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(54) Title: PRODUCTION OF FLU VACCINE IN MYCELIOPHTHORA THERMOPHILA

(57) **Abrégé/Abstract:**

Recombinant expression of influenza virus surface proteins in the fungus *Myceliophthora thermophila* strain C1 is provided. The recombinant proteins are for use in influenza vaccine compositions.

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(54) Title: PRODUCTION OF FLU VACCINE IN MYCELIOPHTHORA THERMOPHILA

(57) Abstract: Recombinant expression of influenza virus surface proteins in the fungus Myceliophthora thermophila strain C1 is provided. The recombinant proteins are for use in influenza vaccine compositions.

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PRODUCTION OF FLU VACCINE IN *MYCELIOPHTHORA THERMOPHILA*

FIELD OF THE INVENTION

The present invention relates to the production of recombinant influenza virus surface proteins in the fungus *Myceliophthora thermophila*. The recombinant proteins are for use in influenza vaccine compositions.

BACKGROUND OF THE INVENTION

Influenza virus is a lipid-enveloped virus with a negative-sense, single-stranded, segmented RNA genome. The envelope of the virion contains two types of surface glycoproteins, hemagglutinin and neuraminidase, which play essential roles in viral infection. The hemagglutinin (HA) mediates attachment of the virus to its host cells and viral entry by membrane fusion. The neuraminidase (NA) is an enzyme which plays important roles in the release of new progenies and the prevention of their aggregation.

Influenza viruses are classified into types A, B and C based on differences in their nucleoproteins and matrix proteins. Each type is further classified into subtypes according to combinations of HA and NA present on their surface. For influenza A viruses, 16 subtypes of HA and 9 subtypes of NA have been identified, of which three HA subtypes (H1, H2 and H3) and two NA subtypes (N1 and N2) are commonly found in humans. For influenza B viruses, only one subtype of HA and one subtype of NA are recognized.

The World Health Organization guidelines for the nomenclature of influenza virus strains are as follows: first, the type of virus is designated (A, B, or C), then the host (if non-human), place of isolation, isolation number, and year of isolation, separated by slashes. For influenza A, the HA and NA subtypes are noted in parentheses. For example, a strain often included in influenza vaccines is: A/New Caledonia/20/1999 (H1N1).

Vaccination of high-risk persons each year before the influenza season is the most effective measure for reducing the impact of influenza. The most common influenza vaccine is composed of inactivated virus particles produced using fertilized chicken eggs. Prior to each influenza season, a special committee selects three virus strains which are thought to represent the most likely viruses to strike in the coming flu season. Samples of the selected viruses are provided to manufacturers as seed virus stocks which possess the desired antigenic characteristics. The seed viruses are injected into fertilized chicken eggs.

These eggs are incubated while the influenza viruses multiply. After a suitable period of time the eggs are opened and the egg white is harvested. This sample contains the viruses. The viruses are purified from the egg material and inactivated. Individual virus stocks are then combined to create the common trivalent influenza vaccine.

5 The production of the vaccine in eggs is associated with a number of drawbacks. First, a huge number of eggs is required, as high as 1 - 2 eggs/dose. In addition, the production is time consuming, expensive and lacks flexibility, for example if changes in the vaccine composition are needed during the flu season. Also, production in eggs results in varying viral yields and is also associated with allergic reactions to egg protein.

10 To avoid the use of eggs, alternative methods for producing influenza viruses have been proposed. These include production of virus particles by propagating the virus in mammalian cell cultures, for example in MDCK cells (Novartis) or PERC.6 cells (Crucell). In addition, production of recombinant hemagglutinin and/or neuraminidase proteins has been suggested, for example in insect cells using a baculovirus expression
15 vector (Flublok®, Protein Sciences Corp.), in plant cells (Medicago Inc.), in bacterial systems (VaxInnate) and in fungi such as *Neurospora crassa* (Intrexon/Neugenes) and *Pichia pastoris* (see for example, Murugan et al., 2013, *Journal of Virological Methods*, 187:20– 25). However, hitherto described methods are relatively expensive and/or their yield is relatively low.

20 US 8,163,533 discloses methods and compositions for rapidly producing multivalent recombinant vaccines using filamentous fungal heterokaryons. Filamentous fungal heterokaryons are generated from combinations of two or more parent strains into which recombinant DNA molecules encoding variants of antigens derived from pathogenic organisms have been introduced. The resulting vaccines are multivalent.

25 WO 2014/151488 discloses methods of improving the stability and maintaining the potency of recombinant hemagglutinin formulations, in particular, recombinant influenza hemagglutinin (rHA). In particular, it was shown that the stability of rHA formulations may be significantly improved by mutating cysteine residues or by formulating with a reducing agent and sodium citrate.

30 *Myceliophthora thermophila* strain C1, previously named *Chrysosporium lucknowense* strain C1, is a filamentous thermophilic fungus discovered in the early 1990's. The wild type C1 naturally produces high levels of cellulases, which made it attractive for production of these enzymes on a commercial scale. Over the years

expression systems and several improved strains of C1 have been developed for producing additional enzymes and other industrial proteins in C1. For example, improved C1 strains characterized by cellulase production at higher levels compared to the wild type C1 isolate have been developed, denoted "High Cellulase" or "HC". In addition, C1 strains which
 5 produce low levels of cellulases have also been developed, denoted "Low Cellulase" or "LC", enabling the commercial production of purer enzymes.

Wild type C1 was deposited in accordance with the Budapest Treaty with the number VKM F-3500 D, deposit date August 29, 1996. HC and LC strains have also been deposited, for example: strain UV13-6, deposit no. VKM F-3632 D, strain NG7C-19,
 10 deposit no. VKM F-3633 D, strain UV18-25, deposit no. VKM F-3631 D. Additional improved C1 strains that have been deposited include (i) HC strain UV18-100f ($\Delta alp1\Delta pyr5$) – deposit no. CBS141147; (ii) HC strain UV18-100f ($\Delta alp1\Delta pep4\Delta alp2\Delta pyr5,\Delta prt1$) deposit no. CBS141143; (iii) LC strain W1L#100I ($\Delta chi1\Delta alp1\Delta alp2\Delta pyr5$) – deposit no. CBS141153; and (iv) LC strain W1L#100I
 15 ($\Delta chi1\Delta alp1\Delta pyr5$) – deposit no. CBS141149.

US 8,268,585 and US 8,871,493 disclose a transformation system in the field of filamentous fungal hosts for expressing and secreting heterologous proteins or polypeptides. Also disclosed is a process for producing large amounts of polypeptide or protein in an economical manner. The system comprises a transformed or transfected
 20 fungal strain of the genus *Chrysosporium*, more particularly of *Chrysosporium lucknowense* and mutants or derivatives thereof. Also disclosed are transformants containing *Chrysosporium* coding sequences, as well expression-regulating sequences of *Chrysosporium* genes.

US 9,175,296 discloses a fungal host strain of *Chrysosporium lucknowense*. Also
 25 disclosed is a method for homologous and/or heterologous production of a pure protein with a purity of higher than 75%, a method for production of artificial protein mixes and a method for simplified screening of strains functionally expressing a desired enzyme. US 9,175,296 further discloses an isolated promoter sequence suitable for the transcriptional control of gene expression in *Chrysosporium lucknowense* and a method for isolating a
 30 fungal host strain of *Chrysosporium lucknowense* wherein the protease secretion is less than 20% of the protease secretion of *Chrysosporium lucknowense* strain UV 18-25.

There is a need for improved methods for producing influenza vaccine compositions, which are cost effective and which provide high yields of effective

immunogenic proteins in a time-constrained manner that meets the production requirements of a seasonal influenza vaccine.

SUMMARY OF THE INVENTION

5 The present invention provides according to some aspects *Myceliophthora thermophila* strain C1 genetically modified to produce the influenza virus surface proteins hemagglutinin and neuraminidase.

As disclosed herein, the influenza virus surface proteins are produced as full length membrane-bound proteins, containing both their ectodomain and transmembrane domain.
10 It was surprisingly found that the full-length membrane-bound form produced by C1 is functional and immunogenic, while modified secreted forms are inactive. The membrane-bound form was particularly effective and elicited an immune response at relatively low concentrations, as exemplified in a mouse model. Advantageously, C1 could produce the membrane-bound form at high yields, suitable for commercial-scale production.

15 The present invention therefore provides an efficient system for producing effective immunogenic influenza virus proteins at high yields, for use in influenza vaccine compositions.

According to one aspect, the present invention provides a *Myceliophthora thermophila* C1 genetically-modified to produce an influenza virus surface protein,
20 comprising an expression construct comprising a nucleic acid sequence encoding the influenza virus surface protein operably linked to at least one C1 regulatory sequence, wherein the influenza virus surface protein comprises its ectodomain and transmembrane domain and is expressed in the C1 as a membrane-bound protein.

In some embodiments, the influenza virus surface protein is hemagglutinin (HA).
25 According to these embodiments, the expression construct further comprises a nucleic acid sequence encoding a C1 signal peptide linked in-frame to the nucleic acid sequence encoding the HA.

In some embodiments, the HA subtype is selected from the group consisting of influenza A-H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and
30 H16; and influenza B subtype.

In some particular embodiments, the HA subtype is a subtype infecting humans selected from influenza A subtypes H1, H2 and H3; and influenza B subtype. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the HA is from an influenza virus strain selected from the group consisting of A/New Caledonia /20/99 (H1N1), A/California/04/2009 (H1N1), A/Uruguay/716/07 (H3N2) (A/Brisbane/10/07-like), B/Florida/04/2006: B Yamagata lineage, A/Puerto Rico/08/1934 (H1N1), and A/Texas/50/2012 (H3N2).

5 In some embodiments, the at least one C1 regulatory sequence comprises a C1 promoter. In some embodiments, the C1 promoter is selected from the group consisting of: *hexI* (Woronin body), *cbhI* (cellobiohydrolase 1) and *chiI* (chitinase 1) promoters. Each possibility represents a separate embodiment of the present invention. In some particular embodiments, the C1 promoter is *hexI* promoter.

10 In some embodiments, the C1 signal peptide is a signal peptide derived from a protein selected from the group consisting of Cbh1 (Cellobiohydrolase 1, C1), Glal (Glucoamylase 1, C1) and GlalA (Glucoamylase A, *Aspergillus*). Each possibility represents a separate embodiment of the present invention. In some particular embodiments, the C1 signal peptide is derived from Cbh1.

15 In some embodiments, the expression construct comprises: *hexI* promoter operably linked to a nucleic acid sequence encoding a Cbh1 signal peptide fused to HA. According to these embodiments, the HA is from an influenza virus strain selected from the group consisting of A/New Caledonia /20/99 (H1N1), A/California/04/2009 (H1N1), B/Florida/04/2006: B Yamagata lineage, A/Puerto Rico/08/1934 (H1N1), and
20 A/Texas/50/2012 (H3N2).

In some embodiments, the influenza virus surface protein is a neuraminidase (NA).

In some embodiments, the NA subtype is selected from the group consisting of influenza A- N1, N2, N3, N4, N5, N6, N7, N8 and N9; and influenza B subtype.

In some particular embodiments, the NA subtype is a subtype infecting humans
25 selected from influenza A subtypes N1 and N2; and influenza B subtype. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the expression construct comprises: *hexI* promoter operably linked to a nucleic acid sequence encoding NA. According to these embodiments, the NA is from the influenza virus strain A/New Caledonia /20/99 (H1N1).

30 In some embodiments, the C1 strain is selected from the group consisting of: W1L#100I (prt- $\Delta alp1\Delta chi1\Delta alp2\Delta pyr5$) deposit no. CBS141153, UV18-100f (prt- $\Delta alp1$, $\Delta pyr5$) deposit no. CBS141147, W1L#100I (prt- $\Delta alp1\Delta chi1\Delta pyr5$) deposit no. CBS141149, and UV18-100f (prt- $\Delta alp1\Delta pep4\Delta alp2\Delta prt1\Delta pyr5$) deposit no.

CBS141143. In some particular embodiments, the C1 strain is W1L#100I (prt- $\Delta alp1 \Delta chi1 \Delta alp2 \Delta pyr5$) deposit no. CBS141153. In additional particular embodiments, the C1 strain is UV18-100f (prt- $\Delta alp1$, $\Delta pyr5$) deposit no. CBS141147.

In some embodiments, the C1 strain is a strain mutated to delete one or more genes
5 encoding an endogenous protease. In some embodiments, the C1 strain is a strain mutated to delete a gene encoding an endogenous chitinase.

According to a further aspect, the present invention provides a method for producing an influenza virus surface protein, the method comprising culturing the genetically-modified *Myceliophthora thermophila* C1 of the present invention under
10 conditions suitable for expressing the influenza virus surface protein; and recovering the influenza virus surface protein.

In some embodiments, recovering the influenza virus surface protein comprises extraction from mycelia.

In some embodiments, the yield of the recovered protein is at least 80%. According
15 to certain exemplary embodiments, the yield is 80%.

According to another aspect, there is provided herein an expression construct for expressing a membrane bound influenza virus surface protein in *Myceliophthora thermophila* C1, the expression construct comprising at least one C1 regulatory sequence operably linked to a nucleic acid sequence encoding an influenza virus surface protein,
20 wherein the influenza virus surface protein comprises its ectodomain and transmembrane domain.

According to yet another aspect, there is provided herein a substantially pure, recombinant influenza virus surface protein produced by the modified *Myceliophthora thermophila* C1 of the present invention, wherein the influenza virus surface protein is
25 purified to 95% purity or greater and is active and immunogenic, and induces a protective immune response when used as a vaccine.

Further provided herein is an influenza vaccine composition comprising an influenza virus surface protein produced the modified *Myceliophthora thermophila* C1 of the present invention.

30 These and further aspects and features of the present invention will become apparent from the detailed description, examples and claims which follow.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1B. Expression constructs designed and tested. Hemagglutinin (HA), first series. P=promoter, ss=signal sequence, T=terminator. The HA type included in each construct is marked in boldface.

5 **Figures 2A-2B.** Expression constructs designed and tested. Hemagglutinin (HA), second series. P=promoter, ss=signal sequence, T=terminator. The HA type included in each construct is marked in boldface. **Figure 2C.** Illustration of an expression vector containing the construct *Phex1*-ssCbh-NC-TMD-*Tcbh1*. **Figure 2D.** Illustration of the fragment used for transformation.

10 **Figure 3.** Expression constructs designed and tested. Hemagglutinin (HA), third series. P=promoter, ss=signal sequence, T=terminator. The HA type included in each construct is marked in boldface.

Figure 4. Expression constructs designed and tested, neuraminidase (NA). P=promoter, ss=signal sequence, T=terminator. The NA included in each construct is
15 marked in boldface.

Figure 5. List of abbreviations.

Figure 6. Exemplary expression vector for heterologous protein production in C1.

Figure 7. Purification procedure of TMD-containing HA.

Figure 8. Flowchart of fractionation study to distinguish between HA localized
20 intracellularly or cell-wall attached.

Figure 9. Biochemical evaluation of rHA-TMD.

Figures 10A-10B. Antibody responses elicited against A/New Caledonia/20/99 (H1N1) produced in C1 on Day 27 (**Figure 10A**) and D49 (**Figure 10B**) following injection to mice.

