



(86) Date de dépôt PCT/PCT Filing Date: 2006/11/08

(87) Date publication PCT/PCT Publication Date: 2007/05/18

(45) Date de délivrance/Issue Date: 2014/02/25

(85) Entrée phase nationale/National Entry: 2008/05/06

(86) N° demande PCT/PCT Application No.: EP 2006/068234

(87) N° publication PCT/PCT Publication No.: 2007/054516

(30) Priorités/Priorities: 2005/11/08 (EP05110453.7);
2005/12/14 (US60/750,156)

(51) Cl.Int./Int.Cl. *C12N 5/10* (2006.01),
C07K 14/01 (2006.01), *C07K 14/075* (2006.01),
C12N 5/16 (2006.01)

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(54) Titre : FACTEUR PROTEIQUE AUGMENTANT LA PRODUCTIVITE, NOUVELLES LIGNEES CELLULAIRES ET
LEURS UTILISATIONS

(54) Title: PRODUCTIVITY AUGMENTING PROTEIN FACTOR, NOVEL CELL LINES AND USES THEREOF

(57) **Abrégé/Abstract:**

The present invention provides a method for preparing a non-adenoviral target virus or target proteins utilizing a potent expression cell line having stably integrated into its genome a gene encoding a specific heterologous regulator protein.



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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 May 2007 (18.05.2007)

PCT

(10) International Publication Number
WO 2007/054516 A1

(51) International Patent Classification:

C12N 5/10 (2006.01) *C07K 14/075* (2006.01)
C07K 14/01 (2006.01) *C12N 5/16* (2006.01)

(21) International Application Number:

PCT/EP2006/068234

(22) International Filing Date:

8 November 2006 (08.11.2006)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

05110453.7 8 November 2005 (08.11.2005) EP
60/750,156 14 December 2005 (14.12.2005) US

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, 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, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, 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, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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WO 2007/054516 A1

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Productivity Augmenting Protein Factor, Novel Cell Lines and Uses Thereof

The present invention provides a method for preparing a non-adenoviral target virus or target proteins utilizing a potent expression cell line having stably
5 integrated into its genome a gene encoding a specific heterologous regulator protein.

Background of the Invention

10 Biopharmaceutical products from eukaryotic cells are an integral part of modern medicine. However, increased productivity and increased safety are important parameters that still require substantial optimization. The most crucial bottleneck in the efforts towards optimization is the cellular substrate itself. A safe transgene that can be introduced into cell lines already released for use in biopharmaceutical
15 processes to increase product yields from the such manipulated cells is extremely valuable.

Adenoviruses are unenveloped (naked) double-stranded DNA viruses that infect a broad spectrum of animals. Among the best characterized members is the human adenovirus serotype 5 (Ad5). This virus is a frequent cause of common cold
20 symptoms and infection often occurs in childhood.

Adenoviruses are amenable to genetic manipulation and replication incompetent adenoviruses, including Ad5 (GenBank accession number for sequence: AC_000008), are used as vectors in gene therapy and for therapeutic vaccination. To obtain replication incompetent viruses large regions of the genomic DNA are
25 substituted with non-viral sequences. The missing viral functions are provided in trans by host cell lines that have been stably transfected with genes that are deleted in the vector. One of the earlier systems that were developed consist of adenoviral vectors deleted in the regulatory E1 region produced on cell lines providing the corresponding E1 proteins.

30 The most common cell line for this purpose is the 293 cell line. This line was generated in 1977 by transfection of fragmented adenoviral genomic DNA into primary human cells (Graham et al., J. Gen. Virol. 36, 59-74 (1977)), well before it

was recognized that adenoviruses may serve as gene therapy vectors. The resulting cell line was subsequently shown to contain nucleotides 1 to 4344 of the genomic DNA (Louis et al., Virology 233, 423-429 (1997)) which includes the E1 region; this characterization demonstrated that the E1 region can be used to immortalize and transform primary cells.

Adjacent to the E1 region follows the gene for pIX ("protein 9"; nucleotides 3609 to 4031 on the genomic DNA). The promoter for pIX is embedded within the E1B component of the E1 region. A mechanism called promoter occlusion allows expression of pIX at a delayed-early time in the viral infection cycle at the onset of viral DNA replication with increasing copy number of viral DNA (Fessler and Young, J. Virol. 72, 4049-4056 (1998)). Although the gene is present within the 4344 nucleotides integrated into 293 cells, pIX expression cannot be detected even with sensitive radioactive labeling methods (Spector et al., J. Virol. 36, 860-871 (1980)).

As described above, replication deficient adenoviral vectors have been generated by deletion of the E1 region from the viral genome. E1 products are essential for viral replication and the function of the E1 region therefore had to be provided in trans via stable E1 transgenes in the adenovirus packaging cell (such as the 293 cell line). Because of the proximity to the E1 region the pIX gene caused frequent recombination between deleted adenoviral vectors and E1 in the host genome. This recombination event generated replication competent adenovirus (RCA), a serious contamination in vector preparations. To suppress this recombination event pIX was deleted from the adenovirus genome. In the course of these experiments it was recognized that pIX stabilizes the adenoviral capsid against thermal and steric stress by increasing the interaction of the main building blocks, the hexons (Colby and Shenk, J. Virol. 39, 977-980 (1981); Ghosh-Choudhury et al., EMBO J. 6, 1733-1739 (1987)). Morphogenesis in the absence of pIX yields temperature-sensitive viruses that cannot deliver genomic DNA molecules larger than 105% of the wild type 35938 base pairs. To still allow a normal packaging capacity and thermal stability the pIX protein was stably introduced into cell lines intended as packaging cells for adenovirus vectors (Krougliak and Graham, Hum. Gene Ther. 6, 1575-1586 (1995); WO 99/57296 and Imler et al., Gene Therapy 3, 75-84 (1996)).

Also with recognition that pIX decorates the surface of virions pIX-fusion proteins have been generated with the purpose to expand the host range of adenovirus

vectors or to follow morphogenesis and the intracellular migration of virus particles (reviewed by Parks, Mol. Ther. 11, 19-25 (2005)).

Although clearly a structural protein, pIX also is implicated as regulatory protein for adenovirus replication. PIX is assumed to function as transactivator of transcription to augment E1A expression, a function possibly even exerted as a virokin by incoming PIX protein from the capsid at the infection step (reviewed by Parks, Mol. Ther. 11, 19-25 (2005)). PIX, together with early protein E4 Orf3, also is described to interact with sub-nuclear inclusions called PML bodies (Puvion-Dutilleul et al., Exp. Cell. Res. 218, 9-16 (1995); Leppard and Everett, J. Gen. Virol. 80, 997-1008 (1999)). These are dynamic aggregates of 250 nm to 500 nm in size and proposed to be involved in regulation of cell differentiation (Wang et al., Science 279, 1547-1551 (1998)), control of apoptosis (Quignon et al., Nature Gen. 20, 259-265 (1998)), and response to viral infection (Möller and Schmitz, Arch Immunol Ther Exp (Warsz) 51, 295-300 (2003)).

Because of its pleotropic effects we introduced the PIX protein into cell lines to examine whether PIX augments cell proliferation or production properties for biopharmaceutical products that are not related to adenovirus or adenoviral vectors. There is a general need for factors that modulate these properties in established cell lines.

For example, attenuated (weakened) viruses are promising vaccine candidates: upon inoculation they mimick a natural infection but allow more time for establishment of the desired protective immune response by the vaccinee. With increasing numbers of immunocompromised patients (for example, due to HIV infection) highly attenuated strains are desirable. Whereas attenuated strains still proceed towards (usually benign) infection highly attenuated strains are blocked at a cellular level even in absence of a functional immune system. A frequently used method to establish and maintain attenuation is to passage viruses on different host tissue. For example, measles and mumps viruses intended for human vaccination are passaged in primary cells, either in embryonated chicken eggs or cultures derived thereof. A new generation of vaccines is based on highly attenuated pox viruses that also depend on primary chicken cells for production. A cell line that can substitute for primary chicken cells and at the same time that even less efficiently protects itself against viral infection due to secondary manipulations such as introduction of the PIX transgene therefore provides a highly

desirable substrate.

For other purposes a mammalian (rather than avian) cell line may be preferred. Such preferred cell lines already exist and have passed the examination by health authorities with respect to safety and risks posed by derived biopharmaceutical products. Here too, a secondary manipulation (such as introduction of the PIX transgene) to increase the spectrum of available applications or production efficiencies without compromising safety features is highly desirable.

Summary of the Invention

When preparing cell lines that have been stably transfected with adenoviral gene pIX (or a chimaeric fusion analogue thereof), we unexpectedly observed that pIX exerts a phenotypical effect in avian and human cells. For avian cells this is especially surprising because avian cells cannot be infected by human adenoviruses and aviadenoviruses (such as CELO or fowl adenovirus type 8) do not encode a PIX homologue (Ojkic and Nagy, J. Gen. Virol. 81, 1833-1837 (2000)). Furthermore, we observed that stable presence of PIX increases susceptibility of the cell to induction by double stranded RNA analogue, probably via toll like receptor 3. Probably in this context, we also unexpectedly observed that the presence of PIX protein increases yields of highly attenuated poxvirus in avian host cells. As poxvirus infected cells suffered less from induction by double stranded RNA analogue we may have found an interaction between PIX protein and anti-interferon genes of poxvirus that has not been described previously. We also observed a surprising increase in the yield of proteinaceous product (not only virus) released by a stably transfected cell line.

Thus, the present invention provides

(1) a method for preparing a non-adenoviral target virus or one or more target proteins, which method comprises

(a) culturing an expression cell being obtainable by infecting or transfecting a host cell, having stably integrated into its genome a gene coding for a Adenovirus PIX or a functional variant thereof as a heterologous regulator protein and stably expressing said regulator protein or the functional variant thereof, with said target virus, or with a vector carrying nucleic acid sequences encoding said target virus, or with a vector carrying nucleic acid sequences encoding said one or more target

proteins (wherein the non-adenoviral target virus does not contain said regulator protein and the target protein(s) differs from said regulator protein or the functional variant thereof), and

(b) isolating the target virus or the target protein(s);

5 (2) a preferred embodiment of (1) above, wherein said PIX protein or the functional variant thereof cooperates with non-cognate viral factors, modulates subcellular distribution of factors other than said protein, modulates transcription and/or cell growth, and enhances the productivity of the cell in the production of a virus not containing said regulator protein and in the production of a protein differing from
10 said regulator protein or the functional variant thereof;

(3) a preferred embodiment of (1) and (2) above, where the adenovirus pIX is provided as a fusion protein with another protein that modulates or expands the activity or the subcellular distribution of the adenovirus pIX, for example is fused to retinoic acid receptor alpha, preferably is a fusion protein having the sequence
15 shown in SEQ ID NO:4, or is fused to a GFP and contains an NLS, preferably is a fusion protein having the sequence shown in SEQ ID NO:23;

(4) a preferred embodiment of (1) to (3) above, wherein the host and expression cell is a vertebrate cell including mammalian cells and avian cells, preferably the mammalian cell is a human brain derived cell, and the avian cell is a duck retina
20 derived cell or a duck somite derived cell;

(5) a preferred embodiment of (1) to (4) above, wherein the host and expression cell is derived from a human brain, preferably from human foetal brain, and most preferably is a NC5T11 cell, and said cell carries a nucleic acid sequence encoding Adenovirus PIX or a functional variant thereof as a heterologous regulator
25 proteinpreferably said heterologous regulator protein is encoded by a nucleic acid shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO: 22;

(6) a preferred embodiment of (5) above, wherein the host cell is a NC5T11#34 cell and the expression cell is derived from the NC5T11#34 cell, said NC5T11#34 cell being deposited with the DSMZ under Accession Number DSM ACC2744;

30 (7) a preferred embodiment of (1) to (4) above, wherein the host and expression cell is an avian cell, preferably is derived from duck retina or duck somite, and said cell carries a nucleic acid sequence encoding Adenovirus PIX or a functional variant thereof as a heterologous regulator protein, preferably said heterologous regulator

protein is encoded by a nucleic acid shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO: 22;

(8) a preferred embodiment of (7) above, wherein the host cell is a CR.PIX (17a11b) cell and the expression cell is derived from the CR.PIX (17a11b) cell, said
5 CR.PIX (17a11b) cell being deposited with the DSMZ under accession number DSM ACC2749;

(9) an expression cell for producing a target virus or one or more target proteins as defined in (1) to (8) above, preferably the expression is as defined in (5) to (8) above;

10 (10) a method for preparing the expression cell as defined in (9) above, which comprises infecting or transfecting a host cell line as defined in (1) to (8) above with a virus, or with a vector carrying nucleic acid sequences encoding said virus or with a vector carrying nucleic acid sequences encoding said one or more target proteins;

15 (11) a host cell as defined in (5) to (8) above;

(12) a method for preparing the host cell of (11) above, which comprises transfecting an appropriate starting cell with a vector carrying said regulator protein or the functional variant thereof;

20 (13) the use of the expression cell as defined in (7) above for preparing a target virus or one or more target proteins;

(14) a fusion protein comprising at least one first domain comprising a regulator protein and at least one second domain comprising a protein or peptide acting as a transcription modulator and/or as a signal for subcellular targeting as defined in (2) or (3) above; and

25 (15) a nucleotide sequence encoding the fusion protein as defined in (14) above.

Short Description of the Figures

Figure 1: History of the cell line NC5T11. A neurospheres of primary cells grown under serumfree conditions. B monolayer of primary cells grown in DMEM/F12 with
30 5% FCS. C primary focus two weeks after transfection with p79. the arrows indicate the border between primary and immortal cells. D homogenous adherent culture of

immortalised cells in DMEM/F12 with 5% FCS. E suspension culture of NC5T11 in shaker tubes in EXcell VPRO.

Figure 2: Immunofluorescence detecting E1A and E1B in NC5T11. After fixation with methanol cells were treated with rat antibodies directed against E1A and E1B 55k respectively followed by texas red conjugated anti rat antibodies. As shown in fig. 2 all cells in the sample show nuclear staining typical for E1A and cytoplasmic staining for E1B. After fixation with methanol cells were treated with rat antibodies directed against E1A and E1B 55k respectively followed by texas red conjugated anti rat antibodies. As shown here, all cells in the sample show nuclear staining typical for E1A and cytoplasmic staining for E1B.

Figure 3: Specific productivity of selected aat clones. Cell clones of NC5T11 transfected with C55 and selected with puromycin were seeded at 7×10^4 cells in 12 well plates, cell number and aat titer was determined on day 1, 2 and 3 after seeding and maximal cell specific productivity per day was calculated.

Figure 4: Expression analysis of NC5T11puro#8 in shaker batch culture. Cells were seeded in 12 ml of EXCELL VPRO at 6×10^4 cells/ml in 50 ml polypropylene shaker tubes (TPP, Switzerland) with and subjected to rotation with a radius of 1 cm and a speed of 160 rpm. Samples of 200 μ l were taken on days 4, 7, 9, 11, 15, 18 and 21. Cell density and viability was determined after staining with trypan blue using a haemocytometer.

Figure 5: Detection of the pIX-RARA cDNA by PCR in a clone pool. A clone pool carrying the pIXRARA gene was generated from NC5T11puro#8 by transfection with F67 and hygromycin selection. RNA was extracted using an RNA extraction kit (Machery Nagel, Germany) and cDNA was synthesized using AMV Reverse Transcriptase (Invitrogen). cDNAs for the pIX fragment alone or the whole pIXRARA gene were amplified with primer 1 and 1 and 164 respectively.

Figure 6: Detection of the pIXs sequences as part of the fusion protein in hygromycin resistant subclones of NC5T11puro#8 and NC5T11(#34, 35, 36, 37, 38) by PCR. DNA was isolated from cell clones grown in 6 wells by lysis in SDS followed by phenol extraction and precipitation. DNA was amplified with Primers 1 and 2 for 28 cycles

Figure 7: Stability of the pIXRARA and pIX genes in stably transfected NC5T11 was determined relative to E1B: E1A+E1B and pIXRARA were introduced in separate

transfections and E1 genes and have been maintained in the absence of selection for > 2 years and are therefore considered stably integrated. Genomic DNA was isolated from selected cell clones at 2 time points: immediately after hyg selection stop (early); after 2 month in the absence of selection pressure (late). PIX and E1B DNA levels were determined by Real Time PCR.

