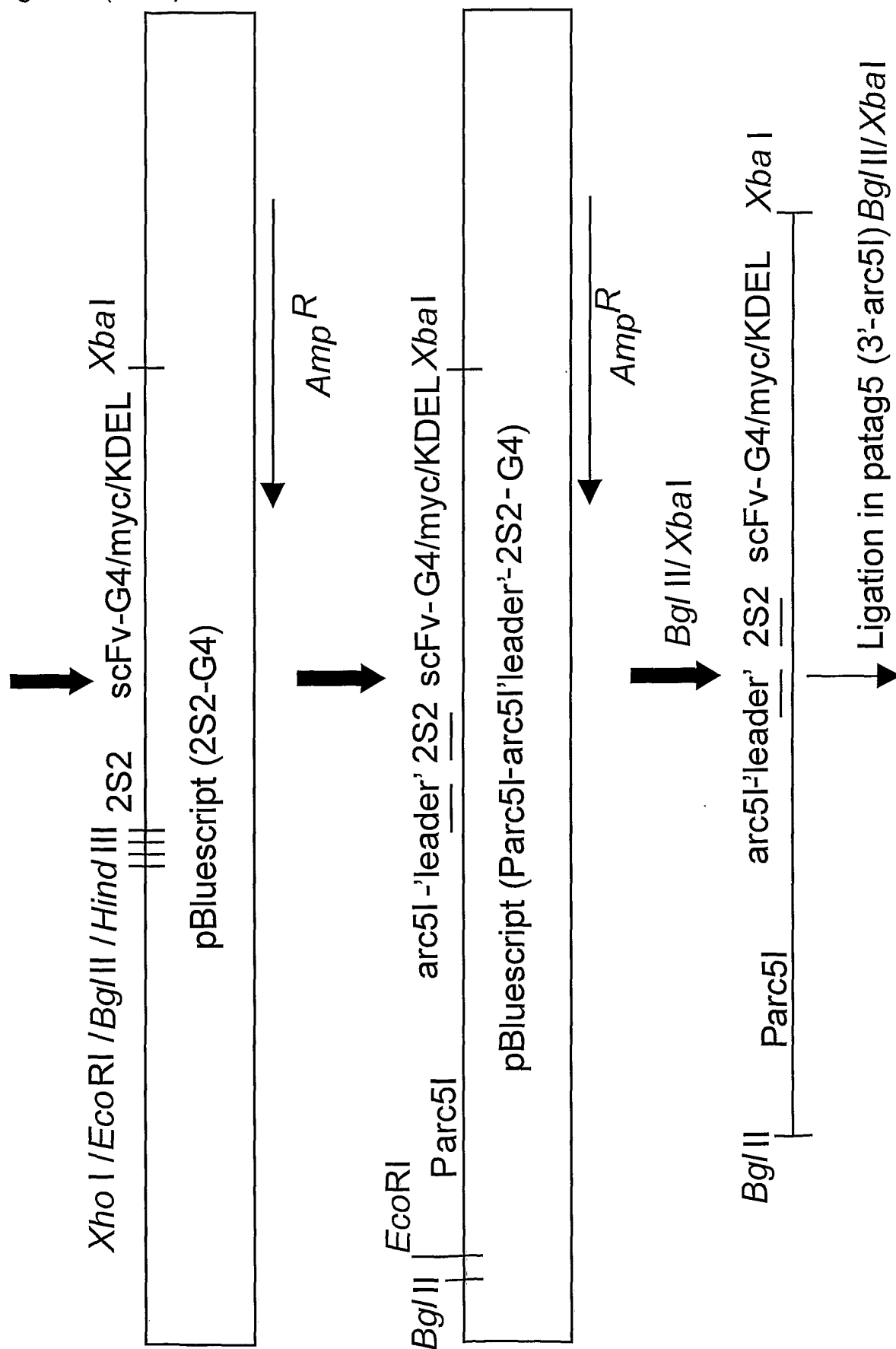


Figure 6



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Figure 6 (cont.)



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Figure 6 (cont.)