25 **Figure 11.** Western blot analysis of HA production in C1 using stirred-tank fermentation.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to recombinant expression of influenza virus
30 surface proteins in the fungus *Myceliophthora thermophila*, particularly in the strain C1. The present invention provides according to some embodiments genetically modified C1 cells expressing influenza virus surface proteins, and methods for producing an influenza vaccine composition using the same.

It is now disclosed that influenza antigens produced in C1 generate an equal, or even better, immune response in mice than the industry standard antigens.

As used herein "C1" or "*Myceliophthora thermophila* C1" refers to *Myceliophthora thermophila* strain C1, previously named *Chrysosporium lucknowense* strain C1 deposited in accordance with the Budapest Treaty with the number VKM F-3500 D, deposit date August 29, 1996. The terms also encompass genetically modified sub-strains thereof which have been mutated, for example, to delete one or more endogenous genes. For example, the C1 strain (sub-strain) may be a strain mutated to delete one or more genes encoding an endogenous protease and/or one or more genes encoding an endogenous chitinase. For example, C1 strains which are encompassed by the present invention include W1L#100I (prt- Δ alp1 Δ chi1 Δ alp2 Δ pyr5) deposit no. CBS141153, UV18-100f (prt- Δ alp1, Δ pyr5) deposit no. CBS141147, W1L#100I (prt- Δ alp1 Δ chi1 Δ pyr5) deposit no. CBS141149, and UV18-100f (prt- Δ alp1 Δ pep4 Δ alp2, Δ prt1 Δ pyr5) deposit no. CBS141143.

It is noted that a recent paper (Marin-Felix et al., 2015, *Mycologica*, 3:619-63) proposed the splitting of the *Myceliophthora* genus based on several criteria such as temperature growth, sexual morph in culture and conidia properties. According to the proposed criteria C1 belongs to the genus *Thermothelomyces* species *heterothallical thermophila*. Thus, according to the Marin-Felix paper, C1 updated name is *Thermothelomyces thermophila* strain C1.

Expression constructs

The terms "expression construct", "DNA construct" or "expression cassette" are used herein interchangeably and refer to an artificially assembled or isolated nucleic acid molecule which includes a nucleic acid sequence encoding a protein of interest and which is assembled such that the protein of interest is expressed in a target host cell. An expression construct typically comprises appropriate regulatory sequences operably linked to the nucleic acid sequence encoding the protein of interest. An expression construct may further include a nucleic acid sequence encoding a selection marker.

The terms "nucleic acid sequence", "nucleotide sequence" and "polynucleotide" are used herein to refer to polymers of deoxyribonucleotides (DNA), ribonucleotides (RNA), and modified forms thereof in the form of a separate fragment or as a component of a larger construct. A nucleic acid sequence may be a coding sequence, i.e., a sequence that

encodes for an end product in the cell, such as a protein. A nucleic acid sequence may also be a regulatory sequence, such as, for example, a promoter.

The terms "peptide", "polypeptide" and "protein" are used herein to refer to a polymer of amino acid residues. The term "peptide" typically indicates an amino acid sequence consisting of 2 to 50 amino acids, while "protein" indicates an amino acid sequence consisting of more than 50 amino acid residues.

A sequence (such as, nucleic acid sequence and amino acid sequence) that is "homologous" to a reference sequence refers herein to percent identity between the sequences, where the percent identity is at least 70%, preferably at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%. Each possibility represents a separate embodiment of the present invention. Homologous nucleic acid sequences include variations related to codon usage and degeneration of the genetic code.

Sequence identity may be determined using a nucleotide/amino acid sequence comparison algorithms, as known in the art.

The term "regulatory sequences" refer to DNA sequences which control the expression (transcription) of coding sequences, such as promoters and terminators.

The term "promoter" is directed to a regulatory DNA sequence which controls or directs the transcription of another DNA sequence in vivo or in vitro. Usually, the promoter is located in the 5' region (that is, precedes, located upstream) of the transcribed sequence. Promoters may be derived in their entirety from a native source, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. Promoters can be constitutive (i.e. promoter activation is not regulated by an inducing agent and hence rate of transcription is constant), or inducible (i.e., promoter activation is regulated by an inducing agent). In most cases the exact boundaries of regulatory sequences have not been completely defined, and in some cases cannot be completely defined, and thus DNA sequences of some variation may have identical promoter activity.

The term "terminator" is directed to another regulatory DNA sequence which regulates transcription termination. A terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence to be transcribed.

The terms "C1 promoter" and "C1 terminator" indicate promoter and terminator sequences suitable for use in C1, i.e., capable of directing gene expression in C1. According to some embodiments, the C1 promoter/terminator is derived from an

endogenous gene of *Myceliophthora thermophila* C1. For example, in some embodiments, the C1 promoter is *hexI* (Woronin body) promoter. An exemplary *hexI* promoter sequence is set forth as SEQ ID NO: 1. In additional embodiments, the C1 promoter is *chiI* (chitinase 1) promoter. An exemplary *chiI* promoter sequence is set forth as
5 SEQ ID NO: 2.

According to some embodiments the C1 promoter/terminator is derived from a gene exogenous to *Myceliophthora thermophila* C1. For example, *bgl* promoter may be used.

The term "operably linked" means that a selected nucleic acid sequence is in
10 proximity with a regulatory element (promoter or terminator) to allow the regulatory element to regulate expression of the selected nucleic acid sequence.

The term "signal peptide" or "signal sequence" are used herein interchangeably and refer to a short peptide (usually 5-30 amino acids long) typically present at the N-terminus of a newly synthesized polypeptide chain that directs the protein to the secretory pathway
15 in the host cell. The signal peptide is typically subsequently removed. A "C1 signal peptide" indicates a signal peptide suitable for use with C1, i.e., capable of directing proteins expressed in C1 into the secretory pathway of C1. According to some embodiments, the C1 signal peptide is derived from an endogenous gene of *Myceliophthora thermophila* C1. For example, in some embodiments, the C1 signal
20 peptide is a signal peptide derived from Glc1 (Glucoamylase 1, C1). An exemplary sequence encoding Glc1 signal peptide is set forth in positions 1-20 of SEQ ID NO: 23. In additional embodiments, the C1 signal peptide is a signal peptide derived from Cbh1 (Cellobiohydrolase 1, C1). An exemplary sequence encoding Cbh1 signal peptide is set forth as SEQ ID NO: 3.

25 According to some embodiments the C1 signal peptide is derived from a gene exogenous to *Myceliophthora thermophila* C1. For example, in some embodiments, the C1 signal peptide is derived from GlcA (Glucoamylase A, *Aspergillus*). An exemplary sequence encoding GlcA signal peptide is set forth in positions 1-18 of SEQ ID NO: 24.

As used herein, the term "in frame", when referring to one or more nucleic acid
30 sequences, indicates that these sequences are linked such that their correct reading frame is preserved.

Expression constructs according to some embodiments of the present invention comprise a C1 promoter sequence and a C1 terminator sequence operably linked to a

nucleic acid sequence encoding a C1 signal peptide and an influenza virus surface protein fused in-frame.

In some embodiments, the expression construct does not contain a nucleic acid sequence encoding a carrier protein fused to the influenza virus surface protein and
5 facilitating secretion thereof.

A particular expression construct may be assembled by a variety of different methods, including conventional molecular biological methods such as polymerase chain reaction (PCR), restriction endonuclease digestion, in vitro and in vivo assembly methods, as well as gene synthesis methods, or a combination thereof.

10

Influenza virus surface proteins

Hemagglutinin, abbreviated "HA", is a type I membrane glycoprotein that mediates attachment of the virus to its host cells via sialic acid-containing receptors on the host cells. The HA molecule is present in the virus as a homotrimer. Each monomer generally
15 comprises two domains, termed HA1 and HA2, where the HA2 domain comprises a transmembrane region, which connects the HA protein to the viral membrane, and a small cytoplasmic tail. The monomer is synthesized as a 75 kDa precursor protein, termed HA0, which assembles at the virus's surface into a trimeric protein. A signal peptide directs the HA0 into the host cell's secretory pathway and is not present in the mature protein. In
20 order to be active, the HA0 precursor must be cleaved by cellular proteases of the host. After cleavage, two subunits corresponding to the HA1 and HA2 domains are generated, linked by a disulfide bond (and anchored to the virus's surface).

Unless defined otherwise, the term "hemagglutinin" or "HA" as used herein refers to influenza virus hemagglutinin, particularly to the full-length protein containing the
25 ectodomain that extends outside the virus particle, the transmembrane domain and the cytoplasmic tail. The term encompasses the HA0 uncleaved form as well as the mature HA1+HA2 form.

The subtype of the HA of the present invention may be influenza A subtype H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 or H16. The HA
30 subtype may also be influenza B subtype. In particular embodiments, the HA subtype is a subtype infecting humans. In some embodiments, the HA subtype is selected from influenza A subtypes H1, H2 and H3. In additional particular embodiments, the HA subtype is influenza B subtype.

Neuraminidase, abbreviated "NA", is a type II membrane-bound enzyme that mediates the release of new viral progenies from a host cell. It is present on the viral surface as a tetramer of four identical monomers, each generally comprising a cytoplasmic domain, a transmembrane domain, a stalk domain, and a globular head domain that carries the enzymatically active site. Unless defined otherwise, the term "neuraminidase" or "NA" as used herein refers to the full length protein.

The subtype of the NA of the present invention may be influenza A subtype N1, N2, N3, N4, N5, N6, N7, N8 or N9, or influenza B subtype. In particular embodiments, the NA subtype is a subtype infecting humans. In some embodiments, the NA subtype is selected from influenza A subtypes N1 and N2. In additional particular embodiments, the NA subtype is influenza B subtype.

Nucleotide and protein sequences of HA and NA from various influenza virus strains are publicly available, for example, at the database of the National Center for Biotechnology Information (NCBI). Exemplary sequences for HA and NA genes/proteins include those from influenza strains A/New Caledonia /20/99 (H1N1), A/California/04/2009 (H1N1), A/Uruguay/716/07 (H3N2) (A/Brisbane/10/07-like), B/Florida/04/2006: B Yamagata lineage, A/Puerto Rico/08/1934 (H1N1), and A/Texas/50/2012 (H3N2). Each possibility represents a separate embodiment of the present invention.

Genetically-modified variants of HA and NA may also be engineered into C1 according to the present invention, such as variants denoted "universal" HA and NA. Universal HA and NA are cross-reactive antigens that stimulate protection against multiple influenza strains, as described, for example, in Carter et al. (2016) *Design and Characterization of a Computationally Optimized Broadly Reactive Hemagglutinin Vaccine for H1N1 Influenza Viruses.*, J Virol. 90(9). 4720-34.

The influenza virus surface proteins are cloned and expressed according to the present invention as membrane-bound proteins comprising their ectodomain and transmembrane domain. The cloned HA/NA genes of the present invention are typically modified by replacement of the natural signal peptide with a C1 signal peptide.

In some embodiments, the HA is from the influenza virus strain A/New Caledonia /20/99 (H1N1) ("HA New Caledonia"). The amino acid sequence of HA New Caledonia with its transmembrane domain, without the natural signal peptide, is set

forth as SEQ ID NO: 4. A nucleotide sequence encoding the HA New Caledonia with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 5.

In some embodiments, the HA is from the influenza virus strain A/Texas/50/2012 (H3N2) ("HA Texas"). The amino acid sequence of HA Texas with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 6. A nucleotide sequence encoding the HA Texas with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 7.

In some embodiments, the HA is from the influenza virus strain A/Puerto Rico/08/1934 (H1N1) ("HA Puerto Rico"). The amino acid sequence of HA Puerto Rico with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 8. A nucleotide sequence encoding the HA Puerto Rico with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 9.

In some embodiments, the HA is from the influenza virus strain B/Florida/04/2006: B Yamagata lineage ("HA Florida"). The amino acid sequence of HA Florida with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 10. A nucleotide sequence encoding the HA Florida with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 11.

In some embodiments, the HA is from the influenza virus strain A/California/04/2009 (H1N1) ("HA California"). The amino acid sequence of HA California with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 12. A nucleotide sequence encoding the HA California with its transmembrane domain, without the natural signal peptide, is set forth as SEQ ID NO: 13.

In some embodiments, the NA is from the influenza virus strain A/New Caledonia/20/99 (H1N1) ("NA New Caledonia"). The amino acid sequence of NA New Caledonia, full length, is set forth as SEQ ID NO: 14. A nucleotide sequence encoding the NA New Caledonia, full length, is set forth as SEQ ID NO: 15.

For expression in C1, the cloned HA/NA genes are preferably codon optimized for C1 expression, meaning that the cloned genes are designed based on the amino acid sequence of an influenza virus surface protein of interest, employing the codon usage of C1.

According to certain exemplary embodiments, the genes are cloned under a C1 promoter and a C1 terminator.

An exemplary expression construct encoding HA New Caledonia with its transmembrane domain under *hex1* promoter and *cbh1* terminator, with a Cbh signal sequence, is set forth as SEQ ID NO: 16. The sequence encoding the HA corresponds to positions 2920-4563 of SEQ ID NO: 16. This segment may be replaced by a nucleotide
 5 sequence encoding a different HA protein or an NA protein, to obtain an expression construct encoding a different HA protein or an NA protein.

An exemplary expression vector comprising the expression construct set forth as SEQ ID NO: 16 is set forth as SEQ ID NO: 17 and illustrated in **Figure 2C**.

10 Genetically-engineered C1

C1 cells genetically engineered to produce influenza virus surface proteins according to the present invention are generated by introducing into C1 cells, particularly into the nucleus of C1 cells, an expression construct comprising a nucleic acid encoding an influenza virus surface protein, as described above. In particular, the genetic modification
 15 according to the present invention means incorporation of the expression construct to the host genome.

In some embodiments, C1 is genetically-engineered to produce a single influenza virus protein. In other embodiments, C1 is genetically-engineered to produce a plurality of different influenza virus proteins. A "plurality" indicates at least two.

20 Introduction of an expression construct into C1 cells, i.e., transformation of C1, can be performed by methods known in the art for transforming filamentous fungi. For example, transformation can be performed using the protoplast transformation method as known in the art and also described in the Examples section below.

To facilitate easy selection of transformed cells, a selection marker may be
 25 transformed into the C1 cells. A "selection marker" indicates a polynucleotide encoding a gene product conferring a specific type of phenotype that is not present in non-transformed cells, such as an antibiotic resistance (resistance markers), ability to utilize a certain resource (utilization/auxotrophic markers) or expression of a reporter protein that can be detected, e.g. by spectral measurements. Auxotrophic markers are typically preferred as a
 30 means of selection in the food or pharmaceutical industry. The selection marker can be on a separate polynucleotide co-transformed with the expression construct, or on the same polynucleotide of the expression construct.

Following transformation, positive transformants are selected by culturing the C1 cells on e.g., selective media according to the chosen selection marker.

Expression of the protein of interest can be detected using standard methods. The detection may be performed by detecting the protein itself, e.g., by various types of staining or by an immunological method, or by detecting its activity, e.g., by a hemagglutination assay. Prior to detection, the protein may be separated using a variety of techniques, such as SDS-PAGE. Exemplary procedures are described in the Examples section below.

Best producers are selected and applied in fermentations to produce high amount of the desired gene product.