Figure 8: Growth retardation induced by RA treatment in pIXRARA recombinant clones of NC5T11 and NC5T11puro#8. Cells were seeded at 2×10^5 in 6 well plates in the presence or absence of 6 $\mu\text{g/ml}$ retinoic acid. Photos showing growth retardation in RA treated clones #12 and #34 but not in NC5T11 were taken 4 days after seeding using phase contrast imaging at a 4x magnification.

Figure 9: PIXRARA prevents inhibition of virus replication by interferon. NC5T11 and NC5T11#34 cells were infected with EMCV an interferon sensitiv virus at an MOI of 0.004 in cells treated with interferon beta. Cell lysates were subjected to titration by plaque formation on A549 cells.

Figure 10: Expression analysis of the cell line NC5T11puro#8 expressing alpha-1-antitrypsin from vector C55 and subclones #10, #12, #14 thereof, carrying the vector F67 (pEFpIX-RARA) in addition to C55. Cells were seeded in EXCELL VPRO (JRH Biosciences) at 6×10^4 cells/ml in 50 ml polypropylene shaker tubes (TPP, Switzerland) with a total volume of 12 ml and subjected to rotation with a radius of 1 cm and a speed of 160 rpm.

Figure 11: Expression of PIX in duck retina CR cells. Left Panel: PCR reaction against PIX gene in genomic DNA of pIX cells. Gel loading from left to right: 1 kb-marker (Invitrogen); PCR on genomic DNA from CRpIX; non-template control; positive control with plasmid used in transfection of CRpIX. Right panel: Western blot for detection of pIX protein in CRpIX cells. Lane 1: 293 cells; lane 2: pIX protein in CRpIX cells.

Figure 12: Stable maintenance of PIX transgene in duck retina cells. Top-left panel: MCX and DXS are independent aliquots of a CRpIX clone cultivated for > 3 months in parallel and without selection. After three months, TaqMan PCR was used to count the copy numbers of E1B and PIX transgene in MCX and DXS. As E1B transgene is maintained independent of PIX the ratio of the two genes is an indication of maintenance of PIX transgene. The ratio did not change between MCX and DXS. Furthermore, the ratio of PIX mRNA to E1B mRNA also did not change

and PIX mRNA was in excess of E1B mRNA indicating expression of PIX. Right panel: MCX and parental (PIX-negative) CR.HS cells were cultivated with or without hygromycin selective pressure for 2 weeks (indicated by black bar on x-axis). At various time points genomic DNA was isolated (indicated by "A", "B", and "C") and subjected to TaqMan quantification (bottom-left). Whereas parental cells were killed by hygromycin MCX cells survived. The ratio of transgenes remained constant, again indicating stable maintenance of PIX transgene also in presence of selective pressure. Doubling time for parental cells is approx. 32 h but only 41 hours for MCX cells.

Figure 13: PIX effect on MVA replication in CS cells. CS and CSpIX cells were infected with MVA and assayed for MVA replication 48 h and 72 h post infection. The yield of MVA was significantly higher in PIX-positive CS cells. Also, as evident in the phase contrast microimage, cytopathic effect appeared delayed for CSpIX cells at 48 h. However, both cultures are susceptible to virus to similar degrees as complete lysis is evident at 72 h post infection.

Figure 14: PIX effect on MVA replication in CR cells. CR and CRpIX cells in suspension were infected with various multiplicities and at various seed cell densities. MVA yield was assayed 48 h post infection and is shown on the y-axis. The size of the bubbles displays seed cell densities. The filled bubbles show values for CRpIX cells, white bubbles show values for parental cells. In all configurations, PIX confers a clear increase in amplification rates of MVA.

Figure 15: Subcellular distribution of PIX-GFP in duck cells. GFP-tagged PIX appears in few cytoplasmic bright spots and diffuse cytoplasmatic staining largely excluding the nucleus. Other clones exhibit stronger, diffuse cytoplasmatic staining and more intense accumulation just outside the nucleus.

Figure 16: Subcellular distribution of PIX-GFP variants in CHO cells. To investigate induced changes of PIX distribution within cells fusion variants were generated, including insertion of a nuclear localization site (NLS) and fusion to retinoic receptor alpha, a cellular protein that already contains an NLS. Shown are CHO cells transiently transfected with expression plasmids for PIX-GFP variants.

Figure 17: Effect of interferon induction in presence of PIX. CSpIX and CRpIX cells were treated with poly I:poly C, a known inductor of type I interferon response and compared to parental cells. CSpIX cells reacted with greater sensitivity to poly

I:poly C than CS cells. CRpIX and CR cells reacted comparably and to a lower extent than CS cells.

Figure 18: Effect of MVA infection and interferon induction in presence of PIX-GFP. CSp9GFP and CRp9GFP cells were treated with poly I:poly C and infected with MVA at an M.O.I. of 0.1 as indicated. Again, CS-derived cells reacted more strongly to interferon induction. Surprisingly, bright PIX bodies appear to decrease in number upon induction or infection in both cell lines whereas overall signal intensity of PIX-GFP increases. Furthermore, the effect of poly I:poly C induction is ameliorated upon parallel infection with MVA as indicated by a greater number of CS cells still adherent 22 h after treatment.

Detailed Description of the Invention

The method of embodiment (1) of the invention utilizes an expression cell having integrated into its genome a gene coding for a heterologous regulator protein, namely pIX or a functional variant thereof. Said regulator protein possesses the following properties:

1. It modulates transcription and, especially if linked to appropriate modulators or regulators, also influences cell growth.
2. It enhances productivity of the cell line with regard to the production of a virus not containing said regulator protein (i.e. the regulator protein is not a substitute for a protein deleted from the virus) and/or with regard to the production of a protein differing from said regulator protein or the functional variant thereof.

According to the invention the heterologous regulator protein is the adenovirus serotype 5 protein pIX (e.g. that having the aa sequence shown in SEQ ID NO:2), mutants (including addition, substitution, and/or deletion mutants) thereof, variants thereof (e.g. variants obtained from an adenovirus of a different serotype), and the like.

“Functional variant of the heterologous regulator protein” according to the invention include homologues from adenovirus other than serotyp 5, all types of mutation (addition, substitution and/or deletion) of particular amino acid residue(s) of the respective wild-type regulator protein, modification by fusion that further active protein or peptide sequences and the like. Particularly preferred are fusion proteins,

namly fusion proteins comprising at least one first domain comprising a regulator protein as defined hereinbefore and at least one second domain comprising a protein or peptide acting as a transcription modulator. In a preferred embodiment of the invention said transcription modulator is a transcription factor including the retinoic acid receptor alpha, which may be present in complete or incomplete (i.e. truncated) form. The modulator may also be a transit peptide, which includes NLF sequences, e.g. that shown in SEQ ID NO:21. According to the invention the first and second domain(s) are either directly or via a peptide linker covalently attached to each other. Suitable peptide linkers include flexible and hydrophilic structures such as poly gly-ser.

The terms "cell" and "cell line" as used in the following detailed description refer to expression cells/expression cell lines and host cells/host cell lines.

According to the invention it is preferred that the heterologous regulator protein or the functional variant thereof is expressed in the cell in an amount of at least 1 pg/ μ g cellular protein, preferably at an amount of at least 10 pg/ μ g cellular protein so that its expression can be determined by Western-blotting.

In the cell of the invention the heterologous regulator protein or the functional variant thereof is preferably under control of a stabile homologous or heterologous promoter. Suitable promoters are constitutive cellular promoter or variants thereof such as the human translocation elongation factor 2 promoter. A particular variant of the human translocation elongation factor 2 promoter utilized within the invention has the sequence shown in SEQ ID NO:12. The sequence shown represents a "short" version of the promoter which is located on human chromosome 19:3,935,325-3,936,638 (human genome assembly May 2004) and provides for a stable medium level expression. For a stronger expression a "longer" version of the promoter located on chr19:3,935,349-3,938,957 (human genome assembly May 2004) may be used.

According to the invention the cell is a vertebrate cell including mammalian cells, avian cells and the like. Suitable mammalian cells are human cells, and rodent cells including mouse, rat, hamster, etc. Particularly preferred mammalian cells according to the invention are cells derived from human brain, particularly from human foetal brain, a mouse NSO or Sp2/0 cell, a BHK or CHO cell. Suitable avian cells are duck, chicken quail and goose cells. Particularly preferred avian cells according to the invention are cells derived from a duck retina-derived or somite-

derived cell.

On the other hand, the cell of the invention may be derived from a primary cell or from a previously immortalized cell. Moreover, the cell may carry further immortalizing (viral) genes including an E1 protein of an adenovirus, such as the E1 protein of mastadenovirus group C type 5, and the like. Particularly preferred according to the present invention is that the cell further carries the adenovirus E1A and/or E1B gene shown in SEQ ID NO:5.

The cell utilized in the method of embodiment (1) of the invention may further carry functional sequences, e.g. sequences required for its application as expression cell such as selection marker sequences, splice donor/acceptor sites and/or recombinase recognition sequences allowing integration of a target nucleic acid sequence to be expressed in the cell, etc.

The "infecting", "transfecting" and "transforming" effected within the method of embodiments (1), (10) and (12) of the invention may be performed according to standard procedures known to the skilled person. Said methods may further encompass suitable selection, isolation and expansion steps, if required.

In the method of embodiment (1) the target virus includes from wild-type, mutated or deleted virus, cold adapted or attenuated virus, vaccine strains, viral vectors carrying heterologous gene(s), viral vectors such as lentivirus, poxvirus, adeno associated virus (aav), herpesvirus, flavivirus and the like.

Further, in the method of embodiment (1) the one or more target proteins include antibodies, recombinant proteins such as erythropoetin, alpha-1-antitrypsin, blood clotting factors VIII and IX and interferons, viral antigens such as influenza HA and NA, and M, HBV-S, herpes G protein and rabies G protein, peptid hormones and the like. Although the method allows the production of cells capable of contemporary expression of more than one target proteins, it is particularly preferred that it encodes only one target protein.

The culturing and isolation of the method of embodiment (1) of the invention may be performed according to standard procedures readily available to the skilled person. Said method may further include standard purification steps as well as subsequent modification steps of the target virus or target protein(s).

Concerning the preferred embodiments of the expression and host cell lines of embodiments (9) and (11), respectively, as well as the production methods for said

cell lines of embodiments (10) and (12), it is referred to the detailed discussion provided hereinbefore in connection with embodiment (1).

According to embodiments (14) and (15) the invention provides for a fusion protein comprising at least one first domain comprising a regulator protein and at least one
5 second domain comprising a protein or peptide acting as a transcription modulator as defined hereinbefore, and for a nucleotide sequence encoding said fusion protein, respectively. The invention also relates to the use of said fusion protein (e.g. in diagnostic and pharmaceutical applications, etc.) and of the nucleotide sequence encoding it of embodiments (14) and (15), respectively, e.g. in all types
10 of vector constructs, cell lines, tissue culture, transgenic animals, etc.

The cell line NC5T11#34 was deposited with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany on November 4, 2005 under accession number DSM ACC2744. The cell line CR.PIX (17a11b) was deposited with the DSMZ-Deutsche
15 Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany on November 24, 2005 under accession number DSM ACC2749.

The invention will be explained in more detail by means of the following examples, which are, however, not to be construed to limit the invention.

Examples

Example 1: Development of the cell line NC5T11

The cell line was developed from a mixture of cells from foetal brain by immortalisation with adenovirus 5 E1A and B genes by nonviral transfection.

25 A tissue sample was taken from the periventricular zone of the brain of a foetus after induced abortion. It was dissected with scissors and homogenised by aspiration with a tissue culture pipette in neuronal culture medium based on DEMEM/F12 containing 20 ng/ml hFGF (Invitrogen, Carlsbad, CA 92008, USA), 20 ng/ml hEGF (Invitrogen), 1x N2 supplement (Invitrogen) and 8 µg/ml heparin
30 (Sigma Aldrich, St. Louis, USA). Cells were sedimented at 200g for 3 min. Viability of cells was assessed using trypan blue and propidium iodide using a flow cytometer (BD Biosciences, Jose, CA 95131, USA). The viability was 75%. 0.5×10^6 cells were seeded into T25 flasks. Cells were incubated at 37 °C and 5% CO₂. The

cells formed neurospheres by day 5 of the culture. Neurospheres are shown in Figure 1A. On day 8 cells were transferred to DMEM/F12 supplemented with 5% FCS to allow for attachment and to stimulate proliferation. Cells formed a homogenous monolayer as shown in Figure 1B and were passaged 1:5 once per
5 week. This primary neural cell culture was named NC5.

After 2 weeks in serum containing medium cells were transfected with vector p79 in subconfluent 6-well plates using Effectene™ (Qiagen, 40724 Hilden, Germany) as a transfection reagent following the manufacturers instructions. The plasmid p79 contains the following elements: The pBluescript™ (Stratagen, USA) serves as
10 plasmid backbone, in which the ampicillin resistance marker was replaced by the kanamycin resistance gene driven by a bacterial promoter which allows for growth and selection in E.coli. The vector harbours a fragment from wild type adenovirus type 5 (SEQ ID NO:5) containing the open reading frames for E1A (splice variants 13S and 12S with or without the CR3 domain respectively) and E1B 55k and 19k as
15 well as sequences upstream of E1A. The E1A gene is preceded by the phosphoglycerate kinase promoter (mouse). The adenovirus sequences are followed by the polyadenylation signal from the Herpes simplex thymidine kinase gene, serving as a replacement for the E1B polyadenylation signal. Elements were obtained from the respective organisms or from donor plasmids by PCR and cloned
20 using conventional recombinant DNA techniques and verified by sequencing. Two days after transfection cells were trypsinized and transferred to a 10 cm dish. Two weeks later foci of small cells with a high nucleus/Cytoplasm ratio and clearly distinguishable borders were formed (Figure 1C) in transfected but not in mock treated cells. From the 11th transfection eight independent foci were isolated with
25 and trypsin using cloning cylinders (Corning, USA), seeded to wells of a 24 well plate and expanded via 12 well plate, 6 well plate to T25 flasks. All isolated foci contained two types of cells: small cells with sharp borders and extended fibroblast like cells.

Three weeks posttransfection 15 additional clones became visible in transfection
30 T11 some of which may originate from remaining cells of already isolated primary clones.

Clones T11a.1 and T11a.6 showed the fastest growth and were cryopreserved in DMEM/F12, 10% DMSO, 25% serum 8 weeks post transfection. At this time all clones still contained a fraction of cells with enlarged cytoplasm resembling

primary phenotype. However, with a split ratio of 1:5 for T11a.1 and T11a.6 they were overgrown and eliminated approximately 3 month after transfection. Immunofluorescence experiments were carried out to associate the changed morphology with expression of E1 A and E1B. After fixation with methanol cells
5 were treated with rat antibodies directed against E1A and E1B 55k respectively followed by texas red conjugated anti rat antibodies. As shown in Fig. 2 all cells in the sample show nuclear staining typical for E1A and cytoplasmic staining for E1B.

Three month after transfection clone T11a.1 and T11a.6 were transferred to serum-free medium ProPER™ (Cambrex, Belgium) by seeding 1.6×10^6 cells after trypsin
10 treatment. Cells were harvested by centrifugation and medium was exchanged by centrifugation once a week. The cell population survived but even six month after transfection cells still showed a low viability and grew with a high doubling time of 60-80 h.

When the cells were transferred back to DMEM/F12 with 5% FCS after 3 month in ProPER™ medium, a homogenous and highly viable culture with a doubling time of 40 h has formed for both clones. T11a.1 was chosen as the more most robust cell clone for further experiments and named NC5T11 (Fig. 1D)

Example 2: Establishment of NC5T11puro clone#8

Protein production in a cell line depends on efficient transfection and selection
20 methods. The suitability of commonly used selection markers G418, puromycin, hygromycin, blasticidin, MTX, histidinol for NC5T11 was unknown. In a first evaluation step different commercially available transfection reagents Lipofectamine™ (Invitrogene), Fugene™ (Roche, Germany), Polyfect™ (Qiagen) and
Effectene were tested following the manufacturers protocols. The plasmid pEGFP-
25 N1 (Clontech, USA) expressing gfp from the human CMV promoter was used to determine transient transfection efficacy. The highest efficiency was obtained with Effectene (Qiagen) reaching 20-50% depending on cell density when applied to adherent cells growing in DMEM/F12 10% FCS.