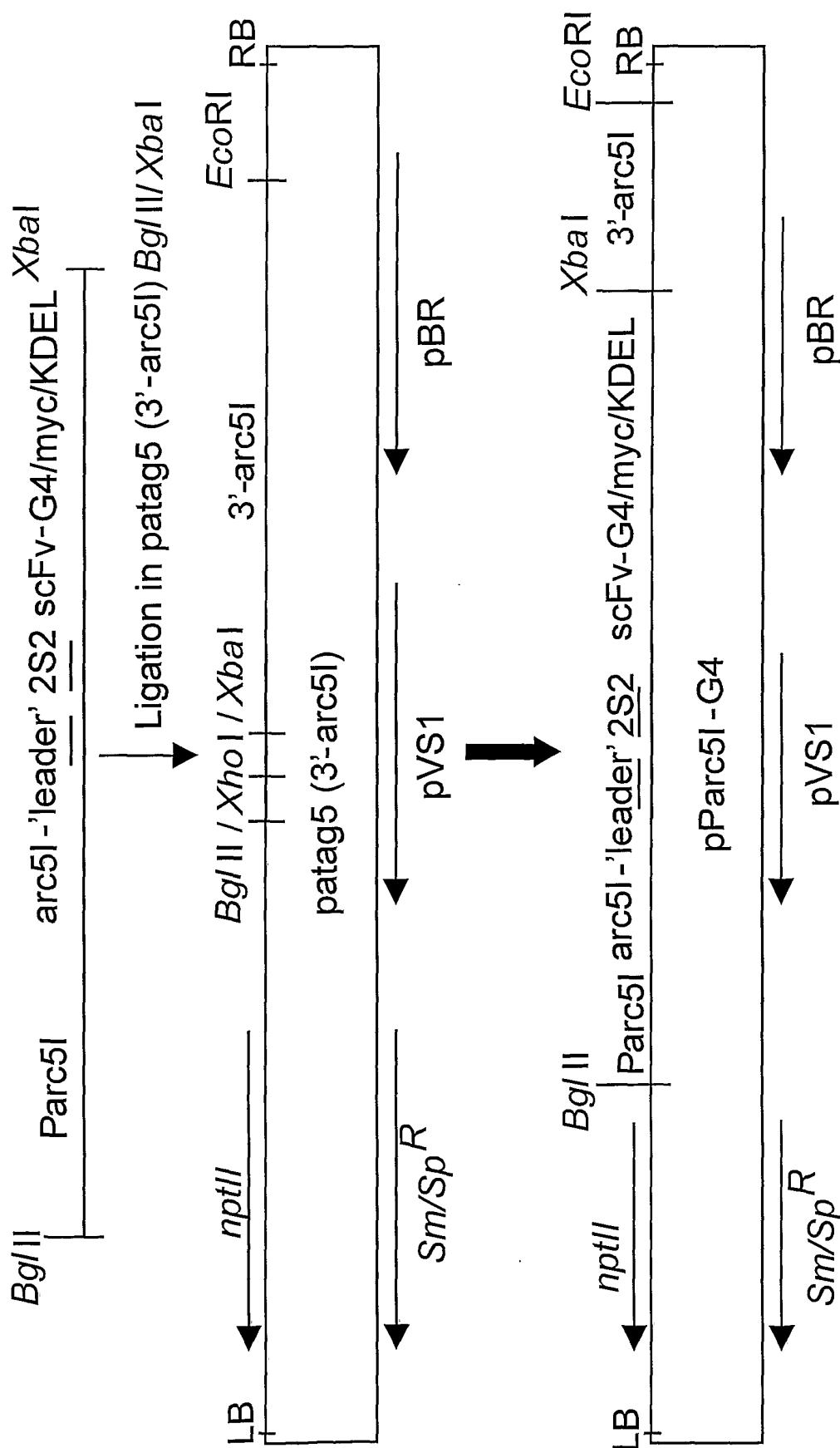
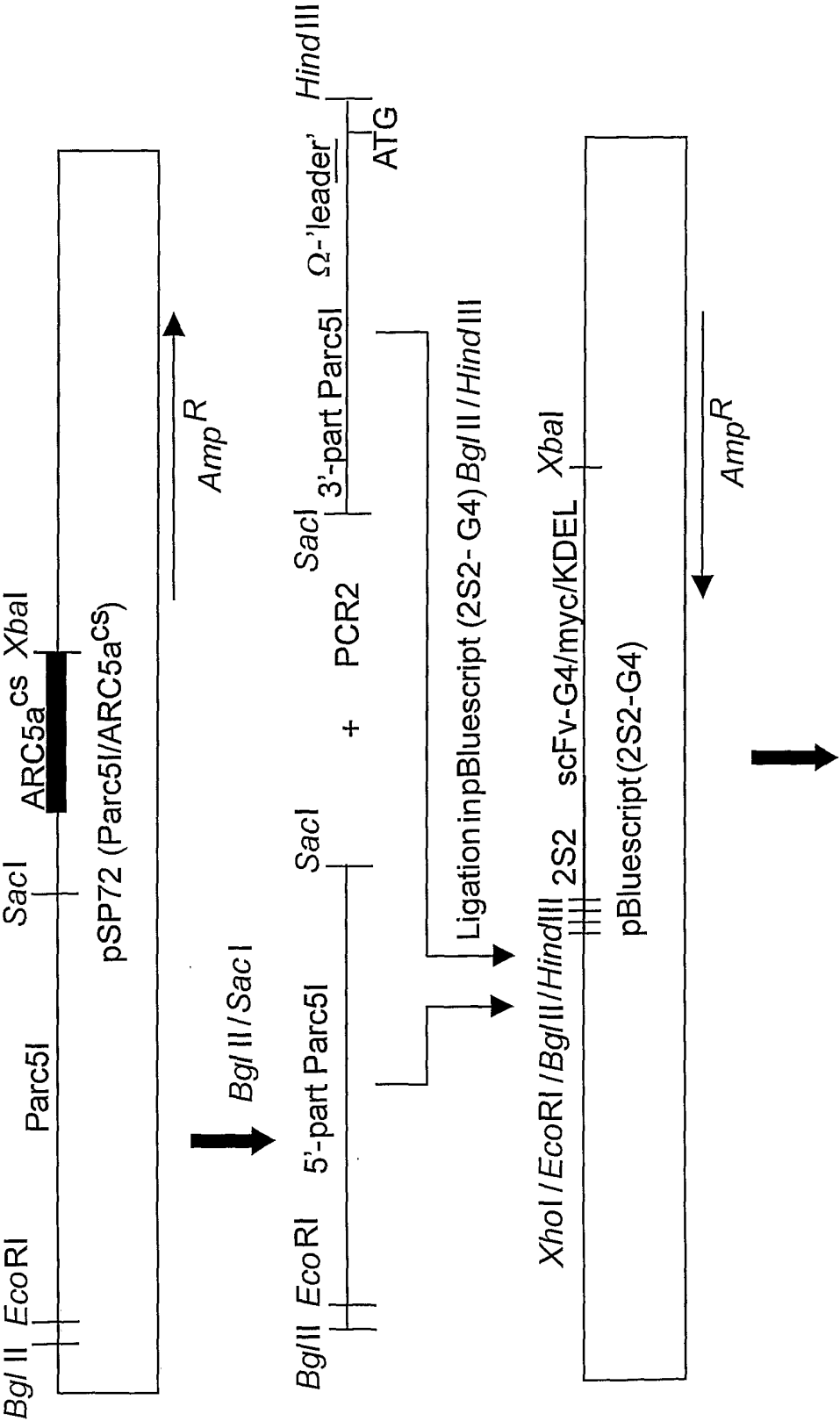
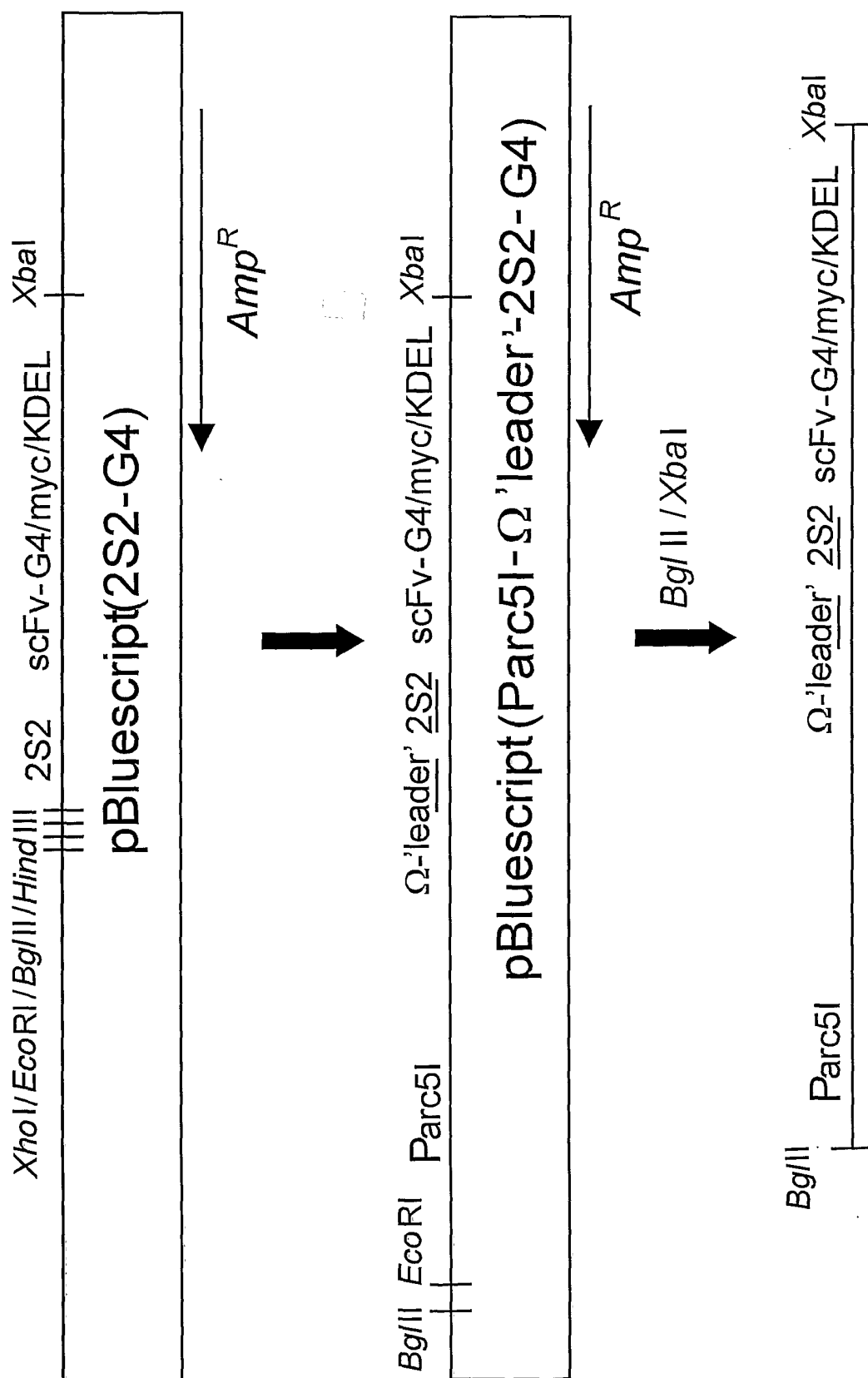


Figure 7



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Figure 7 (cont.)



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Figure 7 (cont.)

