

# Production of Recombinant HA1 subunit from H5N1 Avian Influenza Hemagglutinin in Escherichia coli

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## Research article

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## **Production of Recombinant HA1 subunit from H5N1 Avian Influenza Hemagglutinin in *Escherichia coli***

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### **Abstract**

Influenza A/H5N1 represents a potential risk of a worldwide pandemic event, and as we have witnessed in past influenza outbreaks, the current production chains for vaccination cannot supply the demand for emergencies such as these. Limiting factors include the need for eggs and active virus handling as well as turnaround time and cost. Most of the influenza AH5N1 cases are contained in poultry; however, risk of zoonosis and spread of the virus in humans grows by the day, thus, a mass-produced fast-responding preventive vaccine is required. In this article, we describe the production of a recombinant HA1 subunit of influenza AH5N1 hemagglutinin as a potential key ingredient of a new avian vaccine, using a prokaryotic biotechnological platform. This system is potentially faster, cheaper, and more efficient than current means of vaccine production.

KEYWORDS: avian influenza, recombinant hemagglutinin, avian influenza vaccine

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### **1. Introduction**

A highly pathogenic influenza viral subtype of avian origin, Influenza A virus (IAV) AH5N1, is responsible for the death of millions of birds throughout Africa, Asia, and Europe. Besides being an economic burden to local economies (it is estimated that as much as 0.7% of the global GDP could be lost on a pandemic outbreak), due to this it could be considered as a potential human pandemic threat, having already increased human morbidity and mortality in zoonotic regions [1,2].

The case of the AH1N1 influenza pandemic in 2009 evidenced the need for a reliable system to mass-produce influenza vaccines as a consequence of the lacking response of the traditional live virus production systems to cover for the need for a vaccine not only in the US or Mexico, but worldwide [3]. Moreover, facing the risk of a pandemic event involving influenza, this system should also be able to cover for preventive vaccination programs, i. e., vaccines designed for poultry to avoid zoonosis and the economic hindrance of culling infected birds to deter the disease spread [1].

At the time of writing this article, most programs for vaccination of poultry against IAV include three inactivated viruses in their vaccine formulation; namely, Influenza B, AH3N2, and AH1N1, typically produced by growing the virus in pathogen-free chicken eggs. While for humans, live-attenuated vaccines also produced in pathogen-free chicken eggs are available [4]. The use of pathogen-free chicken eggs vastly increases the cost of vaccine production, since only one dose per egg is obtained, hence, this technology raises both the direct and indirect economic impact of the disease and its prevention. To clarify the situation, to preventively vaccinate the human population only in the US and Mexico, nearly 500 million eggs would be needed. In addition, production of live vaccines is dependent on egg production, and in the event of an AH5N1

outbreak, local availability of chicken egg will decrease, hampering the production of inactivated virus vaccine, accompanied with an increase of costs per unit [5,6].

Vaccination with live viruses relies heavily on the immune system effective response to hemagglutinin (HA), a homotrimeric integral membrane glycoprotein with each monomer consisting of two domains: a hydrophilic domain (HA1) that binds to sialic acid (a polysaccharide frequently found in receptors on the host cell surface) and a hydrophobic domain (HA2) that anchors the protein in the lipid component of the virus envelope. HA1 is not only responsible for fusion of the viral envelope with the cell membrane, it is the main immunogenic agent within the virus as well [7]. Indeed, serum containing IgG capable of recognizing this protein confers immunity against IAV infection, provided that the subtype has a HA identical to that recognized by the vaccine [8–10]. Given the characteristics of the protein, HA is the optimal choice for creating a subunit vaccine, to develop a faster and cheaper technology to cover for the needs of vaccination programs [11].

A number of alternative systems have been proposed to produce influenza vaccines, both for human use and veterinary application [12,13], which include but are not limited to viral propagation in mammalian or insect cells and resorting to recombinant(r) DNA generated proteins [14–16]. rHA production stands out among all of these options due to its reported low-cost, ease of implementation, fast turnaround time, and safety [17–19].

In this article, we describe the design, development, and evaluation of a reliable, fast, and relatively low-cost system for the production of rHA of IAV in *Escherichia coli* and discuss its potential as a platform for fast mass-production of influenza A sub-unitary vaccines for veterinary vaccination applications.

## 2. Materials and methods

### 2.1. Design of expression system and cloning of the HA gene into the expression vector

The gene sequence taken as reference for the design of the rHA expression system was IAV (A/chicken/Egypt/1063/2010(H5N1)) segment 4 HA complete coding sequence (CD) (GenBank accession number HQ198269.2). The sequence was optimized for expression in *E. coli*, using the GeneOptimizer® assisted Sequence Analysis service (LifeTechnologies, Inc. Carlsbad California, USA).