Production of proteins

To produce the protein, the genetically modified C1 cells are cultured under conditions that permit the expression of the nucleic acid encoding the influenza virus surface protein. Exemplary culturing conditions for shake-flask and stirred-tank fermentations are detailed in the Examples section below.

Vaccine compositions

A vaccine or an immunogenic composition according to the present invention comprises an influenza virus surface protein produced as described above and a pharmaceutically acceptable carrier.

The term “immunogenicity” or “immunogenic” relates to the ability of a substance to stimulate or elicit an immune response. Immunogenicity is measured, for example, by determining the presence of antibodies specific for the substance after challenging an immunologically competent organism with the substance. The presence of antibodies specific to the substance and their quantity is detected by methods known in the art, for example using an ELISA assay.

In some embodiments, the vaccine is formulated in an immunizing dosage form including purified HA proteins derived from three strains of influenza virus recommended by the FDA for a particular influenza season. Functional immunity can be measured using assays that quantify antibodies that bind to influenza hemagglutinin, that block the ability of influenza virus to agglutinate red blood cells, or that neutralize the influenza virus.

Protective immune responses with recombinant HA vaccines can also be measured in animals that are susceptible to influenza infection or in human challenge studies.

The vaccines of the present invention comprise recombinant HA and/or NA proteins, and optionally, an adjuvant. As exemplified hereinbelow, it was surprisingly
5 found that a recombinant HA protein produced in C1 according to the present invention elicited a humoral response in mice even when administered without an adjuvant.

The vaccine can be formulated for administration in one of many different modes, including via intramuscular, intranasal, intradermal, oral, intraperitoneal, subcutaneous or transdermal mode of administration

10 In some embodiments, the vaccine is formulated for parenteral administration. In some particular embodiments, the vaccine is formulated for intramuscular administration. In other particular embodiments, the vaccine is formulated for intradermal administration.

In additional particular embodiments, the vaccine is formulated for mucosal delivery, in particular intranasal delivery.

15 The vaccine composition can contain a variety of excipients, including stabilizers, buffers, or preservatives.

According to some embodiments, the vaccine compositions according to the present invention do not contain an adjuvant.

In some applications a pharmaceutically acceptable adjuvant may be included in the
20 vaccine formulation. The choice of the adjuvant is determined in part by the mode of administration of the vaccine.

Non-limiting examples of intranasal adjuvants include chitosan powder, PLA and PLG microspheres, QS-21, calcium phosphate nanoparticles (CAP) and mCTA/LTB (mutant cholera toxin E112K with pentameric B subunit of heat labile enterotoxin).

25 Examples of adjuvants for other modes of administration include inorganic adjuvants in gel form (aluminium hydroxide/aluminium phosphate), calcium phosphate, bacterial adjuvants such as monophosphoryl lipid A and muramyl peptides, particulate adjuvants such as ISCOMS ("immunostimulatory complexes"), liposomes and biodegradable microspheres, adjuvants based on oil emulsions and emulsifiers such as IFA
30 ("Incomplete Freund's adjuvant"), SAF ("Syntex Adjuvant Formulation"), saponines (such as QS-21), squalene/squalane, synthetic adjuvants such as non-ionic block copolymers, muramyl peptide analogs, synthetic lipid A, synthetic polynucleotides and polycationic adjuvants.

Adjuvants are utilized in an adjuvant amount, which can vary with the adjuvant, host animal and immunogen. Typical amounts can vary from about 1 microgram to about 1 mg per immunization. Those skilled in the art know that appropriate concentrations or amounts can be readily determined.

5

List of sequences

Element	SEQ ID NO:	Remarks
<i>PhexI</i> (promoter)	1	
<i>PchiI</i> (promoter)	2	
ssCbh (signal sequence Cbh), nucleotide sequence	3	
HA New Caledonia with TMD, without ss, amino acid sequence	4	TMD corresponds to positions 512-548 of SEQ ID NO: 4.
HA New Caledonia with TMD, without ss, nucleotide sequence	5	Sequence encoding TMD corresponds to positions 1533-1644 of SEQ ID NO: 5. 'Stop' codon is omitted.
HA Texas with TMD, without ss, amino acid sequence	6	
HA Texas with TMD, without ss, nucleotide sequence	7	'Stop' codon is omitted.
HA Puerto Rico with TMD, without ss, amino acid sequence	8	
HA Puerto Rico with TMD, without ss, nucleotide sequence	9	
HA Florida with TMD, without ss, amino acid sequence	10	TMD corresponds to positions 538-569 of SEQ ID NO: 10.
HA Florida with TMD, without ss, nucleotide sequence	11	Sequence encoding TMD corresponds to positions 1612-1707 of SEQ ID NO: [-]. 'Stop' codon is omitted.
HA California with TMD, without ss, amino acid sequence	12	
HA California with TMD, without ss, nucleotide sequence	13	
Neuraminidase (NA) New Caledonia full length amino acid	14	

sequence		
Neuraminidase (NA) New Caledonia full length nucleotide sequence	15	'Stop' codon omitted
<i>PhexI</i> -ssCbh-NC-TMD- <i>TcbhI</i>	16	Construct used for transformation
<i>PhexI</i> -ssCbh-NC-TMD- <i>TcbhI</i> – complete vector sequence	17	
<i>PhexI</i> -ssCbh-NC-8G-T4 foldon amino acid sequence without ss	18	
<i>PhexI</i> -ssCbh-NC-8G-T4 foldon nucleotide sequence including ss	19	
<i>PhexI</i> -ssCbh-FL-8G-T4 foldon amino acid sequence without ss	20	
<i>PhexI</i> -ssCbh-FL-8G-T4 foldon nucleotide sequence including ss	21	
<i>PhexI</i> -ssCbh-NA NC VSAP- <i>TcbhI</i> amino acid sequence	22	Signal sequence corresponds to positions 1-17 of SEQ ID NO: 22; VSAP domain (tetramerization domain) corresponds to positions 18-59 of SEQ ID NO: 22; and NA ecto-domain corresponds to positions 62-449 of SEQ ID NO: 22.
Gla1 from C1, including ss, amino acid sequence	23	Signal sequence corresponds to positions 1-20 of SEQ ID NO: 23
GlaA from <i>Aspergillus niger</i> including ss amino acid sequence	24	Signal sequence corresponds to positions 1-18 of SEQ ID NO: 24
GlaA from <i>Aspergillus niger</i> including ss nucleotide sequence	25	Sequence encoding the signal sequence corresponds to positions 1-54 of SEQ ID NO: 25. 'Stop' codon is omitted. Codon usage should be taken into consideration when cloning for expression in C1.
EG2 from C1, including ss, amino acid sequence	26	Signal sequence corresponds to positions 1-16 of SEQ ID NO: 26
EG2 from C1, including ss, nucleotide sequence	27	Sequence encoding the signal sequence corresponds to positions 1-48 of SEQ ID NO: 27. 'Stop' codon is omitted.

		Codon usage should be taken into consideration when cloning for expression in C1.
GCN4 leucine zipper amino acid sequence	28	The leucine zipper corresponds to positions 252-254, 259-261, 266-268 and 273-275 of SEQ ID NO: 28.
T4 Foldon amino acid sequence	29	
T4 Foldon nucleotide sequence	30	
KEX2 cleavage site amino acid sequence	31	
FLAG-tag amino acid sequence	32	

The following examples are presented in order to more fully illustrate certain embodiments of the invention. They should in no way, however, be construed as limiting the broad scope of the invention. One skilled in the art can readily devise many variations and modifications of the principles disclosed herein without departing from the scope of the invention.

EXAMPLES

Example 1 – Expression of hemagglutinin (HA) and neuraminidase (NA) in *Myceliophthora thermophila* (C1)

Several series of expression constructs were designed for expression of recombinant HA proteins of various influenza virus strains in C1. A list of HA proteins tested in the study is detailed in **Table 1** below. The expression constructs are detailed in **Figures 1-4** (Figures 1-3 - constructs with HA; Figure 4 - constructs with NA. P=promoter, ss=signal sequence, T=terminator). The HA type or NA included in each construct is marked in boldface. A list of abbreviations used to describe the various elements in the constructs is provided in **Figure 5**. Production of the constructs is described in detail below under "Materials and Methods".

Table 1. HA proteins/viral strains

Abbreviation	Viral strain
NC	A/H1N1 A/New Caledonia /20/99
CA	A/H1N1 A/California /04/2009
UR	A/H3N2 A/Uruguay/716/07 (A/Brisbane/10/07-like)
FL	B/Florida/04/2006: B Yamagata lineage
PR	A/H1N1 A Puerto Rico/08/1934
Tex	A/ H3N2 A/Texas/50/2012

Initially, HAs were expressed under *cbh1* (cellobiohydrolase 1) or *chi1* (chitinase 1) promoter, fused to the well-secreted *A. niger* glucoamylase A (GlaA) as a carrier to facilitate secretion of the HAs into the extracellular medium. The HA genes were fused to the part of the *glaA* gene that encodes the catalytic domain of GlaA. A KEX2 cleavage site (VISKR) was designed in between the *glaA* and HA gene to obtain a separate HA protein after cleavage of the site in the Golgi apparatus. The constructs further included a C-terminal FLAG tag for detection and purification purposes. In this initial series of expression constructs, the HAs were expressed without their transmembrane domain (TMD) and cytoplasmic tail.

In a modified version of the expression constructs, the *hex1* (hexagonal peroxisome, Woronin body) promoter was tested, which is considered an early constitutive promoter, induced earlier compared to the other two promoters.

For HA New Caledonia, additional construct variants were made, as follows:

- Construct with *cbh1* or *chi1* promoter, without GlaA carrier protein, but with *cbh1* signal sequence for targeting to the ER.
- Construct with *chi1* promoter and Gla1 (C1 Glucoamylase 1) as a carrier protein.
- Construct with EG2 (Endoglucanase 2) as a carrier protein.
- Construct with *hex1* promoter, GlaA carrier protein and without a FLAG tag.

C1 host strains for expressing the constructs were selected based on a proteolytic stability assay. Briefly, HAs produced with a baculovirus expression system (Protein Sciences Corporation, Meriden USA) were tested for proteolytic stability in end-of-fermentation (EOF) culture medium of several candidate C1 host strains (see **Table 2** below). Less HA degradation was observed in the presence of the culture filtrates of the strains D240 (a high-cellulase (HC) strain) and D389 (a low-cellulase (LC) strain)

compared to the other strains that were tested. D240 and D389 were therefore selected as host strains for producing the recombinant HAs. In later experiments, D382 was also tested for recombinant expression of HA.

Transformation was carried out as described below under "Material and Methods".

5 Following transformation, transformants were collected and cultivated in 96-well plates. The medium was screened for expression of glucoamylase (if applicable) and hemagglutinin. Positive transformants were further cultivated in shake flasks and expression of HA was evaluated using blotting techniques and an HA activity assay. The results are summarized in **Figures 1A-B**.

10 Analysis of the obtained transformants revealed that recombinant HA_NC was better expressed in C1 when the FLAG-tag sequence was omitted. Without this tag high molecular weight species containing HA (130 – 160 kDa) were detected in the extracellular medium of shake flask cultivations, as shown on Western blot with a monoclonal antibody against HA_NC. Based on the signal strength this represents
15 approximately 30 mg/L in the culture medium if extrapolated to a large-scale fermentation, reaching a total extracellular protein level of 10 g/L. The expression level of HA_NC was higher in the LC strain than in the HC strain. In addition, high levels of HA were found intracellularly and/or cell-wall-associated, which is estimated to represent at least as much as what is found in the medium.

20 Highest expression was obtained with transformants containing a construct with the *hexI* promoter.

 A second series of expression constructs were designed, in which the presence of the native transmembrane domain (TMD) of HA or a recombinant C-terminal trimerization domain from T4 bacteriophage fibritin (T4 foldon domain) was tested. The
25 T4 foldon domain was added to facilitate stability of the HA upon extracellular secretion. A linker of eight Gly residues ("8xG") was cloned between the HA and T4 foldon domain. The TMD was added to test its effect on expression levels and activity of the obtained HA (an HA construct containing the TMD was not expected to be secreted, but rather found intracellularly or cell-wall associated). In addition, a modified KEX2 proteolytic site
30 containing a stretch of five Gly residues ("5xG") between HA and GlaA carrier was tested in order to test whether such modified linker would result in obtaining higher levels of separate HA protein. The promoter used for this series was *hexI*, based on the stronger expression obtained using this promoter in the previous series. The various constructs in

the series are detailed in **Figure 2A-2B**. **Figure 2C** illustrates an expression vector containing the construct *Phex1*-ssCbh-NC-TMD-*Tcbh1* (sequence of the expression vector is set forth as SEQ ID NO: 17). **Figure 2D** illustrates the fragment used for transformation.

As seen with the first series, better expression was achieved without the carrier
5 GlaA. When the carrier was present, only Gla-HA fusion proteins were visible, even when the modified proteolytic site between the two moieties was used. The presence of a C-terminal T4-foldon domain had a positive effect on the levels of HA detected extracellularly, however the resulting HA seemed to be inactive (negative results in the hemagglutination assay). To further evaluate the activity of HA fused to a T4 foldon
10 domain, HA expressed from the construct *Phex1*-ssCbh-NC-8xG-T4 foldon-*Tcbh1* was purified and tested in an immunogenicity assay in mice (see Example 2 below). No functional immunogenicity was observed.

The natural transmembrane domain of HA had a positive effect on expression levels (intracellular or cell wall-associated). In addition, the resulting protein was highly
15 active in the HA activity assay. To further evaluate the activity of HA with its TMD, HA expressed from the construct *Phex1*-ssCbh-NC-TMD-*Tcbh1* was purified and tested in an immunogenicity assay in mice (see Example 2 below). Functional immunogenicity was observed, which was particularly effective, as will be described in more detail below.

A third series of expression constructs were designed, in which HAs of different
20 types, including types that were not tested until now, were expressed under the *hex1* promoter with *cbh* signal sequence, no carrier, and with T4 foldon domain or TMD at the C-terminus. In this series the T4 foldon domain was cloned without the Gly linker or with modified linkers between the HA and the T4 foldon domain. In addition, two variants of neuraminidase (NA) were also tested. The various constructs in the series are detailed in
25 **Figures3-4**.

C1 was able to produce HAs of different types. It was demonstrated that between 50 – 100 mg of proteins per liter of each of the HA type could be produced by C1 strains. In one or more cases expression levels of ~ 300 mg per liter were achieved.

As seen with the second series, the presence of a C-terminal T4-foldon domain had
30 a positive effect on the levels of HA detected extracellularly, however the resulting HA seemed to be inactive (negative results in the hemagglutination assay), even with no linker or with modified linkers between the HA and T4 foldon. The natural transmembrane domain of HA again showed a positive effect on expression levels (intracellular or cell

wall-associated). In addition, the resulting proteins were highly active in the HA activity assay. Both type A and type B influenza proteins were successfully expressed and found to be biologically active.

With respect to NA, it was found that it is well expressed in C1 and can be detected
5 both extra- and intra-cellularly.

Materials and methods

HA protein standards

HA protein standards were obtained from Protein Sciences Corporation (Meriden,
10 USA). These proteins were produced in insect cells using the baculovirus expression vector system and purified to >90% purity under conditions that preserve the biological activity and tertiary structure. The protein standards were stored at 4°C as recommended by the manufacturer. In addition, a batch of egg-derived HA from A/H1N1 A/New Caledonia /20/99 (NC) was ordered from the National Institute for Biological Standards
15 and Control (NIBSC). This sample was aliquoted and stored at -20°C as recommended by the manufacturer.