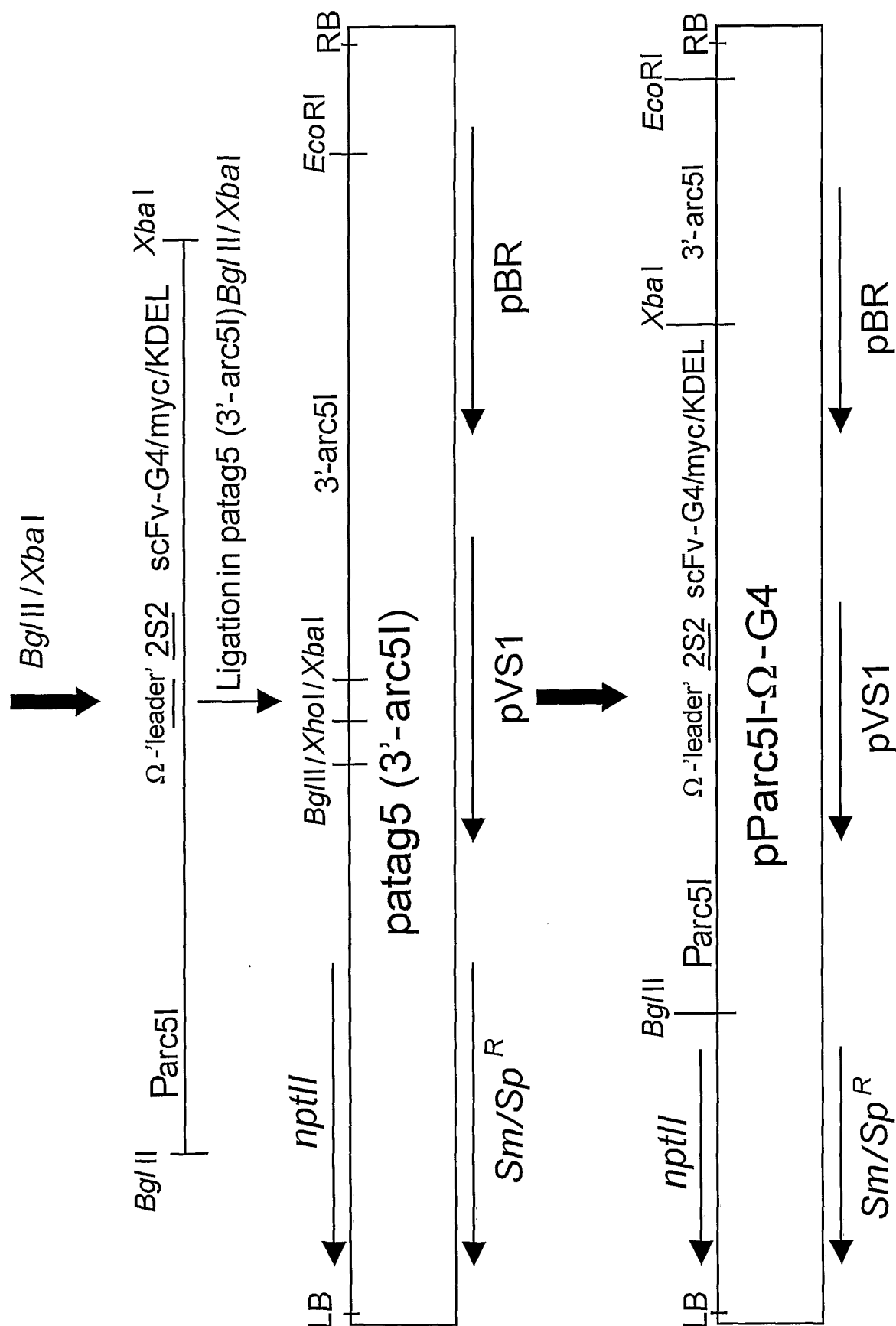
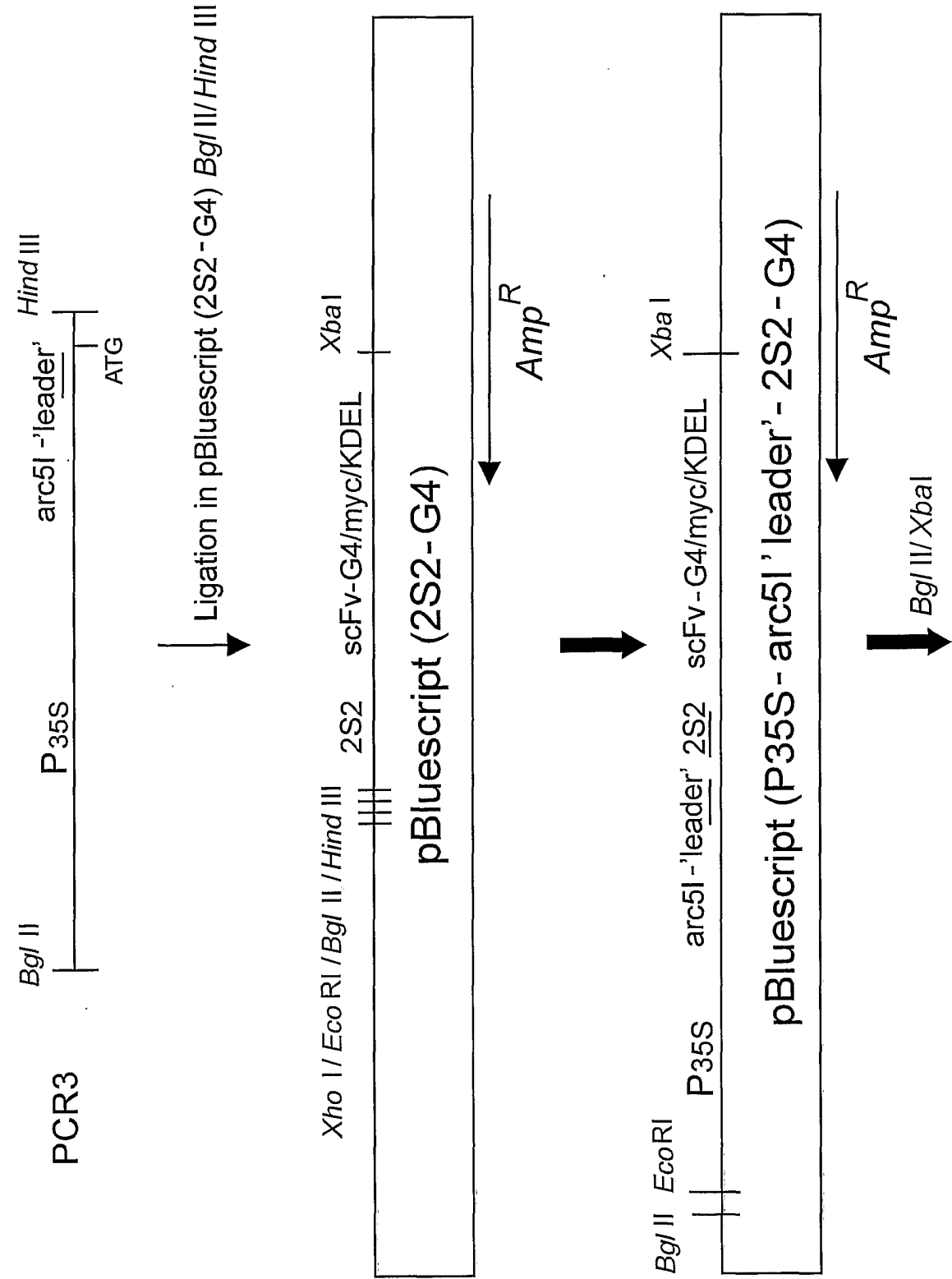


