



(86) **Date de dépôt PCT/PCT Filing Date:** 2010/03/16
(87) **Date publication PCT/PCT Publication Date:** 2010/09/23
(45) **Date de délivrance/Issue Date:** 2018/11/13
(85) **Entrée phase nationale/National Entry:** 2011/09/13
(86) **N° demande PCT/PCT Application No.:** NL 2010/000045
(87) **N° publication PCT/PCT Publication No.:** 2010/107303
(30) **Priorité/Priority:** 2009/03/16 (EP EP 09003750)

(51) **Cl.Int./Int.Cl. C12N 15/113** (2010.01),
C12N 1/14 (2006.01), **C12N 15/00** (2006.01),
C12N 15/80 (2006.01), **C12N 9/24** (2006.01),
C12N 9/58 (2006.01), **C12P 21/02** (2006.01),
C12Q 1/04 (2006.01), **C12Q 1/37** (2006.01)

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(54) **Titre : NOUVEAU SYSTEME DE PRODUCTION FONGIQUE**
(54) **Title: NEW FUNGAL PRODUCTION SYSTEM**

(57) **Abrégé/Abstract:**

The present invention provides a new fungal production system comprising a fungal host strain of *Chrysosporium lucknowense* wherein the endogenous cellulase secretion is less than 20 % of the endogenous cellulase secretion of *Chrysosporium lucknowense* strain UV 18-25. Preferably, also the secretion of endogenous protease, endogenous β -glucanase and endogenous cellobiohydrolase is less than 20 % of the secretion of *Chrysosporium lucknowense* strain UV 18-25. Furthermore, fungal host strains are provided wherein several genes have been disrupted. According to another aspect of the invention a method for homologous and/or heterologous production of a pure protein with a purity of higher than 75 %, comprising expressing a gene encoding said protein in the strains according to the invention have been described. Furthermore, a method for production of artificial protein mixes comprising expressing a gene encoding each of said proteins in a strain according to the invention have been disclosed. Finally a method for simplified screening of strains functionally expressing a desired enzyme by application of said strains have been provided.

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 September 2010 (23.09.2010)

PCT

(10) International Publication Number
WO 2010/107303 A3

(51) International Patent Classification:

C12N 15/00 (2006.01) *C12P 21/02* (2006.01)
C12N 15/09 (2006.01) *C12R 1/645* (2006.01)

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(21) International Application Number:

PCT/NL2010/000045

(22) International Filing Date:

16 March 2010 (16.03.2010)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

EP 09003750 16 March 2009 (16.03.2009) EP

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(81) Designated States (unless otherwise indicated, for every
kind of national protection available): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ,
CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO,
DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP,
KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD,
ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI,
NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD,
SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR,
TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every
kind of regional protection available): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ,
TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE,
ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV,
MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM,
TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW,
ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))
- with (an) indication(s) in relation to deposited biological
material furnished under Rule 13bis separately from the
description (Rules 13bis.4(d)(i) and 48.2(a)(viii))

(88) Date of publication of the international search report:

16 December 2010

(54) Title: CHRYSOSPORIUM LUCKNOWENSE PROTEIN PRODUCTION SYSTEM

(57) Abstract: The present invention provides a new fungal production system comprising a fungal host strain of *Chrysosporium lucknowense* wherein the endogenous cellulase secretion is less than 20 % of the endogenous cellulase secretion of *Chrysosporium lucknowense* strain UV 18-25. Preferably, also the secretion of endogenous protease, endogenous β -glucanase and endogenous cellobiohydrolase is less than 20 % of the secretion of *Chrysosporium lucknowense* strain UV 18-25. Furthermore, fungal host strains are provided wherein several genes have been disrupted. According to another aspect of the invention a method for homologous and/or heterologous production of a pure protein with a purity of higher than 75 %, comprising expressing a gene encoding said protein in the strains according to the invention have been described. Furthermore, a method for production of artificial protein mixes comprising expressing a gene encoding each of said proteins in a strain according to the invention have been disclosed. Finally a method for simplified screening of strains functionally expressing a desired enzyme by application of said strains have been provided.

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New fungal production system

The present invention relates to a fungal host strain of *Chrysosporium lucknowense*. The invention relates furthermore to a method for homologous and/or
5 heterologous production of a pure protein with a purity of higher than 75 %, to a method for production of artificial protein mixes and to a method for simplified screening of strains functionally expressing a desired enzyme. The invention relates furthermore to an isolated promoter sequence suitable for the transcriptional control of gene expression in *Chrysosporium lucknowense* and to a method for isolating a fungal host strain of
10 *Chrysosporium lucknowense* wherein the protease secretion is less than 20 % of the protease secretion of *Chrysosporium lucknowense* strain UV 18-25.

Fungi have been proven to be excellent hosts for the production of a variety of enzymes. Strains like *Aspergillus*, *Trichoderma*, *Penicillium* and recently the fungus *Chrysosporium lucknowense* C1, have been applied in the industrial production of a wide
15 range of enzymes. Super-producing strains have been developed that secrete up to 100 g/L or more protein in the fermentation broth (see for instance in Hans Visser et al., Abstracts, J. of Biotechnology, S211-S241 (2007)). The large protein-secreting capacity of these fungi make it preferred hosts for the targeted production of specific enzymes or enzyme mixes. However, typically, these hosts secrete a mix of many different enzymes,
20 making the crude protein product undefined and yielding, besides the desired enzyme activity, a range of non-relevant or even contra-productive activities. This also holds true for the use of such fungal hosts for production of specific enzyme activities by over-expression of selected genes via genetic modification approaches. Also in these cases the target enzyme will only constitute a minor part of the total secreted protein.

25 A microbial production system able to secrete high amounts of a specific enzyme without the presence of high levels of other proteins would be highly desirable. It would enable simplified screening of hosts functionally expressing a desired enzyme. It would enable production of relatively pure enzyme. It would also enable simplified large scale purification of the desired enzyme. These advantages would greatly contribute to e.g.
30 easy generation of artificial enzyme mixes tailored for different applications, e.g. plant biomass hydrolysis (biofuels and chemicals), textile finishing, applications in paper and pulp industry.

The relatively clean production of specific extracellular enzymes to high levels by micro-organisms that do not intrinsically secrete high levels of protein would be a non-preferred approach. The limited enzyme secreting capacity of such organisms would prevent high level production of the enzyme of interest.

- 5 The object of the present invention comprises the isolation of mutants of a fungal strain with high secretion capacity that unexpectedly no longer produce high levels of many non-desired proteins, while maintaining good growth characteristics, and amenability to genetic modification. These mutant strains were should be able to function as a host for high level production of specific enzymes.
- 10 In order to achieve the intended object of the invention the invention provides a fungal host strain of *Chrysosporium lucknowense* wherein the endogenous cellulase secretion is less than 20 % of the endogenous cellulase secretion of *Chrysosporium lucknowense* strain UV 18-25, preferably less than 15 %, more preferably less than 10 %, specifically less than 5 %, more specifally less than 2 %, more specifically less than 1 %, most
- 15 specifically less than 0,5 %, or less than 0,1%. The strain *Chrysosporium lucknowense* strain UV 18-25 has been described in the international patent application WO 0020555. Preferably, the secretion of one or more of the group consisting of endogenous protease, endogenous β -glucanase and endogenous cellobiohydrolase of the fungal host strain according to the invention is less than 20 % , more preferably less than 15%, most
- 20 preferably less than 10%, especially less than 5%, more especially less than 1% most especially less than 0,5% or 0,1% of the secretion of endogenous protease, endogenous β -glucanase and endogenous cellobiohydrolase respectively of the *Chrysosporium lucknowense* strain UV 18-25. All percentages mentioned apply to the secretion of protease, β -glucanase and cellobiohydrolase independently.
- 25 Preferably the strains according to the present invention are further characterized in that secretion of endogenous cellobiohydrolase 1 (Cbh1) is absent. Most preferably the strain according to the present invention is strain W1L, deposited at the Centraal Bureau Schimmelcultures (CBS) under accession nr 122189 or W1L#100.1 deposited at the CBS under accession number 122190.
- 30 More preferably from the strains according to the present invention the gene encoding endochitinase 1 (*chi1*) has been disrupted. Most preferably, one or more genes selected

from the group consisting of those encoding alkaline protease 1 (*alp1*), alkaline protease 2 (*alp2*), proteinase A (*pep4*), glucoamylase (*Gla1*), exo-chitinase (*Chi2*) and laminarinase (*Lam1*) have been disrupted. Especially, the strains according to the invention are WIL#100.1 Δ *alp1* Δ *pyr5* or WIL#100.1 Δ *alp1* Δ *chi1* Δ *pyr5*.

- 5 The invention also relates to a method for homologous and/or heterologous production of a pure protein with a purity of higher than 75 %, preferably of higher than 80%, more preferably of higher than 85, 90 or 95%, comprising expressing a gene encoding said protein in a strain according to the invention. Especially the invention provides a method for production of artificial protein mixes comprising expressing genes encoding each of
- 10 said proteins of the mix in a strain according to the invention. In this way protein mixes may be prepared for different applications, e.g. plant biomass hydrolysis (bio fuels and chemicals), textile finishing, applications in paper and pulp industry). Furthermore the invention provides a method for simplified screening of strains functionally expressing a desired enzyme by application of strains according to the present invention.
- 15 According to another aspect of the invention an isolated promoter sequence is provided suitable for the transcriptional control of gene expression in *Chrysosporium lucknowense*, selected from the group consisting of
 - a. the *chi1(0.8)* promoter comprising the nucleotide sequence of SEQ ID NO 25,
 - b. the *chi1(1.8)* promoter comprising the nucleotide sequence of SEQ ID NO 26,
 - 20 c. the *hex1* promoter comprising the nucleotide sequence of SEQ ID NO 27,
 - d. the *xyl6* promoter comprising the nucleotide sequence of SEQ ID NO 28, and
 - e. the *gla* promoter comprising the nucleotide sequence of SEQ ID NO 29
 or a transcriptionally active part thereof.

- 25 Also a chimeric gene comprising said promoter sequence and a host comprising said promoter and chimeric gene are provided by the present invention.

Finally a method for isolating a fungal host strain of *Chrysosporium lucknowense* wherein the cellulase and protease secretion is less than 20 % of the cellulase respectively protease secretion of *Chrysosporium lucknowense* strain UV 18-25 , comprising the

- 30 steps of

- (i) plating *Chrysosporium lucknowense* on acid swollen cellulose (ASC) plates,
- (ii) selecting at least one colony showing a reduced cellulose clearing zone,
- (iii) plating the strain selected in step (ii) on skim milk plates, and
- 5 (iv) selecting at least one colony showing a reduced protein degradation halo,
- has been provided. Preferably this method further comprises the steps of mutagenesis prior to steps (i) and/or (iii).

If in this patent application patent hosts are defined by comparing the level of production
10 of various enzymes with the *Chrysosporium lucknowense* strain UV 18-25 then of course the production of one and the same enzyme in the host has to be compared with that in the *Chrysosporium lucknowense* strain UV 18-25.

The proteins mentioned in this patent application as for instance protease, β -glucanase, cellobiohydrolase, proteinase A, β -glucanase, exo-chitinase and laminarinase have been
15 defined as described in WO 2009/0918537 in the name of Dyadic International INC. .

To emphasize the advantages provided by the present invention: it relates to the isolation of novel fungal hosts that have lost their intrinsic capability to secrete high levels variety of background proteins, while retaining the ability to secrete high levels of only few
20 enzyme activities. The invention also relates to the use of these hosts to produce specific enzymes at high levels without co-production of high levels of non-specific proteins. In addition the invention relates to the generation of defined artificial enzyme mixes tailored for different applications.

25 The invention will now be elucidated by the following non-limiting examples.

Legends to the figures mentioned:

Figure 1: Medium samples of UVI8-25, WIL wildtype, WID wildtype and the protease
30 mutants WIL#100.1, WID#50.g and WID#100.b on SDS-PAGE. These strains were

cultivated in medium #1 (low density medium with cellulose) for 282 hours except for lane 8 which is a sample in medium #2 (high density). The medium sample in lane 1 was 2 times diluted and the medium sample in lane 8 was 4 times diluted.

- 5 **Figure 2:** Shake flask culture medium samples of C1 strain W1L#100.1 and derivatives. Lane 1, W1L#100.1; lane 2, W1L#100.1 Δ chi1; lane 3, W1L#100.1 Δ alp1; lane 4, W1L#100.1 Δ alp1 Δ chi1.

Figure 3: Cosmid cloning vector pAopyrGcosarp1.

10

Figure 4: Plasmid pCHI4.8. The plasmid, which was isolated from E. coli clone #5 is shown. This plasmid was used in the construction of the white strain gene expression vectors.

- 15 **Figure 5:** Over-expression of chi1 via the introduction of extra chi1 gene copies in W1L#100.1. Lane 1, W1L#100.1 wild type strain (control); lane 3, W1L#100.1[chi+/pyr5]#3; lane 4, W1L#100.1[chi+/pyr5]#9; lane 5, W1L#100.1[chi+/pyr5]#17.

- 20 **Figure 6:** Plasmid Pcbh1-glaA(II)-Tcbh1. This plasmid was used in the construction of the white strain gene expression vectors.

Figure 7: The pPchi1(1.8)-Tcbh1 NotI gene expression vector.

- 25 **Figure 8:** A schematic map of the pCRS-pPchi1(1.8)-Tcbh1 gene expression vector.

Figure 9: SDS-PAGE analysis of culture supernatant samples of W1L#100.L Δ alp1 Δ pyr5 (B2), and CL10518-expressing transformant strains of W1L#100.L Δ alp1 Δ pyr5 (B3, B4). The arrow indicates the CL10518 position.

30

Figure 10: SDS-PAGE analysis of culture supernatant samples of W1L#100.L $\Delta alp1 \Delta pyr5$ (D1), and of *cbh2*-expressing transformant strains of W1L#100.L $\Delta alp1 \Delta pyr5$ (D3-D5). The arrow indicates the CBH2 position.

- 5 **Figure 11:** SDS-PAGE analysis of culture supernatant samples of W1L#100.L $\Delta alp1 \Delta chi1 \Delta pyr5$ transformed with *pyr5* only (C), and of *pgx*-expressing transformant strains of W1L#100.L $\Delta alp1 \Delta chi1 \Delta pyr5$ (1, 2).

- Figure 12:** SDS-PAGE analysis of culture supernatant samples of W1L#100.L $\Delta alp1 \Delta chi1 \Delta pyr5$ transformed with *xyl1* (31, 34) and with *xyl1* Δcbd . The arrows indicate the protein bands representing the respective xylanase variants.

- Figure 13:** SDS-PAGE analysis of culture supernatant samples of W1L#100.L $\Delta alp1 \Delta chi1 \Delta pyr5$ transformed with *med* with selection marker only (71) and with *abn2* (68-70), M, marker.

- Figure 14:** Analysis of *chi1* transformants. #1, control strain (transformed with selection marker *pyr5* only). M, marker. #65, *chi1* transformant. The arrow marks the Chi1 protein band.

20

Figure 15: Protein gel of purified heterologous *Aspergillus niger* PGII produced by C1. M, Marker proteins. The molecular weights of 3 marker proteins are indicated to the left of the gel. Lane 1, purified PGII.

25

Examples

Example 1: Isolation of C1 mutants with greatly reduced cellulase activity (white strains).

5 C1 strain UV18-25 (described in WO/2000/020555) was mutated using UV light to produce the strain UV26-2 (Appendix 1 to the Examples). UV26-2 exhibited large clearing zones on ASC (acid swollen cellulose) plates, indicating overproduction of cellulase.

10 UV26-2 was not a stable mutant and successive streaking of UV26-2 resulted in generation of cellulase negative mutants as shown by the absence of clearing zones on ASC plates. These colonies exhibited enhanced sporulation and white color, while normal, cellulase-producing, colonies were cream colored and showed no sporulation on ASC plates.

15 Two white colonies (UV26-2W1 and UV26-2W2) were picked from ASC plates along with two normal colonies and cellulase production was evaluated using a shake flask screening procedure. The white colonies produced 6 and 4 U/ml of AzoCMC cellulase activity, while the two normal colonies produced 278 and 294 U/ml of cellulase activity. This confirmed the white colonies to be cellulase-negative mutants.

20 Example 2: Isolation and analysis of protease deficient strains of UV26-2W1L and UV26-2W1D.

The purification of strain UV26-2W1 on RM-ASP medium plates (Appendix 2 to the Examples) resulted in the identification of 2 types of colonies: colonies with light colored spores like UV18-25 (UV26-2W1L further indicated as strain W1L), and
25 colonies with dark (pink) colored spores (UV26-2W1D further indicated as strain W1D).

In additional experiments batches of spores of both W1L and W1D were irradiated with UV (Appendix 1 to the Examples) and used in a direct selection procedure for protease-deficient mutants (Braaksma et al., 2008). Positive clones were analyzed on skim milk plates for their protease activity.

5 After several rounds of purification and selection on skim milk plates, two mutants of W1L (W1L#50.c and W1L#100.l) and three mutants of W1D (W1D#50.g, W1D#50.n and W1D#100.b) with a reduced halo on skim milk plates were selected for cultivation for in vitro degradation assays. In a first cultivation experiment these mutants and their parent strains were cultivated in medium #2 Appendix 1 to the Examples) for 240 hours
10 at 35°C. Apparently, the low cellulase activity in these strains did not allow for growth in high density cellulose based medium. In following cultivation experiments W1L#50.c, W1L#100.l, W1D#50.g and W1D#100.b and their parents were grown in low (#1) and high (#2) density cellulose medium for 240 hours at 35°C. Also UV18-25 was taken as a control. The parent strains 2W1D, 2W1L and UV18-25 were also cultivated in medium
15 #2. None of the W1L or W1D strains grew in high density cellulose medium #2. In medium #1 good growth could be observed for the 'white' strains and their protease-deficient mutants, although the cellulose in the medium was hardly used by the 'white' strains. Unexpectedly, it was noted that the UV26-2W1D parent strain, which showed an unstable growth phenotype on agar plates, did use the cellulose in the medium.

20

The medium samples of the W1L parent strain showed less protease activity on skim milk plates compared to medium samples of W1D parent strain and UV18-25 (Table 1). This is contrary to what was observed when the strains were grown directly on skim milk plates. In that case a large halo could be detected around the colony of UV18-
25 25 and of W1L after 72 hours growth at 30°C, while a small halo could only be detected after 144 hours for W1D. The medium samples of protease mutant W1D #50.g showed a smaller halo on milk plates until 162 hours of cultivation. After 186 hours cultivation, halos were similar as observed for its parent strain.