C1 strains

Candidate C1 production hosts tested in the study are indicated in **Table 2**.
20 HC refers to a C1 High-Cellulase strain and LC to a C1 Low-Cellulase strain. Prt- refers to protease deficient, $\Delta alp1$ refers to gene disruption of the major secreted protease, $\Delta alp2$ refers to gene disruption of a highly expressed vacuolar protease and $\Delta chi1$ refers to gene disruption of the highly expressed extracellular C1 chitinase in C1. All strains are $\Delta pyr5$

25 **Table 2. C1 host strains**

C1 host strain	Deposit info.	Code
HC prt-		D202
HC prt- $\Delta alp1$	"UV18-100f" deposit no. CBS141147	D240
HC prt- $\Delta alp1 \Delta pep4$		D249
HC prt- $\Delta alp1 \Delta pep4 \Delta alp2, \Delta prt1$	"UV18-100f" deposit no. CBS141143	D270
LC prt- $\Delta alp1$		D326

LC prt- $\Delta alp1 \Delta chi1$	"W1L#100I" deposit no. CBS141149	D382
LC prt- $\Delta alp1 \Delta chi1 \Delta alp2$	"W1L#100I" deposit no. CBS141153	D389

Construction of expression vectors

The expression vector used herein for heterologous protein production in C1, pP-ssglaA-glaA-HA-FLAG-tag, is shown in **Figure 6**. This expression vector makes use of the well-secreted *A. niger* glucoamylase A protein as a carrier, facilitating the secretion of a heterologous protein into the extracellular medium. The HA genes were fused to the part of the *glaA* gene that encodes the catalytic domain of glucoamylase A of *A.niger*. A FLAG-tag sequence was fused C-terminally and incorporated for detection and purification purposes. The FLAG-tag is a short, hydrophilic 8-amino acid peptide with the sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK, SEQ ID NO: 32).

Generation of constructs of the first series

For HC transformation experiments the following basic cloning vector was used: pVJ1 (*Pcbh1_glaA_kex2_GeneX_Tcbh1*) digested with *EcoRV* – *EcoRI*. *EcoRV* is located in the *kex2*-site and *EcoRI* is located immediately after the stop codon of GeneX.

This vector was used to ligate the following synthetic gene fragments, codon-optimized for C1 based on 43 well expressed genes, secreted into to the culture medium (all synthetic fragments were sub-cloned by GeneArt® in a pMK or pMS shuttle vector):

- HA_NC_FLAG-tag x *EcoRV* – *EcoRI* (1577bp)
- HA_UR_FLAG-tag x *EcoRV* – *EcoRI* (1586bp)
- HA_FL_FLAG-tag x *EcoRV* – *EcoRI* (1655bp)
- HA_NC x *EcoRV* – *EcoRI* (1553bp)

For LC transformation experiments the following basic cloning vector was used: pVJ6 (*Pchi1_glaA_kex2_Gene-of interest_Tcbh1*) digested with *EcoRV* – *EcoRI*. *EcoRV* is located in the *kex2* site and *EcoRI* is located immediately after the stop codon of the gene of interest.

This vector was used to ligate the following synthetic gene fragments: (all synthetic fragments were sub-cloned by GeneArt® in a pMK or pMS shuttle vector).

- HA_NC_ FLAG-tag x *EcoRV* – *EcoRI* (1577bp)
- HA_UR_ FLAG-tag x *EcoRV* – *EcoRI* (1586bp)

- HA_FL_ FLAG-tag x *EcoRV* – *EcoRI* 1655bp)

- HA_NC x *EcoRV* – *EcoRI* (1553bp)

For HA New Caledonia, additional construct variants were made, as follows:

- * Vector with *cbhI* promoter or *chil* promoter, without GlaA carrier protein, but
5 with *cbhI* signal sequence for targeting to the ER. This vector was generated as follows:

Cloning vector pCBHProm was digested with *SacI* – *BspHI* and the 1819bp fragment, containing the *cbhI* promoter, was isolated.

Cloning vector pVJ1 was digested with *SacI* – *EcoRI* and the 3896bp fragment, containing the *cbhI* terminator and pUC backbone, was isolated.

- 10 These two fragments were ligated in a three way ligation with the synthetic fragment digested with *PciI* – *EcoRI* (1617bp) containing the *sscbhI*-HA_NC_ FLAG-tag.

- Cloning vector pP*chil*-linker-T*cbhI* was digested with *NcoI* – *BspHI* and the 5758bp fragment, containing the *cbhI* promoter – *cbhI* terminator and pUC backbone, was isolated. The vector was used in a ligation with the synthetic fragment digested with
15 *PciI* – *EcoRI* (1617bp) containing the *sscbhI*-HA_NC_ FLAG-tag.

* Vector with *chil* promoter and Gla1 (C1 homologue) as a carrier protein, generated as follows:

- Cloning vector pP*chil*-Gla1-XynB was digested with *BsrGI* – *EcoRI* and the 6609bp fragment, containing the *chil* promoter, part of the Gla1 carrier protein, and *cbhI*
20 terminator, was isolated.

Cloning vector pP*chil*-Gla1-XynB was digested with *BsrGI* – *MabI* and the 783bp fragment, containing part of the Gla1 carrier protein, was isolated.

- These two fragments were ligated in a three way ligation with the synthetic fragment digested with *MabI* – *EcoRI* (1723bp) containing the part of Gla1, a KEX2 site,
25 and HA_NC_ FLAG-tag.

* Vector with EG2 as a carrier protein, generated as follows:

Cloning vector pP*cbhI*_EG2-T*cbhI* was digested with *SacI* – *PciI* and the 3031bp fragment, containing the *cbhI* promoter and part of the EG2 carrier protein, was isolated.

- Cloning vector pVJ1 was digested with *SacI* – *EcoRI* and the 3896bp fragment,
30 containing the *cbhI* terminator and pUC backbone, was isolated.

These two fragments were ligated in a three way ligation with the synthetic fragment digested with *PciI* – *EcoRI* (1669bp) containing the part of EG2, a KEX2 site, and HA_NC_ FLAG-tag.

* Vector with 'early constitutive' promoter *hexI*, GlaA carrier protein and HA_NC without FLAG-tag, generated as follows:

Cloning vector pTcV1011 digested with *SacI* – *EcoRV* and the 1643bp fragment, containing the *hexI* promoter, GlaA carrier protein, and part of the KEX2 site, was
5 isolated.

Cloning vector pVJ1 was digested with *SacI* – *EcoRI* and the 3896bp fragment, containing the *cbhI* terminator and pUC backbone, was isolated.

These two fragments were ligated in a three way ligation with the synthetic fragment digested with *EcoRV* – *EcoRI* (1553bp) containing the *sscbhI*-HA_NC.

10 All vectors were generated in XL1-blue (Stratagene). From all vectors cloning junctions were verified by sequence analysis.

Generation of constructs of the third series

The HA or NA expression cassettes were excised from their vectors using NotI.
15 The *pyr5* selection marker was excised from its vector (DNL35) using BglII. Both fragments were separated from plasmid backbone and purified from gel using the Wizard® SV Gel and PCR Clean up System (Promega). C1 hosts D389 and D382 were co-transformed with a single HA or NA expression cassette and the *pyr5* marker.

Transformation and microtiterplate (MTP) cultivations

The expression vectors were digested with NotI to generate a foreign-DNA free expression cassette. These expression cassettes were co-transformed with the *pyr5* marker in *pyr5*-deficient C1 strains.

Purification streaks of 96 transformants of each strain were made on minimal
25 medium plates containing sucrose and incubated for 4 days at 35°C. Pure colonies were transferred to a 96-well plate containing Caylase medium (mother plate) and incubated at 35°C. After 3 days 3µl of culture per well was transferred to new 96 well plates (daughter plates) containing production medium ((NH₄)₂SO₄, 35 mM, NaCl, 7 mM, KH₂PO₄, 55 mM, Glucose, 0.5 %, MgSO₄, 2 mM, trace element solution, casamino acids, 0.1 %, 30 biotin, 4 µg/L, penicillin 20 g/L, streptomycin 50 g/L, 10 mM. pH set to 5.5 with 10M KOH) and plates were incubated for 72-96 hours. Supernatants were subsequently assayed for the required target expression or enzyme activity. For some constructs, the daughter plate medium used was either production medium or adapted inoculum medium (aIM), the

growth temperatures were 35°, 30°C or a combination of 35°C followed by 30°C, and daughter plates were incubated for 48 and 72 (aIM) hrs.

In case of TMD-containing antigens, 100µl of each culture (well) was transferred from the daughter plate wells to the wells of a 96-wells PCR plate. This was centrifuged
5 for 15 minutes at 4000 rpm. The culture liquid was removed and 100 µL of extraction buffer (50 mM Tris-Cl pH 7.5, 1 mM EDTA, 1% SDS, 0.2% CHAPS) was added per well. This was mixed well and incubated for 5 minutes at 96°C. The plate was subsequently centrifuged for 15 minutes at 4000 rpm and the supernatants transferred to a new 96-wells plate. Twenty µL of this supernatant was used to spot onto PVDF membranes. Spot blots
10 were subsequently blocked with BSA and screened using appropriate antibody. HRP conjugated secondary antibody substrate SuperSignal™ West Dura extended Duration Substrate (34075; ThermoFisher Scientific) was used to visualize bound antibody. Images were made using the Bio-Rad ChemiDoc. Selected transformant culture samples, either microtiter plate or shake flask derived, were further studied using SDS-PAGE and western
15 blotting analyses according to standard techniques. The western blots were screened using the same antibody in a similar way as conducted for the spot blots.

Shake flask cultivation conditions

Shake flask cultivation experiments were carried out in 300 ml flasks containing 50
20 ml medium. LC and HC were standard cultivated in production medium as described above, containing ammonium as nitrogen source and 0.5% glucose. Both strains were also tested in minimal medium (MM) and complete medium (CM)) containing besides 1% glucose, nitrate as nitrogen source, 0.1% casamino acids (in case of CM), 0.5% YE (in case of CM) (and vitamins). Cultivation was carried out at 250 rpm (1 inch / orbit) and
25 35°C.

Spotblot analysis

Most of the transformants obtained were screened by spotblot analysis. After microtitre plate culture, plates were harvested by centrifugation and supernatants were transferred to new 96-well plates. For LC strains 25µl of supernatant was used and for HC
30 strains 12.5µl was used. Samples were denatured at 96°C for 5 min. cooled on ice and transferred to PVDF membrane. Spotblots were stained with mAb αGlaA or mAb αHA_NC (AbCam ab66189).

SDS-PAGE and Western analysis

SDS-PAGE and Western blotting was carried out according to standard procedures. Development of Western blots with alkaline phosphatase was carried out with NBT and BCIP as substrates. Development of Western blots with Horse radish peroxidase was carried out with ECL detection reagents according to supplier (Invitrogen: Novex®ECL #WP20005). The following protocol was used for stripping of the developed Western blots (Mild stripping procedure according to AbCam):

Fresh stripping buffer was prepared (1 L: 15 g glycine, 1 g SDS, 10 ml Tween20, adjust pH to 2.2) and the membrane incubated in the stripping buffer for 10 min at RT (shaking). The buffer was discarded and the procedure repeated with fresh stripping buffer. Next, the membrane was washed twice with PBS and twice with TBS-Tween20.

2D Gel electrophoresis

Prior to 2D SDS-PAGE, samples were 10 – 20 x concentrated using Amicon Ultra 0.5 ml 10K centrifugal filters, subsequently samples were desalted using BioRad micro Biospin 6 chromatography columns, and protein concentration was determined using BCA assay (Pierce). Protein samples were purified using BioRad ReadyPrep 2D cleanup kit according to supplier. After purification, protein pellets (~200 µg) were rehydrated in 200 µl 2-D rehydration buffer 1 (BioRad) containing: 7M Urea, 2M Thiourea, 4% CHAPS, 50 mM DTT, 0.2% Bio-Lyte 3/10 ampholyte (20%), and 0.002% Bromophenol Blue. Rehydrated protein samples were loaded on BioRad Ready Strip IPG strips (11 cm pH 4-7) and incubated O/N at RT. After complete rehydration of IPG strips, the strips were focused according to the supplier (BioRad). The second dimension was run on SDS-PAGE Criterion TGX 4-15% gradients gels (BioRad). Gels were either used for Coomassie Brilliant Blue staining or protein was transferred to PVDF.

Hemagglutination assay

Normally, influenza virus particles have HA on their surface that binds to sialic acid receptors on cells. The virus binds to erythrocytes (red blood cells), causing the formation of a lattice. This property is called hemagglutination. If functional HA is present in a sample containing red blood cells, the lattice is formed which is visible as "staying in solution" instead of precipitation of the red blood cells to the bottom of the vessel.

For rapid detection of HA secretion into the culture medium, cultures were tested for hemagglutinin activity by adding (filtered) fungal culture supernatant, serial diluted [in 1xPBS (without Ca^{2+} and Mg^{2+}), 50 μl final amount] in V bottom 96-well plates, to an equal amount (50 μl) of washed 0.5% chicken red blood cells in 1x PBS (without Ca^{2+} and Mg^{2+}). Plates were then incubated at RT for 1 h. Samples were compared to a known amount of a purified HA standard (Protein Sciences Corp. or NIBSC).

EG2 activity assay

EG2 activity was measured by an azo-CMCase assay (MegaZyme), according to the manufacturer's instructions.

BCA assay

The protein content was quantified by a BCA assay (Pierce), according to the manufacturer's instructions.

Purification of secreted HA

Following shake flask culture the fermentation broth was centrifuged and the supernatant collected. A 35 ml sample of the supernatant was concentrated using Millipore Amicon Ultra filter (with a cut off of 10 kDa) to a total volume of 300 μl (>100 x concentrated). Prior to loading on a column, the sample was cleared by centrifugation (1 min. at 14000 rpm). Gel filtration was performed in PBS and a 100 μl sample was loaded onto a 24-ml Superdex 200 column. Sample was processed with flow rate of 1 ml/min, and fractions of 0.5 ml were collected (ÄKTA Explorer system 1). Fractions were analyzed on Western blot with a monoclonal antibody against HA_NC.

Purification of TMD-containing HA

TMD-containing HAs were extracted from mycelium fragments using an extraction buffer and subsequently purified using AEX-Capto Q ImpRes. See **Figure 7** for the purification procedure.

Deglycosylation experiments

Both extracellular and intracellular fractions were used in deglycosylation experiments. EndoH (Endo- β -Nacetylglucosaminidase; Roche 11 088 726 001)

deglycosylation was performed under conditions as recommended by the supplier. Therefore, reactions were performed O/N at 37°C in a buffer containing: 20 mM NaOAc pH 5.5, 0.5 mM PMSF (phenylmethylsulfonylfluoride), 0.1 M β -mercaptoethanol and 0.02% SDS. Prior to adding 10 mU EndoH the protein samples were denatured for 10 min. at 96°C. Samples were analyzed by SDS-PAGE and Western blotting and stained with the mAb against HA_NC (AbCam ab66189). PNGaseF (Peptide N-glycosidase F from *Elizabethkingia miricola*; Sigma-Aldrich G5166) deglycosylation was performed under similar conditions as EndoH deglycosylation with the only difference that Triton X-100 is added to a final concentration of 0.1%. After denaturation of protein samples 5 U of PNGaseF was added and samples were incubated O/N at 37 °C. Samples were analyzed by SDS-PAGE and Western blotting and stained with the mAb against HA_NC (AbCam ab66189).