To test selection marker genes, expression vectors were constructed containing the
30 human alpha-1-antitrypsin (aat) gene under control of the human CMV promoter followed by the bovine growth hormone PolyA signal and the respective selection marker controlled by a weak promoter. More specifically, to test for puromycin selection, vector C55 was used which contains the puromycin resistance gene

driven by the human PGK promoter followed by the SV40 early polyadenylation signal.

After transfection cells were seeded in DMEM/F12 5% FCS containing 0.75 µg/ml puromycin and selected for 3 weeks with weekly medium exchange. A clone pool
5 was generated and expression was verified by immunofluorescence using a goat polyclonal anti- alpha-1-antitrypsin antibody (Innogenetics, USA) followed by a biotin labelled secondary rabbit anti-goat antibody and a streptavidin-Texas Red conjugate.

To isolate individual cell clones 10000 cells of the clone pool were seeded in a 15
10 cm dish. After 4 weeks clones were isolated using clone cylinders (Corning) and clones #1, 2, 7, 8, 9, 10 were analysed for aat expression. More specifically, cells were seeded at 7×10^4 cells in 12 well plates, cell number and aat expression was determined. Calculated cell specific productivity rates are given in Figure 3. Clone NC5T11puro#8 shows a specific productivity above 100pg/cell•day and was chosen
15 for further development.

Example 3: Shift of NC5T11 and NC5T11puro#8 to production medium and expression analysis in shaker batch culture

The growth rate of both NC5T11 and NC5T11puro#8 in ProPER™ medium (Cambrex) was approximately two times reduced compared to serum containing medium.
20 Therefore, Xcell™ VPRO (JRH Biosciences) was tested as an alternative. Adaptation was done by direct seeding of 4.5×10^6 cells in 5 ml EXCELL VPRO in a T25 flask. After 2 weeks cell growth reinitiated and a weekly split ratio of 1:3 was established. As a next step both clones were adapted to growth in the presence of shear stress. Cells were seeded in 12 ml of EXCELL VPRO at 6×10^4 cells/ml in 50 ml
25 polypropylene shaker tubes (TPP, Switzerland) with and subjected to rotation with a radius of 1 cm and a speed of 160 rpm. After repeated passages batch assays were performed to determine maximal cell densities and product accumulation during exponential and stationary growth phases. The following observations were made: Clone NC511puro showed a high viability which was maintained above 70%
30 until day 18. Maximal cell density did not exceed 1×10^6 . Product accumulation continued during stationary phase (Fig. 5). It was concluded that specific media development or further cell improvement will be required to achieve a high cell density comparable to those obtained with CHO cells. This will likely result in a substantial increased product titers.

Example 4: Construction of the fusion protein pIX-RARA and integration vectors for both pIX-RARA and pIX

Sequences for adenovirus PIX and retinoic acid receptor alpha (RARA) were obtained by PCR from adenovirus type 5 and human genomic DNA respectively
5 using primers

1. AACCAGCGCTACCATGAGCACCAACTCGT (SEQ ID NO:6)
2. AATGGTGGCAACCGCATTGGGAGGGGAGG (SEQ ID NO:7) for PIX and
- 3.CCAATGCGGTTGCCACCATTGAGACCCAGA(SEQ ID NO:8)
4. AAGGAGCGCTGGCGAGGGCTGTGTCCAT (SEQ ID NO:9) for RARA.

10 Both Fragments were amplified separately. An overlap between primers 2 and 3 allowed to connect both fragments via PCR using primers 3 and 4. The fusion gene was cloned into vector pEFpromhyg resulting in pEFpIXRARA. This vector contains the human elongation factor 2 promoter located on chr19:3,935,325-3,936,638 (human genome assembly May 2004; SEQ ID NO:12). It contains upstream
15 elements and the first intron of the EF2gene. The natural start codon of EF2, located 5' to the first intron, was removed by mutagenesis. In the vector, pEFpromhyg, the EF2 sequence is followed by the unique restriction site (AfeI) used for insertion of pIX-RARA. An internal ribosome binding site following the AF1 site allows expression of the hygromycin resistance gene as a second open reading
20 frame in a joined RNA with pIX-RARA. This configuration supports pIX-RARA expression in all hygromycin resistant cells.

The gene for wt pIX was amplified using primers AACCAGCGCTACCATGAGCACCA-
ACTCGT (SEQ ID NO:10) and ACCGAGCGCTTGTTTAAACCGCATTGG (SEQ ID
NO:11) and integrated into the Afe site of pEFpromhyg instead of pIX-RARA to yield
25 pEFpIX.

Example 5: Modification of the cell lines NC5T11 and NC5T11puro#8 with the PIX gene and the PIX-retinoic acid receptor fusion protein

Cell clones NC5T11 and NC5T11puro#8 were prepared for transfection in 6-well plates by seeding 1.3×10^6 cells/well in DMEM/F12, 5% FCS. Plasmid F67
30 (pEFpIXRARA) and plasmid F76 (pEFpIX) were linearised with restriction enzyme Asp700 (Roche) and transfected after ethanol precipitation using Effectene (Qiagen): DNA was mixed with 16 µl Enhancer and 200 µl EC Buffer. After 2 min at

room temperature, 18 µl Effectene were added and liposome formation allowed for 10 min at room temperature. The transfection mix was added to 1 ml culture volume on cells seeded to 80% confluency in 6-well plates on the previous day. High transfection efficiency was confirmed with a parallel transfection of pEGFP-N1 (Clontech, Palo Alto, CA 94303-4230, USA). One day after transfection medium was exchanged to DMEM/F12 with 10% FCS and 75 or 100 µg/ml (NC5T11) and 50 µg/ml µg/ml (NC5T11puro#8) hygromycin was added. Selection medium was replaced two times per week. After 18 days 15-20 clear clones became visible in each of the wells. Clone pools were treated with trypsin and selection was continued for two more weeks. Clone pools were tested for expression of PIXRARA by RTPCR. A strong signal was detected with primers 1 and 2, a weaker signal with primers 1 and 4 in most of the clone pools. To isolate individual cell clones 10000 and 1000 cells of the clone pool were seeded into 15 cm dishes. After 4 weeks clones were isolated using clone cylinders (Corning) For clones resulting from transfection of F76 a different strategy was applied. Clone pools were treated with trypsin, transferred to a 50 ml shaker tube in EXCELL VPRO containing 75 µg/ml hygromycin and after 2 weeks of suspension culture 2000 viable cells were mixed with 12 ml clone matrix (Genetix,UK), seeded into a six-well plate (Greiner)(2 ml/well) and subjected to automated colony picking (clonepixFL, Genetics, UK). Clones originating from NC5T11puro#8 were given numbers #10-21. Clones originating from NC5T11 were given # 34-45. Clones transfected with pEF2PIX were named NC5T11PIXA- NC5T11PIXE. Clones were tested for the presence of the respective vector by PCR. An initial test using primers 1 and 2 is provided in Fig. 6. All clones showing a positive signal were shifted to growth in ExCell VPRO. Readaptation was done by direct seeding of 4.5×10^6 cells in 5 ml EXCELL VPRO in a T25 flask. After 6 weeks in stationary culture cells were transferred to 50 ml polypropylene shaker tubes (TPP, Switzerland) at a seeding density of 6×10^4 cells/ml in EXCELL VPRO with a total volume of 12 ml and subjected to rotation with a radius of 1 cm and a speed of 160 rpm. Clones #10, #12, #14 (NC5T11puro#8pIXRARA) and #34, #43 (NC5T11pIXRARA) and NC5T11PIXB, NC5T11PIXC were chosen for their highest viability under shear stress. Clones #34, 43 and NC5T11PIXC were recloned and analysed for stability of the integrated DNA. Stability was assessed determining the gene ratio between PIX and E1B because E1A+E1B and pIXRARA were introduced in separate transfections and E1 genes were introduced and have been maintained in the absence of selection for

> 2 years and are therefore considered stably integrated. Genomic DNA was isolated from selected cell clones at 2 time points: immediately after hyg selection stop (early); after 2 month in the absence of selection pressure (late). PIX and E1B DNA levels were determined by Real Time PCR™ (ABI 7000, CYBR Green) at 2 DNA concentrations. Examples of this evaluation are shown in Fig. 7. The vector pEF2PIXRARA is stably maintained in subclones of #34 and #43 with approximately 0.4-0.6 copies of PIXRARA per E1B. Clone NC5T11PIXC originally contains 4 copies of pIX per E1B. This number is reduced twofold during the course of the experiment.

Example 6: Retinoic acid dependent growth of NC5T11pIX-RARA

Retinoic acid (RA) is an inducer of cell differentiation. Differentiation is typically associated with reduced cell growth. The response to RA depends on expression of retinoic receptor alpha, a transcription factor activated by RA. RA has a profound effect on PML (promyelocytic leukaemia) reverting the transformed phenotype by growth arrest.

As shown in Fig. 8 RA treatment at a concentration of 6 µg/ml did not have any effect on growth of either NC5T11 or the alpha-1-antitrypsin expressing clone NC5T11puro#8. However, growth of clones #14 (NC5T11puro#8pIXRARA) and #34 (NC5T11pIXRARA) were arrested by treatment with 6 µg/ml for 5 days in adherent culture in DMEM/F12 with 5% FCS.

Example 7: PIXRARA prevents inhibition of virus replication by interferon

NC5 T11 and NC5T11#34 cells were seeded into 6 well plates at 8.0×10^5 cells/well in DMEM/F12 5% FCS. After 12 h interferon beta (rebiif 44 of Serono) was added to wells at a concentration of 1 and 8 IU/ml. After 30 min interferon treated cells and control cells of both NC5 T11 and NC5T11#34 were infected with Encephalomyocarditis virus (EMCV) at a multiplicity of infection (MOI) of 0.004. After 24 h, cultures were harvested and frozen at -80 °C to disrupt the cells. This suspension was thawed and cleared by centrifugation with 800 x g for 10 min. Lysates were subjected to virus titration by plaque assay on A549 cells: In brief, A549 cells were seeded to 24 well plates and grown to reach confluency, incubated for 30 minutes with cleared lysates diluted 10 to 2×10^8 fold, overlaid with 0.2 % low gelling agarose type VII in RPMI 10% FCS and incubated at 37 °C for 24 h. The agarose layer was aspirated, cells were fixed with 2% glutaraldehyde in PBS for 20 min at 20 °C, washed with water and incubated for 30 min at room temperature with 1% Kristallviolett solution in 50% ethanol to count virus plaques. Results

shown in Fig. 9 demonstrate that EMCV replication is rather ineffective in NC5T11 cells and that it is highly sensitive to interferon treatment before infection. At 8 IU/ml no viable virus can be recovered from the culture. In contrast virus replication in NC5T11#34 is only slightly affected by interferon treatment. Moreover, even in the absence of exogenous IFN, higher titers are observed for NC5T11#34. The phenomena shown model typical vaccine processes because many vaccine strains induce an IFN response in producer cells with an intact IFN pathway, infection at a low multiplicity of infection (MOI) preferred in production processes can cause IFN secretion resulting in a block of virus replication of cells that did not get infected initially. Therefore, expression of PIX may allow for high titer virus production starting with low MOI.

Example 8: Stimulation of protein production by pIX-RARA

NC5T11puro#8pIXRARA carrying vector F67 as well as alpha-1-antitrypsin were compared to the starter clone NC5T11puro#8 in there capacity to produce alpha-1 antitrypsin in a shaker batch assay. Cells were seeded in EXCELL VPRO (JRH Biosciences) at 6×10^4 cells/ml in 50 ml polypropylene shaker tubes (TPP, Switzerland) with a total volume of 12 ml and subjected to rotation with a radius of 1cm and a speed of 160 rpm. Culture was continued until day 22. Besides suspension cells, a ring of cell clusters adhering to the tube wall was formed. The cells in these clusters preserved a viability above 70% throughout the process. Because dissociation of these clusters by Trypsin™ or Acutase™ (PAA) was not successful, growth kinetics and maximal cell densities were not determined. Samples were taken at day 9, 13, and 22 and titers were determined using the alpha-1-antitrypsin ELISA . Results are shown in Fig. 10.

Example 9: Generation of duck retina cells expressing pIX

The cell line CR, derived from primary duck retina cells and immortalized according to defined risk guidelines is described elsewhere (patent application WO05042728, plasmid 60E-transfected retina cells). The cells were cultivated in DMEM:F12 medium (Invitrogen, Carlsbad, CA 92008, USA) supplemented to 5% fetal calf serum (Biochrom AG, 12213 Berlin, Germany) at 39 °C and 7.5% CO₂. For passaging, the cells were briefly treated with TrypLE Express (Invitrogen). Plasmid 76F pEFPIX1 was linearized with Ssp I and Xmn I restriction enzymes (both from New England Biolabs, Beverly, MA 01915-5599, USA) and purified to 400 ng/μl by affinity chromatography (gel extraction kit from Qiagen, 40724 Hilden, Germany).

μl (2 μg) of the purified DNA was transfected into CR cells with the Effectene reagents (Qiagen): DNA was mixed with 16 μl Enhancer™ and 200 μl EC Buffer. After 2 min at room temperature, 18 μl Effectene were added and liposome formation allowed for 10 min at room temperature. The transfection mix was added to 1 ml culture volume on cells seeded to 80% confluency in 6-well plates on the previous day. High transfection efficiency was confirmed with a parallel transfection of pEGFP-N1 (Clontech, Palo Alto, CA 94303-4230, USA).

The pIX-transfected cultures were expanded into T75 flasks three days after transfection and selection was initiated with 25 μg/ml hygromycin B (Invitrogen). Medium was replaced once per week with hygromycin B raised to 50 μg/ml. After three weeks, a total of four large foci survived and were re-seeded into a T25 flask: cells were detached with TrypLE™ and collected with 100 x g for 10 min, resuspended in fresh medium and plated into a T25 flask.

Four weeks after transfection, cells from a healthy, sub-confluent culture in a T25 flask were detached with TrypLE into 5 ml of DMEM:F12, 5% FCS. 2 ml thereof were mixed with medium #63032-1000M (JRH Biosciences, KS 66215, USA) a medium free of animal-derived components intended for maintenance of suspension cultures. Hygromycin B was added to 50 μg/ml. Genomic DNA was isolated from 2 ml of the culture and subjected to PCR against the pIX transgene with primers V293 and V294. Negative control was provided by a parallel reaction without DNA, positive control by a parallel reaction with plasmid 76F. The expected PCR product in Fig. 11 (left panel) demonstrates stable insertion of the pIX transgene into the CR cells, now termed "CRpIX".

Expression of the PIX protein was confirmed by Western blotting: 6×10^5 cells were disrupted by boiling in (20 mM Tris pH 7.4, 300 mM NaCl, 1% Na-desoxycholat, 1% Triton® X-100, 0.1% SDS) and protein was separated by gel electrophoresis, then transferred to a nylon membrane. PIX was detected with pIX primary antibody (a generous gift from Dr. W. Seidel, Ernst-Moritz-Arndt-Universität Greifswald, Germany), then reacted with secondary antibody directed against the former antibody and labeled with alkaline phosphatase.

The signal of expected size in Fig. 11 (right panel) confirms expression of pIX in CRpIX cells. No signal was present in the negative control from 293 cells prepared in a parallel reaction.

The cells were cultivated in medium #63032-1000M for two additional passages, then transferred to medium #14561-1000M (also from JRH Biosciences) for two additional passages. All JRH media were supplemented to 1 x with Glutamax I (Invitrogen) and 100 µg/ml hygromycin B.