Strain	Relative halo size (hrs of cultivation)							Protease activity
	114	138	162	186	210	240	282	U/ml
W1L parent	++	++	++	++	++	++	++	943
W1L#100.l	+	+++	+++	+++	+++	+++	+++	119
W1L#100.l Δ alp1	nd	nd	nd	nd	nd	nd	nd	46
W1D parent	+++	+++	+++	+++	+++	+++	+++	nd
W1D#50.g	-	-	-	++	++	++	++	nd
W1D#100.b	++	++	++	++	++	++	++	nd
UV18-25	+++	+++	+++	+++	+++	+++	+++	nd

Table 1: Medium analysis of W1L and W1D parent strains and their selected protease mutants: W1L#100.l, W1D#50.g and W1D#100.b. UV18-25 was taken as control. Protease activities of medium samples of W1L, W1L#100.l and W1L#100.l Δ alp1 were also determined. These strains were cultivated in medium #1 (low cellulose / lactose / pharmamedia). The pH was measured and medium was spotted on skim milk plates to determine their protease activity. The relative size of the halo is a measure for the protease activity in the medium. nd, not determined.

10

Analysis of 282 hours medium samples of these strains on SDS-PAGE gels showed that the 'white strains' produced much less protein than UV18-25 (Fig. 1). In particular the two major 50/70 kDa proteins (Cbh1) were absent in these culture supernatants. In the white strains the 'major' proteins are 75 and 45 kDa. These proteins are present in medium of UV18-25 as minor proteins.

15

From this first screening for protease-less mutants in a UV26-2W1 background the strains W1D#50.g and W1L#100.l were selected for further analysis.

20

Example 3: Comparison of extracellular enzyme activities between UV18-25 and W1L#100.l.

Different enzyme activities in the extracellular protein content of UV18-25 and W1L#100.1 samples were determined (Table 2). Based on these data it was concluded that W1L#100.1 secretes very little specific cellulase activity (less than 1 % of UV18-25) and has very little or no detectable protease activity when compared to UV18-25.

5

Activities	UV18-25	W1L#100.1
CMCase (cellulase)	6.20	0.04
Beta-glucanase	10.2	0.53
Cellobiohydrolase	0.72	0.09
Protease (pH 5)	0.06	0.03
Protease (pH 7)	0.05	0.00
Protease (pH 9)	0.04	0.00

Table 2: Specific activities of samples (U/mg of protein). Protease activities were measured at 3 different pH values.

10 In addition, the levels of hydrolases bearing other substrate specificities (e.g. hemi-cellulose) were reduced as well.

Example 4: Further reduction of protein level: identification of major proteins.

15

As described above, the white strain is missing the extracellular cellulolytic enzyme spectrum when compared to its parental strain. Hence, the extracellular protein content in white strain cultures, as analyzed by SDS-PAGE, is low. This strain characteristic is beneficial with regard to protein production and purification, since the relative amount of any target protein expressed in such strain will be high. Furthermore, the (nearly) absence of cellulase activity makes the white strain an ideal host strain for testing new or modified cellulases. The same is valid for xylanases as no major xylanase activity was detectable.

20

To further reduce the protein background level, several major protein bands present in an SDS-PAGE gel from a W1L#100.1 and derivative strain cultures were excised and identified by N-terminal sequencing and/or MS-MS analysis. The most abundant protein was the endochitinase Chi1 (gene identifier: CL06081, peptides MVYDYAG, MPIYGRS, and MFXEASA). Other major proteins were identified as a glucoamylase (Gla1, CL09507, peptides TGGWSVWPVLK (SEQ ID No 1) and VVGSSSEL(I)GNWDTGR (SEQ ID No 2)), exo-chitinase (Chi2, CL00367, peptides TIDAMAWSK (SEQ ID No 3), NFLPVADILR (SEQ ID No 4), GAYHPSQTYSPEDVEK (SEQ ID No 5), and SWQLVYQHDPTAGLTAEAAK (SEQ ID No 6) and a laminarinase (Lam1, CL08253, peptides PQYESAGSVVPSSFLSVR (SEQ ID No 7) and VSGQVELTDFLVSTQGR (SEQ ID No 8). Also an alkaline protease Alp1 (CL04253) has been identified in W1L#100.1 culture broth. Alp1 degrades extracellular proteins, and may degrade proteins of interest.

Example 5: Further reduction of protein level: disruption of the *chi1*, *chi2*, *gla1* and *lam1* genes.

The vector pChi3-4 (see Example 9, Isolation of the endochitinase 1 encoding gene) was used for the construction of the gene disruption vector. A 1.1-kb *MscI/StuI* fragment was replaced with the *amdS*-rep selection marker or the *pyr5*-rep selection marker, resulting in the vectors p Δ *chi1-amdS* and p Δ *chi1-pyr5*, respectively. The disruption fragment Δ *chi1-amdS* was isolated from p Δ *chi1-amdS* by digestion with *EcoRI*. The disruption fragment Δ *chi1-pyr5* was isolated from p Δ *chi1-pyr5* by digestion with *SmaI*. Transformation of strain W1L#100.1 Δ *pyr5*#172-12 using the disruption fragments resulted in 215 Δ *chi1-pyr5* transformants and 32 Δ *chi1-amdS* transformants. All the obtained transformants were purified and analyzed with colony hybridization. Southern analysis of these transformants confirmed the isolation of one W1L #100.1 transformant with a disrupted *chi* gene (W1L#100.1 Δ *pyr5* Δ *chi1-pyr5*#46 (*pyr5*+)).

Shake flask cultures on C1 low density medium were performed from a selection of W1L#100.1 Δ *pyr5* Δ *chi1* mutant strains. The samples were analyzed on SDS-PAGE to

evaluate the protein profiles for absence of Chi1 protein (Fig. 2, lane 2 versus lane 1). As shown no 45 kDa Chi1 protein is observed in the $\Delta chi1$ mutant strain.

The remaining most prominent extracellular proteins in white strain W1L#100.1 $\Delta alp1\Delta chi1$ and derivative strains correspond to glucoamylase (Gla1), exo-chitinase (Chi2) and laminarinase (Lam1). These enzymes were purified from the culture medium. The enzymatic activities of these proteins were verified using (among others) starch, chitosan and laminarin, respectively, as substrates. Furthermore, mass spectrometry analyses data (see Example 4) combined with C1 genome sequence data revealed the corresponding genes. In order to further reduce the extracellular protein background, the Gla1, Chi2, and Lam1 encoding genes were disrupted and thereby inactivated. Disruption was based on the exchange of the gene promoter and part of the 5' coding sequence by an *amdS* selection marker via homologous recombination using approximately 1.5 kbp upstream and downstream sequences that flank these gene promoter and part of the 5' coding sequence. The gene disruption vectors therefore contained the *amdS* expression cassette plus these flanking 1.5 kb homologous gene sequences. White strains W1L#100.1 $\Delta alp1\Delta chi1$ and derivative strains were transformed with the *glal*, *chi2* and *lam1* gene disruption vectors and transformants were screened for the correct genotype using PCR. As such, white strains with a further reduced extracellular protein composition/content were obtained. Target proteins produced by these strains were more than 80% pure in the crude cell-free culture liquid.

Example 6: Further reduction of protease activity: targeted disruption of genes encoding proteases.

25

In general, protease encoding genes were disrupted using disruption DNA fragments that contained selection markers (*amdS*, *pyr4* or *pyr5*) flanked by approximately 1.5 kb large DNA fragments homologous to regions up- and downstream of the gene to be disrupted. Upon introduction of these disruption DNA fragments into the white host, an homologous recombination exchanged the gene to be disrupted for the selection marker fragment. Corresponding transformants were selected as such. Genes

30

that were disrupted this way either encoded disadvantageous (with regard to target protein stability) protease activities e.g. *alp1*, *alp2*, *pep4*) or significant background protein (*chi1*) or were to be used as selection marker (*pyr4*, *pyr5*). Via this approach numerous white C1-strains have been constructed that can be used as hosts for target protein expression (Table 3).

W1L
W1L SUI ^R #S2 6.14
W1L SUI ^R #S2 6S
W1L#100.1
W1L#100.1 Δpyr5
W1L#100.1 Δalp1
W1L#100.1 Δalp1 Δpyr5
W1L#100.1 Δpep4 Δpyr5
W1L#100.1 Δalp1 Δpep4
W1L#100.1 Δalp1 Δpep4 Δpyr5
W1L#100.1 Δalp1 Δalp2 Δpyr5
W1L#100.1 Δchi1
W1L#100.1 Δalp1 Δchi1
W1L#100.1 Δalp1 Δchi1 Δpyr5
W1L#100.1 Δalp1 Δchi1 Δalp2
W1L#100.1 Δalp1 Δchi1 Δalp2 Δpyr5
W1L#100.1 Δalp1 Δchi1 Δpep4
W1L#100.1 Δalp1 Δchi1 Δgla1Δlam1Δchi2Δpyr5

Table 3: Strain W1L and derivatives.

10 Example 7: Identification of strong promoters for gene expression: chitinase encoding gene (*chi1*).

Several major protein bands were isolated from fermentation samples of W1L#100.1 grown in low density cellulose medium in order to identify and isolate strong promoters that can be used for gene expression in the W1L strain and its derivatives. N-terminal sequencing of a mixture of peptides obtained after CNBr treatment of the major 45 kDa protein of W1L#100.1 resulted in the identification of four different peptides. Three of these peptides (MVYAG, MPIYGRS and MFXEASA) showed homology with an endochitinase of *Aphanocladium album*/*Trichoderma harzianum* (CHI_APHAL P32470).

Based on 3 of these peptide sequences, primers were designed in order to obtain PCR fragments containing a part of the endochitinase encoding gene (Table 4). The PCR primers were designed based on the preferred codon usage of C1.

Primer	Region	Position	Deduced sequence
Endochitpep1C	MVYDYAG (SEQ ID No 9)	240 aa	ATGGTSTACGACTACGCBGG (SEQ ID No 10)
Endochitpep2C Endochitpep2revC	MPIYGRS(S EQ ID No 11)	290 aa	ATGCCSATCTACGGYCG (SEQ ID No 12) CGRCCGTAGATSGGCAT (SEQ ID No 13)
Endochitpep3revC	MFXEASA(S EQ ID No 14)	380 aa	GCSSWVGCCTCCCAGAACAT(SEQ ID No 15)
Primer based on a conserved homologous region of endochitinase:			
Endochit3c	DGIDIDWEV (SEQ ID No 16)	160 aa	GAYGGYATCGAYRTSGAYTGGG (SEQ ID No 17)

Table 4: The designed primers of putative endochitinase based on codon usage of C1.

PCR reactions with these primers were carried out using chromosomal DNA of UV18-25 as template DNA. PCR fragments were cloned and sequence analysis showed that one of the cloned PCR fragments obtained with Endochitpep1c and Endochitpep2revc (173 bp) contained a part of an endochitinase-encoding gene (*chi1*). Hybridization analysis of chromosomal DNA of UV18-25 digested *Bam*HI and *Hind*III

with this *chil* fragment as probe showed a clear hybridization signal confirming that the PCR fragment originated from C1 DNA. This fragment was used to clone the complete gene from the ordered C1-cosmid gene library. The fragment sequence (SEQ ID No 18) was as follows:

5 ATGGGCTACGACTACGCCGGCTCGTGGAGCACCGCGGGCGGGACACCAGGCCA
ACCTGTACCCGACCGCCGACGCGGGCAGGACGCCCTTCTCGACCGACAAGGC
CCTGTCCGACTACGTCGCCGCGGCGTCGACCCGGCCAAGATCGTGCTCGGC
ATGCCCATCTACGGCCG

10 Example 8: Construction of an ordered cosmid library of *Chrysosporium lucknowense* UV18-25 in *E. coli*.

For the construction of the C1 cosmid library the non commercial cosmid cloning vector, pAOpyrGcosarp1 (Fig. 3) was used. This vector carries the *Aspergillus oryzae* pyrG selection marker allowing transformation of a wide range of fungal strains.

15 Moreover, for highly efficient transformation of various *Aspergillus* species (for which a large collection of mutant strains is available for complementation cloning of the corresponding C1 gene) the AMA1-replicator is present in this vector. A unique *Bam*HI cloning site allows cloning of partially *Sau*3A digested genomic DNA from *Chrysosporium* strain UV18-25.

20 A cosmid library of UV18-25 was constructed in *E. coli* and stored as glycerol stocks in the -80 °C. The average insert size was 20-35 kb. In total, 6800 clones were obtained, which represents a genome coverage of about 5 fold. To order these clones in 384-wells plates, dilutions of the glycerol stocks were plated on LB-agar plates, containing ampicillin. 7680 individual colonies were manually picked and inoculated in
25 twenty 384-wells plates. These twenty 384-wells plates represent the ordered cosmid library of UV18-25 in *E. coli*.

The individual colonies in the twenty 384-wells plates were spotted on nylon filters (Hybond), using a Staccato 384-pintool (Zymarks). The ordered cosmid library was

spotted in octuplicate (in total 160 filters). These filters were placed on LB-ampicillin medium plates and incubated at 37 °C to allow colonies to grow on the filters.

Subsequently, the colonies were lysed and the cosmid DNA was bound to the filters using standard procedures.

5

Example 9: Isolation of the endochitinase 1 encoding gene (*chi1*).

The 173-bp *chi1* PCR fragment was used as a radio-active labelled probe for hybridization of duplicate filters of the cosmid library. Hybridization of the cosmid gene library using this *chi1* fragment resulted in 3 positive clones. DNA was isolated from these clones and restriction analysis followed by Southern analysis was carried out. A 4.8 kb *Hind*III fragment and a 3.4 kb *Bgl*II fragment showed hybridization using the *chi1* probe. The 4.8kb *Hind*III and 3.4 kb *Bgl*II fragments containing the *chi1* gene were isolated and subcloned in pMTL24, resulting in the vectors pCHI4.8 (Fig. 4) and pChi3-4, respectively. Subsequently, a *Bgl*II sub-clone was used for sequence analysis to obtain more sequence data. The sequence of the complete *chi1* gene of UV18-25 was obtained. The size of the *chi1* gene is 1529bp in which 2 introns of 111bp and 138bp are present.

Example 10: Overexpression of the endochitinase in W1L#100.1.

For overexpression of endochitinase the 3.4kb *Bgl*II fragment containing the *chi1* gene was isolated from pChi3-4. Putative *chi1* multi-copy strains in W1L#100.1Δ*pyr5* were generated by co-transformation of the 3.4 kb *Bgl*II *chi1* fragment and a *pyr5* selection fragment and correct transformants were confirmed by colony hybridization. Shake-flask cultures on C1 low density medium were performed from a selection of W1L#100.1 *chi1*-multicopy strains. The samples were analyzed on SDS-PAGE to evaluate the protein profiles for the overproduction of Chi1 (Fig. 5). Three of the multicopy *chi1* strains (lanes 3, 4, 5) show a stronger 45 kDa band compared to the parent strain (lane 1) indicating chitinase overexpression and the usefulness of the *chi1* promoter for high gene expression.

30

Therefore, a general C1 expression cloning vector (pPchi1(0.8)-Tcbh1 NotI) was constructed containing the *chi*-promoter (*Pchi*) to direct the overexpression of cloned genes. Initially, the *Eco*RI site upstream of *Pchi*1 in pCHI#4.8 was removed by a partial *Eco*RI digestion and treatment of the linear fragment with Klenow yielding

5 pCHI#4.8Δ*Eco*RI. From this vector the 1.9-kb *Sac*I-*Sph*I fragment was cloned in the corresponding site of pPcbh1-*gla*A(II)-*Tcbh*1 (Fig. 6). In the resulting vector, pPchi-*Tcbh*1 NotI#7.1, the target genes can be inserted into the *Nco*I-*Eco*RI sites. Expression cassettes for transformation into C1 strains can be isolated from these constructs as *Not*I fragments.

10

The 0.8 kb *chi*1 promoter sequence in pCHI#4.8 could be more than sufficient to drive *chi*1 expression. However, a longer *chi*1 promoter was also generated by amplifying the a *Pst*I-*Hind*III fragment (upstream of the *Hind*III site at position -775 relative to the ATG start codon), using one of the previously identified positive cosmid

15 clone as template DNA. The resulting fragment was cloned in pGEM-T-Easy and sequenced. From this plasmid the *Pst*I-*Hind*III fragment was isolated and cloned in the corresponding sites of pPchi1-*xyl*1-*Tcbh*1 yielding pPchi1(1.8)-*xyl*1-*Tcbh*1, in which the promoter size is 1.8 kb. The fragment was also cloned in the corresponding site of pPchi1-*Tcbh*1 NotI#7.1, yielding the general expression vector pPchi1(1.8)-*Tcbh*1 NotI

20 (Fig. 7).

The levels of gene expression directed by the extended chitinase promoter (Pchi1(1.8)) and by the initially used chitinase promoter (Pchi1 (0.8)) were compared by the expression of two reporter genes, *xyl*1 and *alp*1. White strain transformants were

25 generated that either expressed *xyl*1 (encoding a xylanase) or *alp*1 (encoding an alkaline protease) (Table 5).

Reporter	0.8 kb Pchi1 Reporter activity	1.8 kb Pchi1 Reporter activity
Alp1 (R19)	0,7 (A)	1,9 (B)
Xyl1 (R14)	122 (C)	1175 (D)

Table 5: Comparison of the short and extended *Pchi1* promoters in terms of reporter protein expression level. Reporter activity, xylanase activity is expressed as U/ml and alkaline protease activity as U/ mg of protein. A = W1L#100.1[*Pchi1*(0,8)-*alp1*/pyr5]#9, B = W1L#100.1[*Pchi1*(1,8)-*alp1*/pyr5]#22, C = W1L#100.1 Δ *alp1*[*Pchi1*-*xyl1*]#95, D = W1L#100.1 Δ *alp1*[*Pchi1*(1.8)-*xyl1*]#A7.

Surprisingly, the reporter gene expression was higher in case of the extended *chi1* promoter (1.8 kb), which indicates the necessity of the further upstream regions. In conclusion, a *Pchi1* based expression system was developed for high level expression of genes in White C1 strains.

Example 11: Identification of other strong promoters for gene expression.

A different approach for searching strong promoters was performed using the quantitative detection of messenger RNA levels from W1L or W1L#100.1 RNA. The RNA samples were isolated from mycelium which was sampled at different time points during a fed-batch fermentation process. A number of genes were identified as being strongly or stronger expressed. To verify the expression level of these genes, the RNA samples were also separated on gel, blotted and hybridized to probes specific for these genes (Table 6).