In vitro KEX2 processing

Both extracellular and intracellular fractions were used in a KEX2 processing experiments. Samples were tested both in native- and denatured form. KEX2 protease protein derived from *Saccharomyces cerevisiae*, produced in High-5 insect cells (AbCam; ab96554). KEX2 protein cleavage was performed under the following conditions: Fractions were incubated in 50 mM Tris/HCl pH=7.5, 5 mM CaCl₂ 0.5 mM PMSF and 0.1% Triton X-100. Protein samples were either incubated in native or denatured form with 80 mU KEX2 for 4 hrs at 37 °C. Samples were analyzed by SDS-PAGE and Western blotting and stained with the mAb against HA_NC (AbCam ab66189) and the monoclonal against GlaA.

Trypsin digestions

Partial trypsin digestions were performed at RT in 50 mM NaOAc pH=5.5 buffer. Extracellular sample was mixed with assay buffer and trypsin [TPCK trypsin (Pierce); stock solution is 50 mg/ml (aliquots are stored at -70°C). Working solution is 50 ng/ μ l]. Samples were taken at t = 0, t = 2', t = 5', t = 10', t = 15', t = 20', t = 30', t = 45', and t = 60'. Digests were stopped by adding 6x SDS-PAGE loading buffer and denatured at 96°C for 5 min. Samples were analyzed by SDS-PAGE and Western blotting and stained with the mAb against HA_NC (AbCam ab66189) and the monoclonal against GlaA.

Fungal mycelium extracts

The fractionation study used to distinguish between HA localized intracellularly or cell-wall attached was carried out as schematically shown in **Figure 8**. Briefly, extracellular medium (Ex) from shake flask cultures was collected (the amount measured) and the mycelium harvested. Mycelium was washed with cold fresh production medium, weighed and divided into equal portions in wash buffer with or without SDS. This wash step was carried out for 1 hr at RT and wash medium was analyzed on SDS-PAGE. Grinded mycelium was resuspended in the same g/volume ratio extraction buffer as in the original shake flask culture, left in a tube for 10 minute on a rotating platform at RT and subsequently the tube was centrifuged for 5 minutes at 14000 rpm. The supernatant was analyzed on SDS-PAGE. The remaining pellet was resuspended again in the same volume of extraction buffer, now with Triton-X100 added to solubilize the membranes. After removal of the supernatant by centrifugation the final cell pellet was resuspended in SDS-PAGE sample buffer.

Preparation of fungal protoplasts

C1 LC cultures were grown for 2 days in Complete medium as described above. Protoplasts were generated and purified. An extra wash step was performed to reduce the risk of cell wall fragments contaminating the protoplasts.

Example 2 – Immunogenicity of HA produced in *Myceliophthora thermophila* (C1)

Animal tests were carried out to test immunogenicity of a full length recombinant hemagglutinin protein from the A/NewCaledonia/20/99 (H1N1) influenza strain possessing the transmembrane domain (rHA-TMD) produced in C1. Biochemical evaluation of rHA-TMD is shown in **Figure 9**, showing SDS-PAGE, native PAGE and analysis of oligomeric state using TEM Negative staining. The magnification in the TEM image is x29000. Scale bar=200 nm. The white arrow points to a ~40 nm oligomer. The white circle marks a ~15 nm oligomer.

A preceding immunogenicity study was conducted with a secreted form of rHA from the same viral strain produced in C1. The transmembrane domain of this rHA is truncated and it contains additional domains not found in a native HA (an 8-Gly domain and a T4 foldon domain). The rHA-8Gly-T4 construct did not induce functional antibody

responses. It was then proposed that a construct with the transmembrane domain (rHA-TMD) could induce a better immunogenicity.

To this end, eight groups of 8 Balb/C ByJ mice received two intramuscular (IM) injections, given four weeks apart, of 3-fold escalating dosages - ranging from 1 to 30 µg - of rHA-TMD produced in C1. As negative control, 5 mice were immunized with PBS according to the same immunization schedule. Blood samples were collected on Day 27 and Day 49 for antibody response analysis by hemagglutination inhibition (HI) assay.

As will be described in more detail below, the results showed that as early as following a single injection in mice of full length rHA prepared in C1, specific functional antibody responses were induced that were further enhanced following the second injection.

Materials and methods

Tested compositions

Table 3 lists the compositions that were tested in the study. Protein contents quantified by Bradford technique was used for injection dose preparation. All compositions were stored at $+5^{\circ}\text{C} \pm 3^{\circ}\text{C}$ until use.

Table 3. Tested compositions

Composition	Description	Concentration /Purity	Endotoxin Content* (EU/mL)	Purity
rHA-TMD C1	Recombinant HA from A/New Caledonia/20/99 (H1N1) with transmembrane domain produced in C1	BCA (BRD sp): 791 µg/mL µBradford (ARD sp): 627 µg/mL Purity: 80%	12	≥96%
PBS (Eurobio)		N/A		

*Endosafe testing at sp

Animal information

Animal species: mice

Status: SPF

Strain: Balb/c ByJ mouse

Supplier: Charles River

- Age: 9 weeks on D0
- Sex: Female
- Weight: 20-22 g
- Individual identification by coloration
- 5 Care and maintenance: daily

Housing

- Location: 4 animals / cage for all groups / cage in an air-conditioned building complying with L2 biosafety requirements.
- 10 Nb of animals / cage: 4
- Diet: granulated food (M20, SDS, DIETEX France, St Gratien, France)
- Water: Tap water, *ad libitum*, via an automatic watering system
- Quarantine: N/A
- Acclimation: Mice assigned for the study were acclimated to their designed
- 15 housing for 5 days before immunization.

Group definition

The group definition is summarized in the **Table 4**.

20

Table 4. Groups

Group	Test composition		Injections at W0 and W4	
	Name	Quantity	Route	Volume injected (100 µL / injection)
A (8 mice)	rHA-TMD C1	1µg	IM	2 x 50µL
B (8 mice)	rHA-TMD C1	3.33µg	IM	2 x 50µL
C (8 mice)	rHA-TMD C1	10µg	IM	2 x 50µL
D (8 mice)	rHA-TMD C1	30µg	IM	2 x 50µL
M (5 mice)	PBS	-	IM	2 x 50µL

Study schedule

The study schedule is detailed in the **Table 5**.

Table 5. Study schedule

Day	Group number	Number of animals	Interventions	Specific characteristics
D(-1)	A to M	88	Weighing and shaving	N/A
D0	A to M	88	Prime IM vaccination	Immunization with rHAs
D27	A to M	88	Weighing, blood sampling	Blood sampling at submandibular vein
D28	A to M	88	Boost IM vaccination	Immunization with rHAs
D49	A to M	88	Blood samplings and animal sacrifice	Final bleeding by exsanguination Animal sacrifice by cervical dislocation

5

Clinical monitoring

Animals were observed daily on working days (from Monday to Friday) following the immunizations and weighted on days -1 and 27.

10

Biological sampling

Blood samples were taken under isoflurane anesthesia from the retro-orbital sinus (ROS) or submandibular vein at D27 and after exsanguination by carotid section at D49 from all the animals. At D49, the anesthesia was performed by Imalgène (1.6 mg of Ketamine) and Rompun (0.32 mg of Xylazine) administrated under a volume of 200µL by intraperitoneal route.

15

For humoral response assays, at D27, 200 µL of blood were collected in vials containing clot activator and serum separator (BD Microtainer SST ref 365951). After two hours at +37 °C, the blood was centrifuged at 10,000 rpm for 5 minutes and sera were stored at -20 °C until analysis.

20

At D49: 1mL of blood was collected in vials containing clot activator and serum separator (BD Vacutainer SST II Advance ref 367957). After 2 days at +5°C, the blood was centrifuged at 2000g during 20 minutes and serum was stored at -20°C until analysis.

Hemagglutination inhibition (HI) assay

The assay is based on the ability of influenza virus to agglutinate red blood cells. A serum containing functional antibodies against HA inhibits the hemagglutination activity. In the assay, sera from influenza-immunized animals are titrated with influenza virus in order to characterize the concentration of functional anti-HA antibodies.

Reagents:

- 96-well V-bottom plates, (NUNC)
- RDE (Receptor Destroying Enzyme): Neuraminidase from *Vibrio cholerae* Type III at 10 mU/mL (Sigma)
- PBS without Ca⁺⁺ and Mg⁺⁺ (Gibco)
- TPCK trypsin (Thermo Scientific)
- Chicken red blood cells ("cRBCs"): 10% in PBS for serum treatment, 0.5% in PBS for HI assays (Sanofi Pasteur)
- Influenza strain A/New Caledonia/20/99, clarified allantoic fluid 1900 Hemagglutination Unit (HAU)/50 μ L

The titration is performed as follows: Serial dilutions (2 fold) of the virus were performed in PBS in order to calibrate the viral suspension and obtain a concentration of 4 HAU/50 μ L. Calibrated virus (50 μ L) was then added to a V-shaped 96 well plate on 50 μ L of serum serial dilutions (2 fold) in PBS starting from 1/10, and incubated one hour at room temperature. Chicken red blood cells (0.5% in PBS) (50 μ L) were then added to each well and inhibition of hemagglutination (red point) or hemagglutination (pink network) was visually read after one hour at room temperature.

In order to eliminate serum non-specific inhibitors directed against the HA, each serum was treated prior to the HI assay with a receptor-destroying enzyme (RDE), chicken red blood cells and TPCK trypsin. Briefly, 10 mU/mL of RDE was added to each serum (5 volume of RDE for 1 volume of serum). The mix was then incubated 18 h at +37 °C, followed by 1h inactivation at +56 °C. To cool, the mixture "serum-RDE" was placed between 30 min to 4 hours at +4 °C. The "serum-RDE" mixture was then absorbed on 10 % cRBCs in PBS for 30 min (5 volumes of cRBCs for 1 volume of serum), at room temperature, and then centrifuged at +5°C, 10 min at 700 g. The supernatant was collected and the trypsin treatment was carried out by mixing 10 volumes of RDE-RBC –heat inactivated serum (dilution serum 1:10) with 1 volume of 0.4 % trypsin (w/v in saline) at

+56 °C for 30 min. The HI was then performed and the final serum dilution still considered at 1:10.

Serial dilutions (2 fold) of the virus were performed in PBS in order to calibrate the viral suspension and obtain a concentration of 4 HAU/50 µL. Calibrated virus (50µL) was then added to a V-shaped 96 well plate on 50µL of serum serial dilutions (2 fold) in PBS starting from 1/10, and incubated one hour at room temperature. Chicken red blood cells (0.5% in PBS) (50 µL) were then added to each well and inhibition of hemagglutination (red point) or hemagglutination (pink network) was visually read after one hour at room temperature.

The HI titer value is the inverse of the last dilution of serum that completely inhibited hemagglutination. A value of 5, corresponding to half of the initial dilution (1/10), was arbitrary given to all sera determined negative in order to perform statistical analysis.

The absence of non-specific agglutinins was controlled for each serum (4 serial dilutions of each serum and cRBCs without virus). A control of the cRBCs (only cRBCs and PBS) and a control of the presence of 4 HAU of the working dilution of virus were performed on each plate.

Observations and deviations

At D28, the day after blood sampling from the submandibular vein, a mouse from group A was found sick likely because it did not recover from this intervention and was thus euthanized.

Results

Clinical monitoring

An increase of mean body weight was observed in all groups from D1 to D27.

Humoral Response

The antibody responses elicited against A/New Caledonia/20/99 (H1N1) were measured in individual sera collected from all animals at D27 and D49 by HI assay. The results are presented in **Figure 10A-10B**.

After the first injection of rHA, no or low HI responses were induced at the two lowest doses of C1-rHA, precluding statistical analysis. Still, the results strongly suggested that C1-rHA indeed induced antibody responses.

Following the booster injection, the responses were further enhanced. Significant
5 dose-dependent HI effects were induced by C1-rHA with mean HI titers ranging from 108 to 830 for 1 and 30 µg dosages, respectively.

It is noted that the full length recombinant HA produced in C1 did not induce any negative clinical signs in the mice.

C1 can easily produce levels of 1 g/L of HAs and other antigens in 5 days
10 fermentation therefore:

In seasonal influenza vaccine—total doses distributed = 146M/year

Each 0.5 mL dose is formulated to contain: 15 µg of HA for each strain.

Thus, 3 X 1000L scale fermentation runs will be able to supply the annual global
HA/strain needs against Influenza of 2,175 g.

15

Example 3 – Stirred-tank fermentation of HA

Experiments were carried out to measure production levels of the rHA-TMD described above on a larger scale, in a stirred-tank fermenter with batch and fed-batch technologies. To this end, MiniforsTM 3L bioreactors were used to culture C1 strain D389
20 expressing the rHA-TMD.

Table 6 summarizes the fermentations conditions of an initial set of experiments, carried out for 50 hrs. "Batch" - concentration of the indicated carbohydrate at the beginning of fermentation. "Fed-batch" - concentration of the carbohydrate in the feed. Fermentations were carried out at pH 7.5 with starting volume 1.5 liters.

25

Table 6. Fermentation conditions 50 hrs

Run #	Temp. (°C)	Batch	Fed-batch
R465F2	25	4% xylose	none
R465F4	35	1% xylose	47% xylose.
R465F5	35	1% glucose	50% glucose.
R465F6	35	4.7% glucose	none

Following fermentation, mycelia were collected and HA was extracted.

To evaluate mycelia concentration, the mycelial dry weight was measured. In brief, a certain amount of the fermentation broth was collected and washed a few times to remove media and media components, such as fermentation intermediates and secreted proteins. The resulting material was then dried for 24 h at 90°C and weighted. All fermentation samples were found to contain about the same mycelium concentration.

Quantification of HA was carried out by comparing Western blot signals obtained for the extracted HA in comparison to HA standards of known amounts (**Figure 13**).

The production level of the best batch was calculated to be approximately 375 mg/l which is 170 mg/l/day

Table 7 summarizes the fermentations conditions and resulting HA production levels of a second set of experiments, carried out for 98-137 hrs. The table shows end-of-fermentation (EOF) results.