The 568-amino acid (aa) sequence encoded by the aforementioned gene (accession number ADM85860.1) was analyzed using the Simple Modular Architecture Research Tool (SMART)[20] to identify the domains present in the protein. Subsequently, its signal peptide (aa 1 to 16) and its transmembranal region (aa 532 to 568) were removed from the sequence to avoid interference with the three-dimensional structure of the protein, leaving a 515-aa chain. A methionine-coding codon was added at the beginning and two stop codons at the end of the corresponding synthetic nucleotide sequence to generate the first CD to be expressed, which was called HAq. A second sequence, named HAp, coding only the globular subunit of the HA1 was generated, comprising the aa residues 17 to 338 from the reference sequence, plus a start methionine-coding codon and two stop codons as well (see Table 1). The putative three-dimensional structures of both constructs were predicted using the SWISS-MODEL tool [21]. The theoretical molecular weight and isoelectric point of the proteins to be encoded by HAq and HAp sequences were inferred informatically by using the tool ProtParam, included in the ExpASy protein informatics package [22].

The two sequences were synthesized by GeneArt (Invitrogen, Carlsbad California, USA), and cloned by PX' Therapeutics (Grenoble, France) into the expression vector pET-30b(+), carrying kanamycin resistance and the T7 promoter under the regulation of an element of the lac operon, rendering the plasmids named pVIT\_HAp and pVIT\_HAq. Figure 1 shows a

simplified map of these plasmids. The recombinant plasmids were then propagated in *Escherichia coli* BLR (DE3) (Novagen, Merck, Darmstadt, Germany), with genotype F- ompT hsdSB(rB<sup>-</sup> mB<sup>-</sup>) gal lac ile dem  $\Delta$  (srI<sup>-</sup>recA) 306::Tn10 (tetR) (DE3), for which Ca<sup>2+</sup> competent cells were prepared and transformed by heat shock. Transformed cells were cultured in Luria-Bertani agar medium containing 50  $\mu$ g/ml of kanamycin seeds. Then, they were grown overnight and the colonies obtained were analysed by polymerase chain reaction (PCR) for verification of successful transformation. Oligonucleotides required to verify by PCR the presence of our synthetic constructs in the plasmids containing bacteria were designed and tested *in silico* using OligoAnalyzer 3.1 (Integrated DNA Technologies®, Coralville Iowa, USA) and PrimerBlast3.1 [23]. The primers and their physicochemical properties are described in Table 2. Amplicons were analyzed by agarose gel electrophoresis.

### 2.2. Bacterial culture, expression induction, and harvesting.

The expression of HAq and Hap was screened in a set of 20 different clones each, which had been PCR-verified for the presence of pVIT-HAq and pVIT-HAp plasmids, respectively. For this, 5-mL Luria-Bertani broth containing 50  $\mu$ g/mL of kanamycin were inoculated and incubated at 37°C and 300 rpm. After 1.5 hours, 1 mM of IPTG was added to induce the expression and the cultures were incubated for 20 h more at 25°C and 300 rpm. After this time, samples of each culture were taken and analyzed by Polyacrilamide Gel Electrophoresis under denaturing conditions (SDS-PAGE) (Shapiro, Viñuela, and Maize, 1967).

Clones with the highest expression levels served as inoculums for 10 mL tubes containing Modified Riesenbergs Medium (MRM: 4.8 g/L of glucose, 140.65 mg/L of MgSO<sub>4</sub>, 58.95 mg/L of C<sub>6</sub>H<sub>11</sub>FeNO<sub>7</sub>, 14.74 mg/L of MnCl<sub>2</sub>•4H<sub>2</sub>O, 9.33 mg/L of C<sub>4</sub>H<sub>6</sub>O<sub>4</sub>Zn•2H<sub>2</sub>O, 2.95 mg/L of H<sub>3</sub>BO<sub>4</sub>, 2.89 mg/L of Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O, 1.85 mg/L of CaCl<sub>2</sub>, 6.48 mg/L of EDTA, 13.07 g/L of KH<sub>2</sub>PO<sub>4</sub>, 3.93 g/L of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub>, 1.67 g/L of C<sub>4</sub>H<sub>2</sub>O<sub>2</sub>, 4.91 g/L of yeast extract, and 1.3 mL/L of ammonia) [24] adapted for batch culture supplemented with 30 $\mu$ g/mL kanamycin. At an OD<sub>600</sub> of 1.5, each of the inoculums were added to new tubes containing 90 mL of MRM, in 500 mL baffled beakers, which, once reaching an OD<sub>600</sub> of 1.5, were used each to inoculate 400 mL of MRM in a one-liter BioFlo® Bioreactor (Eppendorf, Hamburg, Germany). The cultures were kept at 37°C with stirring conditions required to keep dissolved oxygen constant until an OD<sub>600</sub> of 1.5 was reached. Once this density was reached, temperature was kept at 25°C 1M IPTG was added to a final concentration of 0.5mM, to attain the induction stage, constantly monitoring the process throughout 20 h. Cells harvesting was performed centrifuging at 13,000g for 25 min at 4°C. The recovered biomasses were resuspended in 50 mL of depleted MRM (10x more concentrated than the original harvest) and then preserved at -20°C.