<u>Strain / feed</u>	<u>chi1</u>	<u>pep4</u>	<u>his2a</u>	<u>hex1</u>	<u>bgl1</u>	<u>xyl6</u>	<u>cbh1</u>
<u>W1L / glucose</u>							
Batch	0	4	2	21	0	0.3	0
Day 1 feed	47	5	3	15	8	1	0
Day 2 feed	52	3	3	17	8	1	0
<u>W1L#100.1 / xylose</u>							
Batch	0	0.2	2	12	0	0	0
Day 1 feed	40	3	2	12	3	19	0
Day 2 feed	36	4	6	17	5	21	0
<u>W1L#100.1 / glucose</u>							
Batch	0	0.1	3	23	0	0.1	0
Batch	61	0.5	2	17	7	0.2	0
Day 1 feed	59	0.2	2	14	6	1	0
Day 2 feed							

Table 6: Quantification of the expression signals of the different genes in controlled fed-batch fermentations. Probe hybridisation signals were quantified using a densitometer. The signal of the probe on the Northern blot was correlated with the signal of this probe on a C1 genomic DNA Southern blot. Therefore, the values in the table represent the northern hybridization signal level relative to the hybridization signal level from the Southern blot (which was set at 1). Gene sequences are given below.

The *cbh1* promoter, which is a strong promoter in UV18.25 strains, is not active in the white strains. The *chi1* promoter was the strongest both under glucose and xylose feed conditions. The *hex1* promoter is a strong constitutive promoter during all phases of fermentation and under both sugar feed conditions. The *xyl6* promoter is highly active under the xylose feed condition only. The *pep4*, *his2a* and *bgl1* promoters are moderately active. For high level gene expression in the white strains the *chi1*, *hex1* and *xyl6* promoters are very useful. Alternative promoters that also give high expression are those of the *pep4*, *his2a* and *bgl1* genes. Additional northern experiments also indicated that the promoters of the *xyl 4* and *xyl8* genes can be used for high level gene expression in the white strains when grown on xylose. Glucoamylase (Gla1, gene identifier: CL09507) has

been shown to be a major protein in white C1 strains. The *glal* promoter is therefore also a good candidate to be used for the high level expression of genes of interest in white strains. It was shown that glucoamylase was highly abundant in a white strain grown in the presence of starch. This indicated that the *glal* promoter is strong and inducible by starch and its degradation products, like maltose.

The nucleotide sequences of Pchi1(0.8), Pchi1(1.8), Phex1, Pxyl6 and Pglal are given below. Note that the ATG start codons of the corresponding coding regions are given in bold italics.

Example 12: White strain gene expression system

Two expression vectors were designed for expression of genes in W1L and derivatives: pPchi1(1.8)-Tcbh1 *NotI* was as described above. Additionally, pCRS Pchi1-Tcbh1 (Fig. 8) was constructed by placing the C1 repeat sequence in front of the Pchi1 promoter in pPchi1(1.8)-Tcbh1 *NotI*. These vectors have been designed in such a way that they also can be combined resulting in a single vector that contains multiple expression cassettes. The multiple cassettes can be excised from the vector as a single linear DNA fragment.

Many genes have been cloned and expressed in W1L or derivatives. The general procedure was as follows:

- Genes were identified by purification and characterization of the gene products (reverse genetics) and / or by genome mining. The genes were amplified by PCR or synthesized chemically. Amplification of genes by PCR was performed by using proof-reading PCR DNA polymerases (Phusion or Supertaq plus). The amplified genes were cloned into PCR cloning vectors i.e. pGEM-T-Easy (Promega) or pJet1 (Fermentas) and sequenced to verify the correctness of the sequence. Subsequently,

the genes were released from the PCR cloning vector and ligated into the *NcoI* and *EcoRI* sites of the expression vector(s).

- Special care was taken when designing the PCR primers. The ATG-start codon of the gene to be expressed was part of the *NcoI* restriction site in the white strain expression vectors. Therefore, the 5' (ATG) PCR primers contained restriction sites, which are compatible to the restricted *NcoI* site of the vector. These sites were i.e. *NcoI* itself (C↓CATGG), or compatible sites that are cut within the recognition site (*BspHI*, T↓CATGA; *PciI*, A↓CATGT), or compatible sites that are cut outside the recognition site (*BsaI*, GGTCTC(1/5); *BspMI*, ACCTGC(4/8); *Esp3I*, CGTCTC(1/5)).
- In some cases, restriction sites additional to those that were going to be used for cloning of the genes were encountered in the genes. In these cases, the genes were amplified by fusion PCR, where the fusion of the two PCR fragments was selected to take place at the undesired additional restriction site. The undesired restriction site was removed by using fusion primers containing single substitution mutations in the undesired restriction site sequence. In case the undesired restriction site was present within a protein coding region, the substituted nucleotide was selected in such a manner that the mutant codon encoded the same amino acid as the original codon.
- The expression cassettes were released from the *E. coli* DNA vector backbone by *NotI* restriction. The expression cassette was subsequently transformed in to W1L derivatives simultaneously with a selection marker i.e. *pyr5* or *amdS* in co-transformation experiments.
- Positive and high producing transformants were selected by SDS-PAGE or enzyme assay analysis of the growth medium. Best producers were applied in fermentations to produce high amount of the desired gene product.
- The following proteins have been produced using the white strain gene expression system and corresponding genes

In the international patent application WO 2009/018537 has been described:

Abf1, Abf2, Abn1, Axe1, Bgl1 (=Bgl3A), Cbh1, Cbh2, Cbh4, Chi1, Eg2, Eg5, FaeA1, FaeA2, FaeB2, Gal1 (=Gal53A), Gla1 (=Gla15A), Pme1, Xyl1, Xyl1(cd), Xyl2, Xyl3, Xyl3-cbd (=xyl3(cd)), Xyl4, Xyl5, Xyl6.

5 In the international patent application WO 2009/033071 has been described:

Abf3, Abn2, Abn3, Abn4, Abn5, Abn7, Abn9, Agu1, Axe2, Axe3, Bga2, Bxl1, Bxl2, Abf5(formerly known as Bxl3), GH61 genes (gene identifiers: CL09768, CL10518, CL05022, CL04725, CL04750, CL06230, CL05366), Gln, Pgx1, Rga1, Rgx1, Xgl1, Xyl7, Xyl8, Xyl9, Xyl10, Xyl11.

10

Alp1: The Alp1 DNA sequence is given by SEQ ID NO 30. The Alp1 amino acid sequence is given in SEQ ID NO 31.

The alp1 gene was expressed and Alp1 showed protease activity (Table 5). The
15 “Protease colorimetric detection kit” (Sigma, product number PC0100) was used to determine Alp1 protease activity.

Example 13: Expression of C1 GH61-family-encoding proteins genes.

20 A GH61 protein-encoding gene (identifier CL10518) was overexpressed in C1 strains W1L#100.L Δ alp1 Δ pyr5 and W1L#100.L Δ alp1 Δ chi1 Δ pyr5. Culture supernatant samples were analyzed by SDS-PAGE and protein bands were stained with Coomassie brilliant blue (CBB). The CL10518 protein is $\pm$ 26 kDa (Fig. 9). A standard fed batch fermentation was conducted, which yielded 13 g protein per liter fermentation filtrate,
25 based on a BCA protein determination assay.

The functional analysis of this protein has been described in International patent application WO 2009/033071.

Example 14: Expression of a C1 cellulase-encoding gene: cbh2.

The CBH2 encoding gene (identifier CL09854) was overexpressed in C1 strain W1L#100.L Δ alp1 Δ pyr5. Culture supernatant samples were analyzed by SDS-PAGE and protein bands were stained with coomassie brilliant blue (CBB). The CBH2 protein
5 migrates at about 55 kDa (Fig. 10). A standard fed batch fermentation was conducted, which yielded 10 g protein per liter fermentation filtrate, based on a BCA protein determination assay.

The functional analysis of this protein has been described in International patent
10 application WO 2009/018537.

Example 15: Expression of C1 exo-polygalacturonase encoding gene pgx.

The PGX encoding gene (identifier CL10389) was overexpressed in C1 strain
15 W1L#100.L Δ alp1 Δ chi1 Δ pyr5. Culture supernatant samples were analyzed by SDS-PAGE and protein bands were stained with coomassie brilliant blue (CBB). The PGX protein migrates at about 60 kDa (Fig. 11). A standard fed batch fermentation was conducted, which yielded 9 g protein per liter fermentation filtrate, based on a BCA protein determination assay.

20

The following assay was used to measure polygalacturonase activity. This assay measures the amount of reducing sugars released from polygalacturonic acid (PGA) by the action of a polygalacturonase. One unit of activity was defined as 1 μ mole of reducing sugars liberated per minute under the specified reaction conditions.

25

Reagents

- Sodium acetate buffer (0.2 M, pH 5.0) is prepared as follows. 16.4 g of anhydrous sodium acetate or 27.2 g of sodium acetate * 3H₂O is dissolved in distilled water so that the final volume of the solution to be 1000 mL (Solution A). In a separate
30 flask, 12.0 g (11.44 mL) of glacial acetic acid is mixed with distilled water to make the total volume of 1000 mL (Solution B). The final 0.2 M sodium acetate buffer,

pH 5.0, is prepared by mixing Solution A with Solution B until the pH of the resulting solution is equal to 5.0.

- Polygalacturonic acid (PGA) was purchased from Sigma (St. Louis, USA).
- Reagent A: 10 g of *p*-Hydroxy benzoic acid hydrazide (PAHBAH) suspended in 60 mL water. 10 mL of concentrated hydrochloric acid was added and the volume is adjusted to 200 ml. Reagent B: 24.9 g of trisodium citrate was dissolved in 500 ml of water. 2.2 g of calcium chloride was added as well as 40 g sodium hydroxide. The volume was adjusted to 2 L with water. Both reagents were stored at room temperature. Working Reagent: 10 ml of Reagent A was added to 90 ml of Reagent B. This solution was prepared freshly every day, and store on ice between uses.

Using the above reagents, the assay is performed as detailed below.

Enzyme Sample

- 50 μ L of PGA (10.0 mg/mL in 0.2 M sodium acetate buffer pH 5.0) was mixed with 30 μ L 0.2 M sodium acetate buffer pH 5.0 and 20 μ L of the enzyme sample and incubated at 40 °C for 75 minutes. To 25 μ L of this reaction mixture, 125 μ L of working solution was added. The samples were heated for 5 minutes at 99°C. After cooling down, the samples were analyzed by measuring the absorbance at 410 nm (A_{410}) as A_S (enzyme sample).

Substrate Blank

- 50 μ L of PGA (10.0 mg/mL in 0.2 M sodium acetate buffer pH 5.0) was mixed with 50 μ L 0.2 M sodium acetate buffer pH 5.0 and incubated at 40 °C for 75 minutes. To 25 μ L of this reaction mixture, 125 μ L of working solution was added. The samples were heated for 5 minutes at 99°C. After cooling down, the samples were analyzed by measuring the absorbance at 410 nm (A_{410}) as A_{SB} (substrate blank sample).

30 Calculation of Activity

- Activity is calculated as follows: determine polygalacturonase activity by reference to a standard curve of galacturonic acid.

$$\text{Activity (IU/ml)} = \Delta A_{410} / \text{SC} * \text{DF}$$

where $\Delta A_{410} = A_S$ (enzyme sample) - A_{SB} (substrate blank), SC is the slope of the standard curve and DF is the enzyme dilution factor.

The ΔA_{410} of Pgx1 (CL10389) was found to be 0.78 with a DF of 1 for enzyme produced in microtiter plate cultures. No standard curve was analyzed, therefore no reliable activity can be calculated. The only conclusion to be drawn is that the enzyme was found to be active towards polygalacturonic acid, and therefore it is suggested that it is a polygalacturonase.

The functional analysis of this protein has been described in International patent application WO 2009/033071.

Example 16: Expression of a C1 xylanase encoding gene with and without its carbohydrate binding domain: Xyl1.

The Xyl1 encoding gene (identifier CL00649) was overexpressed in C1 strain W1L#100.L $\Delta alp1 \Delta chi1 \Delta pyr5$. Two Xyl1 variants were produced: either full length Xyl1 or Xyl1 without its carbohydrate binding domain (cbd). Culture supernatant samples were analyzed by SDS-PAGE and protein bands were stained with coomassie brilliant blue (CBB) (Fig 12). The Xyl1 protein migrates at about 40 kDa, while its CBD-less counterpart migrates at about 30 kDa. Standard fed batch fermentations were conducted, which yielded up to 33 g protein per liter fermentation filtrate, based on a Bradford protein determination assay. Xylanase activities of these filtrates reached up to 3,500 U/mL.

The following assay is used to measure the xylanase activity towards AZO-wheat arabinoxylan. This substrate is insoluble in buffered solutions, but rapidly hydrates to

form gel particles which are readily and rapidly hydrolysed by specific endo-xylanases releasing soluble dye-labeled fragments.

Reagents

- 5 - Sodium acetate buffer (0.2 M, pH 5.0) is prepared as follows. 16.4 g of anhydrous sodium acetate or 27.2 g of sodium acetate * 3H₂O is dissolved in distilled water so that the final volume of the solution to be 1000 mL (Solution A). In a separate flask, 12.0 g (11.44 mL) of glacial acetic acid is mixed with distilled water to make the total volume of 1000 mL (Solution B). The final 0.2 M sodium acetate buffer, pH 5.0, is prepared by mixing Solution A with Solution B until the pH of the resulting solution is equal to 5.0.
- 10 - AZO-wheat arabinoxylan (AZO-WAX) from Megazyme (Bray, Ireland, Cat. # I-AWAXP) is used as the assay substrate. 1 g of AZO-WAX is suspended in 3 mL ethanol and adjusted to 100 mL with 0.2 M sodium acetate buffer pH 5.0 using
- 15 magnetic stirrer. 96% Ethanol is used to terminate the enzymatic reaction.

Using the above reagents, the assay is performed as detailed below:

Enzyme Sample

- 20 - 0.2 mL of 10 mg/mL AZO-WAX stock solution was preheated at 40° C for 10 minutes. This preheated stock solution was mixed with 0.2 mL of the enzyme sample (preheated at 40° C for 10 min) and incubated at 40 °C for 10 minutes. After exactly 10 minutes of incubation, 1.0 mL of 96% ethanol was added and then the absorbance at 590 nm (A_{590}) was measured as A_S (enzyme sample).

25

Substrate Blank

- 0.2 mL of 10 mg/mL AZO-WAX stock solution was preheated at 40° C for 10 minutes. This preheated stock solution was mixed with 200 µl of 0.2 M sodium acetate buffer pH 5.0 (preheated at 40° C for 10 min) and incubated at 40 °C for 10
- 30 minutes. After exactly 10 minutes of incubation, 1.0 mL of 96% ethanol is added and then the absorbance at 590 nm (A_{590}) is measured as A_{SB} (substrate blank).

Calculation of Activity

- Activity is calculated as follows: determine endo-xylanase activity by reference to a standard curve, produced from an endo-xylanase with known activity towards
5 AZO-WAX.

$$\text{Activity (IU/ml)} = \Delta A_{590} / \text{SC} * \text{DF}$$

where $\Delta A_{590} = A_S$ (enzyme sample) - A_{SB} (substrate blank), SC is the slope of the standard curve and DF is the enzyme dilution factor.

- 10 The functional analysis of this protein has been described in International patent application WO 2009/018537.

Example 17: Expression of the C1 arabinase 2 encoding gene *abn2*.

- 15 The Abn2 encoding gene (identifier CL03602) was overexpressed in C1 strain W1L#100.L $\Delta alp1 \Delta chi1 \Delta pyr5$. Culture supernatant samples were analyzed by SDS-PAGE and protein bands were stained with coomassie brilliant blue (CBB). The Abn2 protein migrates at about 50 kDa (Fig. 13). A standard fed batch fermentation was conducted, which yielded 7 g protein per liter fermentation filtrate, based on a BCA
20 protein determination assay.

The functional analysis of this protein has been described in International patent application WO 2009/033071.

- 25 Example 18: Expression of the C1 endo-chitinase encoding gene *chi1*.

- The Chi1 encoding gene (identifier CL06081) was overexpressed in C1 strain W1L#100.L $\Delta alp1 \Delta pyr5$. Culture supernatant samples were analyzed by SDS-PAGE and protein bands were stained with coomassie brilliant blue (CBB). The Chi1 protein
30 migrates at about 40 kDa (Fig. 14). A standard fed batch fermentation was conducted,

which yielded 12 g protein per liter fermentation filtrate, based on a BCA protein determination assay.

The functional analysis of this protein has been described in International patent
5 application WO 2009/018537.

Example 19: Expression of a heterologous gene: *Aspergillus niger* poly-galacturonase II.

The *Aspergillus niger* poly-galacturonase II encoding-gene (accession number
10 X58893) was expressed in C1 strain W1L#100.1Δalp1Δchi1Δpyr5. After fermentation the enzyme was purified using ion exchange chromatography and size exclusion chromatography. The purified endo-PGII migrated at about 40 kDa on SDS-PAGE gel (Fig. 15).

15 This heterologous enzyme was functional as was shown by activity on poly-galacturonic acid using the following assay.

Reducing sugars assay: PAHBAH method

20 Stock solutions:

- Substrate: 1% (w/v) polygalacturonic acid in H₂O.
- Reagent A: *p*-Hydroxy benzoic acid hydrazide (PAHBAH) (10 grams) is added to 60 ml of water and slurried. To this is added 10 ml of concentrated HCl and the volume is made up to 200 ml. Reagent B: Dissolve trisodium citrate (24.9 g) in 500
25 ml of water, add calcium chloride (2.20 g) and dissolve, add sodium hydroxide (40.0 g) and dissolve. Adjust the volume to 2 litres. The solution should be clear. Store both reagents at room temperature. Working Reagent: add 10 ml of Reagent A to 90 ml of Reagent B. Prepare this solution fresh daily, and store on ice between uses.

Assay:

1. 50 µl substrate
2. 30 µl 0.2M HAc/NaOH pH 5.0
3. 20 µl sample / enzyme (microplate undiluted; fermentor >20 x diluted)
- 5 4. Incubate at 37°C for 10 minutes
5. 25 µl assay mix + 125 µl Working Reagent (in PCR microplate) or 50 µl assay mix + 250 µl Working reagent (in 1.5-ml tube)
6. Heat @ 99°C for 5 minutes in Thermal PCR Cycler (PCR microplate) or in boiling water (1.5-ml tube)
- 10 7. Transfer 100 µl to NUNC microplate and measure extinction @ 410nm

Example 20: Generation of artificial enzyme mixes for efficient plant biomass saccharification.