Table 7. Fermentation conditions and HA production level

Run #	Time (h)	Temp (°C)	C-source	Feed rate (%)	DM* (g/L)	HA (mg/L)	HA** (mg/g DM)	C-source (g/L)
R481F1	98	35	glucose	1	37.9	47.3	1.2	0.8
R481F2	98	35	xylose	1	34.0	35.0	1.0	0.5
R481F5	98	30	xylose	1	25.0	62.9	2.5	66.9
R481F6	98	35-25	glucose	1	33.6	29.0	0.9	17.2
R481F7	98	35-25	xylose	1	13.3	27.6	2.1	42.2
R482F1	102	35	glucose	1	56.6	50.6	0.9	0.1
R482F2	102	30	glucose	1	61.1	49.0	0.8	0.2
R483F1	140	30	glucose	1	47.6	124	5.4	0.1
R483F2	140	30	xylose	1	46.7	171.4	3.7	0.1

R483F4	140	35-25	glucose	1	56.5	62.8	1.1	0.1
R483F5	140	35-25	xylose	1	51.7	86.0	1.7	1.2
R484F2	137	30	glucose	1	48.9	51.8	1.2	0.1
R484F5	137	35	glucose	2	33.6	106.8	4.0	6.5
R484F6	137	35	xylose	2	56.7	169,6	3.6	12.8
R484F7	137	30	glucose	2	76.6	413.4	5.4	0.1

* Dry cell mass

** Estimated.

- 5 The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and therefore, such adaptations and modifications should and are intended to be comprehended within the
- 10 meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed chemical structures and functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A *Myceliophthora thermophila* C1 genetically-modified to produce an influenza virus surface protein, comprising an expression construct comprising a nucleic acid sequence encoding the influenza virus surface protein operably linked to at least one C1 regulatory sequence, wherein the influenza virus surface protein comprises its ectodomain and transmembrane domain and is expressed in the C1 as a membrane-bound protein.
2. The C1 of claim 1, wherein the influenza virus surface protein is hemagglutinin (HA).
3. The C1 of claim 2, wherein the expression construct further comprises a nucleic acid sequence encoding a C1 signal peptide linked in-frame to the nucleic acid sequence encoding the HA.
4. The C1 of claim 2, wherein the HA subtype is selected from the group consisting of influenza A-H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16; and influenza B subtype.
5. The C1 of claim 2, wherein the HA subtype is a subtype infecting humans selected from influenza A subtypes H1, H2 and H3; and influenza B subtype.
6. The C1 of claim 2, wherein the HA is from an influenza virus strain selected from the group consisting of A/New Caledonia /20/99 (H1N1), A/California/04/2009 (H1N1), A/Uruguay/716/07 (H3N2) (A/Brisbane/10/07-like), B/Florida/04/2006: B Yamagata lineage, A/Puerto Rico/08/1934 (H1N1), and A/Texas/50/2012 (H3N2).
7. The C1 of claim 1, wherein the at least one C1 regulatory sequence comprises a C1 promoter.
8. The C1 of claim 7, wherein the C1 promoter is selected from the group consisting of: *hexI* (Woronin body), *cbhI* (cellobiohydrolase 1) and *chiI* (chitinase 1) promoters.
9. The C1 of claim 7, wherein the C1 promoter is *hexI* promoter.

10. The C1 of claim 3, wherein the C1 signal peptide is a signal peptide derived from a protein selected from the group consisting of Gla1 (Glucoamylase 1, C1), GlaA (Glucoamylase A, *Aspergillus*) and Cbh1 (Cellobiohydrolase 1, C1).
11. The C1 of claim 10, wherein the C1 signal peptide is derived from Cbh1.
12. The C1 of claim 1, wherein the expression construct comprises: *hex1* promoter operably linked to a nucleic acid sequence encoding a Cbh1 signal peptide fused to HA.
13. The C1 of claim 12, wherein the HA is from an influenza virus strain selected from the group consisting of A/New Caledonia /20/99 (H1N1), A/California/04/2009 (H1N1), B/Florida/04/2006: B Yamagata lineage, A/Puerto Rico/08/1934 (H1N1), and A/Texas/50/2012 (H3N2).
14. The C1 of claim 1, wherein the influenza virus surface protein is a neuraminidase (NA).
15. The C1 of claim 14, wherein the NA subtype is selected from the group consisting of influenza A- N1, N2, N3, N4, N5, N6, N7, N8 and N9; and influenza B subtype.
16. The C1 of claim 14, wherein the NA subtype is a subtype infecting humans selected from influenza A subtypes N1 and N2; and influenza B subtype.
17. The C1 of claim 14, wherein the expression construct comprises: *hex1* promoter operably linked to a nucleic acid sequence encoding NA.
18. The C1 of claim 17, wherein the NA is from the influenza virus strain A/New Caledonia /20/99 (H1N1).
19. The C1 of claim 1, wherein the C1 strain is selected from the group consisting of: W1L#100I (prt- $\Delta alp1 \Delta chi1 \Delta alp2 \Delta pyr5$) deposit no. CBS141153, UV18-100f (prt- $\Delta alp1, \Delta pyr5$) deposit no. CBS141147, W1L#100I (prt- $\Delta alp1 \Delta chi1 \Delta pyr5$) deposit no. CBS141149, and UV18-100f (prt- $\Delta alp1 \Delta pep4 \Delta alp2, \Delta prt1 \Delta pyr5$) deposit no. CBS141143.
20. A method for producing an influenza virus surface protein, the method comprising culturing the *Myceliophthora thermophila* C1 of claim 1 under conditions suitable for

expressing the influenza virus surface protein; and recovering the influenza virus surface protein.

21. The method of claim 20, wherein recovering the influenza virus surface protein comprises extraction from mycelia.
22. An expression construct for expressing a membrane bound influenza virus surface protein in *Myceliophthora thermophila* C1, the expression construct comprising at least one C1 regulatory sequence operably linked to a nucleic acid sequence encoding an influenza virus surface protein, wherein the influenza virus surface protein comprises its ectodomain and transmembrane domain.
23. A substantially pure, recombinant influenza virus surface protein produced by the modified *Myceliophthora thermophila* C1 of claim 1, wherein the influenza virus surface protein is purified to 95% purity or greater and is active and immunogenic, and induces a protective immune response when used as a vaccine.
24. An influenza vaccine composition comprising an influenza virus surface protein produced the modified *Myceliophthora thermophila* C1 of claim 1.

	Construct	Strain	Expression		Remarks 1	Remarks 2	HA Assay
			Gla1/A	HA			
	<i>Pchi1</i> -ssGla1-Gla1-Kex2-NC-Flag- <i>Tcbh1</i>	D389	+++++	+/-	Only Gla-HA fusion product visible		-
	<i>Pchi1</i> -ssGla1-Gla1-Kex2-NC-Flag- <i>Tcbh1</i>	D382	+++++	-			-
	<i>Pcbh1</i> -ssGlaA-GlaA-Kex2-NC-Flag- <i>Tcbh1</i>	D240	+++++	-			-
	<i>Pchi1</i> -ssGlaA-GlaA-Kex2-NC-Flag- <i>Tcbh1</i>	D389	+++++	+	Only Gla-HA fusion product visible		-
	<i>Pchi1</i> -ssGlaA-GlaA-Kex2-NC-Flag- <i>Tcbh1</i>	D382	+++++	-			-
	<i>Pcbh1</i> -ssGlaA-GlaA-Kex2-NC- <i>Tcbh1</i>	D240	+++++	+	Only Gla-HA fusion product visible		-
	<i>Pchi1</i> -ssGlaA-GlaA-Kex2-NC- <i>Tcbh1</i>	D389	+++++	++	Only Gla-HA fusion product visible	Mainly intracellular accumulation	-
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-NC- <i>Tcbh1</i>	D389	+++++	+++	Only Gla-HA fusion product visible	Mainly intracellular accumulation	-
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-NC- <i>Tcbh1</i>	D240	+++++	+	Only Gla-HA fusion product visible		-

Figure 1A

Construct	Strain	Expression		Remarks 1	Remarks 2	HA Assay
		Gla1/A	HA			
<i>Pchl1</i> -ssCbh-NC-Flag- <i>Tcbh1</i>	D389		-			-
<i>Pcbh1</i> -ssCbh-NC-Flag- <i>Tcbh1</i>	D240		-			-
<i>Pcbh1</i> -ssEg2-Eg2-NC-Flag- <i>Tcbh1</i>	D240	Eg2 +++	-			-
<i>Pcbh1</i> -ssGlaA-GlaA-Kex2- UR -Flag- <i>Tcbh1</i>	D240	+++++	-			-
<i>Pchl1</i> -ssGlaA-GlaA-Kex2- UR -Flag- <i>Tcbh1</i>	D389	+++++	-			-
<i>Pcbh1</i> -ssGlaA-GlaA-Kex2- FL -Flag- <i>Tcbh1</i>	D240	+++++	-			-
<i>Pchl1</i> -ssGlaA-GlaA-Kex2- FL -Flag- <i>Tcbh1</i>	D389	+++++	-			-

Figure 1B

	Construct	Strain	Expression		Remarks 1	Remarks 2	HA Assay
			Gla1/A	HA			
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-NC-8xG-T4 foldon- <i>Tcbh1</i>	D389	+++++	+	Only Gla-HA fusion visible		-
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-NC-TMD- <i>Tcbh1</i>	D389	+++++	-		Intra-cell. not checked	
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG-NC-8xG-T4 foldon- <i>Tcbh1</i>	D389	+++++	+	Only Gla-HA fusion visible		-
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG-NC-TMD- <i>Tcbh1</i>	D389	+++++	-		Intra-cell. not checked	
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG-NC- <i>Tcbh1</i>	D389	+++++	+/-	Only Gla-HA fusion visible		-
	<i>Phex1</i> -ssCbh-NC-8xG-T4 foldon- <i>Tcbh1</i>	D389		+++		Extra- and intra-cellular expression	-
	<i>Phex1</i> -ssCbh-NC-TMD- <i>Tcbh1</i>	D389		+++++		Intra-cellular expression	+++++
	<i>Phex1</i> -ssCbh-NC-TMD- <i>Tcbh1</i>	D382		+++	Much more HA0 processing than D389	Intra-cellular expression	+++
	<i>Phex1</i> -ssCbh-NC- <i>Tcbh1</i>	D389		+++++	Not stable, only HA1 visible		-
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG-NC HA1- <i>Tcbh1</i>	D389	+++++	+	Only Gla-HA fusion visible		-
	<i>Phex1</i> (RI-)-ssGlaA-GlaA-Kex2-NC- <i>Tcbh1</i>	D389	+++++	+	Only Gla-HA fusion visible		-

Figure 2A

	Construct	Strain	Expression		Remarks 1	Remarks 2	HA Assay
			Gla1/A	HA			
	<i>Phex1</i> -ssGlaA-GlaA-Kex2- FL -8xG-T4 foldon- <i>Tcbh1</i>	D389	+++++	-			
	<i>Phex1</i> -ssGlaA-GlaA-Kex2- FL -8xG-T4 foldon- <i>Tcbh1</i>	D382	+++++	-			
	<i>Phex1</i> -ssGlaA-GlaA-Kex2- FL -TMD- <i>Tcbh1</i>	D389	+++++	-		Intra-cell. not checked	
	<i>Phex1</i> -ssGlaA-GlaA-Kex2- FL -TMD- <i>Tcbh1</i>	D382	+++++	-		Intra-cell. not checked	
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG- FL -8xG-T4 foldon- <i>Tcbh1</i>	D389	+++++				
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG- FL -8xG-T4 foldon- <i>Tcbh1</i>	D382	+++++				
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG- FL -TMD- <i>Tcbh1</i>	D389	+++++			Intra-cell. not checked	
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG- FL -TMD- <i>Tcbh1</i>	D382	+++++			Intra-cell. not checked	
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG- FL -TMD- <i>Tcbh1</i>	D389	+++++	-			
	<i>Phex1</i> -ssCbh- FL - <i>Tcbh1</i>	D389		-			
	<i>Phex1</i> -ssCbh- FL -8xG-T4 foldon- <i>Tcbh1</i>	D389		+++++		Extra- and intra-cellular expression	-
	<i>Phex1</i> -ssGlaA-GlaA-Kex2-5xG- FL (wt)- <i>Tcbh1</i>	D389	+++++	-			

Figure 2B

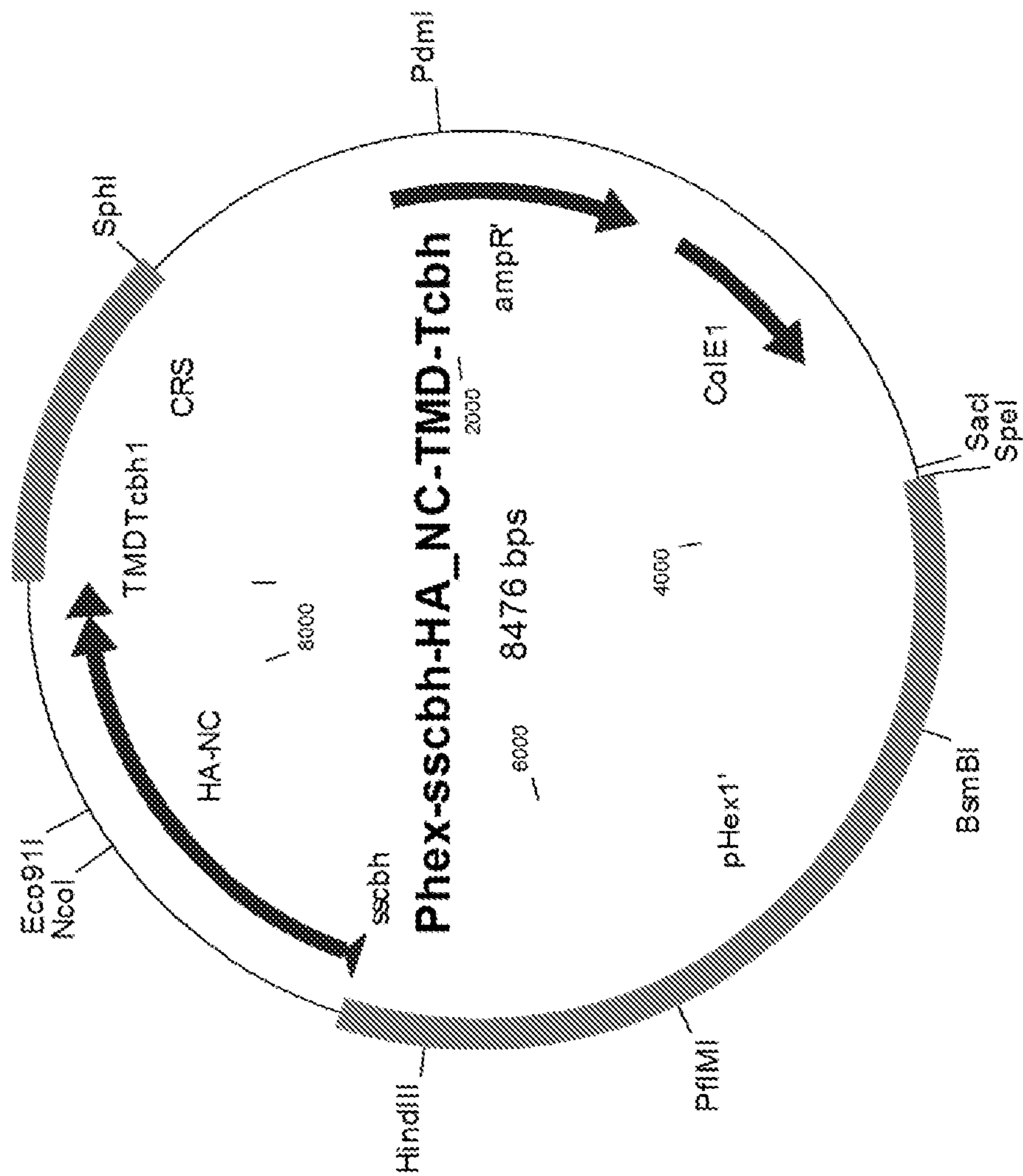
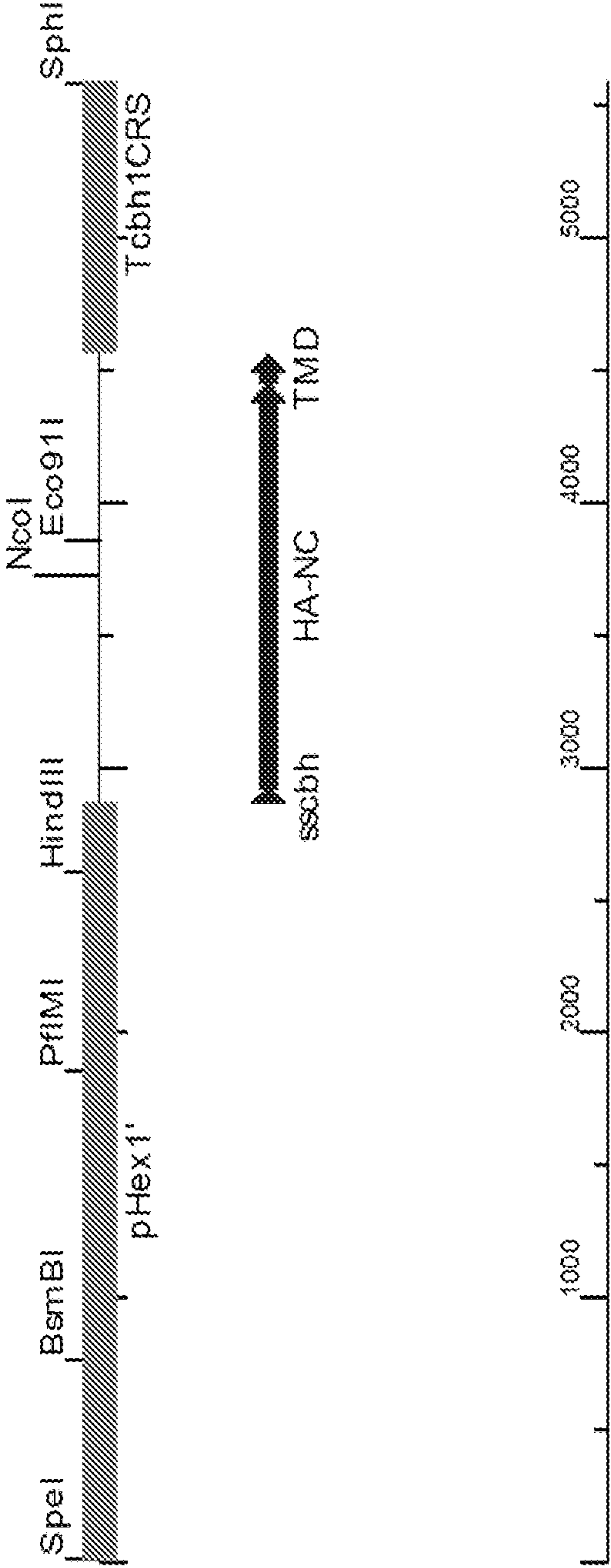