5 With the second passage in medium #14561-1000M a small fraction (approx. 2% of a 5 ml suspension culture) was resuspended thoroughly into DMEM:F12, FCS medium and plated into a 15 cm-diameter petri dish with. After 6 days, eleven foci were removed to individual cavities of a 12-well plate and maintained in DMEM:F12, FCS medium. For clone transfer, the medium was aspirated and cloning
10 disks (Sigma, MO, USA) soaked in TrypLE, briefly applied directly to the clones, then transferred to the cavities filled with medium. Individual clones were expanded for further experiments and determination of growth properties.

To analyze maintenance of PIX in the stably transfected cells genomic DNA was isolated from 1×10^6 cells with the QIAamp™ DNA Blood Kit (Qiagen) and number of
15 E1B and PIX molecules was determined in 25 ng and 50 ng of genomic DNA in an ABI 7000 TaqMan reaction with SYBR Green (ABI) detection chemistry. Primers used for quantification were gTggTTgCTTCATgCTAgTg (SEQ ID NO:13) and TCTTCAGCAggTgACAgTTg (SEQ ID NO:14) for E1B, ACCTACgAgACCGTgTCTg (SEQ ID NO:15) and gAgCCgTCAACTTgTCATC (SEQ ID NO:16) for PIX. As E1B was
20 introduced independently and must be maintained for the cells to survive this gene serves as an internal marker to standardize PIX copy number and gene expression strength.

Fig. 12 demonstrates stable maintenance of PIX transgene in CR suspension cells even in absence of selection pressure. It also demonstrates that PIX-positive cells
25 suffer from a decrease in growth rate. We consistently observed decreased proliferation for all of our cells (avian and human cells) upon stable transfection with a PIX-expression plasmid demonstrating an impact of PIX on the biochemistry of the host cell. Furthermore, we observed a tendency in PIX-positive, adherent CR cells to avoid growth into confluent layers (increased contact inhibition).

30 Example 10: Generation of duck somite cells expressing pIX

The cell line CS is derived from embryonal somites, also described in patent application WO05042728. Generation of PIX-positive CS cells was performed in parallel to the above described procedure for the CR line. Contrary to CRpIX, no

suspension cultures were established for CSpIX. Strict anchorage dependency is a feature of the CS cells. A cell that does not proliferate in suspension can be considered to be less tumorigenic as the potential to metastasize is severely impaired. PIX protein does not change this property in CS cells. Although pleiotropic this protein therefore appears not to impact on the transformation phenotype. This observation supports our assumption that PIX can safely be applied in biopharmaceutical processes.

Example 11: PIX effect on MVA replication in CS cells

CS cells stably transfected for PIX expression and parental CS cells as reference were seeded into a 6-well plate at 2×10^6 cells/cavity and infected with MVA at an m.o.i. of 0.1 on the following day. Pictures to document the differences in the advance of CPE were taken 48 h and 72 h post infection. Virus yield in the supernatant 48 h post infection and complete yield (supernatant together with lysed cell pellet) 72 h post infection was determined in a microfocus assay. The results are shown in Fig. 13: the presence of PIX in the CS cells appears to delay the onset of CPE at 48 h post infection when compared to the parental reference. 72 h post infection both cultures are lysed completely suggesting that the differences in the extent of CPE is not due to a mixture of MVA-refractory and MVA-susceptible cells in the CS.PIX population.

To our knowledge, no PIX-positive cell has ever been tested against viruses other than cognate adenovirus.

Example 12: PIX effect on MVA replication in CR cells

The positive effect of the PIX protein was also quantified on CR cells. Contrary to CS cells, the CR cells are adapted to suspension. Suspension cultures are preferred for many industrial applications. We therefore determined the PIX effect in such a system as opposed to adherent culture.

For CR cells the PIX-supportive effect is not as pronounced as in CS cells but we consistently observe higher titers for MVA. MVA yields were optimized in a series of experiments and found to be greatest 48 h post infection at medium cell densities. Fig. 14 shows a comparison of suspension clone CR.HS (selected out of several clones as the cell line with greatest MVA yields) and PIX(+) cell line CR.MCX. The abscissa indicates m.o.i. (0.01, 0.05 or 0.1, respectively), the ordinate indicates burst and the size of the bubbles indicates cell densities (8 , 2 , or 0.8×10^6 cells/ml,

respectively). Burst is the ration of output virus (or yield) to input virus (the inoculum dependent on cell number and m.o.i.) and thus a measurement of amplification efficiency. In all configurations, the PIX(+) cell lines exceeds the PIX(-) line in performance.

5 Example 13: Nuclear exclusion of PIX

A PIX-GFP fusion gene was generated to visualize distribution of the PIX protein in life cells and to overcome weak binding activity of the available antibody: Plasmid 76F pEFPIX1 was treated with Acc I und Dra I restriction enzymes and termini were blunted with Klenow polymerase (all from New England Biolabs, Beverly, MA
10 01915-5599, USA). Out of the complex banding pattern, the desired fragment of 447 bp containing the PIX coding sequence was isolated by agarose gel extraction (gel extraction kit from Qiagen, 40724 Hilden, Germany). The Dra I restriction enzyme recognizes the sequence "ttTAAa" to cut after the last thymidine residue, wherein the central "TAA" triplett is the stop codon of the PIX open reading frame.
15 The 447 bp fragment therefore is devoid of a stop codon. Fusion of this fragment to the gene for EGFP in the plasmid pEGFP-N1 opened with Sma I (also New England Biolabs) generates a continous fusion gene of PIX followed by EGFP. The resulting plasmid was named p9GFP, the expressed fusion protein will be named PIX-GFP in this text.

20 CS and CR cells were transfected with p9GFP and selected with 300 µg/mL geneticin (Invitrogen) for stable PIX-GFP expression. Two populations of different PIX-GFP expression were observed within 2 weeks: one population had usually 2 to 5 bright spots of PIX-GFP in the cytoplasm, the other population had a more uniform expression of PIX-GFP in the cytoplasm and more diffuse accumulation of
25 GFP signal in a region proximal to the nucleus. In both cases, the cell nuclei appeared as a dark region suggesting that no or very little chimeric protein enters the nucleus. Representative examples for both types of clones in CRpIXGFP are shown in Fig. 15.

Consistent with this result we found a nuclear export sequence in the PIX open
30 reading frame by applying the search algorithm NetNES (Cour et al., Protein Eng. Des. Sel. 17, 527-536 (2004)) to the PIX primary sequence. The NES identified by this program is 313-GCACAATTGGATTCTTTGACCCGGGAACTT-342 (SEQ ID NO:17) (translated: AQLDSLREL; SEQ ID NO:18). To our knowledge, it is the first time

that such a signal has been described in the PIX protein. Conversely, a nuclear localisation sequence (NLS) has not been described in the literature for PIX and we cannot detect such a signal (for example, using the algorithm provided by the Columbia University at <http://cubic.bioc.columbia.edu/cgi/var/nair/resonline.pl>).

- 5 The cytoplasmic localisation of PIX-GFP in our duck cells therefore is consistent with the primary sequence of PIX.

Example 14: PIX GFP fusion variants

The surprising nuclear exclusion of PIX GFP was investigated with additional PIX-fusion constructs. A PIX-GFP-NLS expression plasmid was derived from the above
10 described p9GFP construct by insertion of synthetic oligonucleotides i185 and i186. These oligos were denatured at 80 °C for 5 min and allowed to anneal in 10 mM MgCl₂ by gradual cooling to room temperature to yield:

5'-GATGTACAAAGATCCGAAGAAGAACCGCAAAGGTTAACGCGGCCGCAC-3' (SEQ ID NO:19)

15 3'-CTACATGTTTCTAGGCTTCTTCTTGGCGTTTCCAATTGCGCCGGCGTG-5' (SEQ ID NO:20)

This double stranded oligonucleotide was digested with BsrG I and Not I and inserted into the same sites of p9GFP. Translated, the insertion adds a NLS sequence (PKKNRK; SEQ ID NO:21) to the PIX-GFP fusion protein that resembles
20 the simian virus 40 NLS. A new Hpa I site introduced with this insert serves as diagnostic marker to confirm successful cloning. The resulting plasmid is called p9 GFP NLS, the expressed protein PIX-GFP-NLS and the open reading frame coding for the protein PIX-GFP-NLS are shown in SEQ ID NOs:23 and 22, respectively.

A GFP-tagged PIX-RARA fusion protein was generated via PCR amplification of a
25 fragment containing PIX-RARA with primers EBR 44A (5'-GGATCCTTCCTCCTCGGGCGGGTGT-3'; SEQ ID NO:24), V293 (5'-AACCAGCGCTACCATGAGCACCAACTCGT 3'; SEQ ID NO:25) and plasmid #67F pEF PIX RARA NEO as template. The amplicon of 1926 bp was treated with polynucleotide kinase and inserted into pEGFP-C2 (Clontech) linearized with EcoR I
30 and blunted with Klenow enzyme (all enzymes from New England Biolabs). The resulting plasmid is called p9 GFP RARA, the expressed protein PIX GFP RARA.

Fig. 16 shows the intracellular distribution of the various GFP-tagged proteins in

transiently transfected CHO cells.

Example 15: PIX and interferon

Many viruses induce the innate cellular immune response via TLR-3 (toll like receptor 3). TLR-3 recognizes double stranded RNA, a hallmark pattern of viral replication. Among the functions of TLR-3 is activation of NFkB and type I interferon (Alexopoulou et al., Nature 413, 732-738 (2001)). Interferon mediates an antiviral state in the host cell. The effect of interferon induction via an artificial double stranded RNA, poly I:poly C (polyinosinic-polycytidylic acid, Sigma) was examined. Surprisingly the CR and CRpIX cells displayed very little poly I:poly C sensitivity whereas CS cells clearly responded to the inductor. Unexpectedly, PIX-positive CS cells responded faster and at lower concentrations than parental CS cells (Fig. 17). To our knowledge, a connection between PIX and interferon has not been described in the current literature.

The PIX-supportive effect for MVA was stronger in CS cells than CR cells, and the CS cells responded to double stranded RNA surrogate better than CR cells. It is known that MVA induces interferon and that this virus also is equipped with proteins that alleviate the interferon response. The effect of poly I:poly C inductor on MVA in CRp9GFP and CSp9GFP cells therefore determined. Fig. 18 suggests that MVA infected cells suffer less from poly I:poly C than uninfected control. This observation is consistent with the fact that MVA both induces and interferes with the cellular innate immune response and connects with our unexpected observation that PIX from a human adenovirus in avian cells cooperates with antiviral proteins of a highly attenuated pox virus to increase yields of the latter virus.

Sequence Listing –Free Text

SEQ ID NO	Description
1, 2	adenoviral pIX protein
3, 4	fusion protein pIX-RARA;
5	Ad5 E1A+ E1B
6 – 11	primer
12	“short” human translation elongation factor 2 promoter
13 - 16	primer

17 - 18	NES
19 - 20	synthetic oligonucleotides encoding NLS
21	NLS
22 - 23	fusion protein pIX-GFP-NLS
5 24 - 25	primer

DEMANDES OU BREVETS VOLUMINEUX

**LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVETS
COMPREND PLUS D'UN TOME.**

CECI EST LE TOME __1__ DE __2__

NOTE: Pour les tomes additionels, veuillez contacter le Bureau Canadien des Brevets.

JUMBO APPLICATIONS / PATENTS

**THIS SECTION OF THE APPLICATION / PATENT CONTAINS MORE
THAN ONE VOLUME.**

THIS IS VOLUME __1__ OF __2__

NOTE: For additional volumes please contact the Canadian Patent Office.

CLAIMS

1. A method for preparing a non-adenoviral target virus or one or more target protein(s) which is (are) not related to adenovirus, which method
5 comprises
- (a) culturing a vertebrate expression cell obtained by infecting or transfecting a vertebrate host cell, having stably integrated into its genome a gene coding for adenovirus pIX or a functional variant thereof as a heterologous regulator protein and stably
10 expressing said regulator protein or the functional variant thereof, with said virus, or with a vector carrying nucleic acid sequences encoding said virus, or with a vector carrying nucleic acid sequences encoding said one or more target protein(s), and
- (b) isolating the target virus or the target protein(s).
15
2. The method of claim 1, wherein
- (i) the heterologous regulator protein modulates transcription and/or cell growth, and enhances the productivity of the cell in the production of a virus not containing said regulator protein
20 and in the production of a protein differing from said regulator protein or the functional variant thereof; and/or
- (ii) the functional variant of the heterologous regulator protein is a fusion protein which comprises at least one first domain comprising an adenovirus pIX regulator protein and at least one
25 second domain that modulates or expands the activity or the subcellular distribution of the adenovirus pIX; and/or
- (iii) said at least one second domain in (ii) above is selected from a transcription factor, a fluorescence marker protein, an enzyme, and a transit peptide; and/or
- (iv) said first and second domains in (ii) and (iii) above being directly
30 or via a peptide linker covalently attached to each other; and/or
- (v) the heterologous regulator protein or the functional variant thereof is expressed in the host cell and the expression cell in an amount of at least 1 pg/ μ g cellular protein; and/or

- (vi) the gene encoding the heterologous regulator protein or the functional variant thereof is under control of a stabile homologous or heterologous promoter.
- 5 3. The method of claim 2, wherein the transcription factor is retinoic acid receptor alpha (RARA), the fluorescence marker protein is GFP or DsRed, the enzyme is LacZ, or the transit peptide is a NLS.
- 10 4. The method of claims 1 to 3, wherein the heterologous regulator protein
- (i) is the adenovirus pIX protein having the sequence shown in SEQ ID NO:2; or
- (ii) is a fusion protein between adenovirus pIX and Retinoic acid receptor alpha,; or
- 15 (iii) is a fusion protein between adenovirus pIX, GFP and a NLS sequence; or
- (iv) is a fusion protein between adenovirus pIX and GFP, or
- (v) is a fusion protein between adenovirus pIX and an isolated NLS sequence.
- 20 5. The method of claim 4, wherein
- (i) the fusion protein between adenovirus pIX and Retinoic acid receptor alpha has a sequence shown in SEQ ID NO: 4; or
- (ii) the fusion protein between adenovirus pIX, GFP and a nls sequence has a sequence shown in SEQ ID NO: 23.
- 25 6. The method of any one of claims 1 to 5, wherein
- (i) the host and expression cell is derived from a primary cell or a previously immortalized cell; and/or
- (ii) the host and expression cell carries further immortalizing viral genes including an E1 protein of an adenovirus; and/or
- 30 (iii) the cell carries further functional sequences selected from selection marker sequences, splice donor/acceptor sites and recombinase recognition sequences allowing integration of a target nucleic acid sequence to be expressed in the cell.
- 35

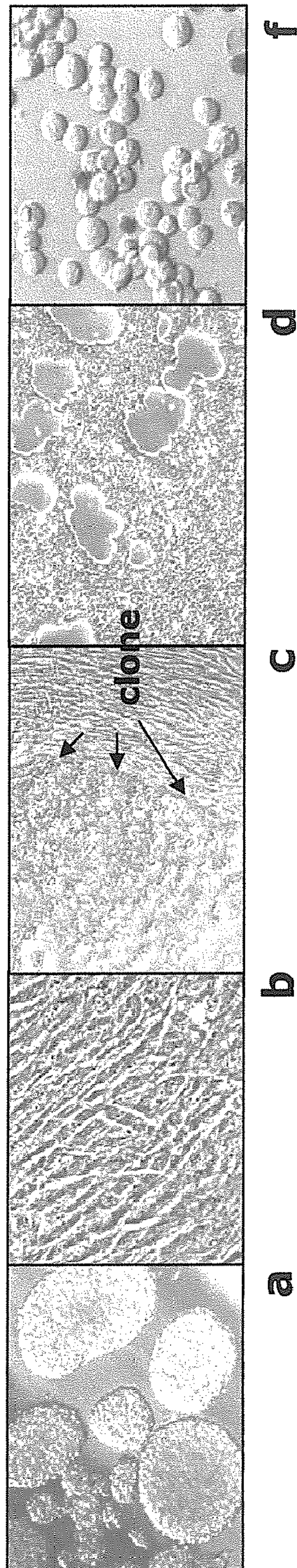
7. The method of any one of claims 1 to 6, wherein
- (i) the target virus is selected from a wild-type, mutated or deleted virus, a cold adapted or attenuated virus, a vaccine strain, a viral vector; or
- 5 (ii) the one or more target protein(s) is (are) selected from antibodies, viral antigens, and peptide hormones.
8. The method of claim 7, wherein the viral vector is selected from a lentivirus, a poxvirus, an adeno associated virus (aav), a herpesvirus
- 10 and a flavivirus.
9. The method of claim 8, wherein the poxvirus is selected from a cowpox virus, a fowlpox and a canarypox virus.
- 15 10. The method of claim 9, wherein the cowpox virus is a Modified Vaccinia Ankara (MVA) virus.
11. The method of claim 7, wherein
- (i) the recombinant proteins are selected from erythropoietin,
- 20 alpha-1-antitrypsin, blood clotting factors VIII and IX, and interferons, or
- (ii) the viral antigens are selected from influenza HA and NA, HBV-S, herpes G protein and rabies G protein.
- 25 12. The method of any one of claims 1 to 11, wherein the host and expression cell is derived from human brain.
13. The method of claim 12, wherein the host and expression cell is derived from human foetal brain.
- 30 14. The method of claim 13, wherein the host cell is a NC5T11#34 cell and the expression cell is derived from the NC5T11#34 cell, said NC5T11#34 cell being deposited with the DSMZ under accession number DSM ACC2744.