Figure 8



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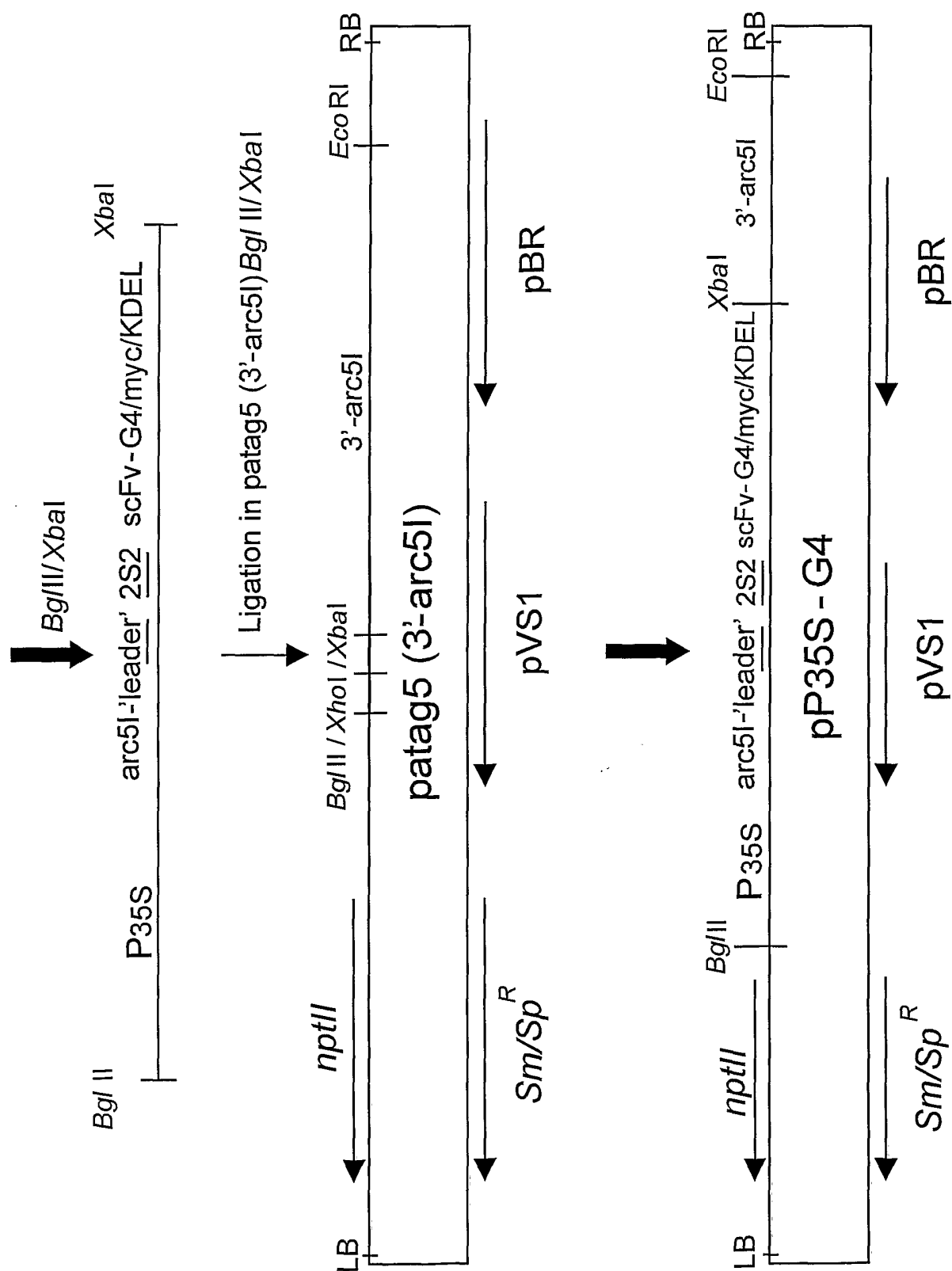
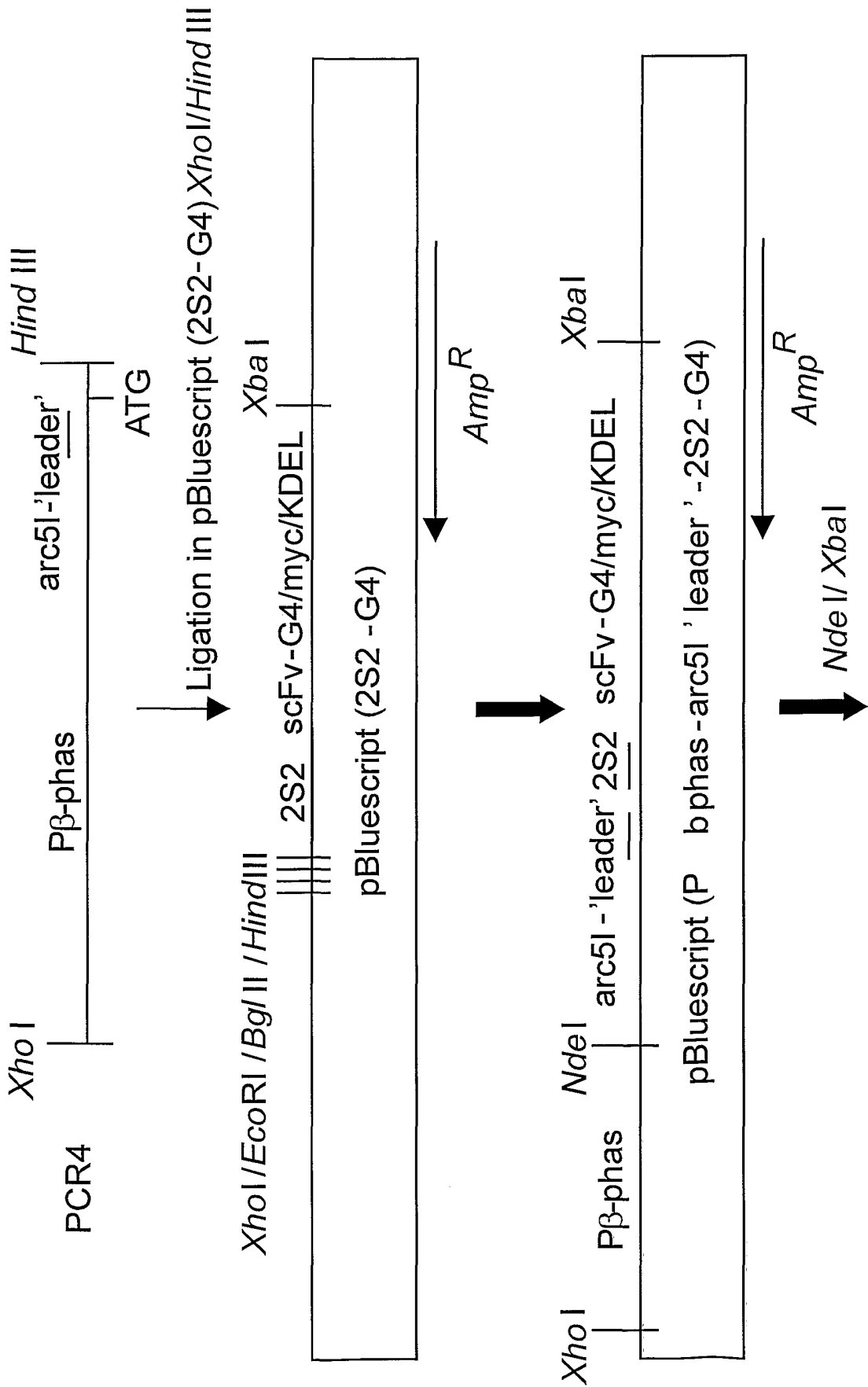
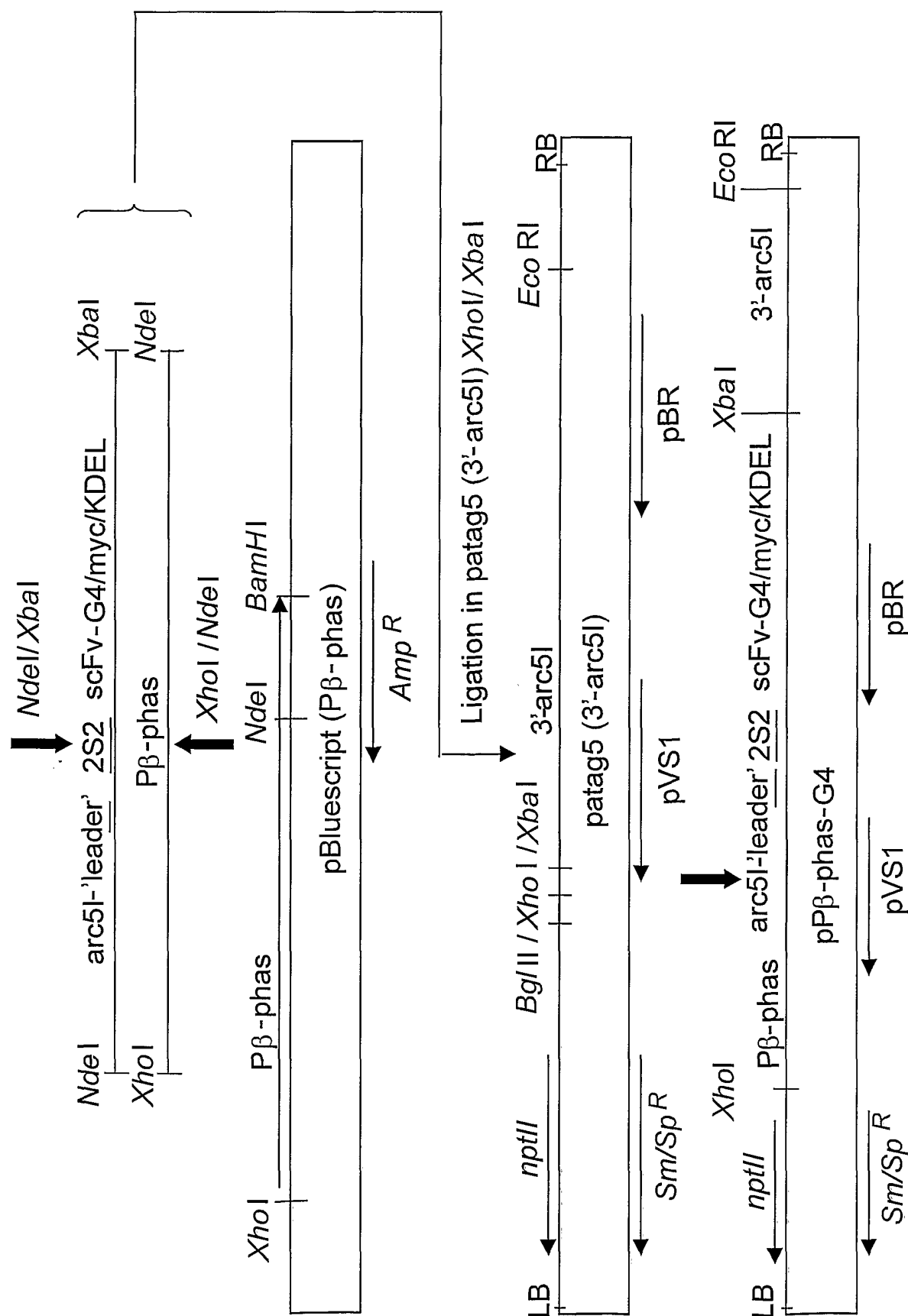