15

An artificial enzyme mixture was created by mixing crude protein from C1 UV18-25Δalp1 with crude protein from white strains expressing C1-β-glucosidase Bgl1, C1-arabinofuranosidase Abf3 and Abn7, C1-xylanase Xyl2 and C1-β-xylosidase Bxl1, being W1L#100.LΔalp1Δpyr5[Bgl1/pyr5], W1L#100.LΔalp1Δchi1Δpyr5[Abf3/pyr5],
 20 W1L#100.LΔalp1Δchi1Δpyr5[Abn7/pyr5], W1L#100.LΔalp1Δpyr5[Xyl2/pyr5], and W1L#100.LΔalp1Δchi1[Bxl1/AmdS], respectively. The ratio of the different components on a protein basis was 10 (UV18-25Δalp1): 1 (white strain proteins).

The saccharification efficiency of the crude protein from UV18-25Δalp1 alone was tested on wheat bran substrate and compared to the efficiency of the artificial mixture. 10
 25 mg protein/g dry matter wheat bran was used. Conditions were as follows: temperature 50°C, pH 5.0, time 72 hours.

It was shown that the enzyme mixture from UV18-25Δalp1 alone liberated approximately 30% of the glucose, approximately 5% of the xylose and 12% of the arabinose from the wheat bran. The artificial mixture liberated at least 60% of the
 30 glucose, 60% of the xylose and 25% of the arabinose from the wheat bran.

Example 21: Construction of gene libraries in white C1 strainW1L#100.1 Δ alp1 Δ chi1 Δ pyr5 and screening of xylanases.

A gene library from genomic DNA of C1 strain UV18-25 was constructed in C1-
5 strain W1L#100.1 Δ alp1 Δ chi1 Δ pyr5 by methods previously described by Verdoes *et al.*
(2007). The library was screened for xylanase activity as described by Example 4 in
chapter 4, which yielded several positive clones that expressed different xylanases. SDS-
page analysis revealed the presence of extra protein bands in the positive clones. PCR
analysis using different primer combinations based on the sequence of the known C1
10 xylanases and the vector sequence revealed the presence of 3 different C1-xylanases. This
result was confirmed by Southern analysis.

Appendix 1 to the Examples: UV mutation procedure for C1 strains

- 15 1. Spread parent strain onto PDA (potato dextrose agar) plates and incubate at 35°C for
14 days to obtain spores.
2. Scrape spores into 0.9% saline and filter through cotton to remove mycelia. Dilute
the resulting spore suspension to 1x10⁶ spores/ml using saline. Remove a small
aliquot of spore suspension, dilute in saline and spread plate to PDA to determine the
20 initial viable spore count.
3. Add 10 ml spore suspension to a sterile glass Petri dish containing a paper clip and
stir on a magnetic stir plate. Remove the glass top and irradiate with UV light to
obtain 90-99.9% kill. Use a Pen-Ray lamp as the UV light source (254 nm) and
warm it up for at least 10 minutes prior to irradiating the spore suspension.
- 25 4. Spread plate to ASC selective plates (Appendic 2 to the Examples) with room lights
off, using a volume to obtain less than 30 colonies on each plate.

5. Invert plates, put in red plastic bags and incubate at 30°C for 6-7 days to grow and allow clearing zones to develop.
6. Determine % kill for the mutation as the difference between the initial viable plate count and a plate count on PDA after UV irradiation.

5

Appendix 2 to the Examples: Media

ASC Selective Agar Plates

Component	Amount	
Deionized water	800	ml
K ₂ HPO ₄	1.0	g
KCl	0.1	g
NaCl	0.1	g
MgSO ₄ -7H ₂ O	0.3	g
FeCl ₃ -6H ₂ O	0.016	g
(NH ₄) ₂ SO ₄	1.9	g
20 g/l ASC	200	ml
Noble Agar	15	g

10

Adjust pH to 7.5 with HCl and sterilize 30 minutes at 121°C. After sterilization add 20 ml of 25 g/l DOC (deoxycholic acid), sterile filtered. Pour about 20 ml/ plate. Spread UV-mutated spores to ASC plates and incubate for 7-14 days to allow colony growth and cellulose clearing.

15

RM-ASP medium

Component	Amount	
Bacto Peptone	2	g
Bacto Yeast Extract	1	g
50x AspA (+N)	20	mL
Glucose	10	g
1000x trace elements	1	mL
MgSO ₄ -7 H ₂ O	0.493	g
Water	Bring total volume to 1 L.	

- 5 Adjust pH to 6.5 prior to autoclaving. Sterilize glucose separately as a 50% solution.

50x AspA (+N)		
Component	Amount	
NaNO ₃ (or (NH ₄) ₂ SO ₄)	300 (or 233)	g
KCl	26	g
KH ₂ PO ₄	76	g
KOH 10 N	22.5	mL
Water	Bring total volume to 1 L.	
1000x trace elements		
Component	Amount	
ZnSO ₄ -7H ₂ O	2.2	g
H ₃ BO ₄	1.1	g
MnSO ₄ -H ₂ O	0.427	g
FeSO ₄ -7H ₂ O	0.5	g
CoCl ₂ -6H ₂ O	0.17	g
CuSO ₄ -5H ₂ O	0.16	g
Na ₂ MoO ₄ -2H ₂ O	0.15	g
EDTA	5	g
Water	Bring total volume to 100 mL.	

Low and high density cellulose media

Component (g/L)	Low density cellulose medium (#1)	High density cellulose medium (#2)
BisTris	15.7	15.7
K ₂ HP0 ₄	0.22	0.66
KH ₂ P0 ₄	0.08	0.24
(NH ₄) ₂ SO ₄	4	12
Na ₃ Citrate·H ₂ O	4	12
MgSO ₄ ·7H ₂ O	0.03	0.09
CaCl ₂	0.4	0.8
Yeast Extract	0.05	0.15
Pharmamedia	5	15
Lactose·H ₂ O	5	15
Cellulose	20	80

Adjust to pH 7.0.

chl sequence: see WO 2009/018537.

pep4 DNA sequence (SEQ ID No 19):

1	gctggctcaccggttatttgctcccgaggaagtccaggtcctcctcgcagttggacaaac
61	tctgcttcgcagcctgcaactttgactcaaggagcgccctcgccctcgatgggtaag
5 121	acagcatgacgttggcctgccaaatgtcagcctctagaagcacactcccactctcggttg
181	aaaggttcctaccccaagccacaagtaaacctcgctccgctcgccggtatctcgcccttcgc
241	atagagagtgtcggttcaattcgaatggtgtctctatcggtatcagattcgccctgccacaa
301	tcaaccgccgatcagcaccatggccgctcatcgagagtggcaacgcctcgccctaccgtc
361	ctcagcttcaaaaagcggacagcctccagcggtttccgaatgtcgggcattttgtccttt
10 421	agtcccgctaccctccgctgcaggttctgctccatgaactggattttcctgcacgccgac
481	cacgtatcagccgaacgcgcgtccgtcaaggctggatttcaatcttaaccgggagagctca
541	cgcaatcatctcttggaaaccgacgcagcgctcggtcaacatctgctcgtagctgacata
601	gtcctcgaccttgctcgacgaatggcgcatagcgaatgccacgaggattggaggggtgtggc
661	gtccctgtctcgtagcaggtggtagtcagcaataacagccagagtgcataatgctagaatg
15 721	gcgcccgcgggggaggggaaagtttggttaccttgctgctgcttcttctgtgctcgcc
781	atcttggacaaattctcacatggtgcagtggaaggatactgcaagcgactgttaaccoga
841	gccaacggagtgacgtcggttttggtacctagtttaggtcaagccgttctcaagctgctg
901	gcaaaaaattcatggcggggtcgagtgggcagcgaggtactcctcgtagggagcaaggtg
961	aagatgtggggtagcaggggtcgacgctacaaagtactttgtatccggattgctgtgtgg
20 1021	tacgaagcgcccggtgtgttggtatgctctctgtatgtacggagtactgtaccttctccat
1081	gcgctgccccattctctattttggttgccactgcttcggttcgtagtgtatgtacagcagta
1141	caactatctacgacacctgcactgactagtgcgtagaattctttagtttctcgagtacgg
1201	cgctaacgcttcgcgcagcaagcaccttcttctgattgtgttactgtgctcaaacctcgc
1261	cagccagctgcggtgtccacaagcccgccggtgcccaaccgccatttgcacccgggtcc
25 1321	catgaatctgtggacgacccatccctctctgtaccgctcgcggtatcagcccagaatga
1381	tagcgggaagacaaaacgcagtgattcggattacgctcgcaggaaatggggggagtagctt
1441	gatagctctccacggcgaggggtgtctcaggctgaggtgtcaactagttgtatgtacactc
1501	aggacgaggcattctgcgttttgaaacaccaatcttccaataccggaggtgttgtatgca
1561	ggatcacttgaatatgtttgcaccattattactgtacctggatgattcggaacagggcga
30 1621	gcatgattggctcgccccgttttgctaccgcatcgcagcgctcgccgggaagcagccacgt
1681	agagcactgccaaacgtttcaagagacaccccatatggagtaaattggagtaatctgtat
1741	ccttcagagccgtcaatcaaactattgtttctcagcaggatggcccggttgctcatggggg
1801	atgtaccttggtaggtagttcggttggtgatgacttccttggtatgagcctgctgcgcatga
1861	aggtgccggggggcccggttggtgcctaaaactaactgtaaacagacgcacgggtggcga
35 1921	cgacgtagccgaaccggtgtagcgagcttccccggccactacgtaatcggggcgatgca
1981	ctgcaggaacacctcacacctgacctaccccttcgcctccgcatccgtcccaaccgcgt
2041	tcccaacctttccatcaactacttccgagactcgacatcaccttttcgcgctcggtgtctc

2101 atcgtcggttatcatcaccatcggcgatagatttgttcgcttcgatcgtcgcatcgccttg
 2161 acttccattcgtccttcacgccgaccgaccggaccagacagtgcgccccaaATGAAGGATG
 2221 CTTTTTTGCTGACCGCAGCTGTGCTGCTCGGCTCCGCCCAGGGAGCAGTTCACAAAATGA
 2281 AGCTGCAGAAGATCCCTCTCTCTGAGCAGCTTgtacgtctgaccccgttcaagcacgcgt
 5 2341 cagcggctactgaccttatcgcgtccagGAGGCGGTTCATCAACACCCAGCTCGAGCA
 2401 TCTCGGCCAAAAATACATGGGGTTGCGCCACGTGAATCTCAAGCCGATGCCATCTTTAA
 2461 GGGCATGGTTGCCGACGTCAAGGGCAACCATCCTATTCCCATCTCCAACCTTCATGAACGC
 2521 ACAGTgtatgtgacgccactgtggtggcatggatggctcgtcctcaattcggagactgac
 2581 actggagcaccctagACTTCTCCGAGATCACGATTGGAACACCCCC**TCAGTCATTCAAGG**
 10 2641 **TGGTCCTCGATACCGGTAGCTCCAACCTGTGGGTTCATCAGTCGAGTGCGGCTCGATTG**
 2701 CTTGTTACCTGCACTCGAAGTATGACTCATCTGCCTCGTCCACCTACAAGAAGAACGGAA
 2761 CCTCGTTCGAGATCCGCTACGGGTCAGGCAGCCTGAG**CGGGTTTGCTCTCTCAGGACACAG**
 2821 TGTCCATCGGCGATATCACTATCCAGGGCCAGGACTTTGCCGAGGCGACCAGCGAGCCCG
 2881 GTCTTGCCTTTGCCTTTGGCCGTTTCGACGGTATCCTTGGCCTTGGCTACGACCGGATCT
 15 2941 CAGTCAACGGCATCGTCCCGCCTTTTTACAAGATGGTCGAGCAGAAGCTCATCGATGAGC
 3001 CCGTCTTCGCCTTCTACCTGGCCGATACCAATGGCCAGTCTGAGGTTGTCTTTGGCCGTG
 3061 TTGACCACGACAAGTACAAGGGCAAGATCACCACCATTCCGTTGAGGCGCAAGGCCTACT
 3121 GGGAGGTTGACTTCGATGCCATTTCTTACGGCGACGACACTGCCGAGCTTGAGAACACTG
 3181 GCATCATCCTGGACACCGGTACTTCTCTGATCGCTCTGCCAGCCAGCTCGCCGAGATGC
 20 3241 TCAACGCTCAGATCGGCGCTAAGAAGAGCTACACTGGCCAGTACACCATCGACTGCAACA
 3301 AGCGCGACTCCCTCAAGGATGTACGTTCAACCTGGCTGGCTACAATTTACGCTCGGCC
 3361 CCTACGACTACGTTCTCGAGGTCCAGGGCAGCTGCATTTCTACCTTTATGGGCATGGATT
 3421 TCCCGGCTCCTACTGGGCCACTTGCGATCCTGGGCGATGCCTTCCTCCGGAGGTATTACT
 3481 CCATTTATGACCTTGGCGCCGACACCGTCCGTCTGGCTGAGGCCAAGtgattgaaggatg
 25 3541 ggcggcagggaaagacgatgggtaatacggggagctctgggaatcgggctttggactgtgg
 3601 tctgtatctagttgctcaagagagttgtcgtttgattttgttataggatctgtctaggaa
 3661 ccttagcaggagtgaattttttcgtgtacgagcatcggcgggctgaagtgggttgataa
 3721 caagtctggacttgagtacgcaggcagttgcacaatctgcttcgccgaggagagcaaagg
 3781 cgtcctctttgaaaaagcctacctacgcgtcacaggggtataattttttgagtttgacct
 30 3841 acgcctgtcccataccaaccgcgtcccaatccccgtcaacccttgcaatgtcattaccc
 3901 gtggatgtatcacgtagcagaagccgacatccacacgcttcaaccttcctatccagaca
 3961 atgacatggtaagctcatttttttaagggtcgccgtcctcctccttcacgtgattcatt
 4021 ttccttgccgcttgtggcgcatccctgacttcatgccgtacggatcaaagggtgcaaac
 4081 ttgccccgcacctcttttctgccgccatcatcatcaccatcatcgccgtttgtcgccctgc
 35 4141 gcagcatgtagcacggacgacgccttgctgtagtcaaacggctcctgctcggcatcgtca
 4201 tcatggccttcctcctgttcgcccagaggtctgttcgtcggctgccgaggtcgcgggagg
 4261 gcagatgtctgctgctgctgctgctgctgctgcttctgggctttcttgggcgctcgaagt
 4321 gccttcctggcttgagccttgagttcctttgtccctttatgtctccgttttgagccagt

4381 tgcctctgccaagagctgagcacgcttgaactcttctcgagcagccttcttggcttgtttc
 4441 ttgacctgcttggcgcccttgatcatcaccacctcaacttctgctcgacactaggagac
 4501 ttccgggtggtctttgcctgcggaactatctccacctatctcgatgtcggaactgcttcg
 4561 gcttcggatgctgactcaacatcaacatccctagacttccgctttcgaccagccttcaga
 5 4621 gtgaaaccttcttcttcgagaacagggagacccttggtgtcttggttcagcgacacgcctg

Pep4 amino acid sequence (SEQ ID No 20):

1 MKDAFLLTAA VLLGSAQGAV HKMKLQKIPL SEQLEAVPIN TQLEHLGQKY MGLRPRESQA
 10 61 DAIFKGMVAD VKGNHPIPI S NFMNAQYFSE ITIGTPPQSF KVVLDTGSSN LWVPSVECGS
 121 IACYLHISKYD SSASSTYKKN GTSFEIRYGS GSLSGFVSQD TVSIGDITI Q QDFAEATSE
 181 PGLAFAFGRF DGILGLGYDR ISVNGIVPPF YKMVEQKLID EPVFAFYLA D TNGQSEVVFG
 241 GVDHDKYK GK MTTIPLRRKA YWEVDFDAIS YGDDTAEL EN TGIILDTGTS LIALPSQLAE
 301 MLNAQIGAKK SYTGQYTIDC NKRDSLKDVT FNLAGYNFTL GPYDYVLEVQ GSCISTFMGM
 15 361 DFPAPTGPLA ILGDAFLRRY YSIYDLGADT VGLAEAK

his2a DNA sequence (SEQ ID No 21):

20 1 cattcatggg tttagaggccc gattttgaac gtatatccta ggctatattc ggggtaagat
 61 actggaagcg ctgggcccga tgactagcta tttcaagtgc ccaagagccc atcatatccta
 121 acttgtggcc taagatctag ccaaatcatt cattgggttac cccagactcg acgaacctga
 181 tattcgaatc cagggcaagt caaatcgccg agtaagactt gacaaacccg gaaccaaga
 241 actgcgcaat ctgggagcag gtttccgacc agcatggaaa cccccgatg gaaaaccac
 25 301 acatacgggg atggggacta acgccggaca aatcaaaaac cctggaggat tgggtaacga
 361 tggggaagtg cgacgggcac tcaacccttc aagcgttgca ggaccttgta cagccaagca
 421 gaatgacgga aaccgatgag caaaccggga atctgatgat cctggaacag aatcatctgt
 481 cttgggtacc gacgttgag tgagagtgtg caaattagca ggatcaagca actatactac
 541 ctaaatcagg tcgatcagtt atcagccctt gcaaaccaga cttgatggag ggaagagggtg
 30 601 aaagctgtga ttgagggagg aagctgagaa ttggtggtgg ttgttttgct cagccagggt
 661 gtaggacgag aagaacgcgt tcgagatttc ggagagcagg ctgtcctaga gcattatattt
 721 cctggccttg agcaaaactta agccagtttt tttttccccc tcgggagggga agtcgctttg
 781 aatttgaagc ttgcggggcg agagctcggc tccataagca tccaatcaaa tgagcctgaa
 841 gcagtcgacc gatttttttt tatctgggtg taatcgcaac catgcacata accgttttgg