15. The method of any one of claims 1 to 11, wherein the host and expression cell is an avian cell.
16. The method of claim 15, wherein the host and expression cell is an avian cell derived from duck retina or duck somite.
17. The method of claim 16, wherein the host cell is a CR.pIX (17a11b) cell and the expression cell is derived from the CR.pIX (17a11b) cell, said CR.pIX (17a11b) cell being deposited with the DSMZ under accession number DSM ACC2749.
18. A vertebrate expression cell for producing a non-adenoviral target virus or one or more target protein(s) which is (are) not related to adenovirus as defined in any one of claims 1 to 17, which
 - (i) stably expresses adenovirus pIX or a functional variant thereof as a heterologous regulator protein, and
 - (ii) is infected or transfected with a non-adenoviral target virus, with a vector carrying nucleic acid sequences encoding said virus, or with a vector carrying nucleic acid sequences encoding one or more target protein(s) which is (are) not related to adenovirus.
19. The vertebrate expression cell of claim 18 which is a cell as defined in claims 12 to 17.
20. A method for preparing the vertebrate expression cell of claim 18 or 19, which comprises infecting or transfecting a vertebrate host cell as defined in any one of claims 1 to 17, with a non-adenoviral virus, with a vector carrying nucleic acid sequences encoding said virus, or with a vector carrying nucleic acid sequences encoding one or more target protein(s) which is (are) not related to adenovirus.
21. An avian host cell or a human brain derived host cell, which has stably integrated into its genome a gene coding for human adenovirus pIX or a functional variant thereof as a heterologous regulator protein and

stably expresses said human adenovirus pIX or the functional variant thereof as a heterologous regulator protein.

22. A method for preparing the avian host cell or the human brain derived
5 host cell of claim 21, which comprises transfecting an appropriate
avian starting cell or human brain derived starting cell with a vector
carrying nucleic acid sequences encoding adenovirus pIX or a
functional variant thereof as a heterologous regulator protein.
- 10 23. Use of the vertebrate expression cell of claims 18 or 19 for preparing a
non-adenoviral target virus or one or more target protein(s) which is
(are) not related to adenovirus.

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**Fig. 1**

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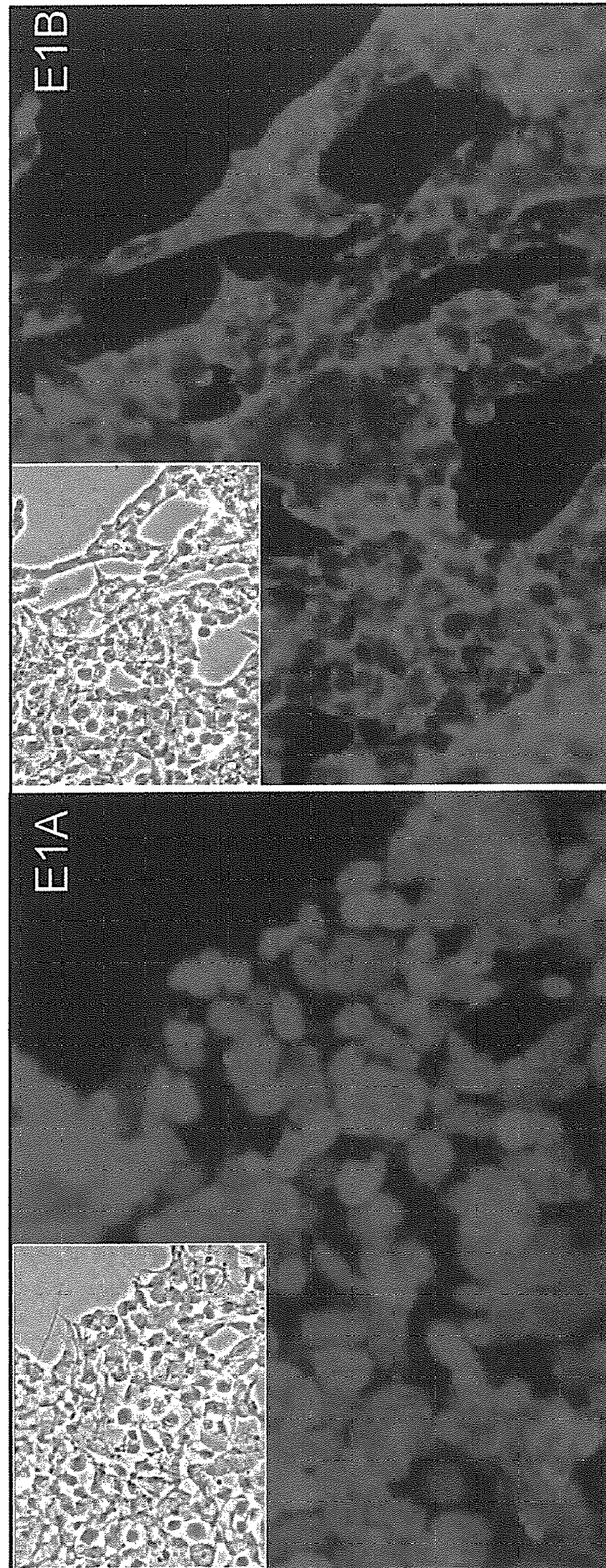


Fig. 2

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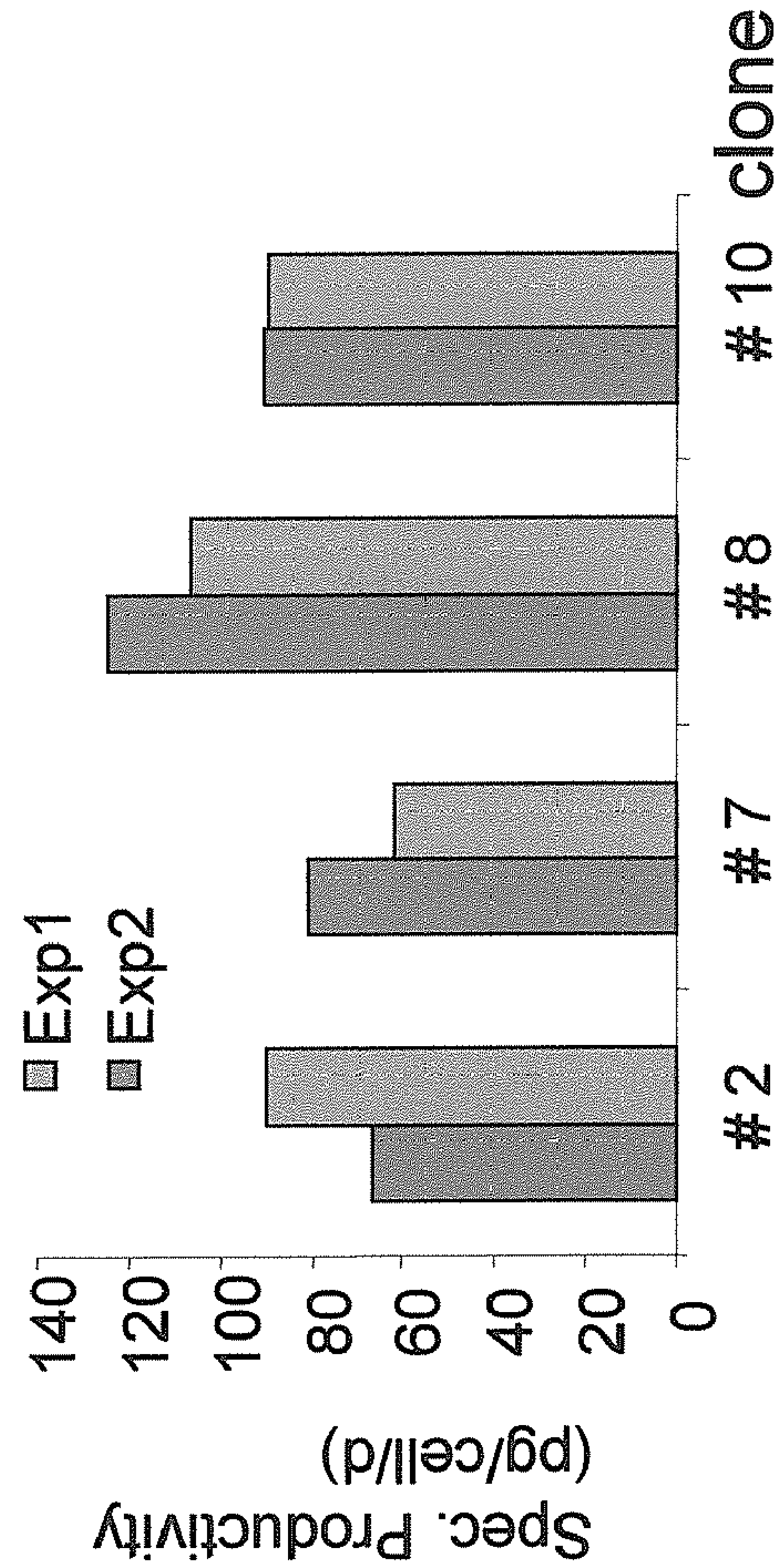


Fig. 3

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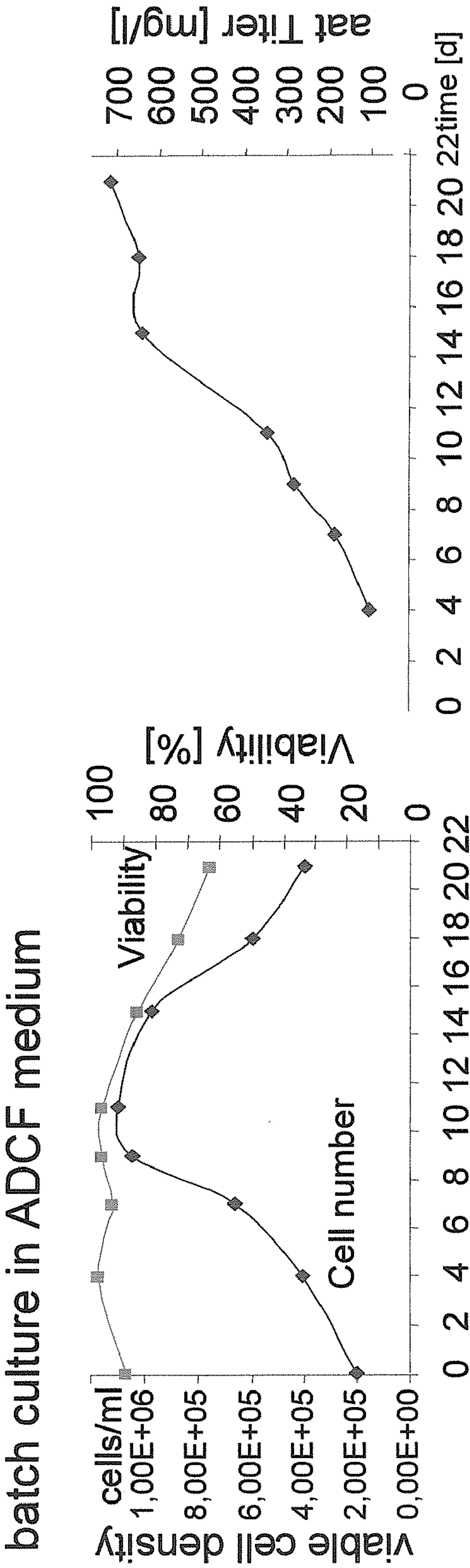


Fig. 4

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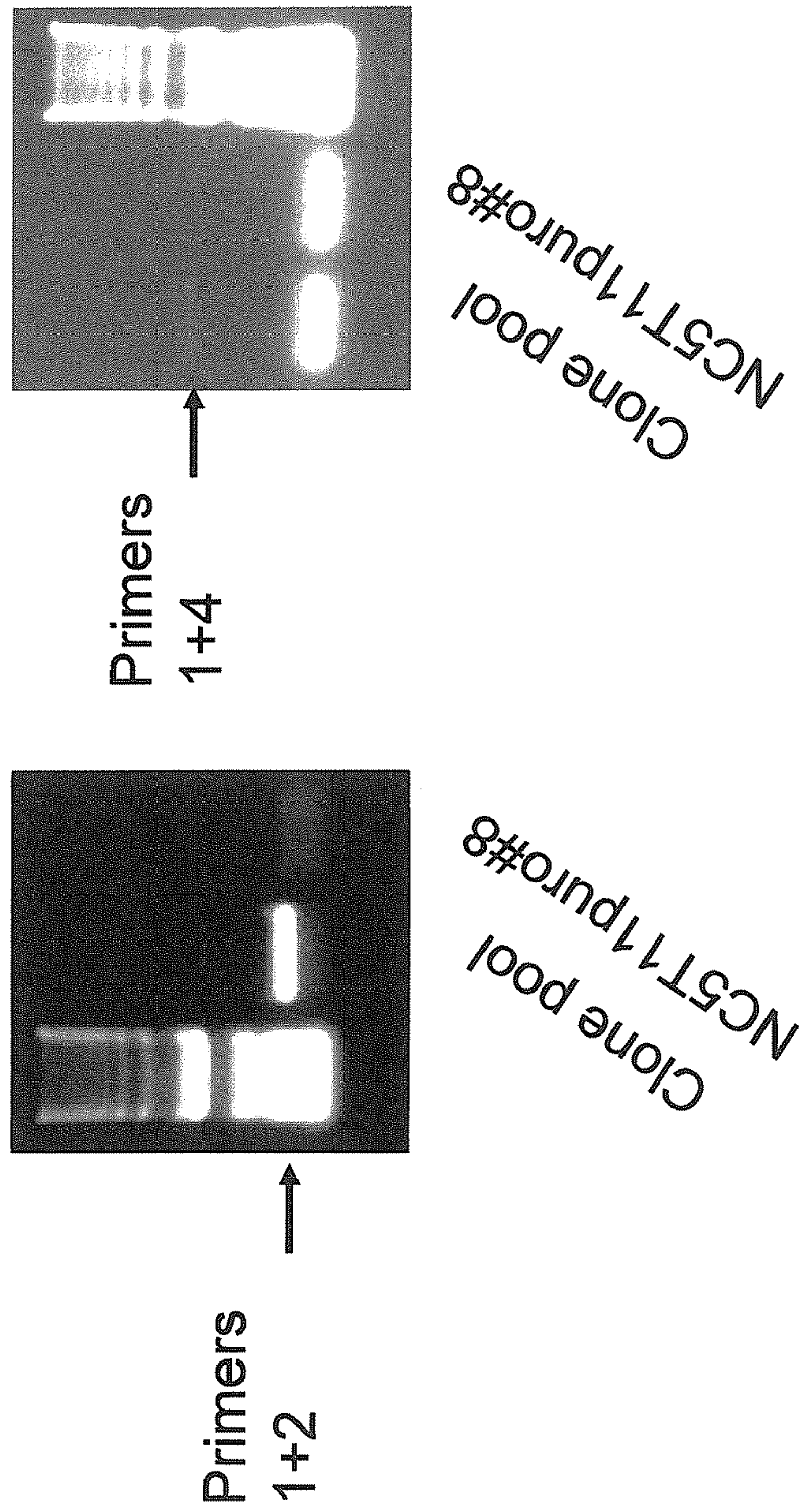