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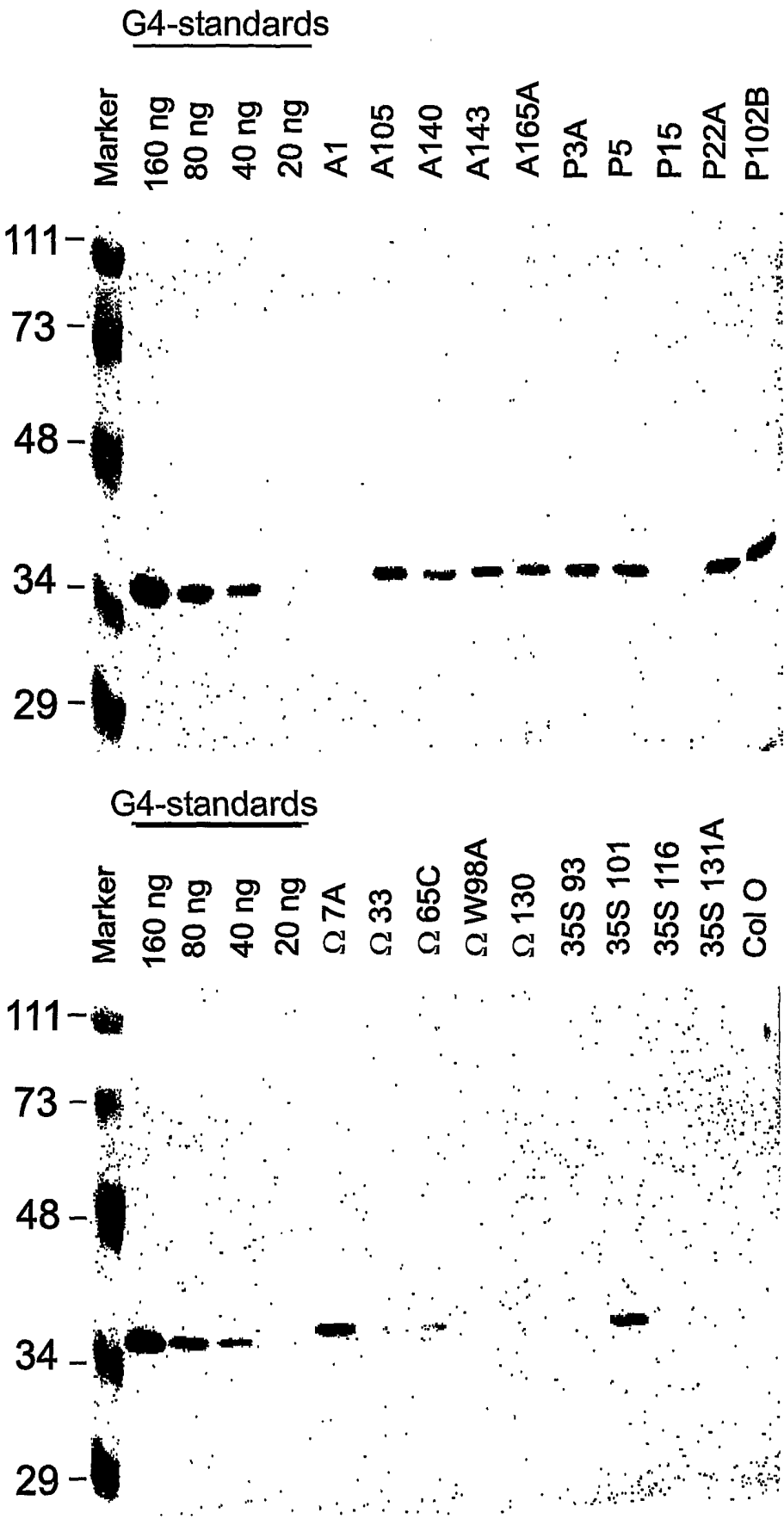
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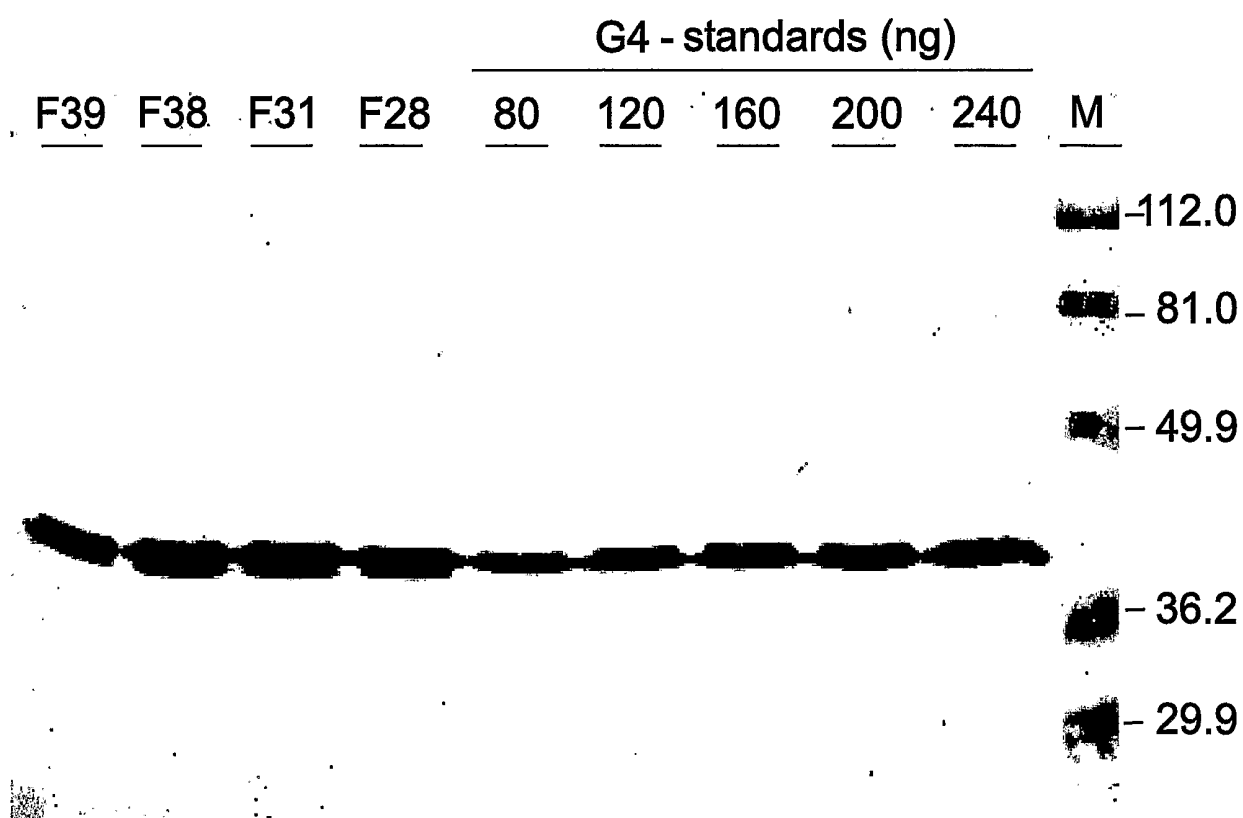
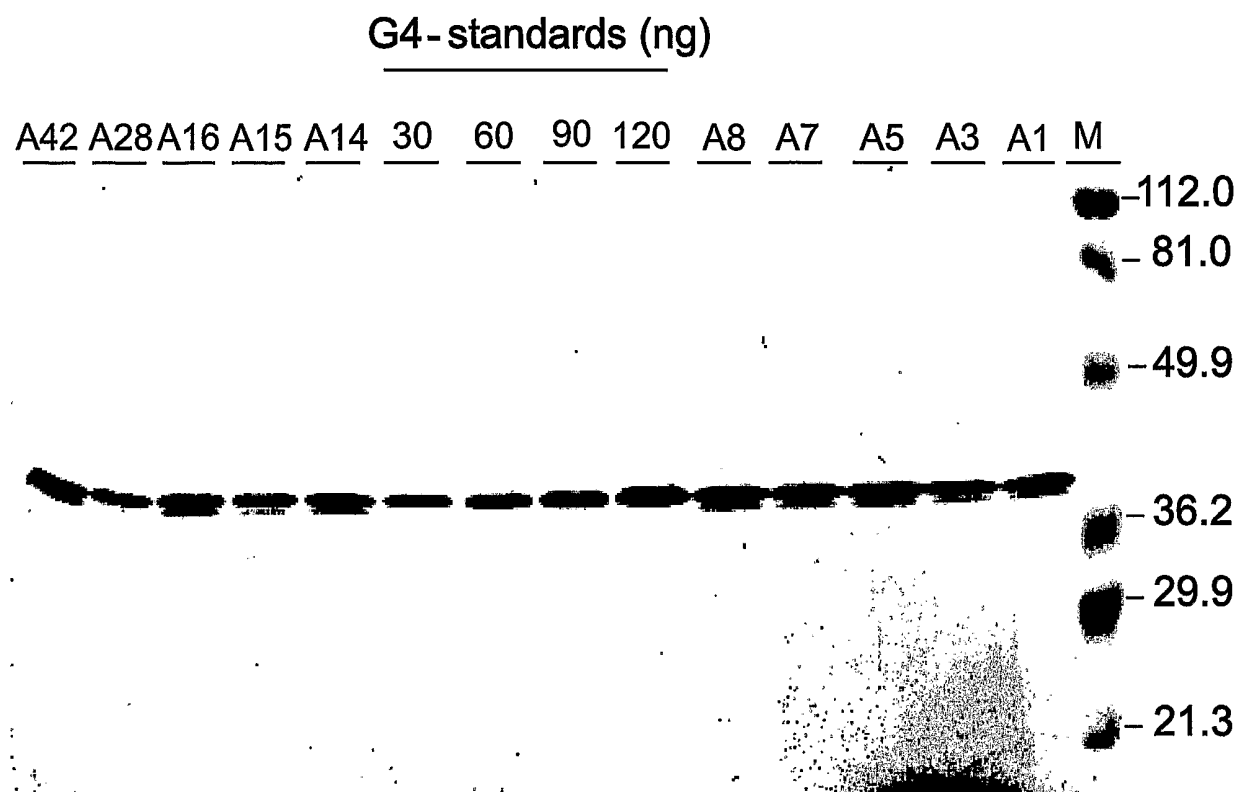
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Figure 10