Figure 2C

NotI fragment used for transformation:



Fragment 2 (5589 bps)

Figure 2D

Construct	Strain	Expression		Remarks 1	Remarks 2	HA Assay
		Gla1/A	HA			
<i>Phex1</i> -ssCbh-NC-T4 foldon- <i>Tcbh1</i>	D389		+++		Intra-cellular expression	-
<i>Phex1</i> -ssCbh-NC-GCN4- <i>Tcbh1</i>	D389		++++		Intra-cellular expression	-
<i>Phex1</i> -ssCbh-FL-TMD- <i>Tcbh1</i>	D389		++++		Intra-cellular expression	++++
<i>Phex1</i> -ssCbh-FL-TMD- <i>Tcbh1</i>	D270		+			
<i>Phex1</i> -ssCbh-NC-GGS1x-T4 foldon- <i>Tcbh1</i>	D389		+++++		Extra- and intra-cellular expression	-
<i>Phex1</i> -ssCbh-NC-GGS2x-T4 foldon- <i>Tcbh1</i>	D389		+++	Less stable than GGS1x	Extra- and intra-cellular expression	-
<i>Phex1</i> -ssCbh-FL-TMDnc- <i>Tcbh1</i>	D389		++		Intra-cellular expression	+++
<i>Phex1</i> -ssCbh-CA-TMD- <i>Tcbh1</i>	D389		+			+
<i>Phex1</i> -ssCbh-PR8-TMD- <i>Tcbh1</i>	D389		+			+
<i>Phex1</i> -ssCbh-TeX-TMD- <i>Tcbh1</i>	D389		++++		Intra-cellular expression	++++

Figure 3

Construct		Strain	Expression	Remarks 1	Remarks 2
<i>Phex1</i> -NA NC full length- <i>Tcbh1</i>		D389	++		Extra- and intra-cellular expression
<i>Phex1</i> -NA NC full length- <i>Tcbh1</i>		D382	++		Intra-cellular expression
<i>Phex1</i> -ssC'bh-NA NC VSAP- <i>Tcbh1</i>		D389	++		Extra- and intra-cellular expression
<i>Phex1</i> -ssC'bh-NA NC VSAP- <i>Tcbh1</i>		D382	++		Extra- cellular expression

Figure 4

Strains:

D240	HC strain	UV18-100f $\Delta alp1 \Delta pyr5$
D270	HC strain	UV18-100f $\Delta alp1 \Delta pep4 \Delta alp2 \Delta pyr5$
D389	LC strain	W1L#100l $\Delta chi1 \Delta alp1 \Delta alp2 \Delta pyr5$
D382	LC strain	W1L#100l $\Delta chi1 \Delta alp1 \Delta pyr5$

Promoters:

Chi1	Chitinase 1
Cbh1	Cellobiohydrolase 1
Hex1	Woronin body
Hex1 (RI-)	Internal EcoRI site mutated

Elements:

kex2	Kex2 protease site
sscbh1	cellobiohydrolase ER signal seq.
Gla1	Glucoamylase 1 (C1)
GlaA	Glucoamylase A (<i>Aspergillus</i>)
Flag	Flag-tag sequence
Eg2	Endoglucanase 2
T4 Foldon	T4 Foldon of bacteriophage
GCN4	GCN4 leucine zipper (yeast)
VSAP	Human vasodilator stimulated phosphoprotein

Figure 5

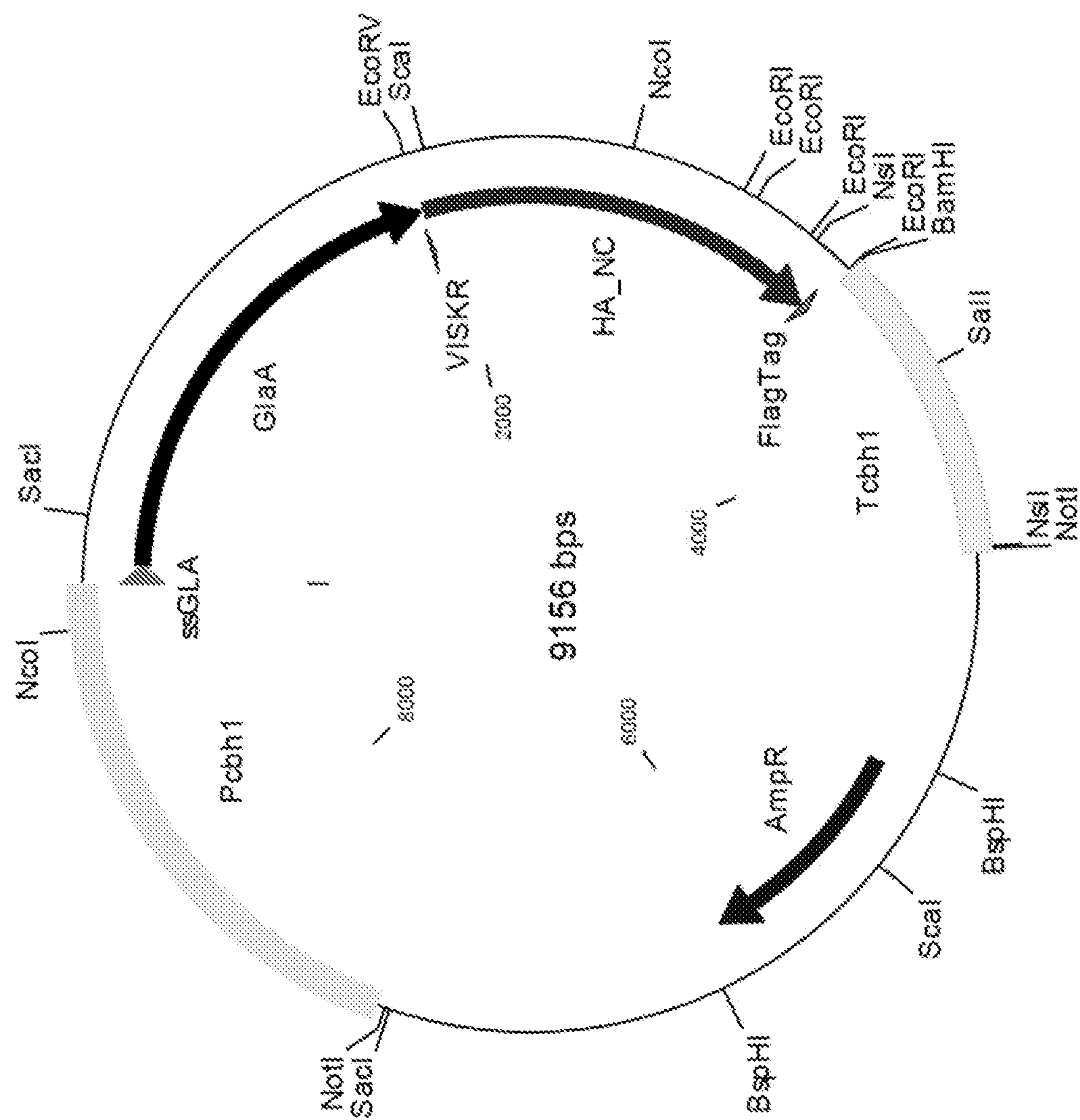


Figure 6

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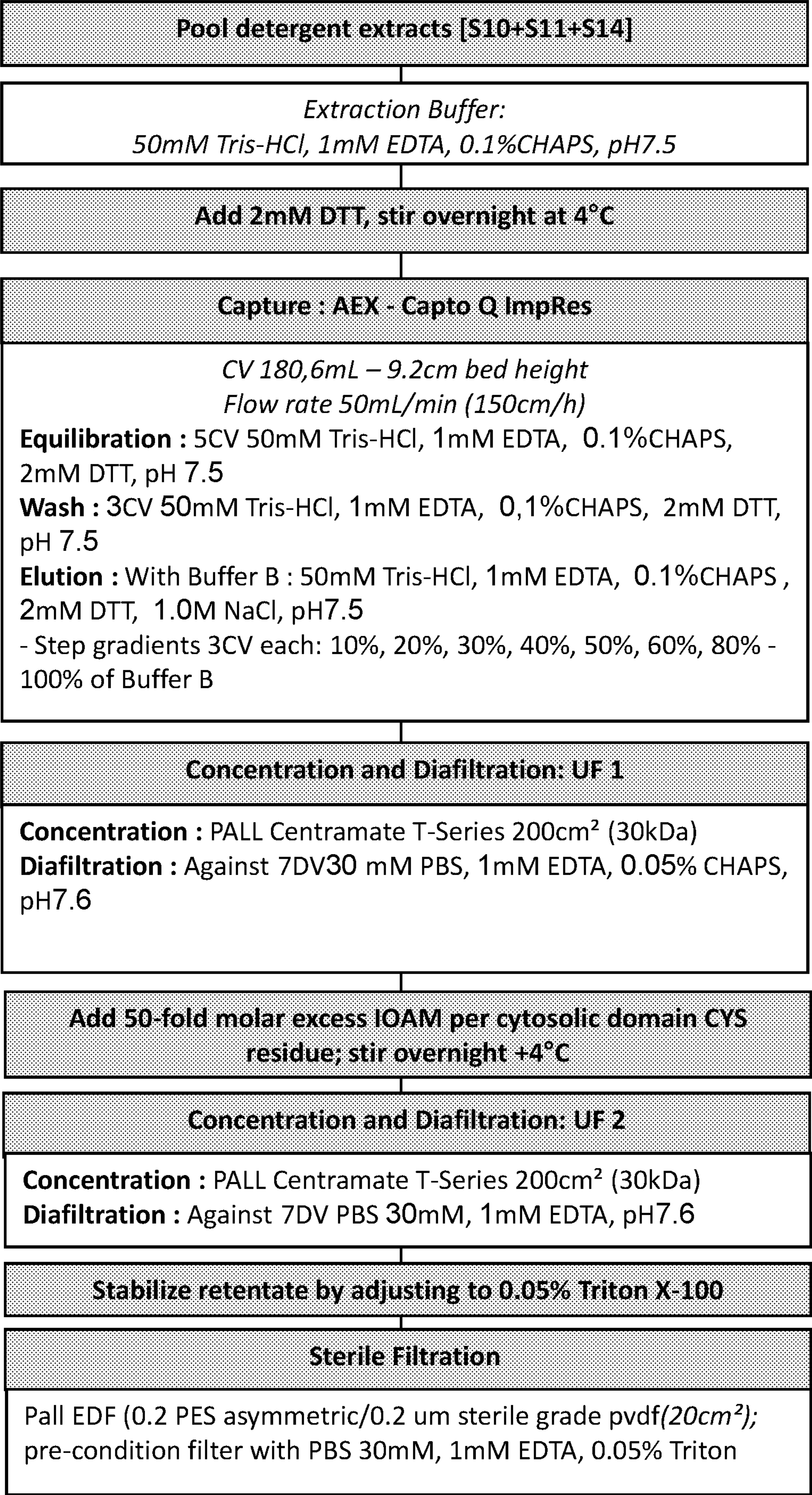