Fig. 5

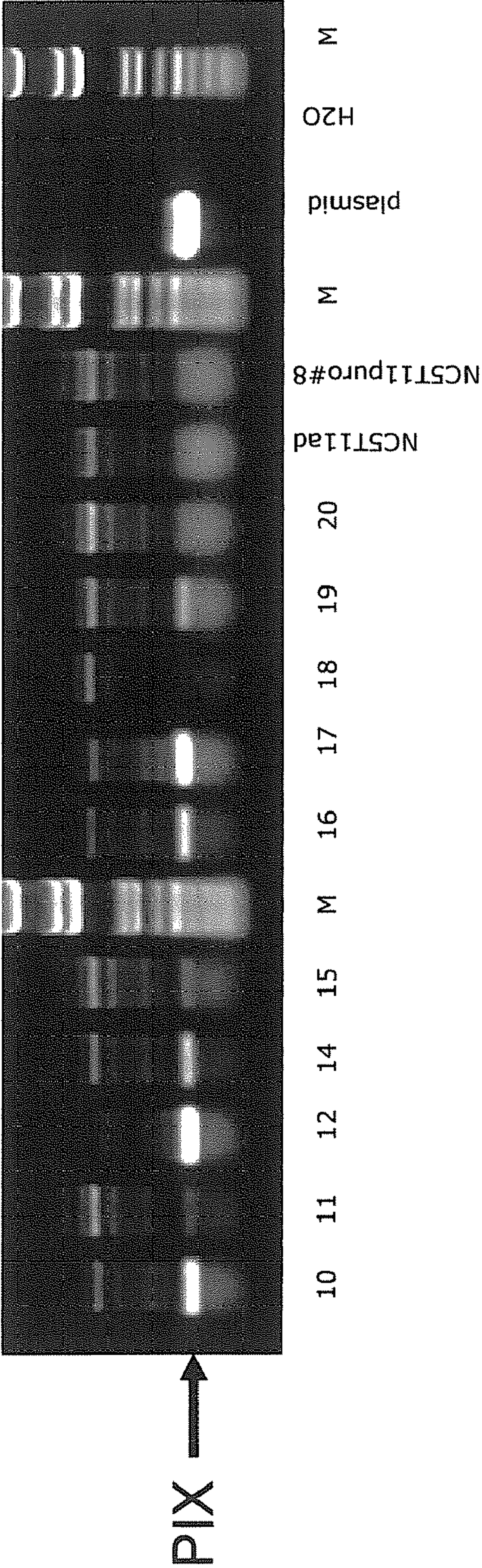
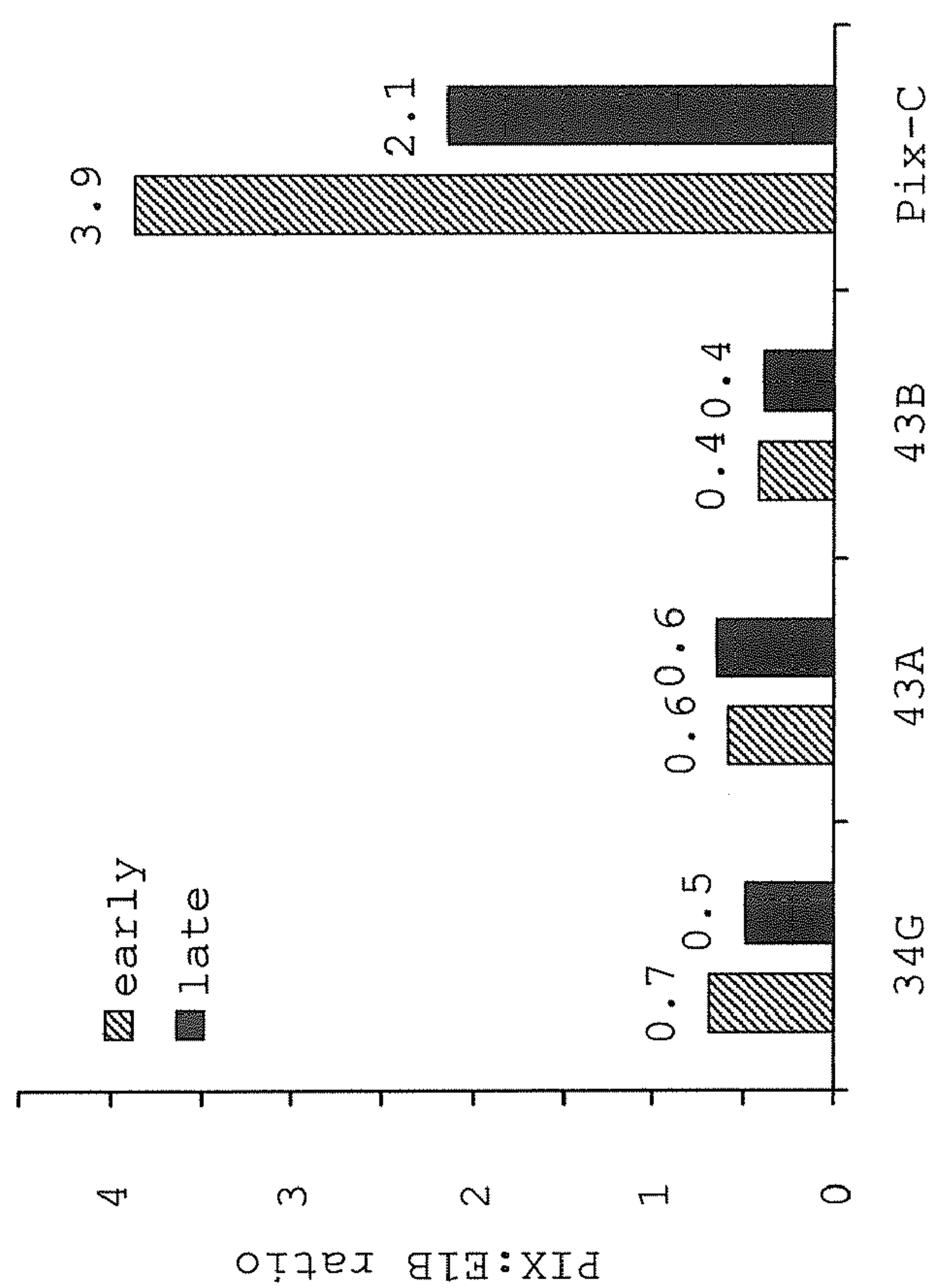


Fig. 6

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**Fig. 7**

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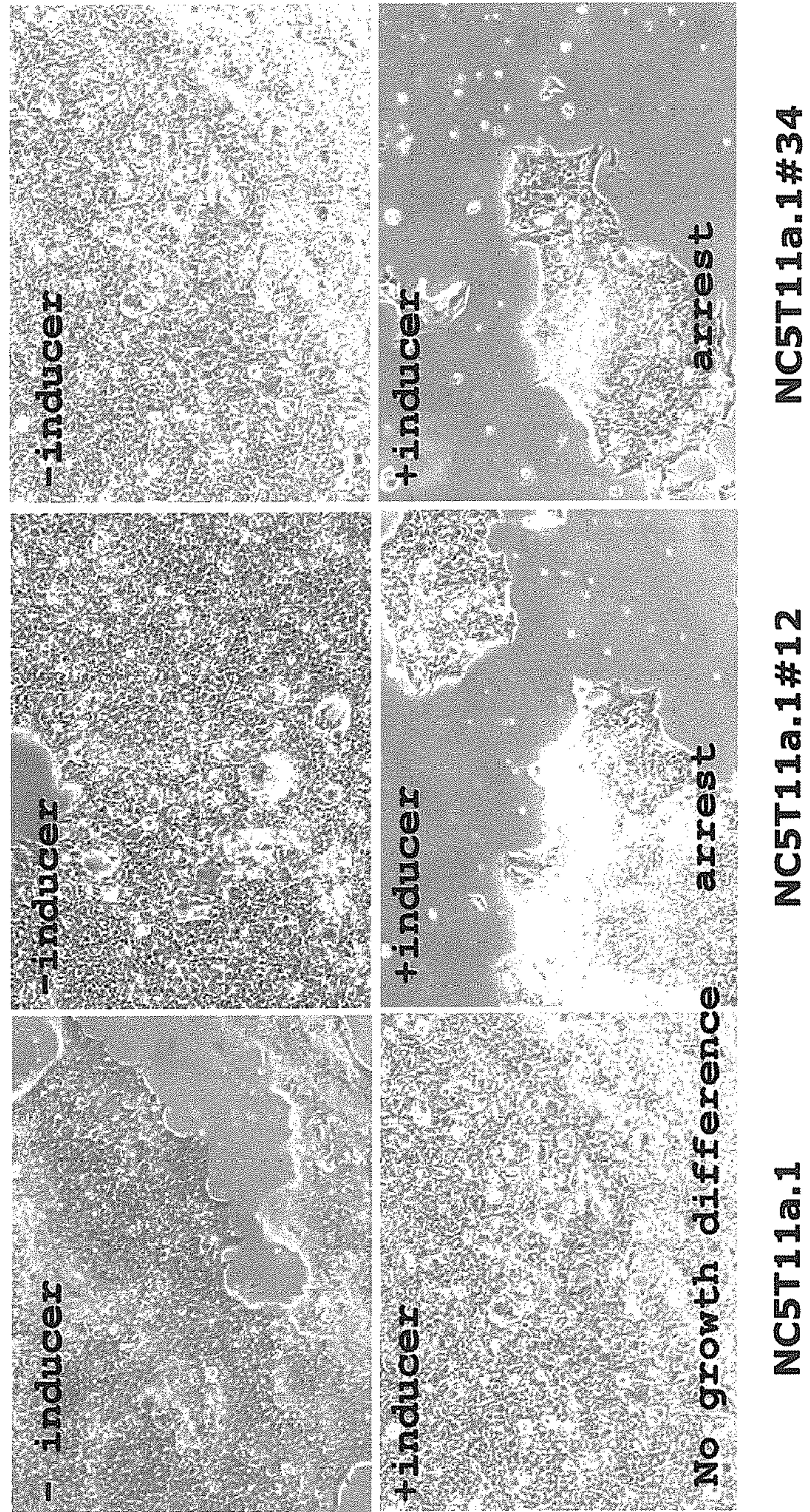


Fig. 8

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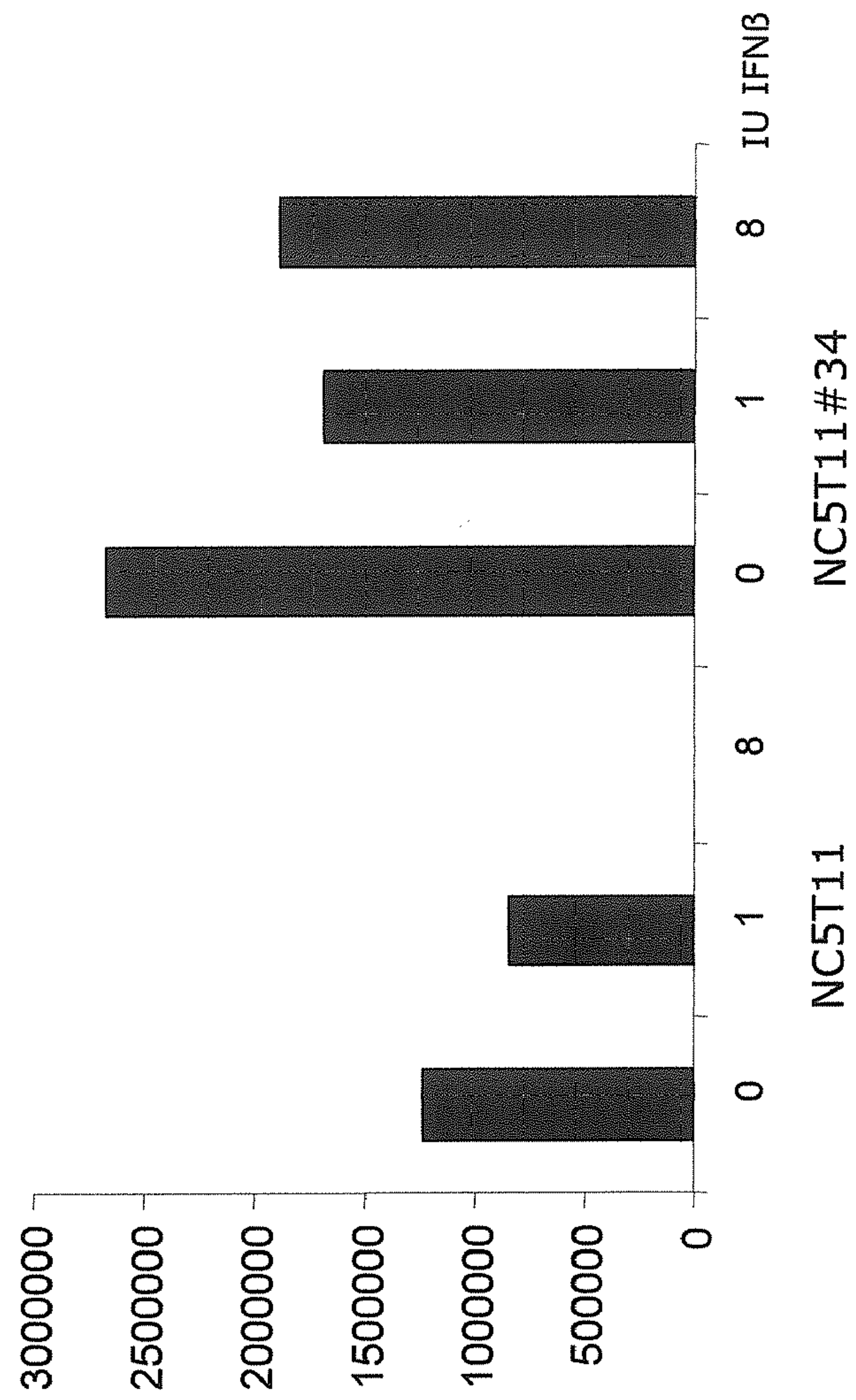


Fig. 9

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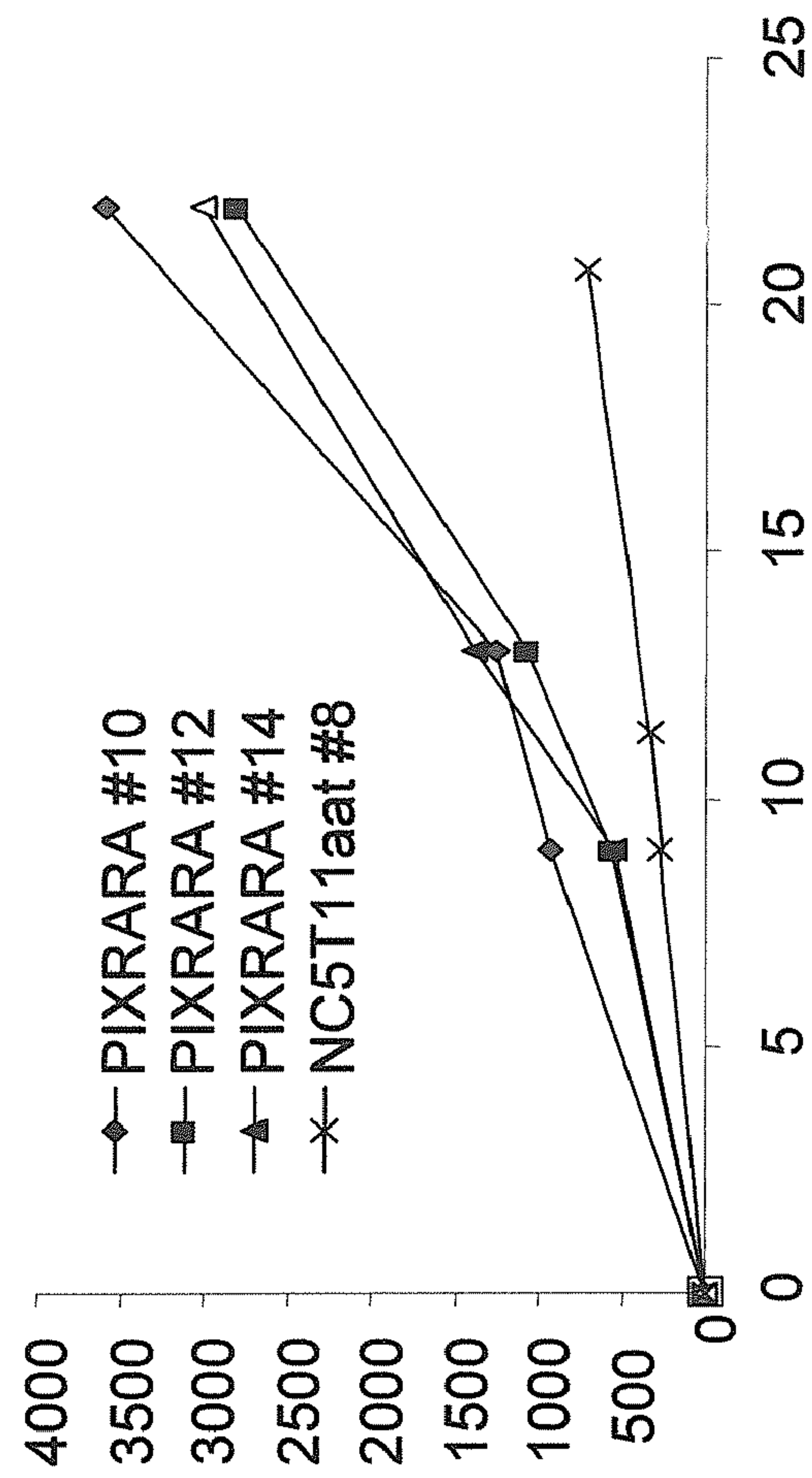