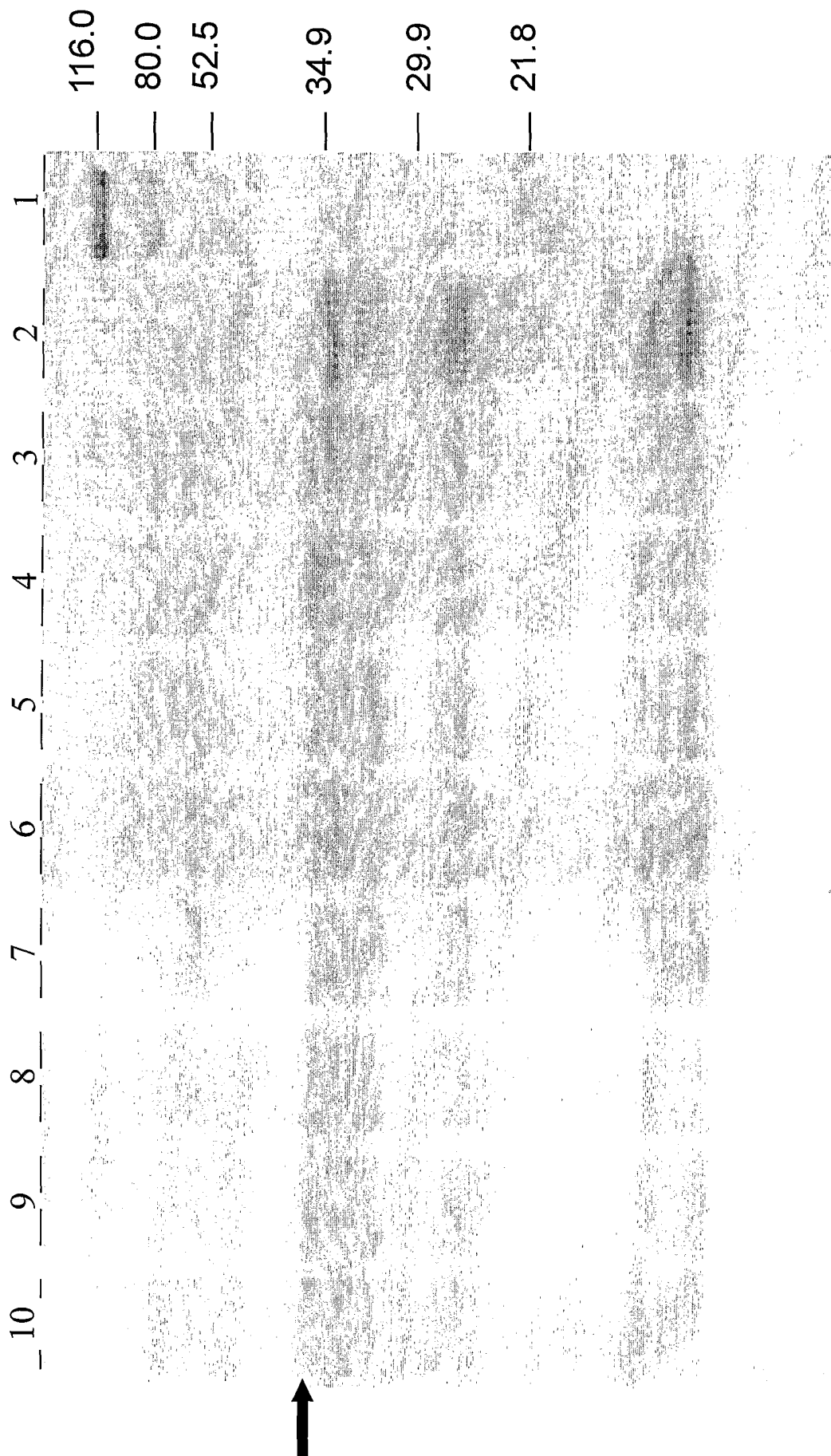
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Figure 11



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Figure 12



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Figure 13

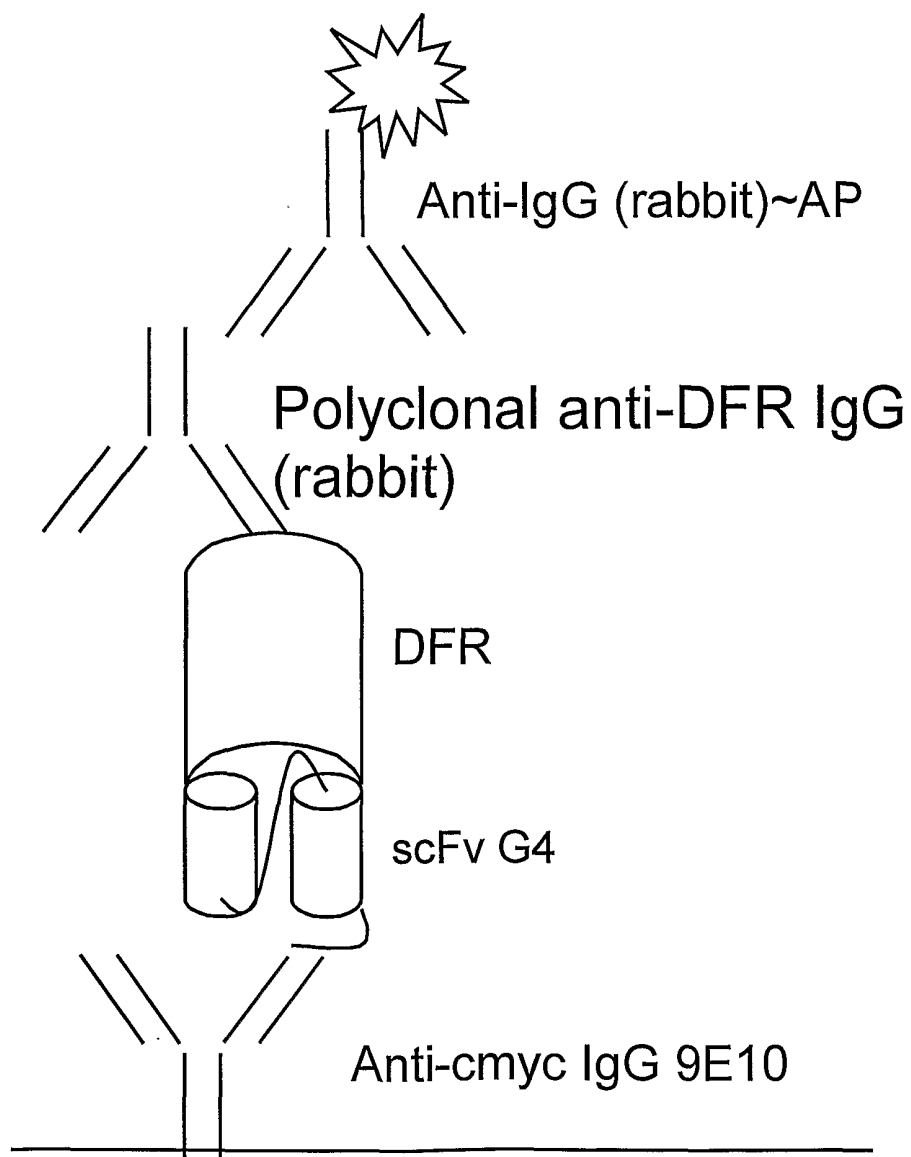
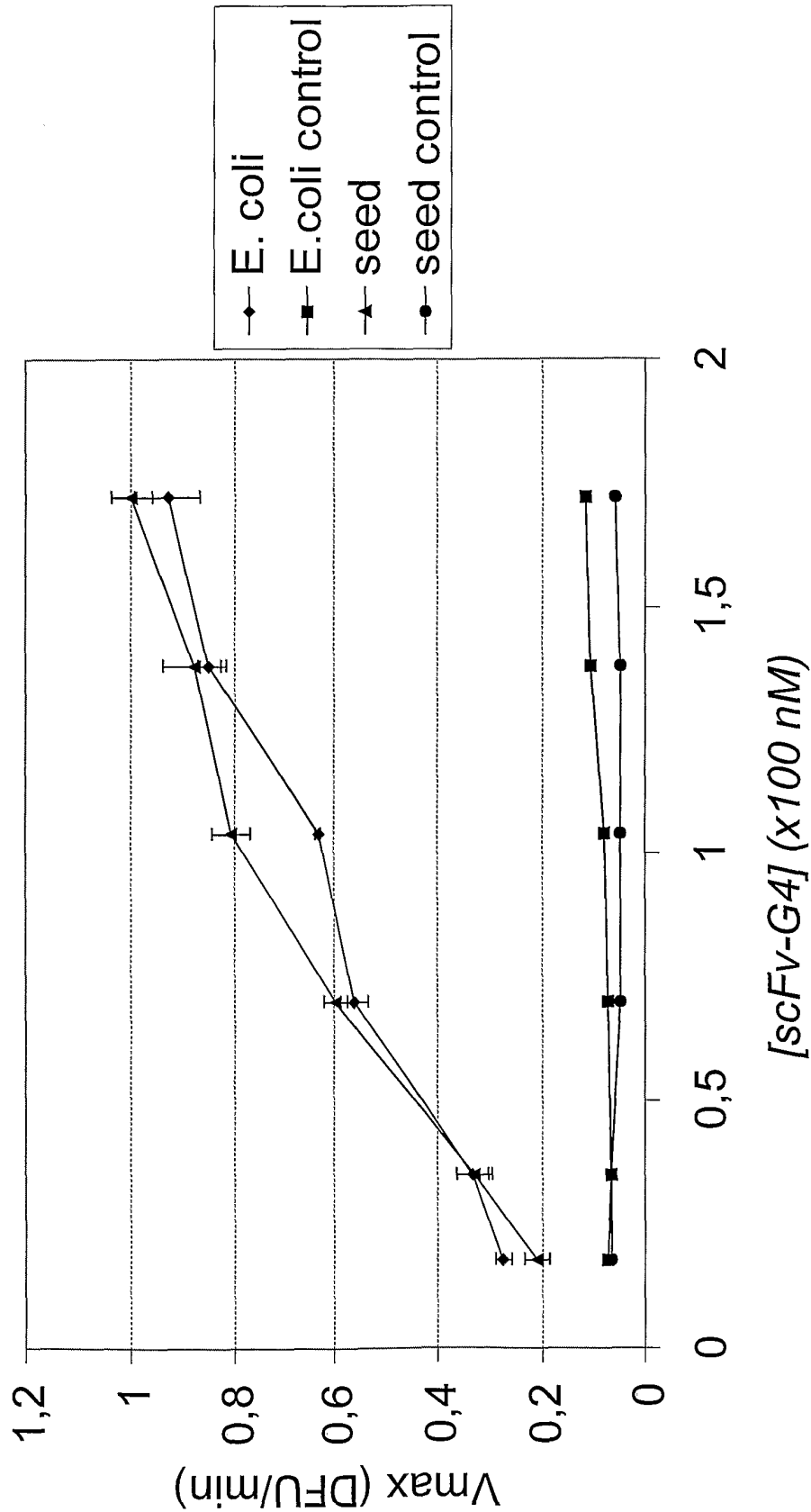
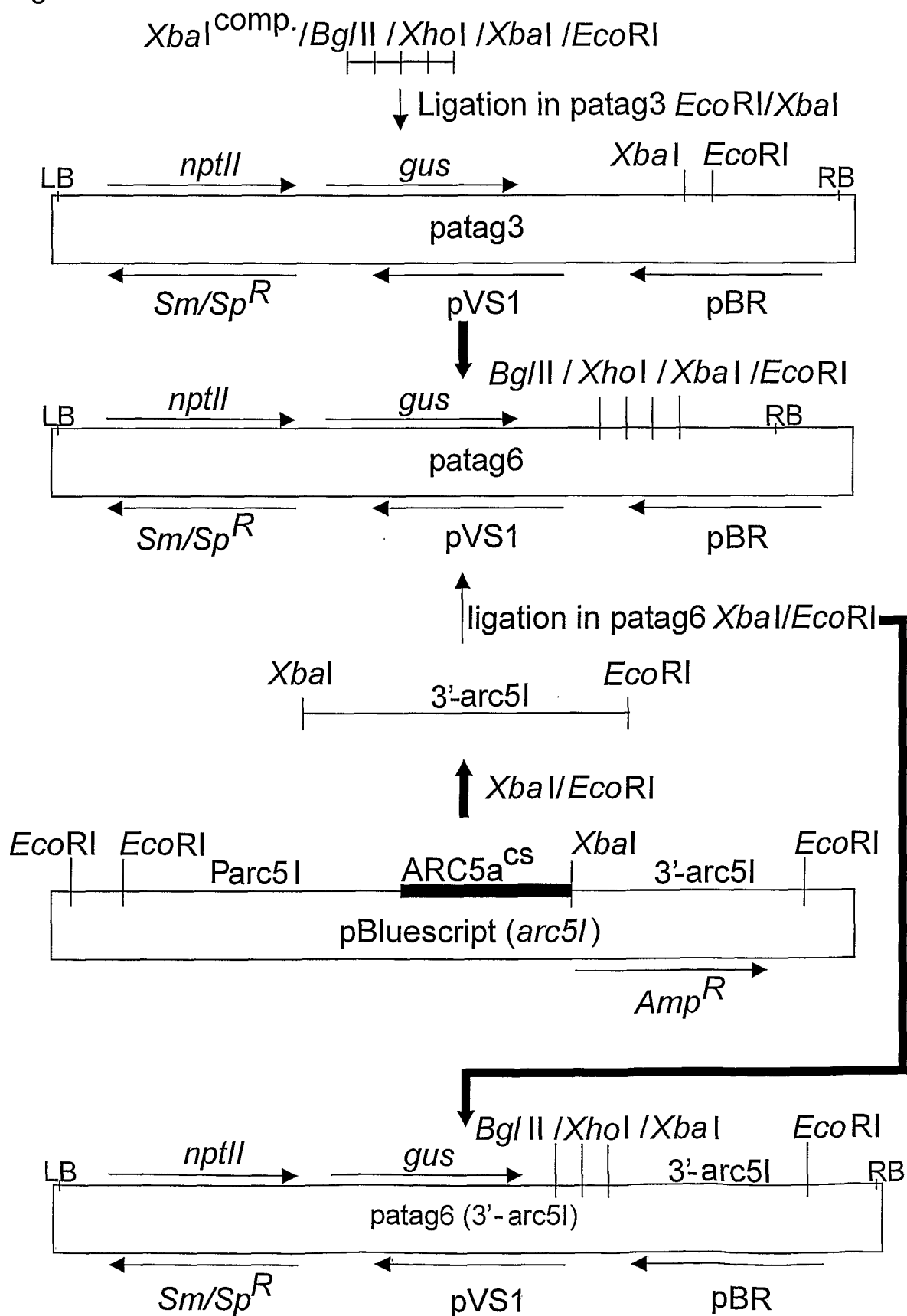