901 gactagctcc aacagctctg atcaacaacc tgagaaaggc gcgagtgatc cgtgatccca
 961 cacccttacg cgaaaactac ttaactccca cctccccac cgcggtcaa cttcttccaa
 1021 ctcccactca accaacttcc gttttcccat caatcactgc attcgcgct caagctcttc
 1081 ctcgccctta caccaaccac ataacttttt tatectttga caaggacat caatcaaaAT
 5 1141 GACTGGCGGC GGCAAGTCCG GTGGCAAGGC GAGCGGTTC AAGAACGCGC AATCgtaggt
 1201 gcccttttcg cgtcatctac ccgcgccttc gtgcagttgg gcatggttca gccttgaact
 1261 ccagatgccc gttccggtgc tcttacagtt ggctaacttt ttgtagTCGT TCATCTAAGG
 1321 CCGGTCTTGC GTTCCCTGTC GGTCTGTTC ACCGCCTTCT CCGGAAGGGC AACTACGCCC
 1381 AGCGTGTCGG TGCCGGTGCT CCCGTTTACC TGGCTGCCGT TCTCGAGTAT CTGCCGCTG
 10 1441 AAATTCTGGA GCTGGCTGGC AACGCCGCTC GCGACAACAA GAAGACGCGT ATCATCCCGC
 1501 GTCATTGCA ACTCGCTATC AGGAACGATG AGGAGTTGAA CAAGCTTCTC GGGCACGTCA
 1561 CCATCGCCCA GGGTGGTGTC CTTCCCAACA TCCACCAGAg tacgttgct taccagacga
 1621 tctctaatagc gcaaatactaa ctttggttcc agACCTTCTG CCGAAGAAGA CCGGCAAGAC
 1681 CGGCAAGAAC TTGTCGCAGG AGCTCtgatt ttcgcggttg ggtttttttg ctttattttc
 15 1741 tggtcggcac gctgggttca tgatatcggg gtcacggttt cgggtcattg gttgcttttt
 1801 gcgcgtgttt gggctgtaca ttaattccat gatgggcatg gtcaggtta tgaatgagaa
 1861 tatectctga acatccaaat cctgacacag tttgctcgag ttgatgtctg cattggaagc
 1921 gactcgttga cggtagcg tagagtcttg tcgcttacga aattcttgca tcgcacagat
 1981 taccagtag tgccatagta ctctttaaga tgataagtgc atttgagccc ggcacgcac
 20 2041 agactttccc atgccttgat atatgcgaat tcctatgtac aagagattcg tcgcgaaaga
 2101 gcccgtaaaa acttgagcgg ggggggagct gcaaaagcct gtcagctaata tcgagtgaga
 2161 cgcgcaaagc aagccaactt acgatccagg tggggcgccg ggaggtttct ctcgattttc

His2A amino acid sequence (SEQ ID No 22):

25 1 MTGGGKSGGK ASGSKNAQSR SSKAGLAFPV GRVHRLLRKG NYAQRVGAGA PVYLAHVLEY
 61 LAEILELAG NAARDNKKTR IIPRHLQLAI RNDEELNKLL GHVTIAQGGV LPNIHQNLPL
 121 KKTGKTGKNL SQEL

hex1 DNA sequence (SEQ ID No 23):

30 1 gtcaacttactccgagtctcgcatcgagttcgatactgagcaccgtactcacaactccgt
 61 cattgacgttgctgagggcgagtatcgtgcccgtgtccagcccaaccacgcgaagcaagc

121 ttccgtagtcggtaccacogtcaacggatcgcggttcagccacagccgcaaggccagcag
 181 caccacotccacccacacogacgagtagacacogtcgatccccctagccacogccccgteta
 241 caagaaggagtcggttgaagtcgccggtaccactggtgacccccctgctcctcgttcgac
 301 ctaccacgagcaggtgaacattggtgaagagaccggtgacgctcacggttacgctcctca
 5 361 acccaacaacaacaacaagATGGGCTACTACGACGAGGACGgtaagcatcttccttcccc
 421 tttgatggtgttccttaccogtgacatccatcggtcgatgctttcttagccacacacaa
 481 gtggtgtgacaagtgcogtgctcacgcccgatatcagGCCACTACCACTCTTTCGCCATG
 541 GATTGCACAAGTTGGCTGACCGTATTGCGCATCCTGAAGGCCATGACCGCGTTGAGGTGA
 601 GCGAGGTTCTGTGAGACCCGCCGCACCCGCGCTCCGTCTTCGGAGGCGTACACGCCGAACA
 10 661 CGGTCACCATTCGGTGCCACCACATCCGCCTCGGCGACATCCTGATCCTCCAGGGCCGCC
 721 CCTGCCAGGTCATCCGTATCTCGACCTCGGCTGCCACTGGCCAGCACCGCTATCTTGGTG
 781 TCGACCTCTTCACCAAGCAGCTCCATGAGGAGTCGTCTCGTCTCGAACCTGCTCCCA
 841 GCGTCGTCTGCCAGACGATGCTTGGCCCTGTTTTCAAGCAGTACCGCGTCTCGACATGC
 901 AGGACGGCCACATCGTCGCCATGACCGAGACGGGCGATGTCAAGCAGAACCTGCCCGTCA
 15 961 TCGACCAGAGCAACCTCTGGGGCCGCCTCAAGCAGGCCTTCGAGACTGGCCGCGGCAGCG
 1021 TCCGTGTCCTGGTCTGTTTCTGACAACGGCAACGAGATGGCTGTTGACATGAAGGTCTGTC
 1081 ACGGCTCGCGCCTCTAAgtcaagccggcaggccttcatgcaagctttggggctacgagtc
 1141 gggcggcattgggtttgcgtttgatgcatcttggttacggcgtgtatgtcatttgaagat
 1201 tgaaagctgcgccttggtcgactcctggcgccggatggatatacatgttcctcgggagga
 20 1261 tatgaagggttcatgtcgctagtttcacgtgtatatgatgactgtaatggatggatgttt
 1321 atggccaactttgcgattgatatcttgaaccttttttctggtcgtgtgagtgaacagtga
 1381 ttaagtgagagtgaggtatgcacogtttatcacaagggtgccttgatatccaccttcaa
 1441 cgggcgtggggaatcgaagtccttcccctacagtaagtagcctctcttgaatgatctgaa
 1501 acgcaaccttccgagccactaccacacctaactacgaaacaaccactttcctgttccag
 25 1561 gaagctccagttctcccgctaccctcccctcccgcogttcagggtgtacgcttatctccc
 1621 aacctcatcttcgagaggtctaataccgtacacacttaacagtgcacctgacatagctaa
 1681 ccatcatcactctagttcattagccogtcccgccatccogtcaattacattcccggctgtt

30 Hex1 amino acid sequence (SEQ ID No 24):

1 MGYYDEDGHY HSFRLHGLHKL ADRIAHPEGH DRVEVSEVRE TRRTRAPSSE AYTENTVTIP
 61 CHHIRLGDIL ILQGRPCQVI RISTSAATGQ HRYLGVDLFT KQLHEESSFV SNPAPSVVVQ
 121 TMLGPVFKQY RVLDMQDGI VAMTETGDVK QNLPVIDQSN LWGRLKQAFE TGRGSVRVLV
 35 181 VSDNGNEMAV DMKVHGSRL

bgl1: see WO 2009/018537. Note that bgl1 = Bgl3A.

xyl6: see WO 2009/018537.

cbh1: see WO 2009/018537. Note that cbh1 = CBH1a .

5

Pchi1(0.8) (SEQ I No 25):

AGCTTGACCCTTTCAGAGCTAGGTTTCATTAGGCCTTCGAAAACAACCCAAGGCCCCGTC
GCAACCATCACAACCGGCCGATAACCAGATCTCGGTAGGTCCGATAAGGATCCAAAATGG
TGTCGGCTGACGTTGCATGTGCCCAGGCAGGAGGATGATCCCCAGGGTTGTTGCCGGCAG
10 CTCCCGCACGTCGGGGAGGGGGAGGGGGAGGGGAAAGCCCTAACTAACGTTTCGTTCTATC
ACGGGCCGACCGGGCCATGCTTTCGGCTTGTGAGCGGTGGGGTCAAGGGCAACAAGAAAT
GCTAAGTGCGGGACGAAGACACGCGGGCATGAGGTCTCAGGGTGACCTGCGCAAAACCAA
GTCCCACCTCGCCATGCCTCCAGCAGCAACGTTGCCGTAGAAGGGTCAGGGGGTTTGTGTGT
AGACCCACGACCATGCTGCCGGCGAGCGGAGGGTTGGCTTGCTACAGGCGCTGAAGGGTC
15 AACTCGGTGCCCAAAGTGGCTACCAAGCGTGCCATCAAGGGAAATGAGATGATGGTGGCT
CGTGGGCAAAGAAAAGACAAGGGAGGTGACTCTAGAGAGATGCTCTCGAGTTCACGGGT
TAAGAGCACTGTGATCGTTTACAAAGCCGGCGTACTCCTCTAGAGCATCTATCATCAACA
TCACCAGAAAGGTCNTAGACCAGGTGGTTGCCATATCCAGTCGCAAAAGAGCCAAAGAGC
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Pchi1(1.8)(SEQ ID No 26):

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Alp1 amino acid sequence (SEQ ID No 31):

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 5 241 MSLGGSYSAS INNAAAALVR SGVFLAVAAG NENQNAANSS PASEASACTV GATDRNDAKA
 301 SYSNYGSVVD IQAPGSNLS TWIGSTSATN TISGTSMAST HIAGLGAYLL ALEGSKTPAE
 361 LCNYIKSTGN AAITGVPSGT TNRIAFNGNP SA

10 List of references

- Braaksma, M. and P. J. Punt. 2008. *Aspergillus* as a cell factory for protein production: controlling protease activity in fungal production, p. 441-455. In G. H. Goldman and S. A. Osmani (ed.), *The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods*, CRC Press, Boca Raton.
- Verdoes, J.C., Punt, P.J., Burlingame, R., Bartels, J., van Dijk, R., Slump, E., Meens, M., Joosten, R. and Emalfarb, M., 2007. A dedicated vector for efficient library construction and high throughput screening in the hyphal fungus *Chrysosporium lucknowense*. *Industrial Biotechnology* 3, 48-57.

48a

SEQUENCE LISTING IN ELECTRONIC FORM

This description contains a sequence listing in electronic form in ASCII text format. A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office. The sequences in the sequence listing in electronic form are reproduced in the following Table.

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<141> 2010-03-16

<150> EP 09003750

<151> 2009-03-16

<160> 31

<170> PatentIn version 3.3

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<213> Chrysosporium lucknowense

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<213> Chrysosporium lucknowense

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<220>

48d

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17

<210> 14

<211> 7

<212> PRT

<213> Chrysosporium lucknowense

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<400> 14

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<213> Chrysosporium lucknowense

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22

<210> 18

<211> 173

<212> DNA

48e

<213> Chrysosporium lucknowense

<400> 18

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<211> 4680

<212> DNA

<213> Chrysosporium lucknowense

<400> 19

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48f

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<210> 20

<211> 397

<212> PRT

<213> Chrysosporium lucknowense

<400> 20

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20          25          30
Gln Leu Glu Ala Val Pro Ile Asn Thr Gln Leu Glu His Leu Gly Gln
35          40          45
Lys Tyr Met Gly Leu Arg Pro Arg Glu Ser Gln Ala Asp Ala Ile Phe
50          55          60
Lys Gly Met Val Ala Asp Val Lys Gly Asn His Pro Ile Pro Ile Ser
65          70          75          80
Asn Phe Met Asn Ala Gln Tyr Phe Ser Glu Ile Thr Ile Gly Thr Pro
85          90          95

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48g

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Tyr	Asp	Ser	Ser	Ala	Ser	Ser	Thr	Tyr	Lys	Lys	Asn	Gly	Thr	Ser	Phe
	130					135					140				
Glu	Ile	Arg	Tyr	Gly	Ser	Gly	Ser	Leu	Ser	Gly	Phe	Val	Ser	Gln	Asp
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Thr	Val	Ser	Ile	Gly	Asp	Ile	Thr	Ile	Gln	Gly	Gln	Asp	Phe	Ala	Glu
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Ala	Thr	Ser	Glu	Pro	Gly	Leu	Ala	Phe	Ala	Phe	Gly	Arg	Phe	Asp	Gly
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		275					280					285			
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			340				345						350		
Cys	Ile	Ser	Thr	Phe	Met	Gly	Met	Asp	Phe	Pro	Ala	Pro	Thr	Gly	Pro
		355					360					365			
Leu	Ala	Ile	Leu	Gly	Asp	Ala	Phe	Leu	Arg	Arg	Tyr	Tyr	Ser	Ile	Tyr
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<210> 21

<211> 2220

<212> DNA

<213> Chrysosporium lucknowense

<400> 21

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48h

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<210> 22
 <211> 134
 <212> PRT
 <213> Chrysosporium lucknowense

<400> 22

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          20          25          30
Val His Arg Leu Leu Arg Lys Gly Asn Tyr Ala Gln Arg Val Gly Ala
          35          40          45
Gly Ala Pro Val Tyr Leu Ala Ala Val Leu Glu Tyr Leu Ala Ala Glu
          50          55          60
Ile Leu Glu Leu Ala Gly Asn Ala Ala Arg Asp Asn Lys Lys Thr Arg
65          70          75          80
Ile Ile Pro Arg His Leu Gln Leu Ala Ile Arg Asn Asp Glu Glu Leu
          85          90          95
Asn Lys Leu Leu Gly His Val Thr Ile Ala Gln Gly Gly Val Leu Pro
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Asn Ile His Gln Asn Leu Leu Pro Lys Lys Thr Gly Lys Thr Gly Lys
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          130

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<210> 23
 <211> 1740
 <212> DNA

48i

<213> Chrysosporium lucknowense

<400> 23

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<210> 24

<211> 200

<212> PRT

<213> Chrysosporium lucknowense

<400> 24

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20          25          30
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35          40          45
Ser Glu Ala Tyr Thr Pro Asn Thr Val Thr Ile Pro Cys His His Ile
50          55          60
Arg Leu Gly Asp Ile Leu Ile Leu Gln Gly Arg Pro Cys Gln Val Ile
65          70          75          80
Arg Ile Ser Thr Ser Ala Ala Thr Gly Gln His Arg Tyr Leu Gly Val
85          90          95
Asp Leu Phe Thr Lys Gln Leu His Glu Glu Ser Ser Phe Val Ser Asn
100         105         110
Pro Ala Pro Ser Val Val Val Gln Thr Met Leu Gly Pro Val Phe Lys
115         120         125

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48j

Gln Tyr Arg Val Leu Asp Met Gln Asp Gly His Ile Val Ala Met Thr
 130 135 140
 Glu Thr Gly Asp Val Lys Gln Asn Leu Pro Val Ile Asp Gln Ser Asn
 145 150 155 160
 Leu Trp Gly Arg Leu Lys Gln Ala Phe Glu Thr Gly Arg Gly Ser Val
 165 170 175
 Arg Val Leu Val Val Ser Asp Asn Gly Asn Glu Met Ala Val Asp Met
 180 185 190
 Lys Val Val His Gly Ser Arg Leu
 195 200

<210> 25
 <211> 779
 <212> DNA
 <213> Chrysosporium lucknowense

<220>
 <221> misc_feature
 <222> (675)..(675)
 <223> n = any nucleotide

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<211> 1803

<212> DNA

<213> Chrysosporium lucknowense

<400> 29

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[illegible]

Claims

1. A strain of *Chrysosporium lucknowense* selected from W1L deposited at CBS under accession number 122189; W1L#100.1 deposited at CBS under accession number 122190; or a strain of either W1L or W1L#100.1 modified by gene disruption.
2. The strain of claim 1, in which the gene encoding endochitinase 1 (*chi1*) has been disrupted.
3. The strain of claim 1 or 2, in which one or more genes encoding alkaline protease 1 (*alp1*), alkaline protease 2 (*alp2*), proteinase A (*pep4*), glucoamylase (*Gla1*), exo-chitinase (*Chi2*) and laminarinase (*Lam1*) have been disrupted.
4. The strain of claim 3, which is:
W1L#100.1 Δ *alp1* Δ *pyr5*; or
W1L#100.1 Δ *alp1* Δ *Chi1* Δ *pyr5*.
5. A method for producing a homologous or heterologous protein, comprising expressing a gene encoding said homologous or heterologous protein in the strain of any one of claims 1 to 4.
6. The method of claim 5, wherein the protein has a purity of higher than 75%.
7. A method for producing a protein mixture comprising homologous and/or heterologous proteins, the method comprising expressing genes encoding said proteins in the strain of any one of claims 1 to 4.
8. A method for isolating a genetically modified strain of *Chrysosporium lucknowense* strain UV 18-25, wherein the cellulase and protease secretion is less than 20% of the endogenous cellulase and protease secretion of unmodified *Chrysosporium lucknowense* strain UV 18-25, comprising the steps of:
 - (i) mutagenizing *Chrysosporium lucknowense* strain UV 18-25;

- (ii) plating the mutagenized *Chrysosporium lucknowense* of step (i) on acid swollen cellulose (ASC) plates;
- (iii) selecting at least one colony showing a reduced cellulose clearing zone, enhanced sporulation and white color;
- (iv) plating the strain selected in step (iii) on skim milk plates; and
- (v) selecting from the plates of step (iv) at least one colony showing a reduced protein degradation halo;

thereby isolating said genetically modified strain.

9. The method of claim 8, further comprising a step of mutagenizing the strain selected in step (iii).

10. A method for isolating a genetically modified strain of *Chrysosporium lucknowense* strain UV 18-25, wherein cellulase secretion is less than 20% of endogenous cellulase secretion of unmodified *Chrysosporium lucknowense* strain UV 18-25, comprising the steps of:

- (i) mutagenizing *Chrysosporium lucknowense* strain UV 18-25;
- (ii) plating the mutagenized strain of step (i) on acid swollen cellulose (ASC) plates; and
- (iii) selecting a colony showing a reduced cellulose clearing zone, enhanced sporulation and white color from the plates of step (ii),

thereby isolating said genetically modified strain.