Fig. 10

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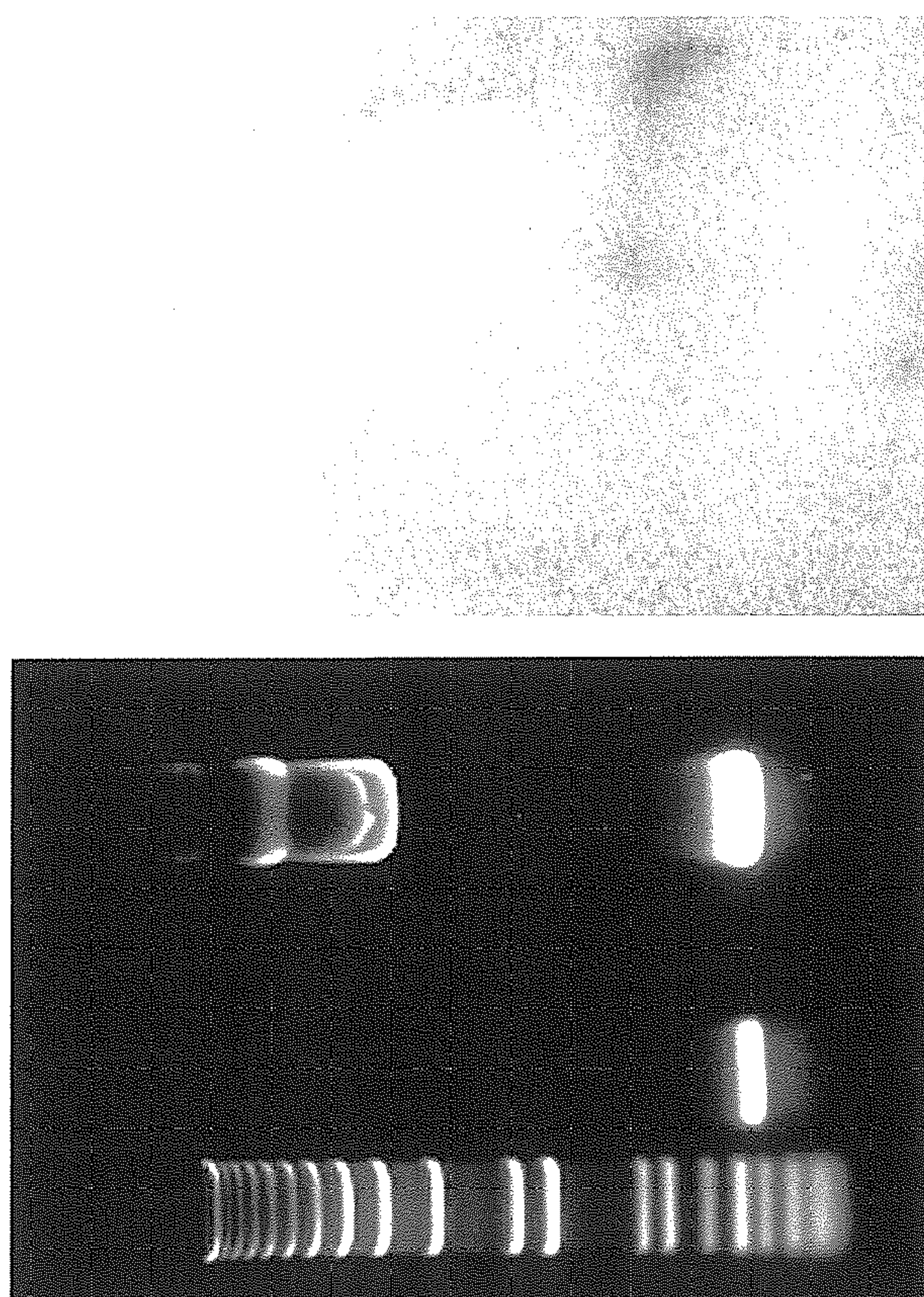


Fig. 11

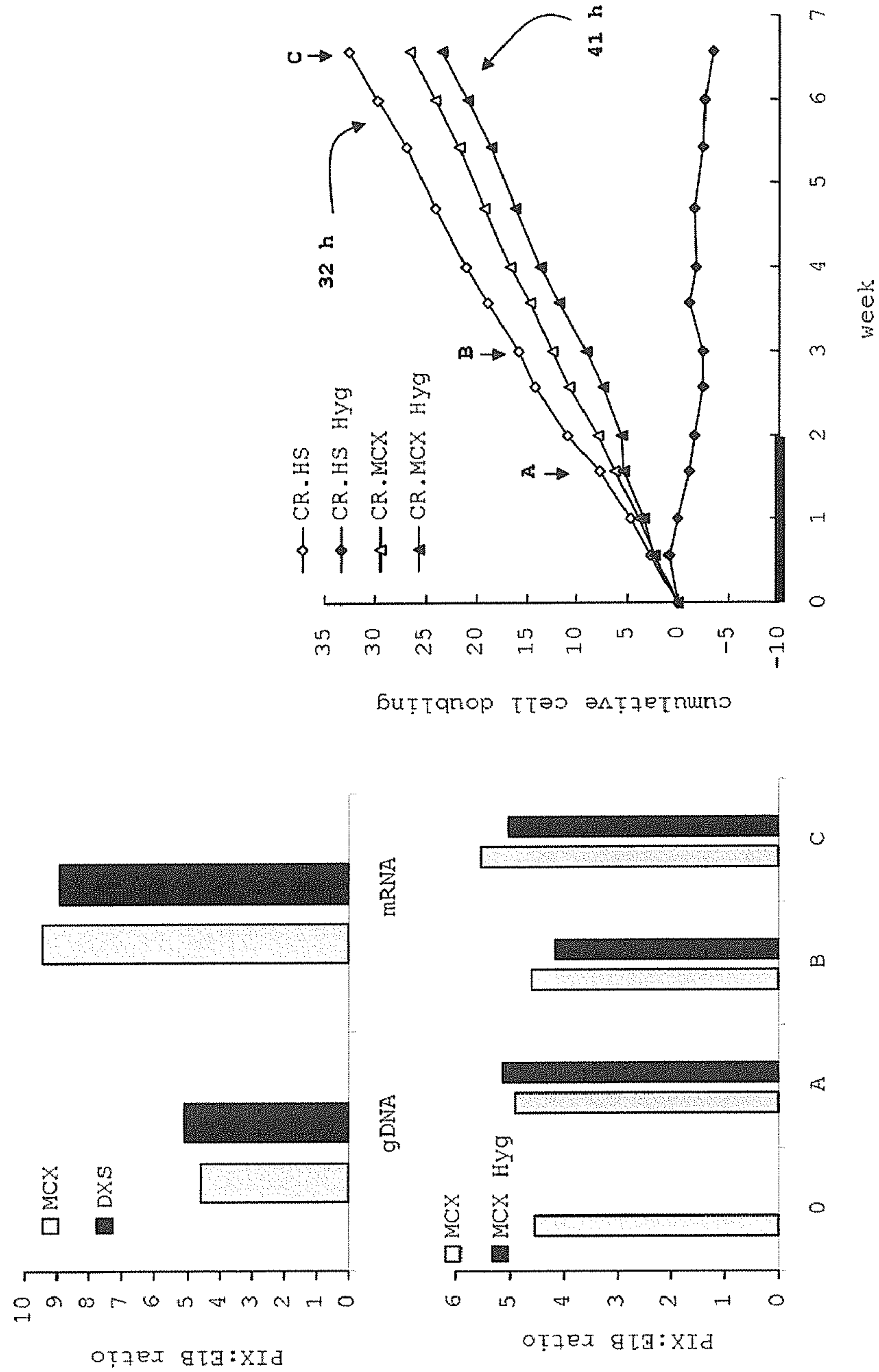
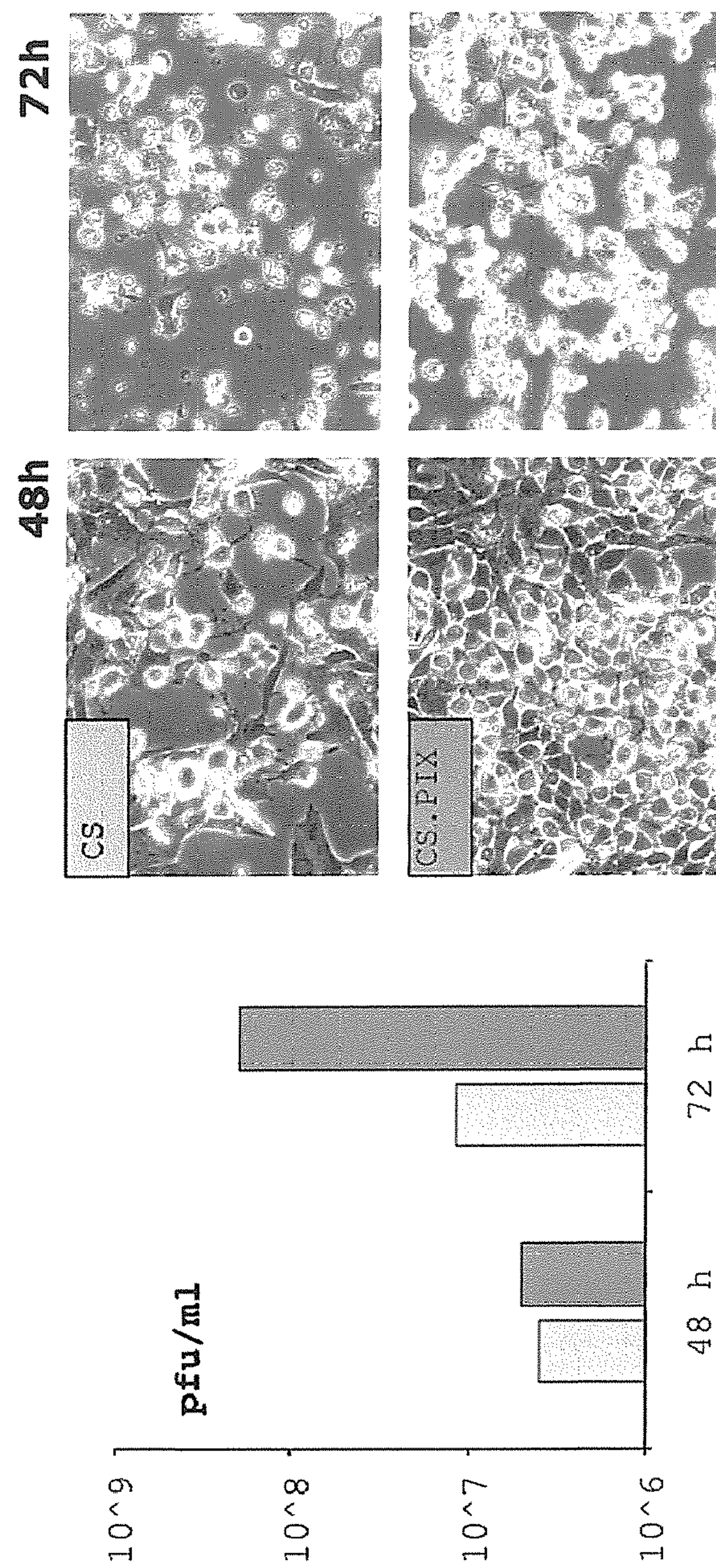
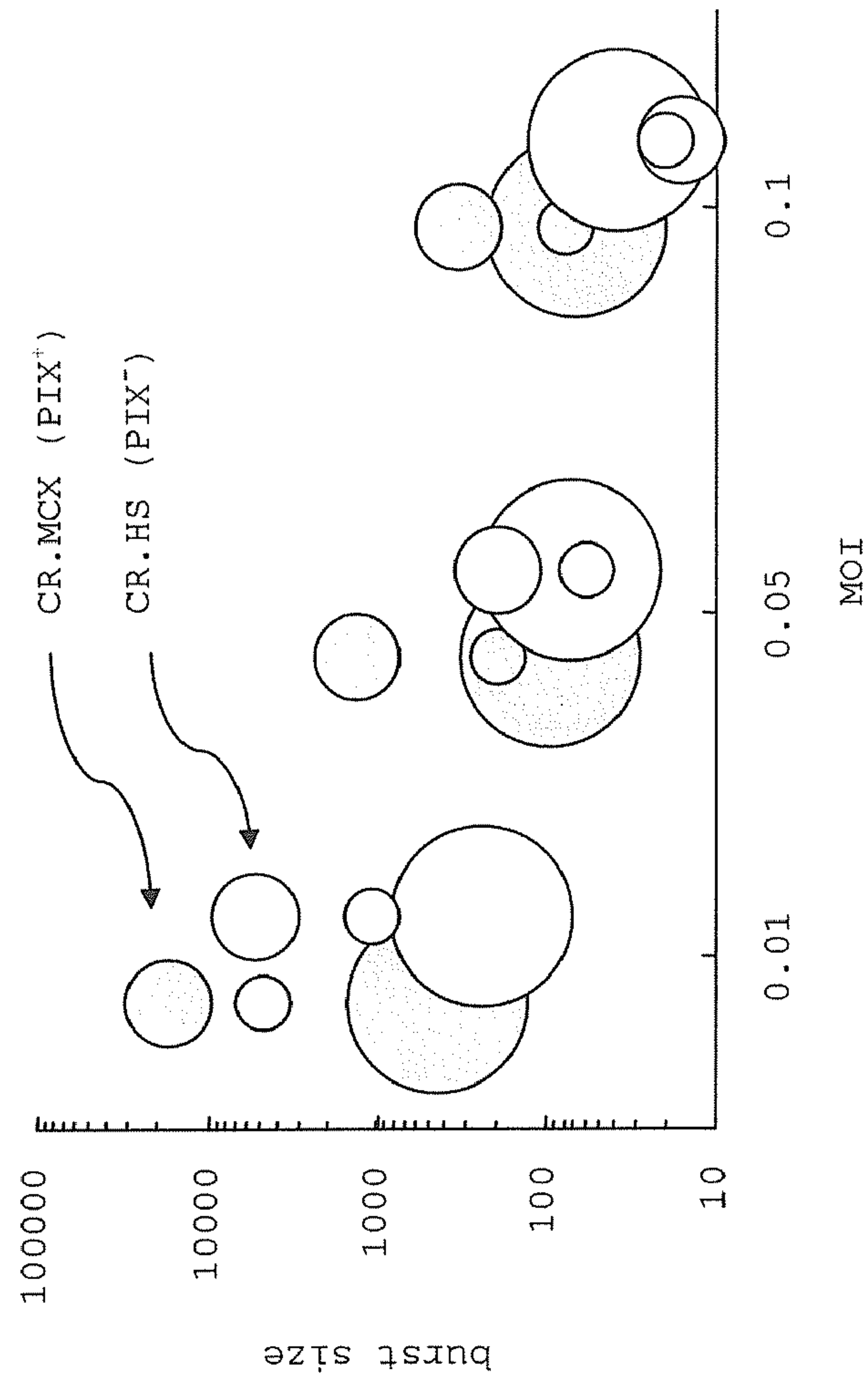