Figure 7

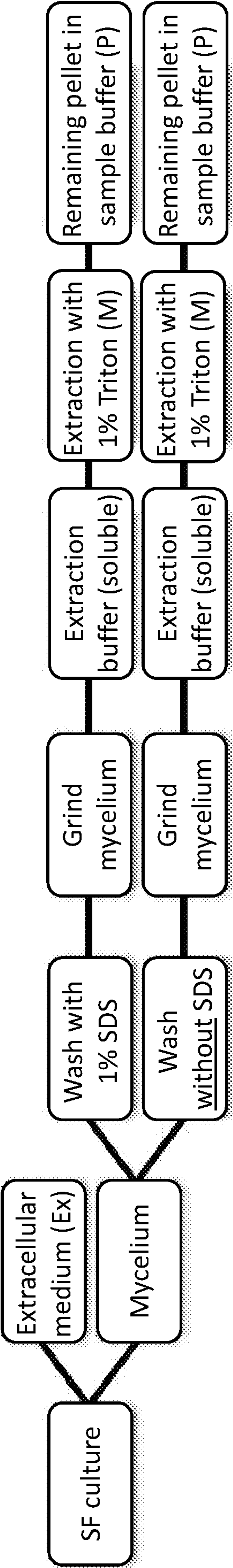


Figure 8

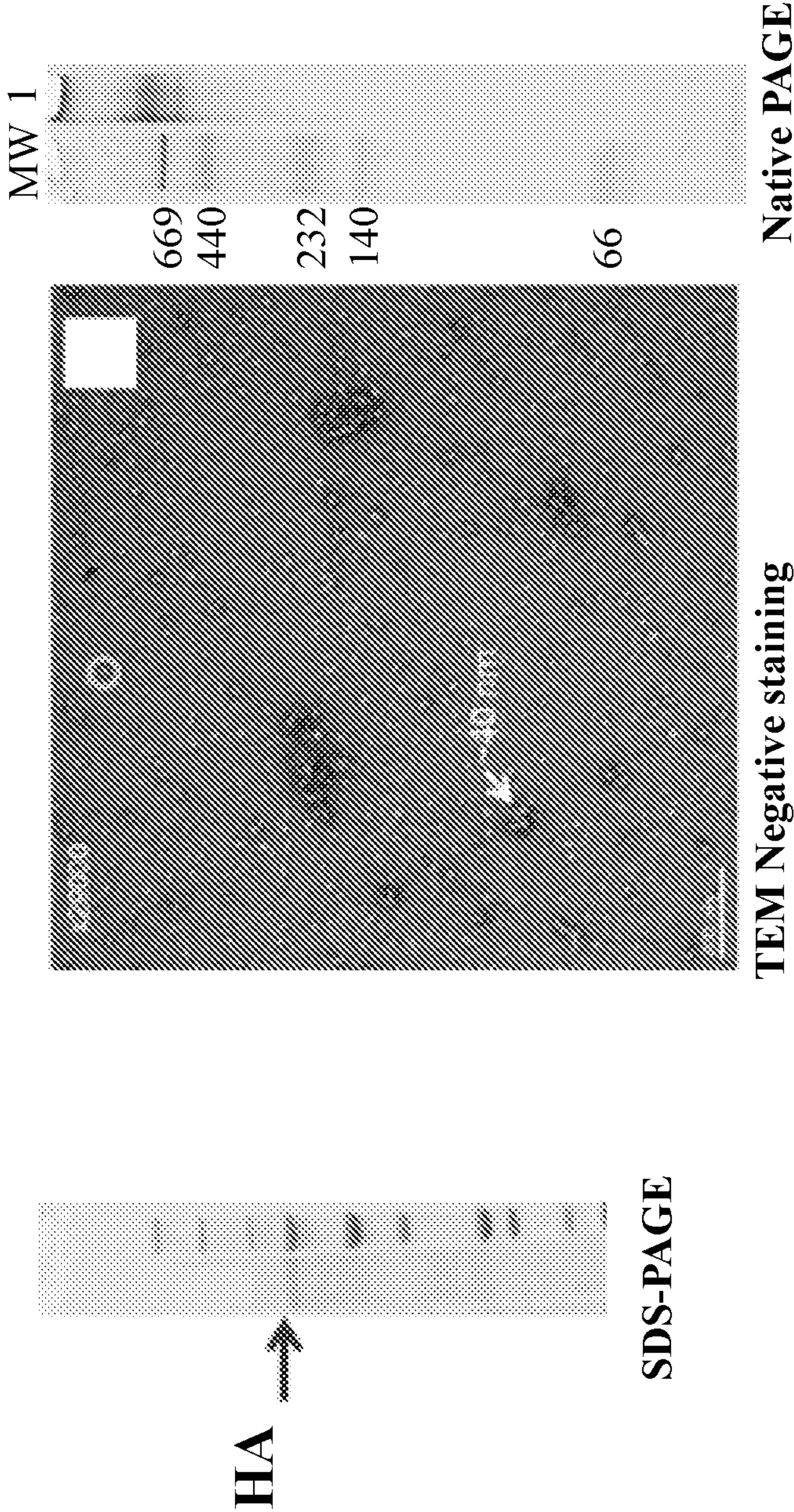


Figure 9

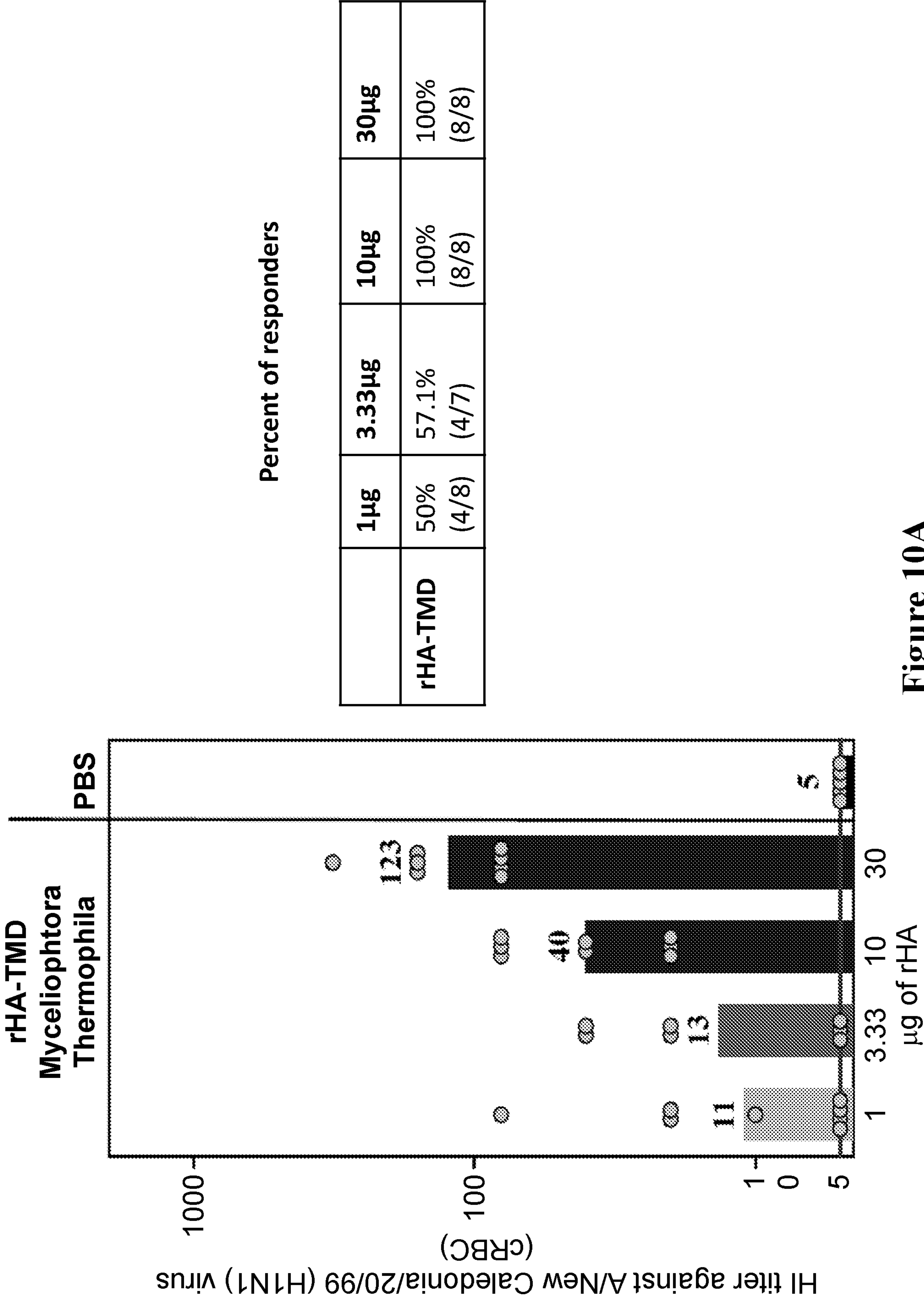


Figure 10A

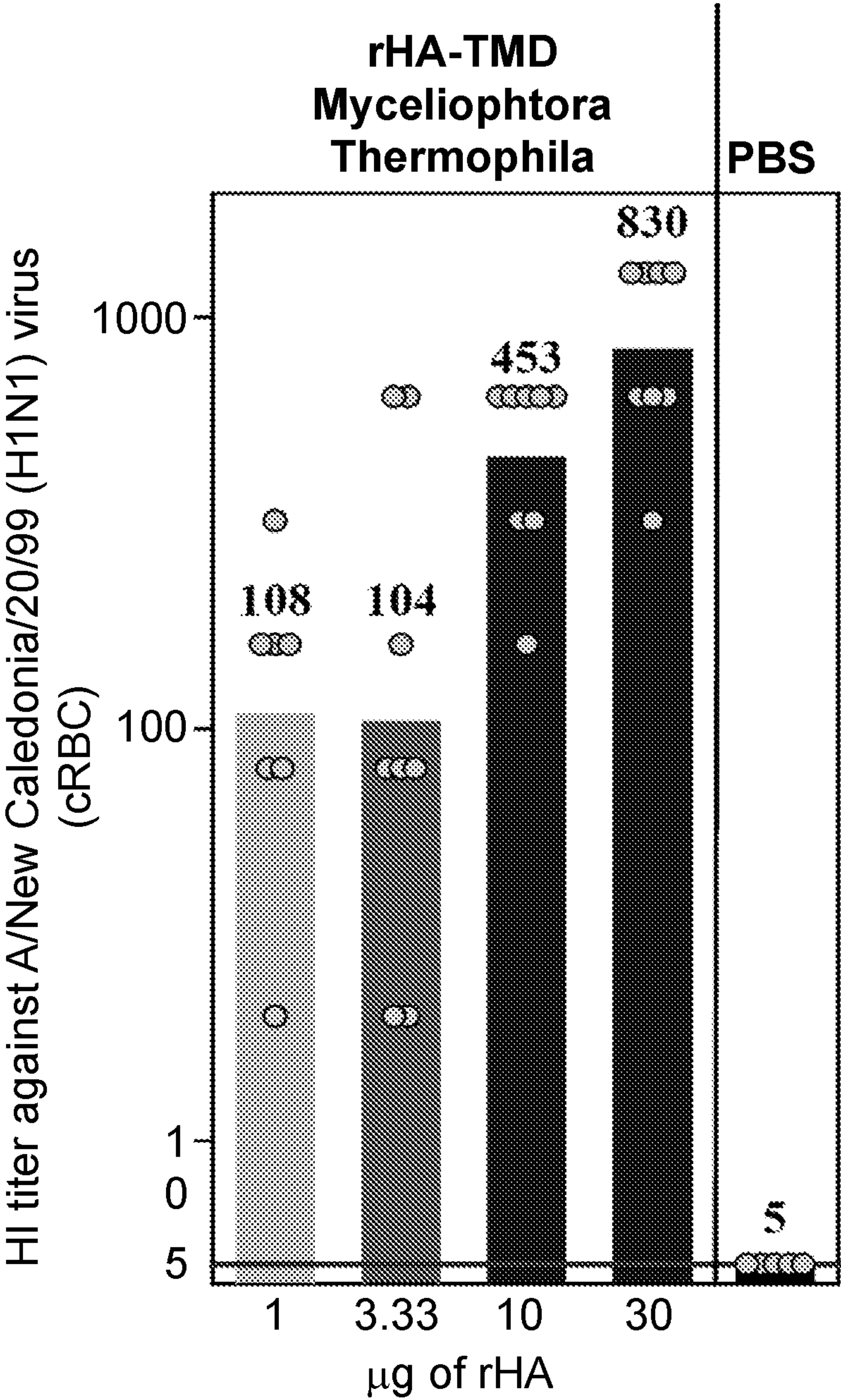


Figure 10B

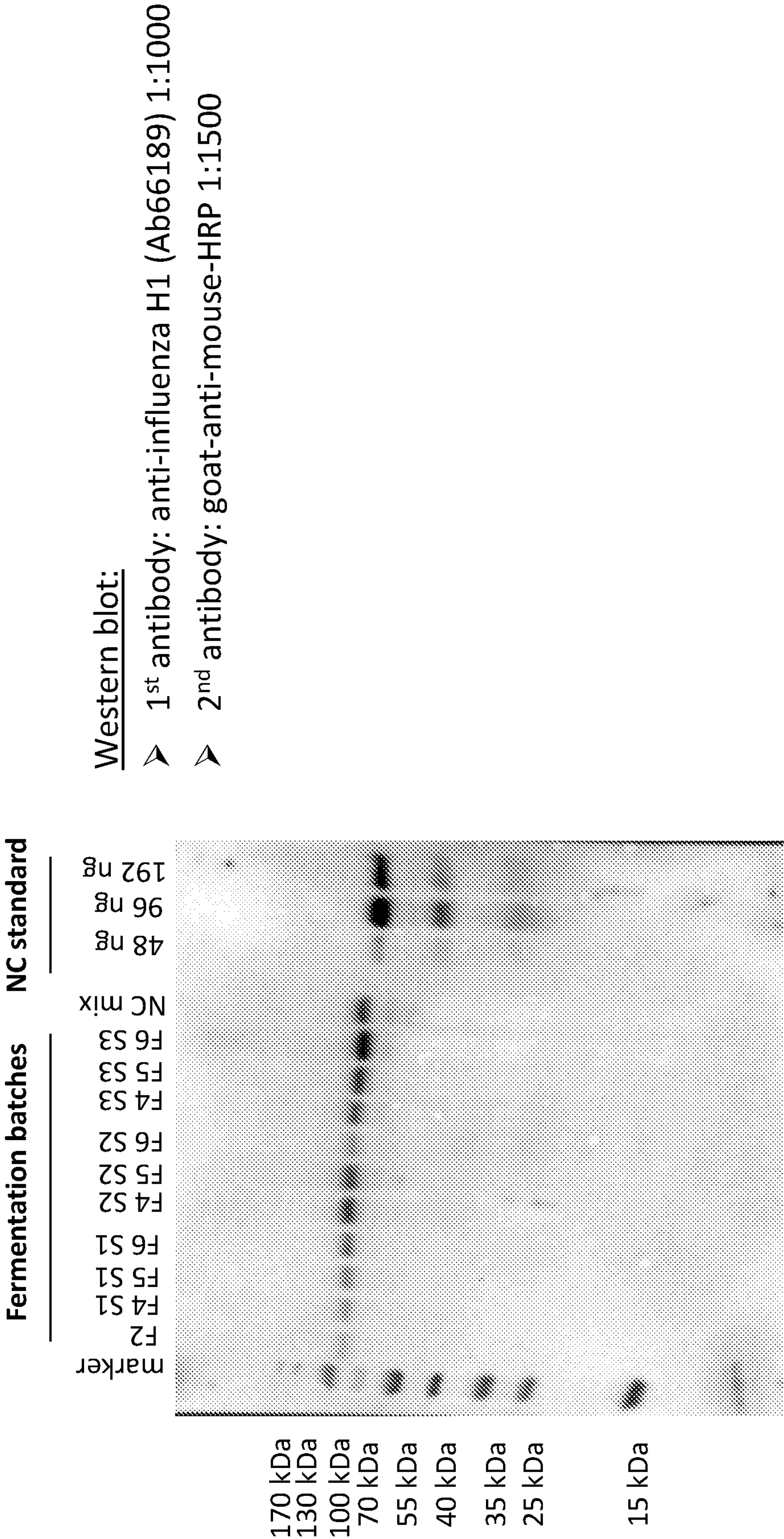


Figure 11