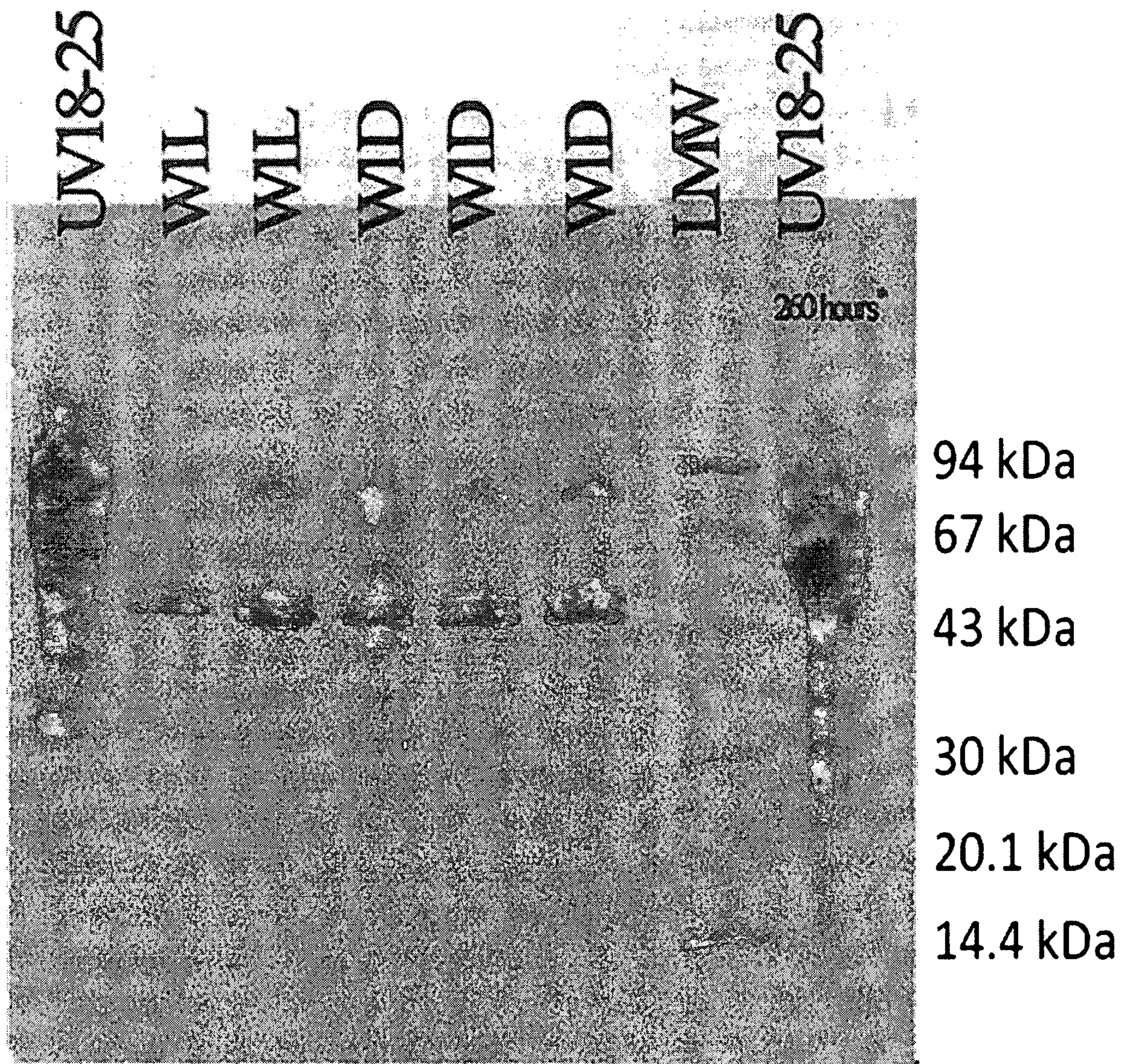


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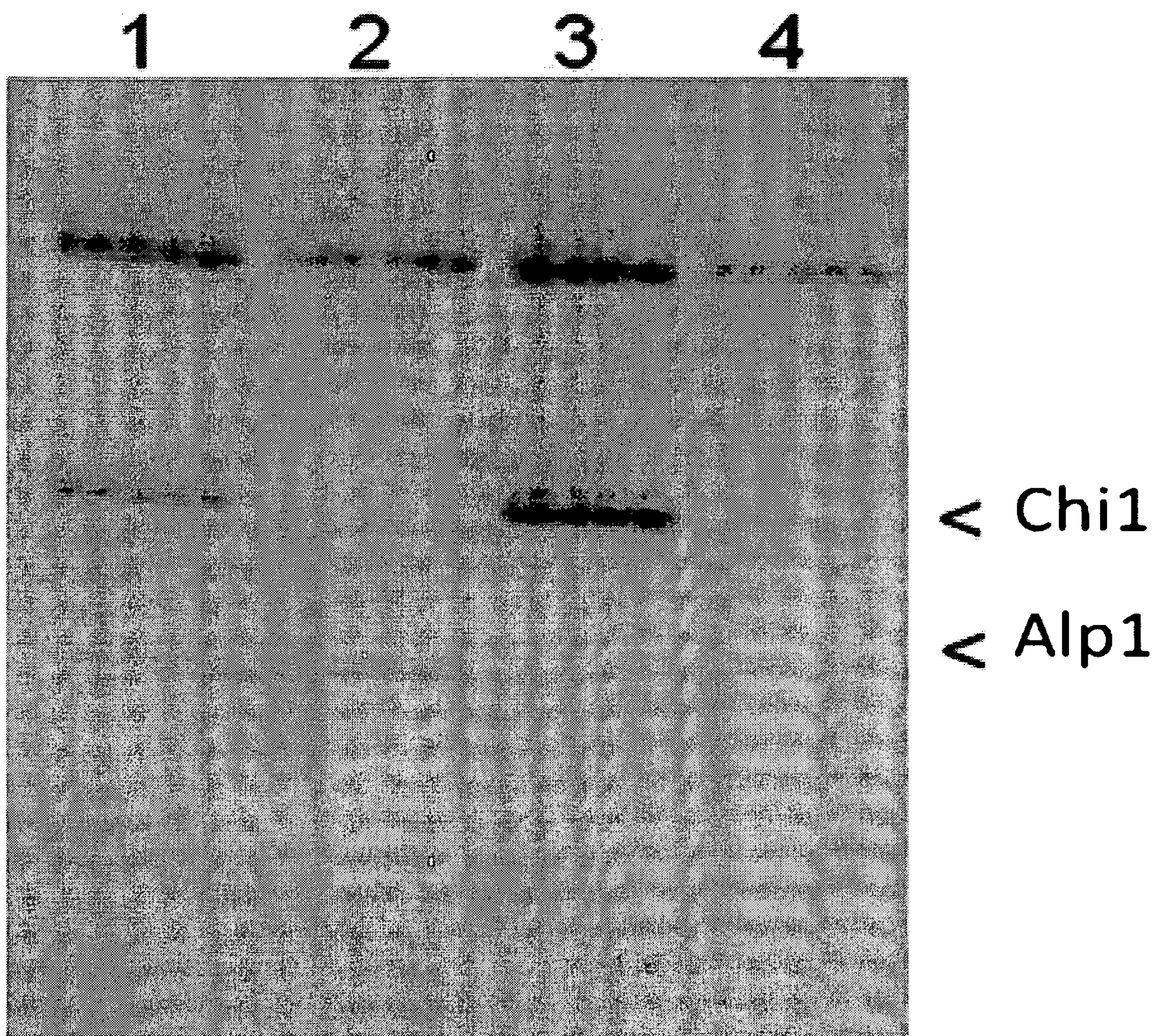


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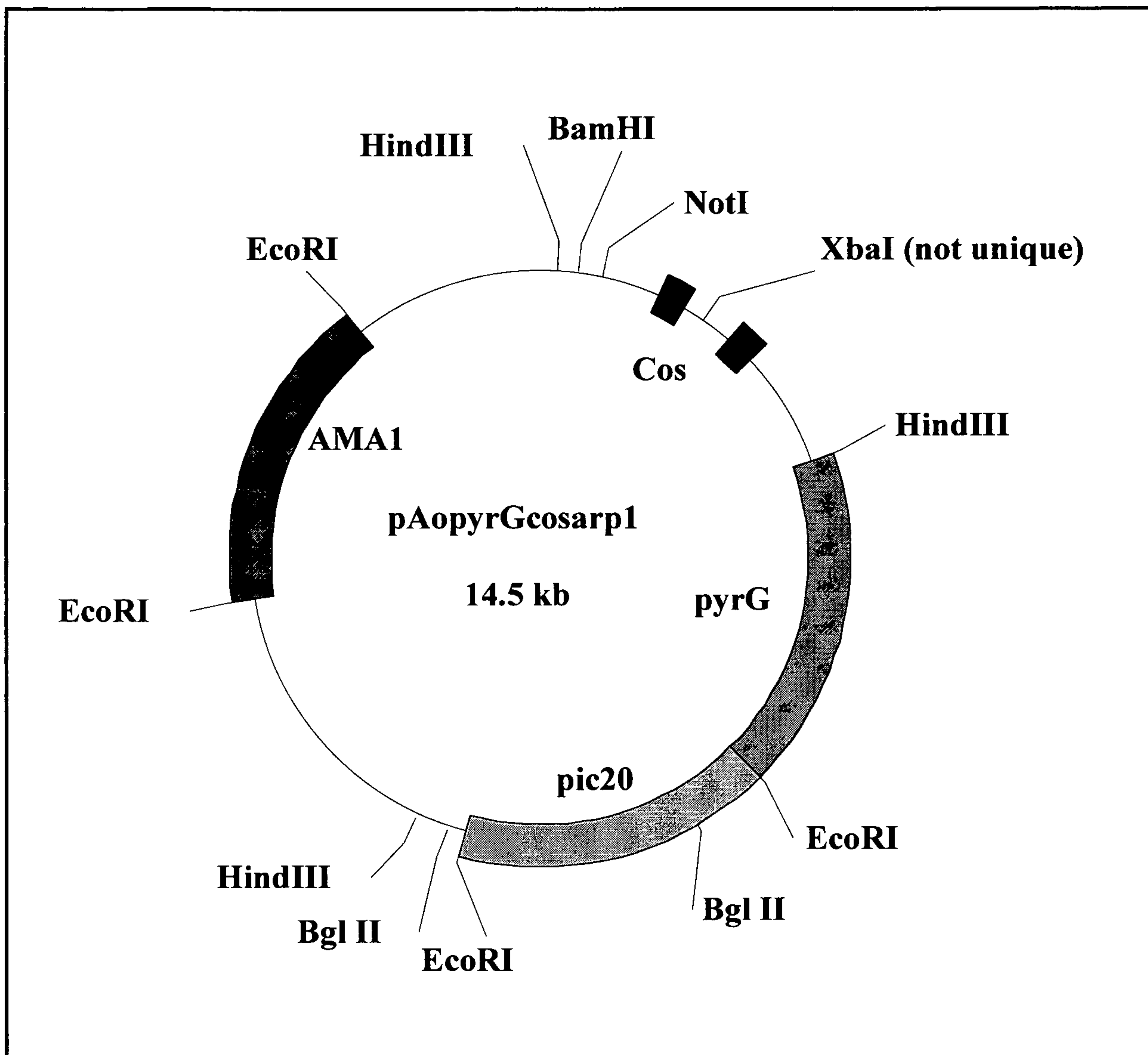


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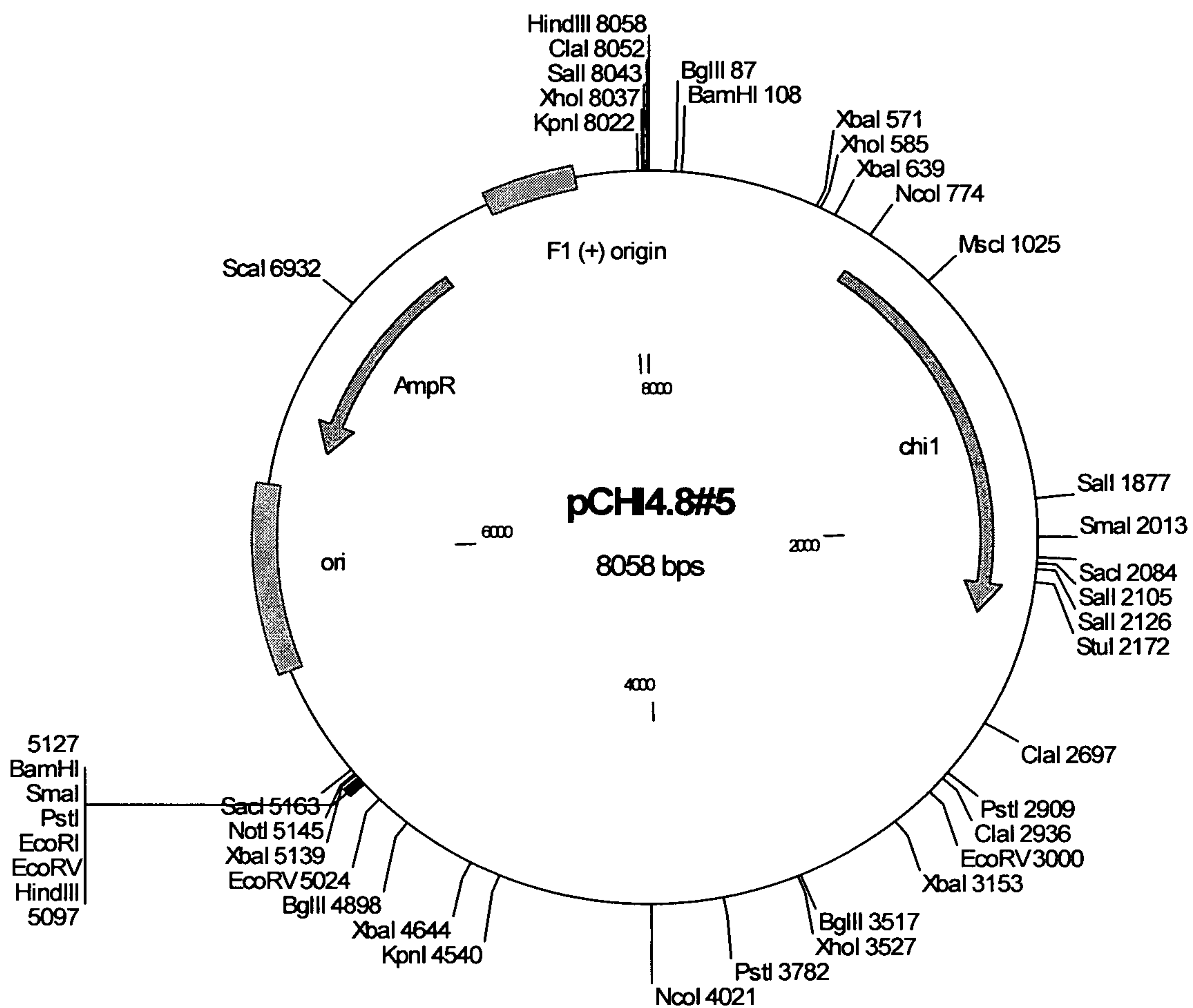


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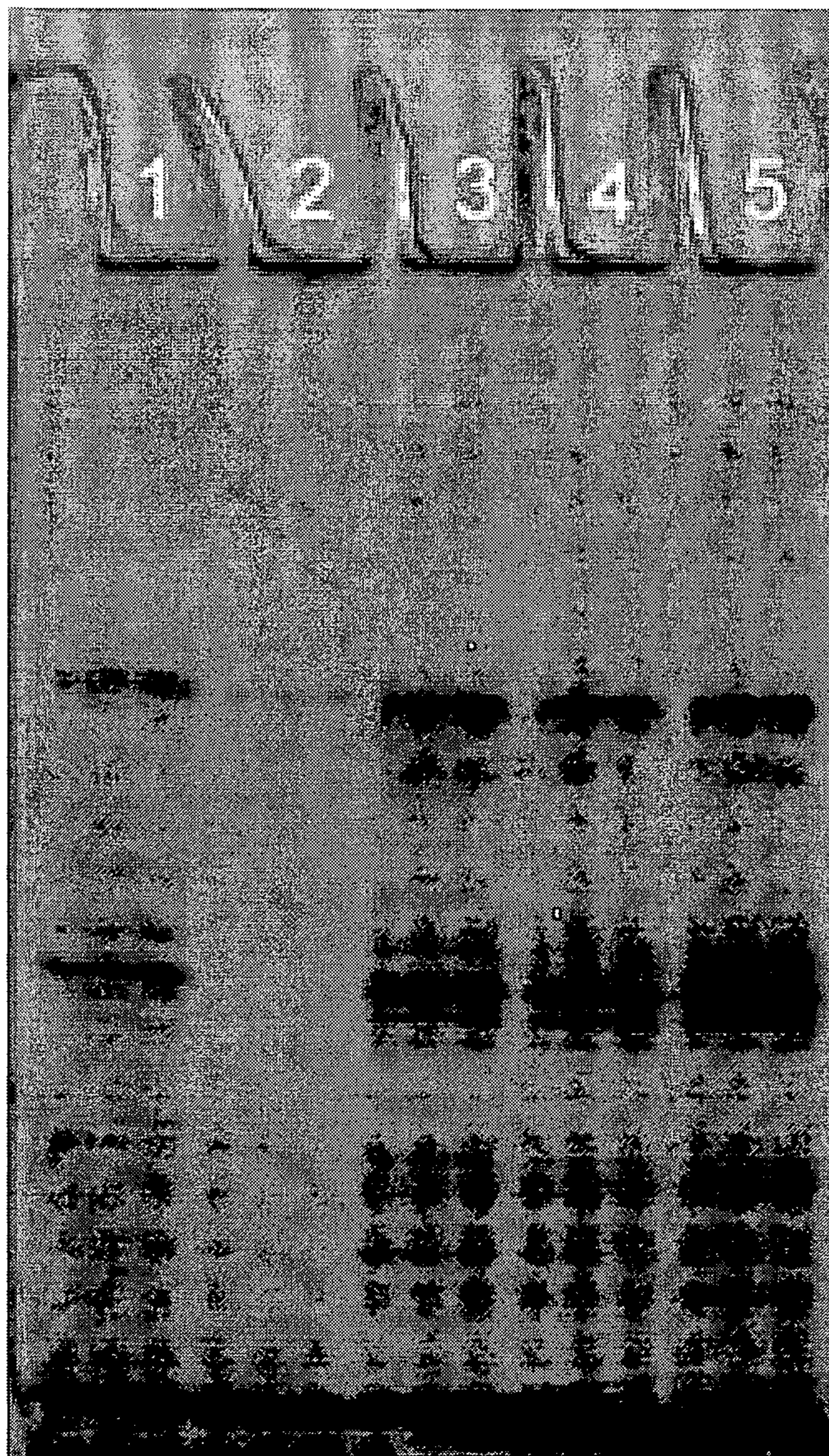