Figure 14



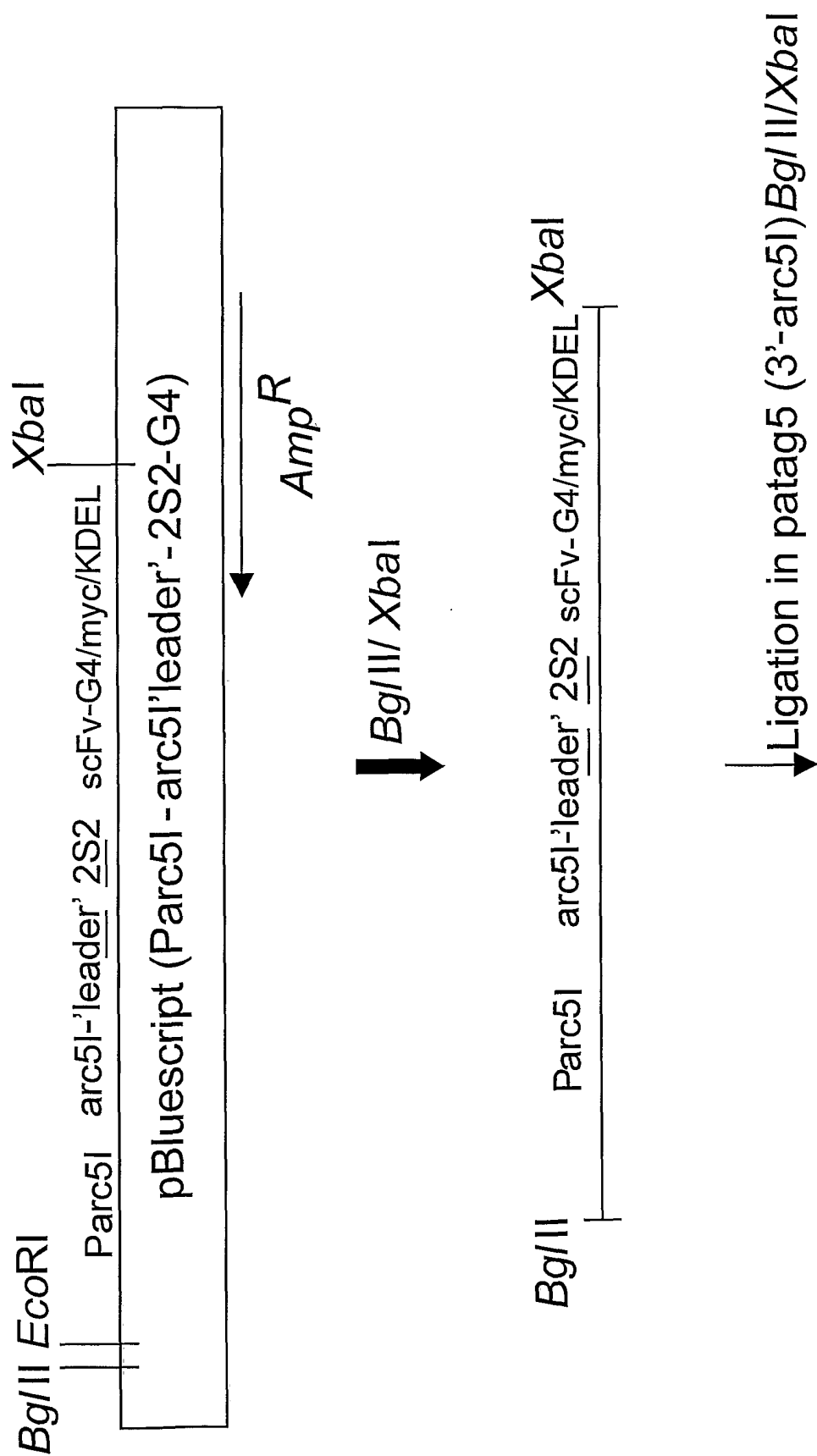
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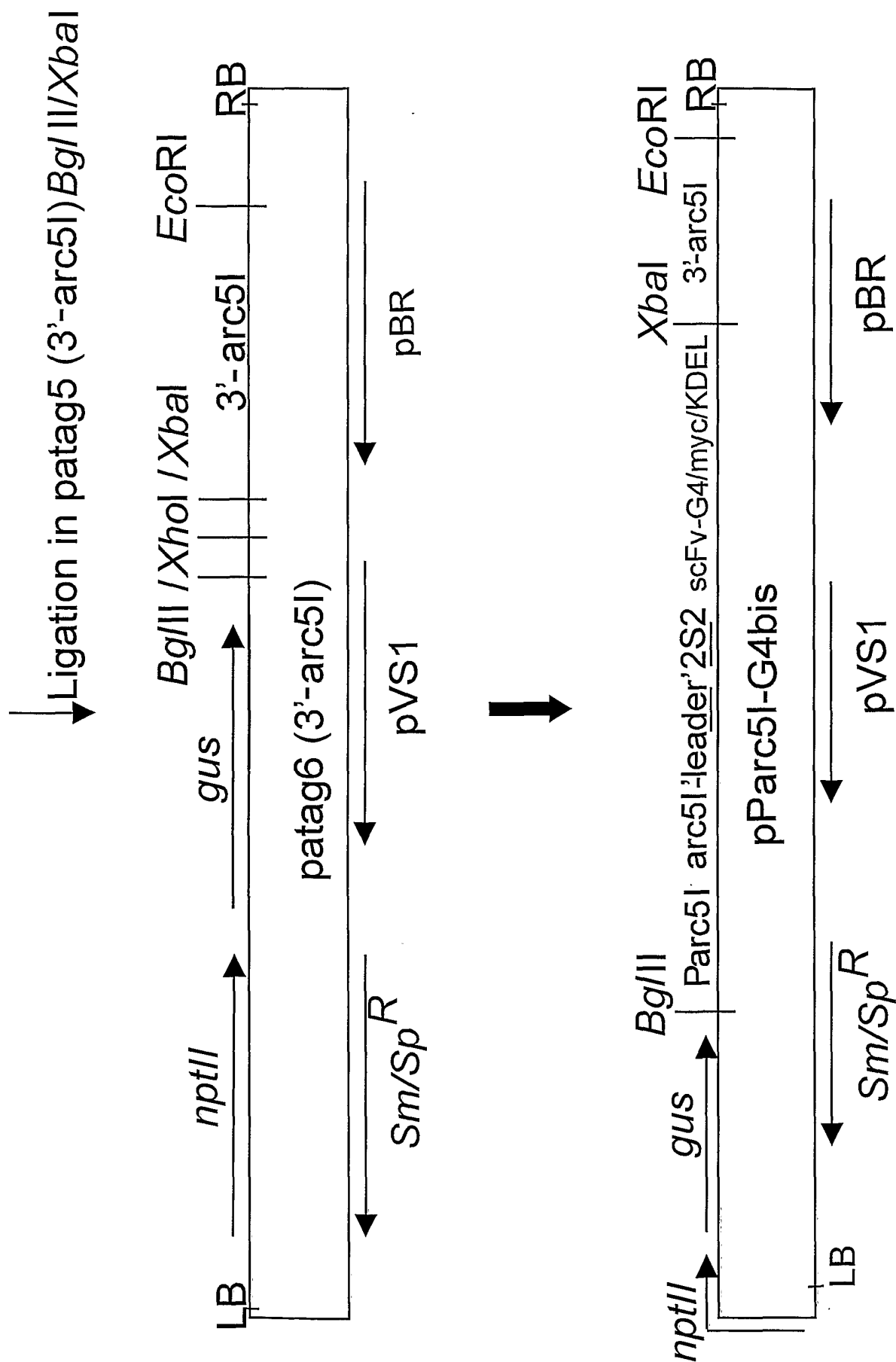
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Figure 16