Fig. 12

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**Fig. 13**

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**Fig. 14**

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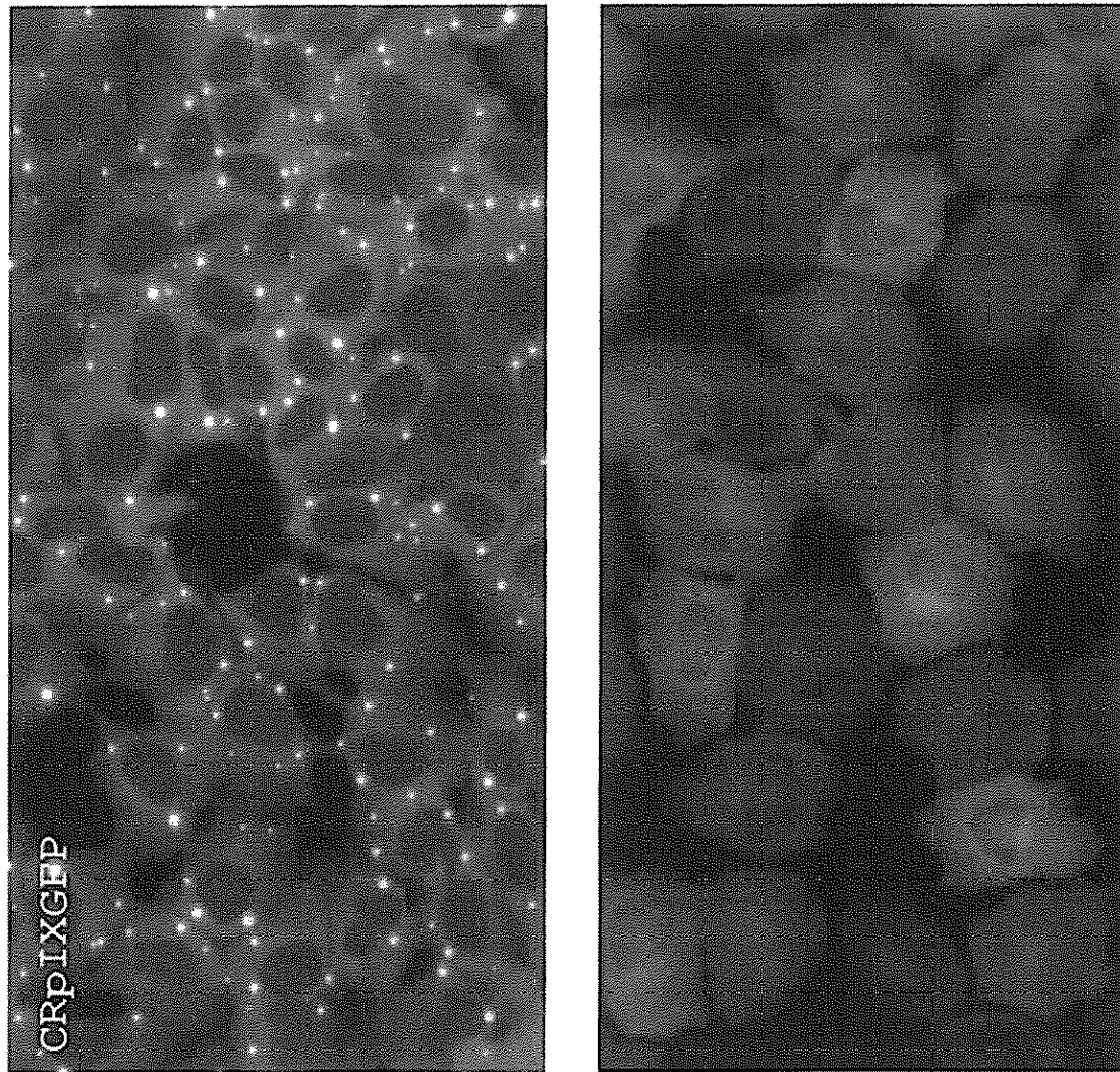
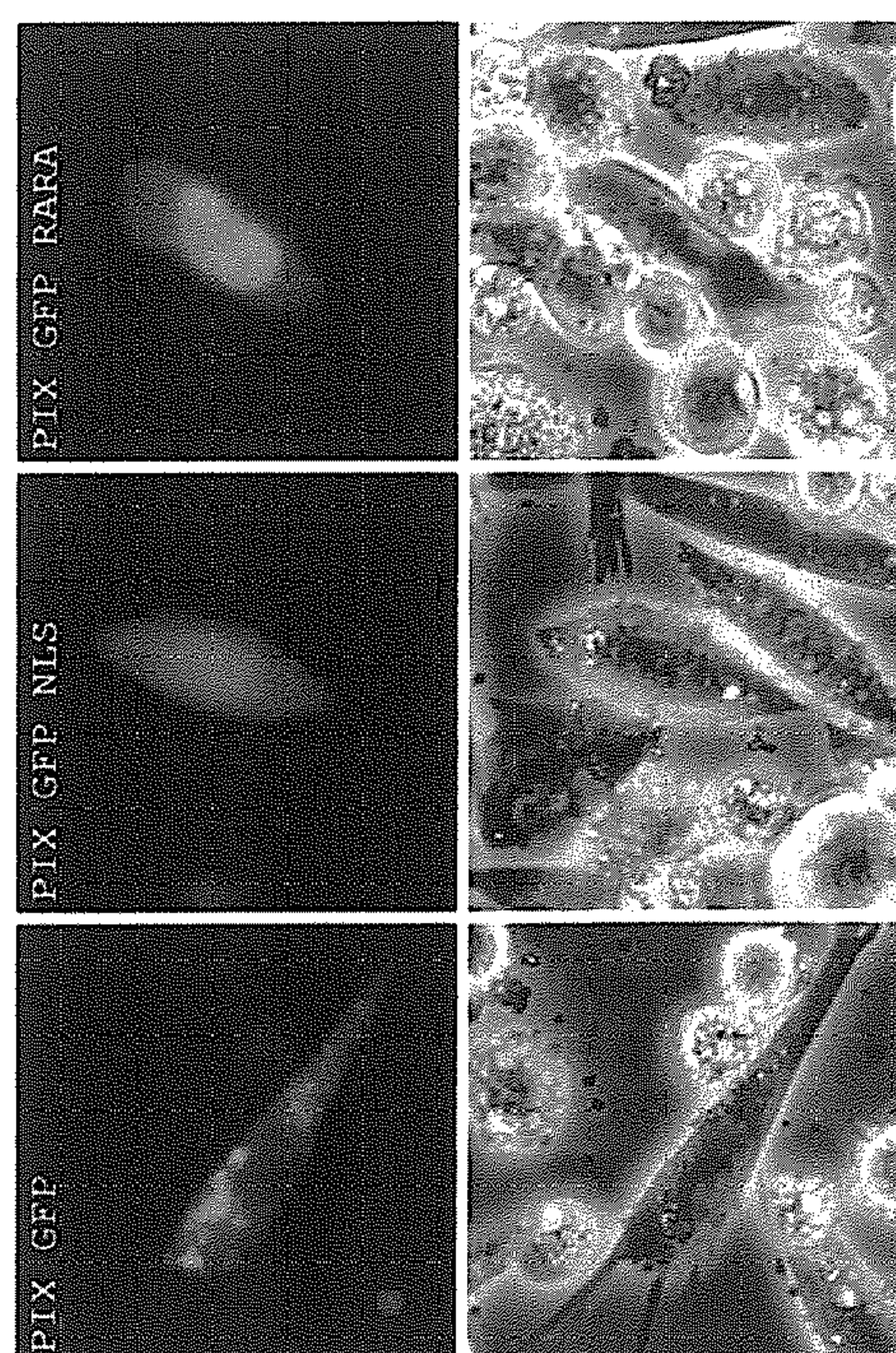
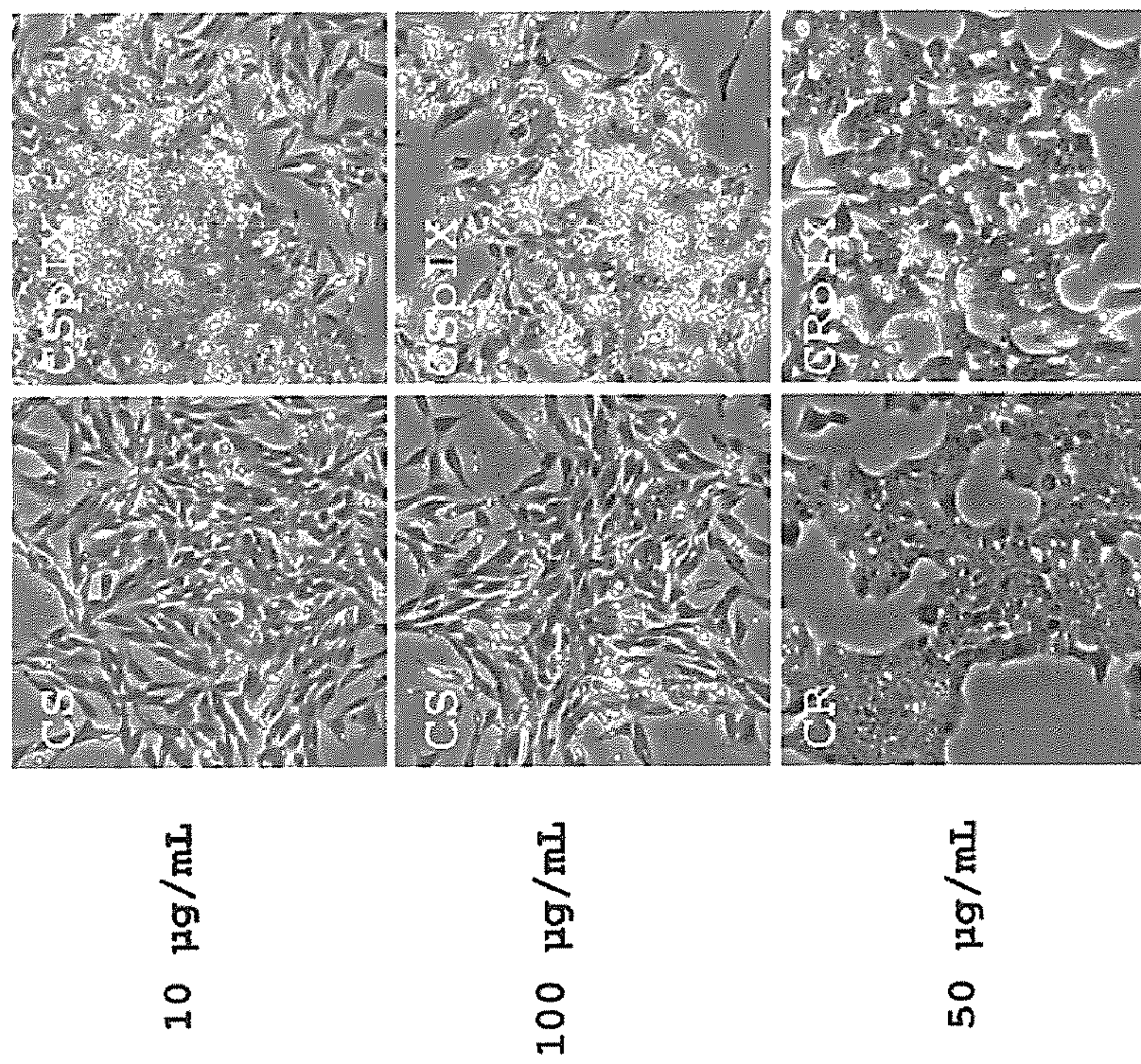


Fig. 15

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**Fig. 16**

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**Fig. 17**

-18/18-

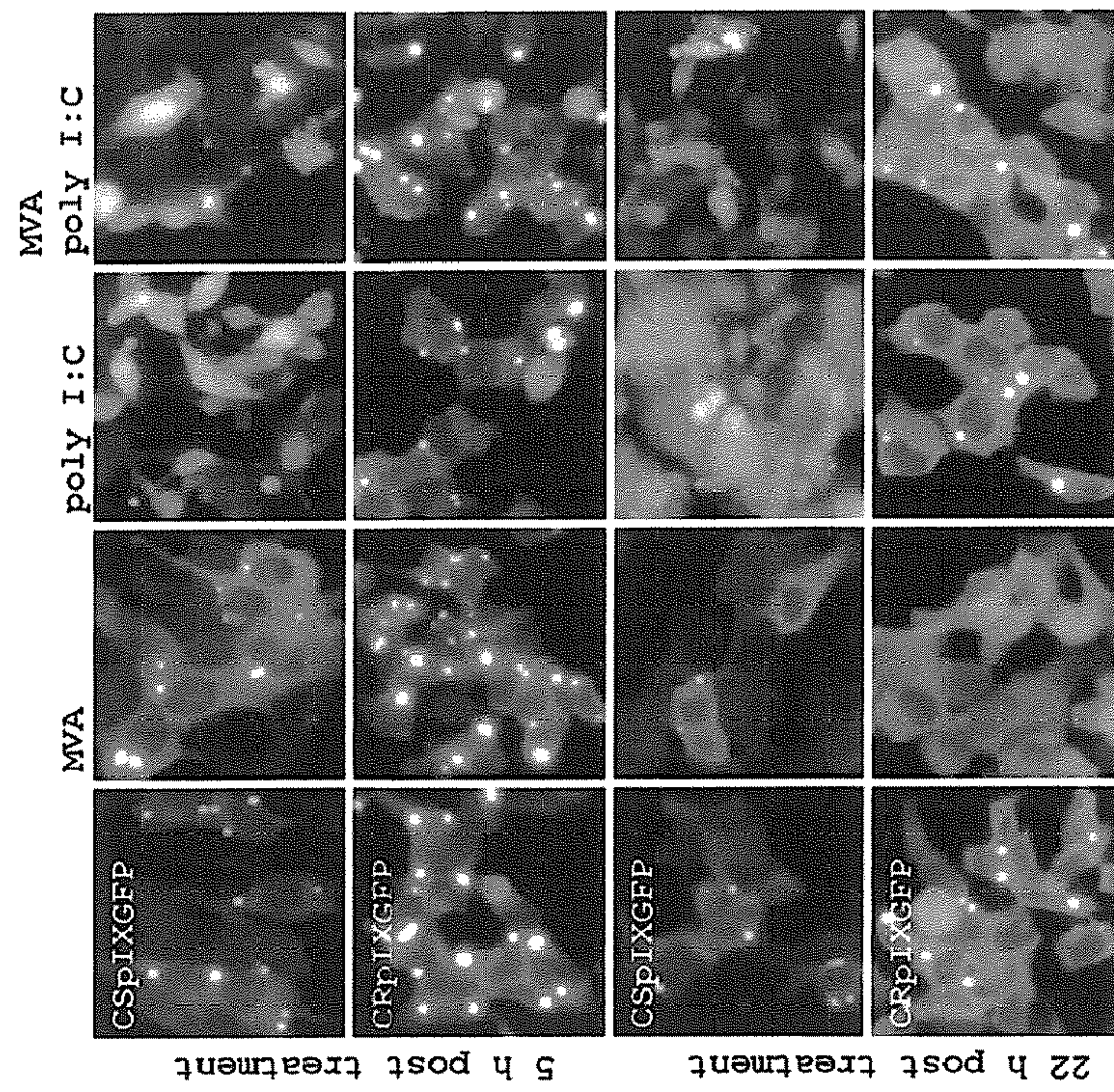


Fig. 18