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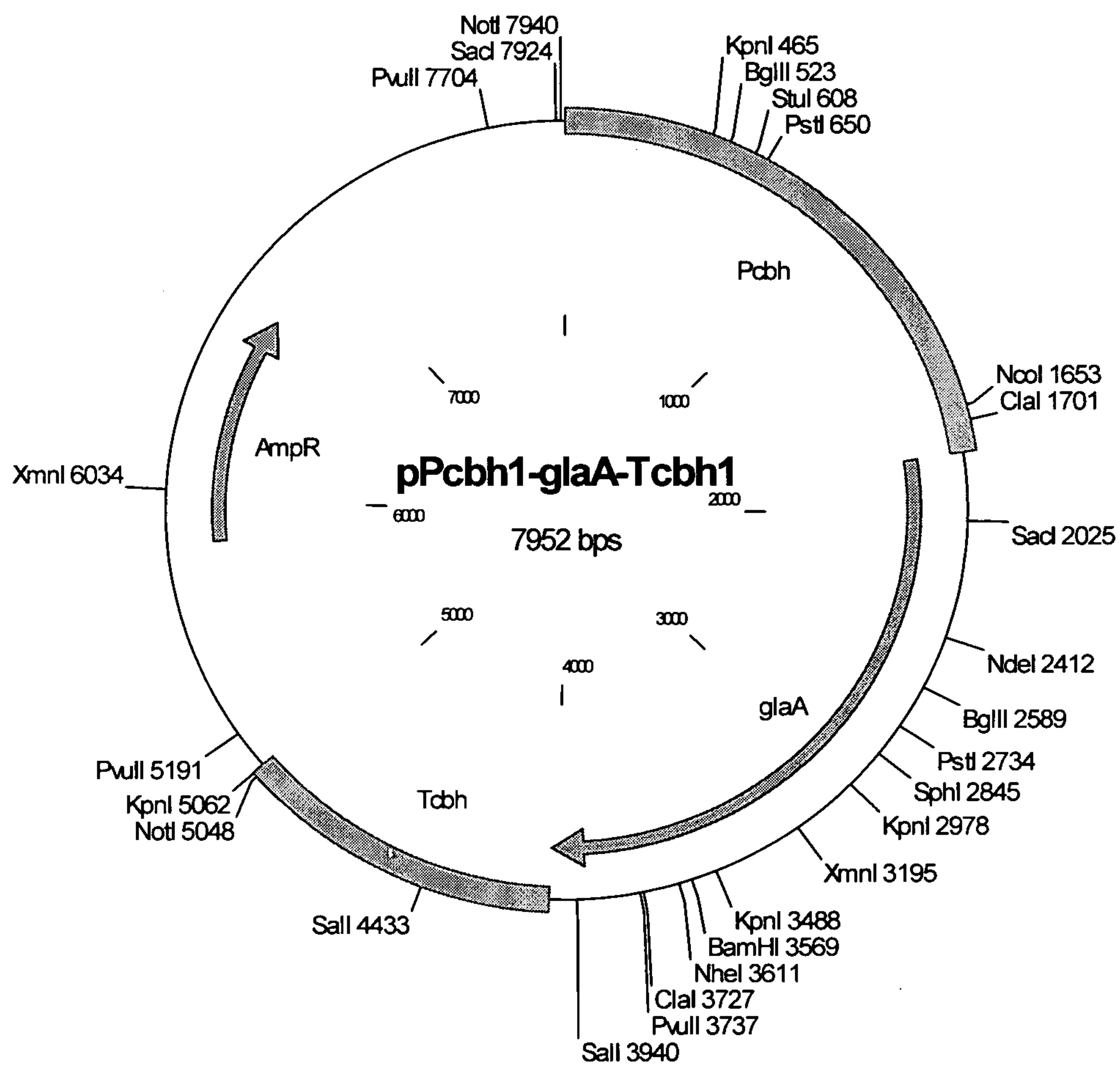


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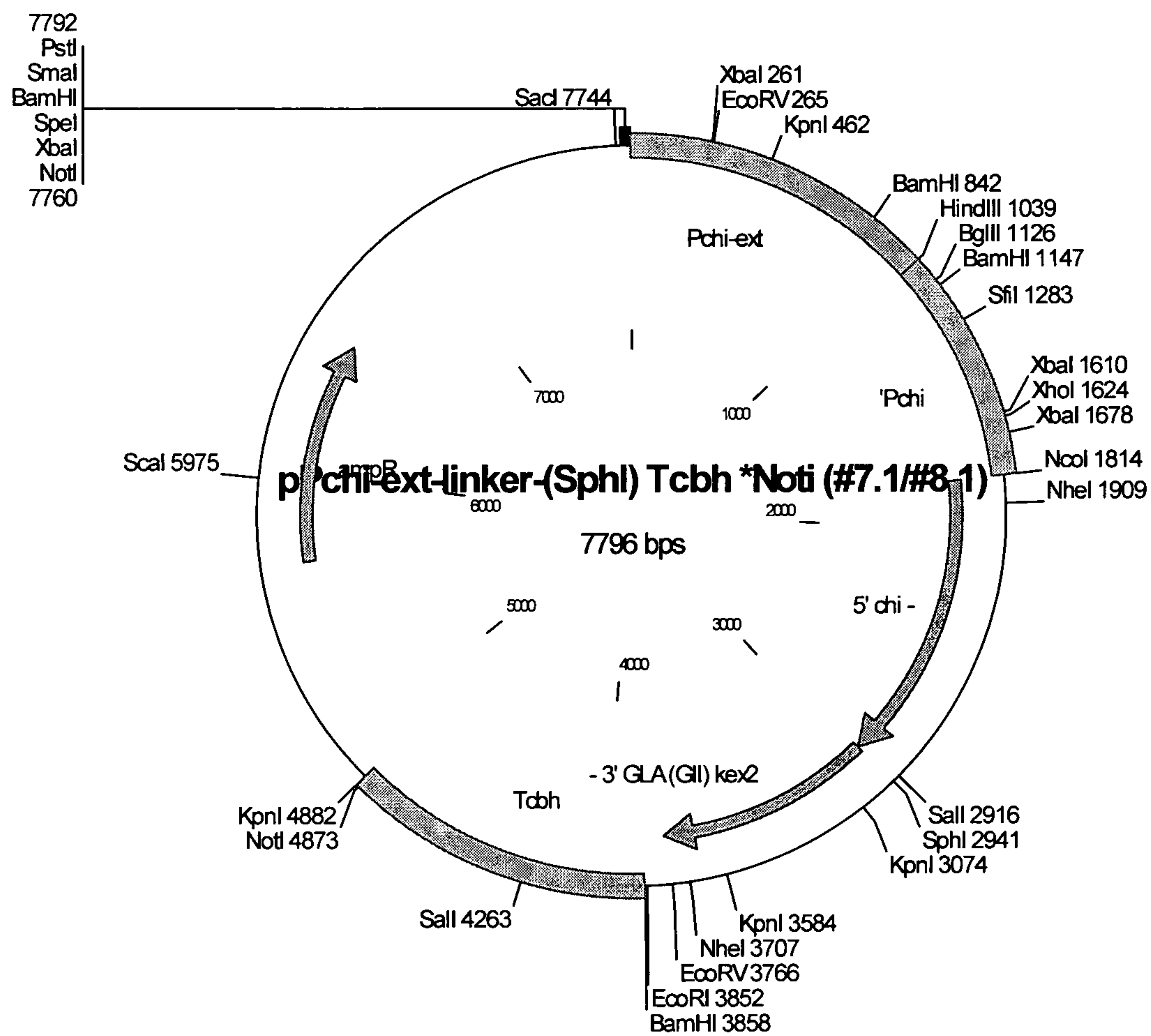


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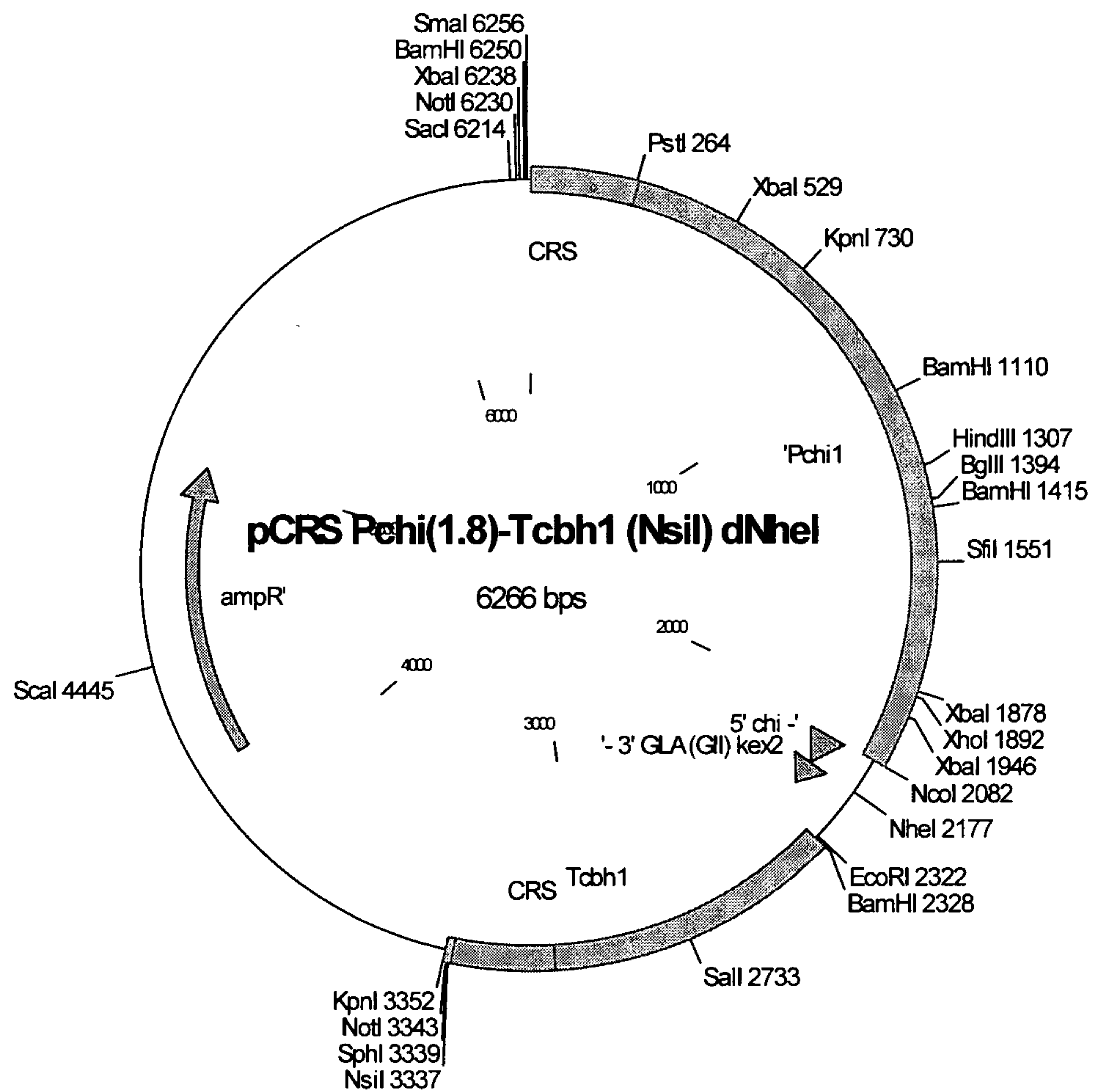


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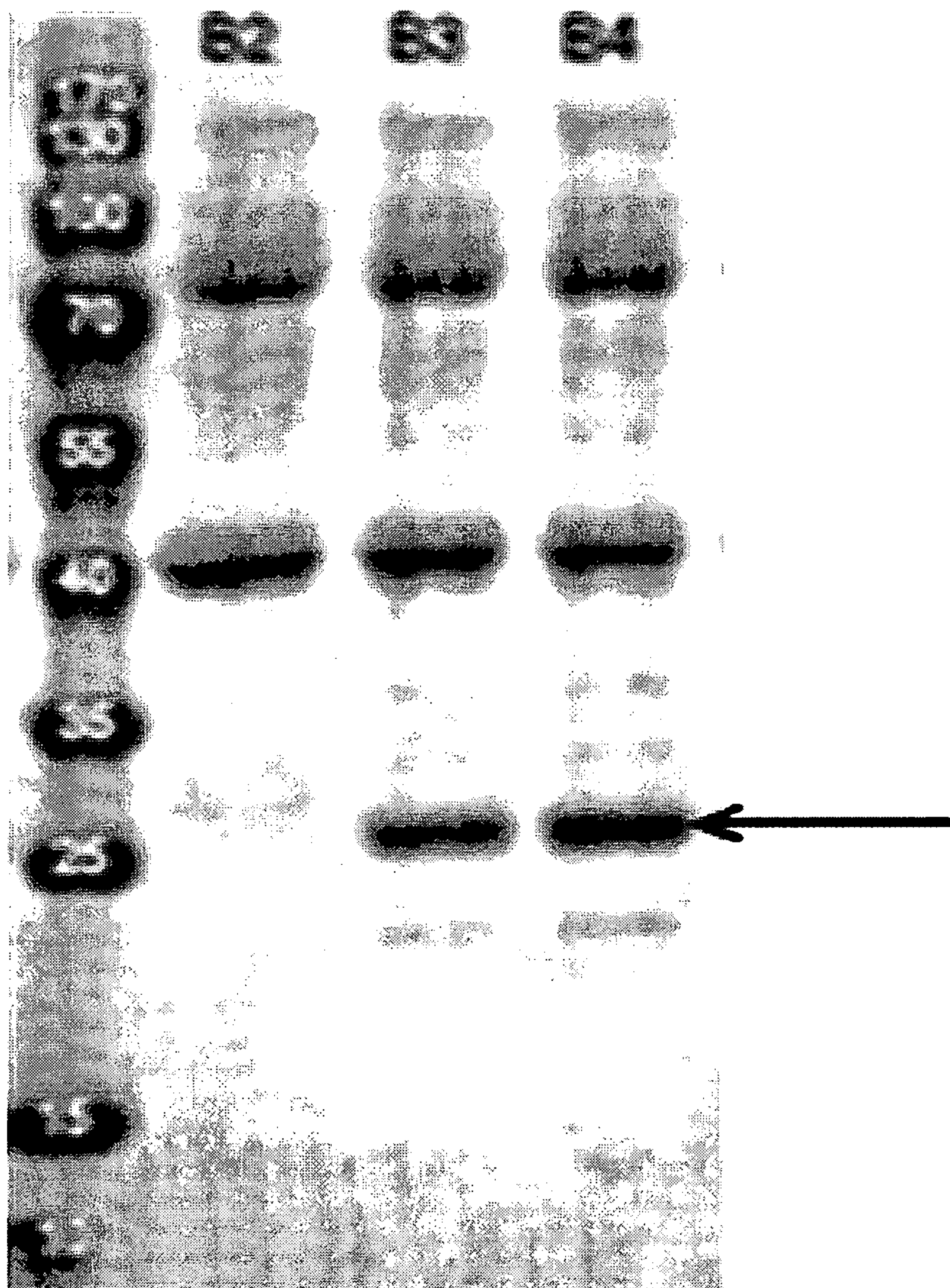


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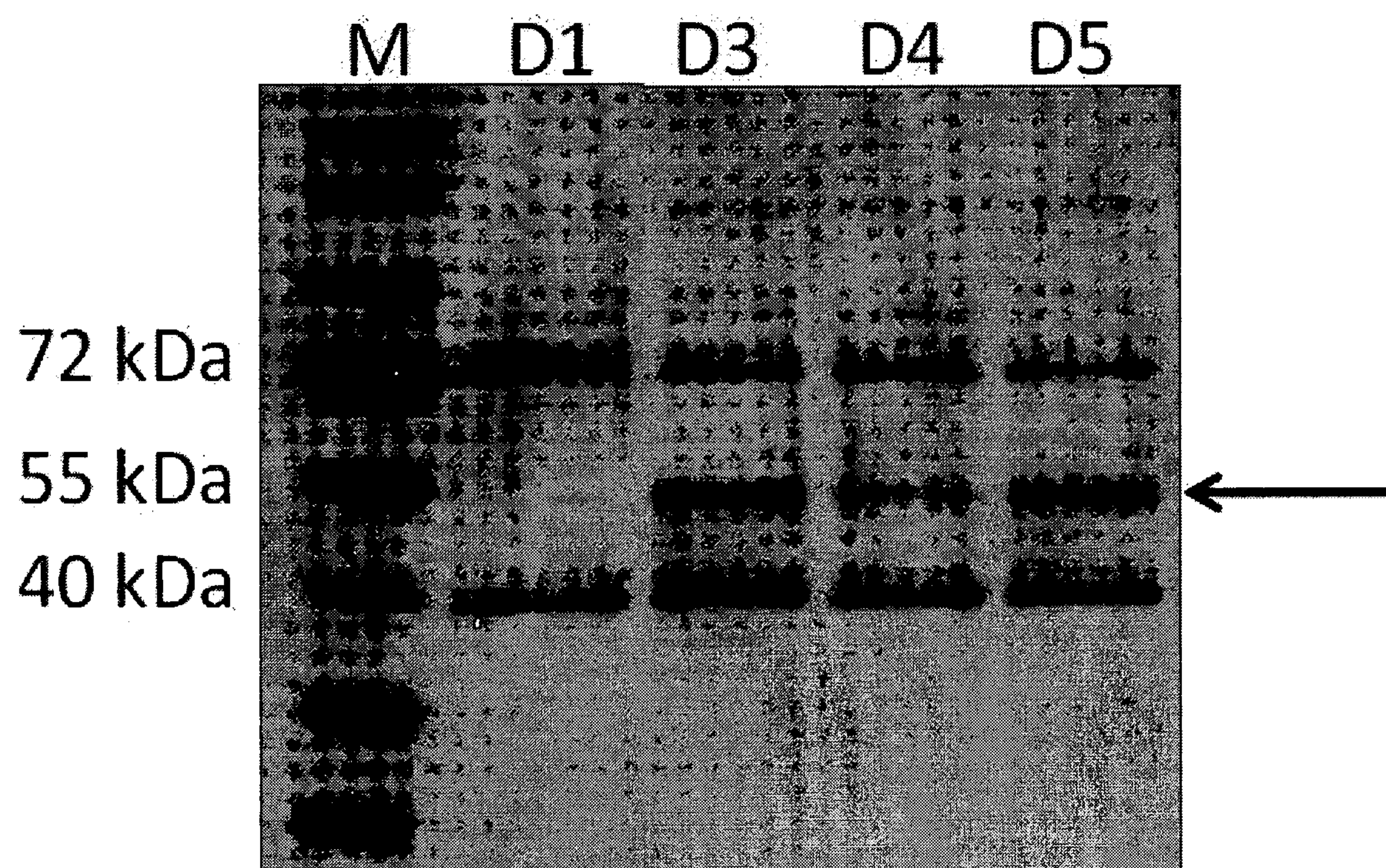