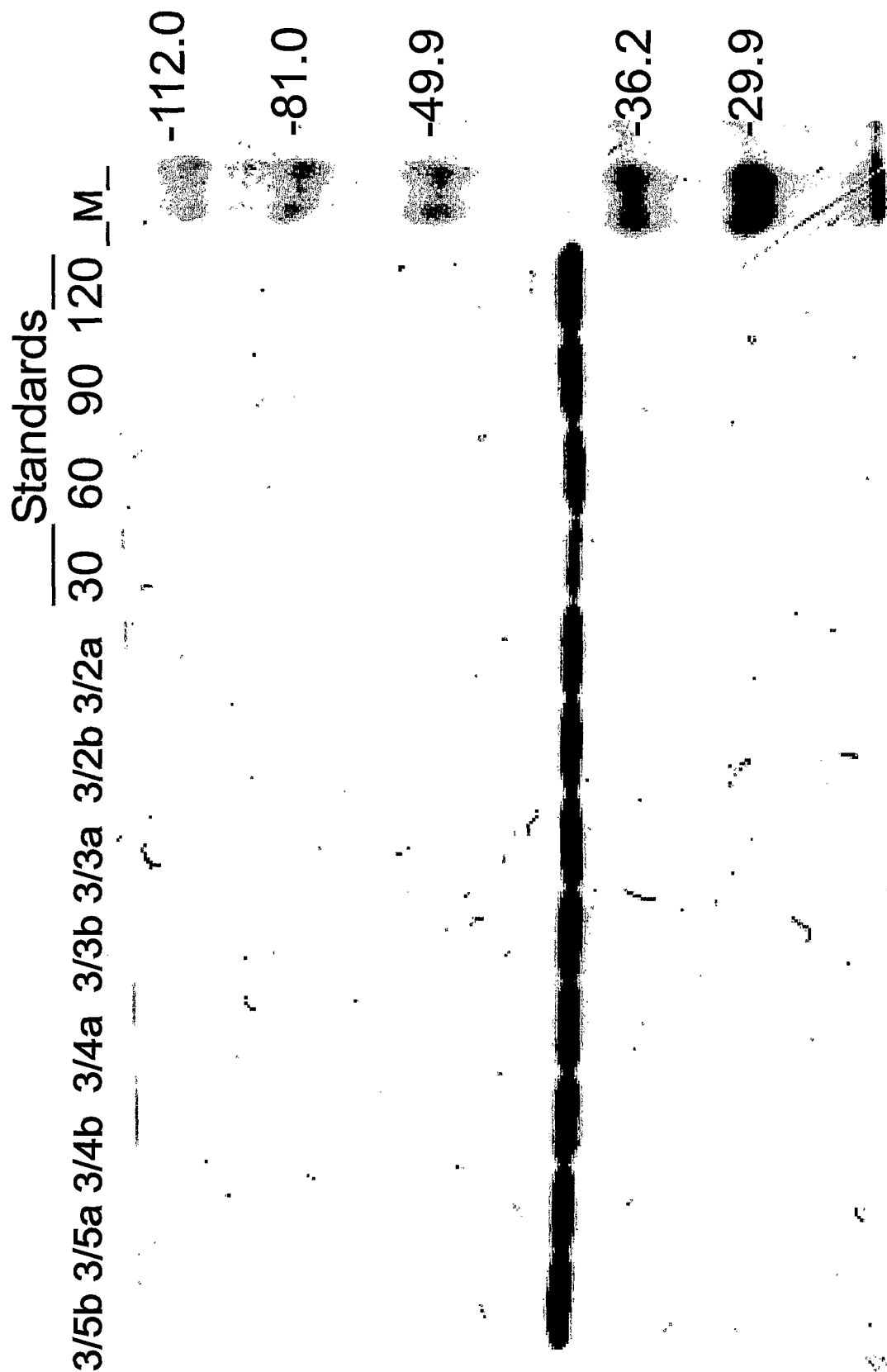
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Figure 17



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**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™
LMBP-COLLECTION**Page 2 of Form BCCM™/LMBP/BP/4/00-16 Receipt in the case of an original deposit

II. Scientific description and/or proposed taxonomic designation

The microorganism identified under I above was accompanied by:

(mark with a cross the applicable box(es))

- | | | |
|------------------------------------|---|--|
| – a scientific description | yes ☒ | no ☐ |
| – a proposed taxonomic designation | yes ☐ | no ☒ |

III. Receipt and acceptance

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on (date of original deposit) : June 19, 2000

IV. International Depositary Authority

Belgian Coordinated Collections of Microorganisms (BCCM™)
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™
LMBP-COLLECTION**

Page 1 of Form BCCM™/LMBP/BP/9/00-16 Viability statement

**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure**

**Viability statement issued pursuant to Rule 10.2 by the International Depositary
Authority BCCM™/LMBP identified on the following page**

International Form BCCM™/LMBP/BP/9/00-16

To : Party to whom the viability statement is issued:

Name : Philippe Jacobs

Address : Rijvisschestraat 120
B-9052 Zwijnaarde

I. Depositor:

I.1 Name : Depicker Ann

I.2 Address : K.L. Ledeganckstraat 35
9000 Gent

II. Identification of the microorganism:

II.1 Accession number given by the International Depositary Authority:

LMBP 4128

**II.2 Date of the original deposit (or where a new deposit or a transfer has been
made, the most recent relevant date) : June 19, 2000**

III. Viability statement.

The viability of the microorganism identified under II above was tested on

: June 22, 2000

(Give date. In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test).

On that date, the said microorganism was: (mark the applicable box with a cross)

☒ **viable**

☐ **no longer viable**

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM™
LMBP-COLLECTION**

Page 2 of Form BCCM™/LMBP/BP/9/00-16 Viability statement

IV. Conditions under which the viability test has been performed:

(Fill in if the information has been requested and if the results of the test were negative).

V. International Depositary Authority

**Belgian Coordinated Collections of Microorganisms (BCCM™)
Laboratorium voor Moleculaire Biologie - Plasmidencollectie (LMBP)
Universiteit Gent
K.L. Ledeganckstraat 35
B-9000 Gent, Belgium**

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

**BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS - BCCM
LMBP-COLLECTION**

Page 1 of Form BCCMTM/LMBP/BP/4/00-16 Receipt in the case of an original deposit

**Budapest Treaty on the International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure**

**Receipt in the case of an original deposit issued pursuant to Rule 7.1 by the
International Depositary Authority BCCMTM/LMBP identified at the bottom of next page**

International Form BCCMTM/LMBP/BP/4/00-16

To : Name of the depositor : Depicker Ann

Address : K.L. Ledeganckstraat 35
9000 Gent

I. Identification of the microorganism:

I.1 Identification reference given by the depositor:

MC1061(pParc5I-G4)

I.2 Accession number given by the International Depositary Authority:

LMBP 4128