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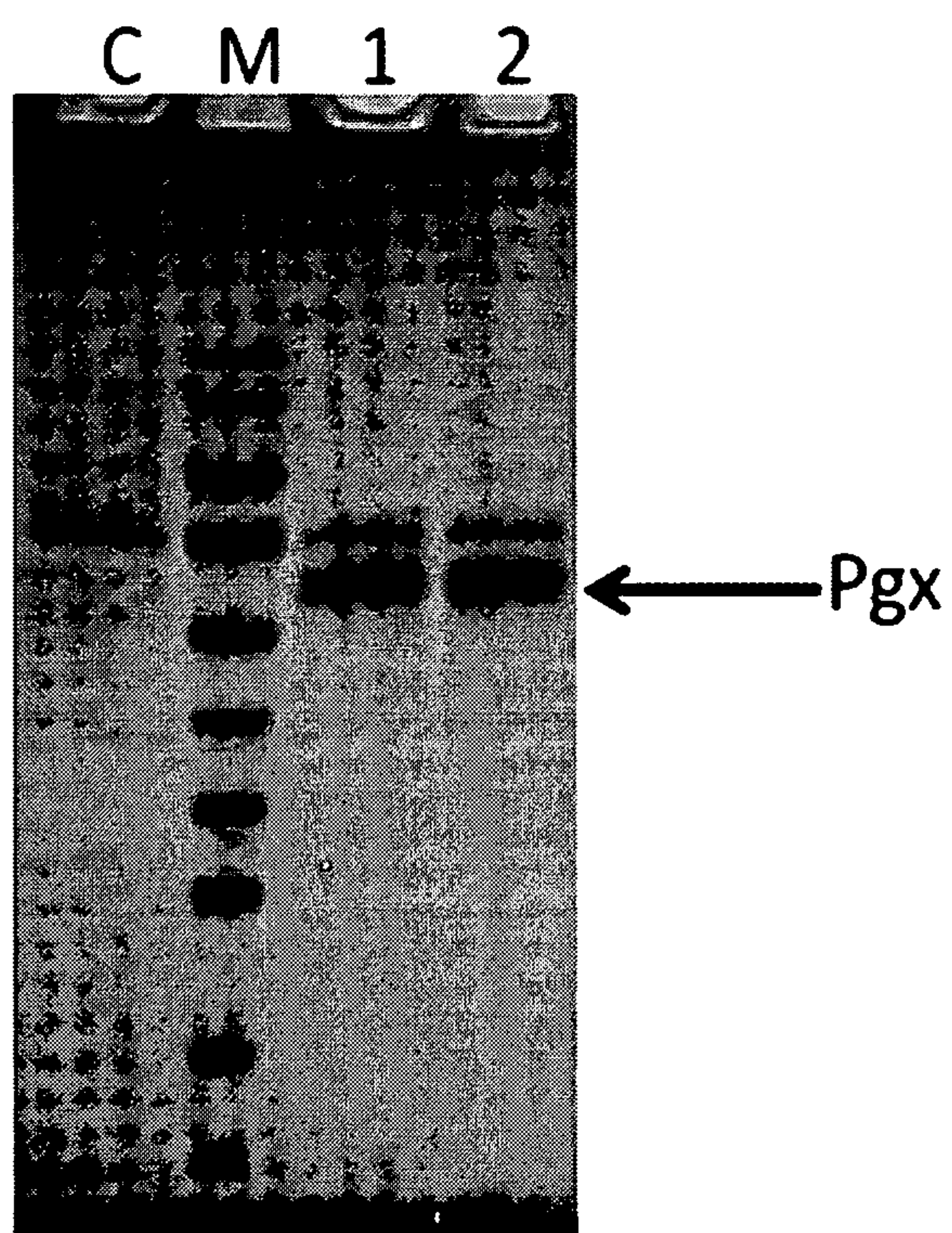


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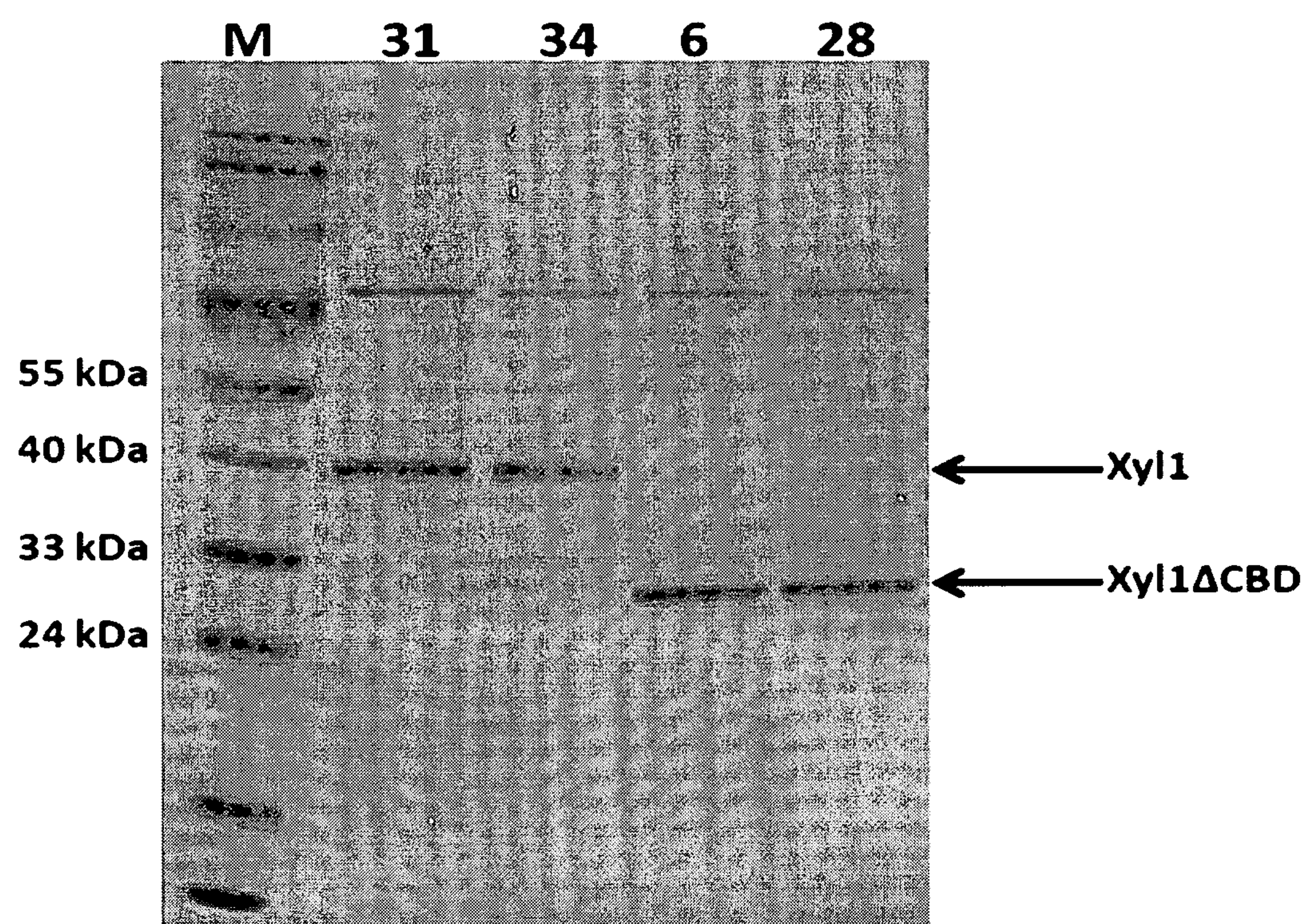


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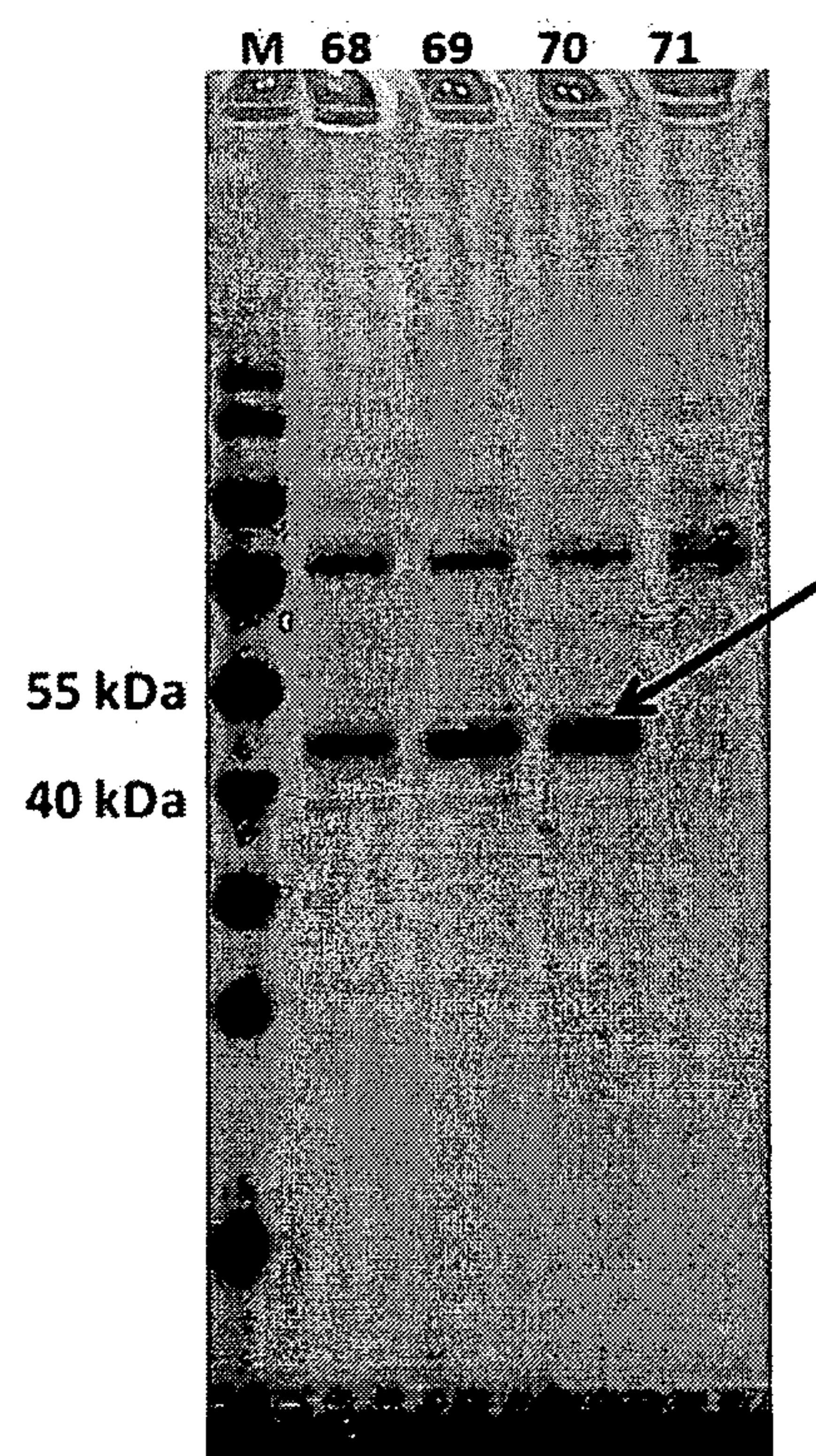


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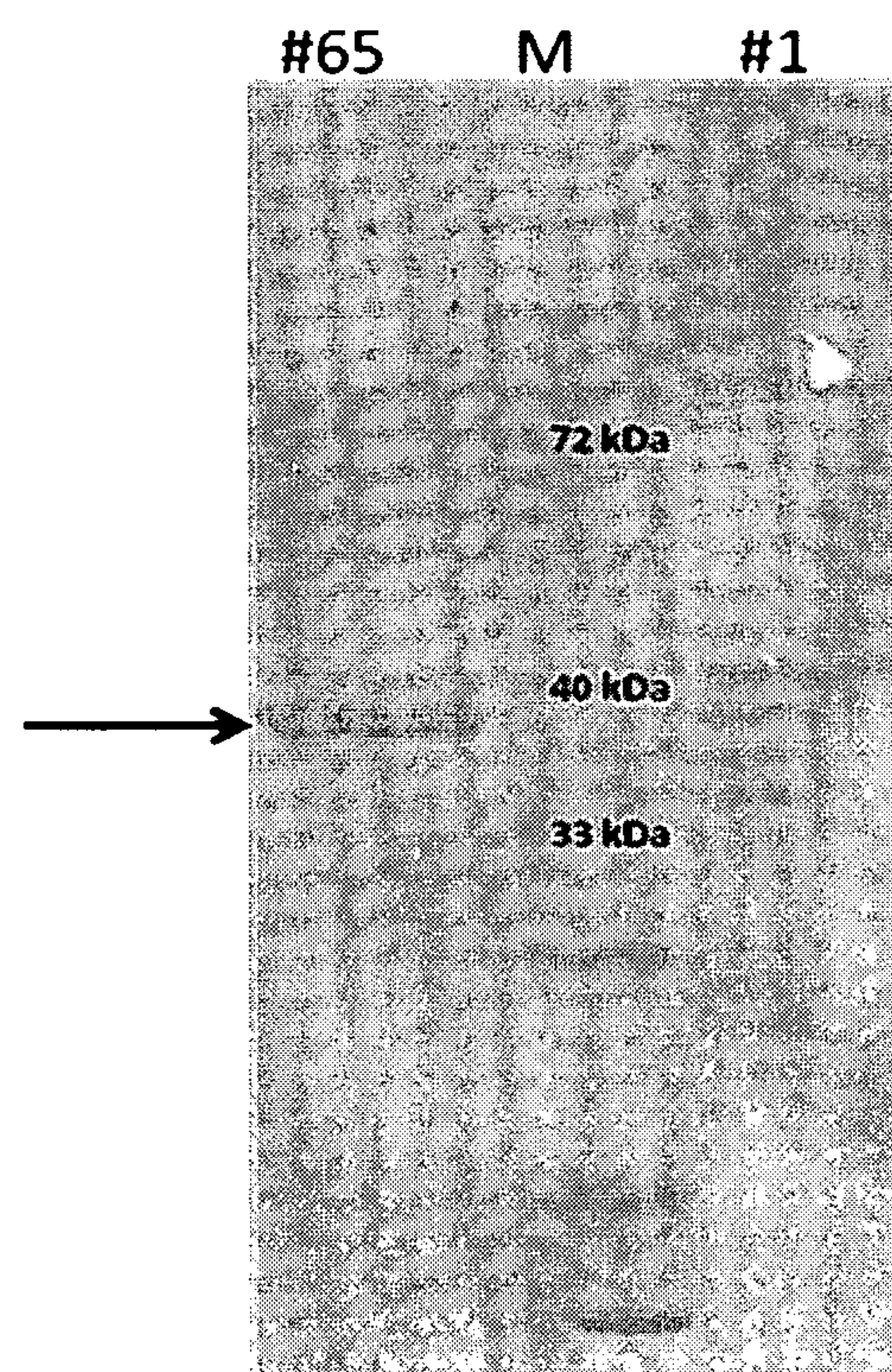


Fig. 14

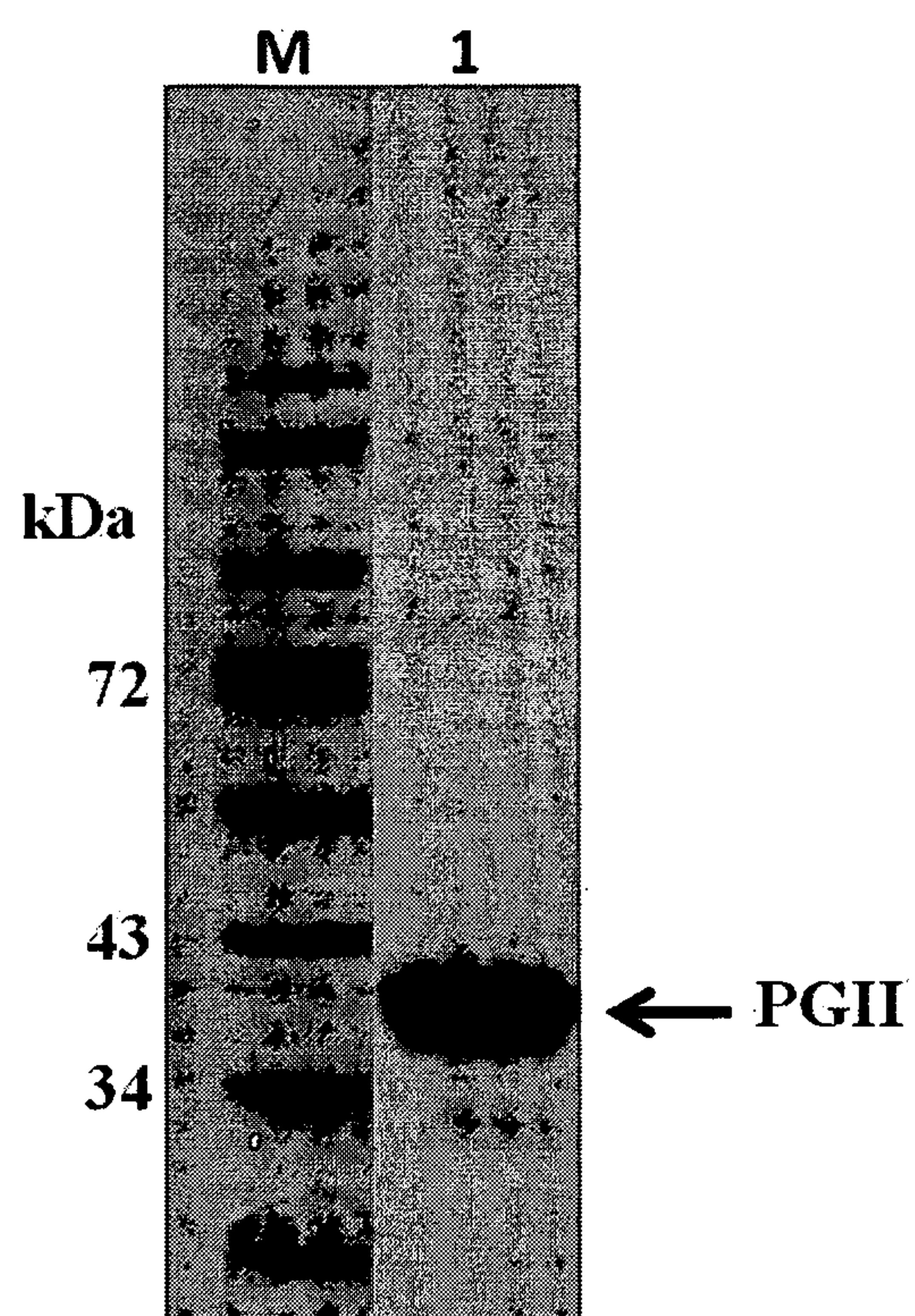


Fig.15