### 2.3. Lysis and preparation of inclusion bodies

From the concentrated harvest, aliquots (10% from the total volume) were taken and centrifuged for 20 min at 12,000g at 4°C. Pellets were resuspended in 30 mL of 50 mM potassium phosphate (monobasic) solution to wash every pellet, obtaining an OD<sub>600</sub> of approximately 10. Samples were centrifuged again for 20 min at 12,000g, at 4°C. The supernatants were discarded, and the pellet were resuspended in 3 mL of lysis buffer (50 mM Tris, 5 mM EDTA, 1 mg/mL lysozyme, 0.5 M, NaCl, 1  $\mu$ g/mL pepstatin A, and 1  $\mu$ g/mL leupeptin, pH 7.5) and distributed in 1 mL aliquots (to facilitate handling and speed up freezing/thawing). The aliquots were subjected to six freeze-thaw cycles by placing the samples in a tube rack, inside an ultra-freezer (REVCO, Thermo Electron Corporation Waltham, Massachusetts, USA) at -70°C for 5 min. Thawing immediately at 42°C with agitation (300 rpm) in thermomixer (vortex vigorously to mix well). The lysate were centrifuged 5 min at 12,000g at 4°C and the supernatant were discarded to recover the pellet containing the inclusion bodies.

#### 2.4. Solubilization and refolding of recombinant rHA

Solubilization of inclusion bodies found in the lysates were carried out as follows: the pellets obtained in the previous stage were resuspended and pooled in 6 mL solubilization buffer (1 mM glycine, 1 µg/mg pepstatin A, 1 µg/mL leupeptin, 50 mM sodium phosphate (monobasic), 5 mM β-mercaptoethanol, and 8 M urea) and incubated overnight at 6-8°C, and then centrifuging at 12,000g for 20 min at 6-8°C, collecting the supernatant and preserving it at -20°C until further processing. In order to allow the rHA to properly fold, 900 µL refolding buffer (400 mM arginine, 2 mM EDTA, and 4 mM CHAPS in PBS at pH 7.3) were gradually added to 100 µL of solubilized protein suspensions under a light vortexing speed and after this, speed was gradually increased for 10 sec to ensure homogeneity. The refolded protein suspensions were then centrifuged at 15,000g for 30 min at 6°C, separated from the scarce pellet formed, and incubated overnight at 6-8°C. These supernatants were stored at -20°C. Recovery percentage were estimated using native SDS-PAGE as described elsewhere [25] and measured as described previously [26].

#### 2.5. Immunological assay

An Enzyme Linked Immunosorbent Assay (ELISA) was carried out according to manufacturer instructions using the H5N1 (Avian Flu) Hemagglutinin Elisa Pair Set SEK002 (Sino Biological Inc., Beijing, China). Standard of HA provided by the kit was used to read a curve of serial dilutions to calculate the concentration of rHA in the unknown samples. The samples included in the assay were direct and diluted aliquots from the solubilized rHA, the protein diluted in refolding buffer, a rHA control provided by PX Therapeutics, and an inactivated IAV H5N1.

### 3. Results

#### 3.1. Rational design of HAp and HAq.

Prior to decide the CDs to be synthesized to render the expression cassettes for rHA form AIV, structural analysis of the reference sequence (Influenza A/chicken/Egypt/1063/2010 [H5N1] hemagglutinin, accession number: ADM85860.1) using SMART showed the following four distinct features. 1. A signal peptide (ranging from position 1 to 16 of the reference sequence). 2. An influenza hemagglutinin headpiece (SCOP: d2viau\_) from the viral protein domain superfamily. 3. A second viral protein domain (PDB: JUL4 [B]) ranging from position 346 to position 523 corresponding to the HA2 domain, and 4. A transmembrane region ranging from residues 533 to 555 (Figure 2). In order to maximize three-dimensional similarity between native hemagglutinin and the recombinant protein, two sequences were designed taking these features into account: *HAp*, which contained only the viral protein domain, and *HAq*, which comprised both the viral protein domain and the HA2 domain. Signal peptide was removed to ensure epitope structure, because this sequence is not found in functional hemagglutinin. The transmembrane region was also removed to improve stability and decrease hydrophobicity. Physicochemical properties of HAq and HAp are shown in Table 3.

Once the theoretical properties of both proteins were inferred bioinformatically, both sequences were modeled three-dimensionally to characterize their behavior in three-dimensional space considering primary structure. These models are shown in Figure 3. Results of this analysis were matched with previously reported three-dimensional models and crystallographic analysis of isolated native hemagglutinins [SMTL ID: 4kth.2 (Structure of A/Hubei/1/2010 H5 HA); 2wr1.1 (Structure of influenza H2 hemagglutinin with human receptor)]. This theoretical data supported the hypothesis that these synthetic constructs could have the same structure as their native counterparts.

### 3.1. Expression of recombinant hemagglutinin

After expression screening of transformed bacteria, the clones containing pVIT-HAp demonstrated a much higher expression level than those containing pVIT-HAq (data not shown) and were thus chosen for subsequent production of the rHA. The rationale for this situation, implies that it is easier to express small versions of the rHA for bacterial cells. In this way it is possible to increase the yields for rHA expression using the same culture media and expression strains. Additional to this, (See Figure 6) Riesenberg media demonstrate the higher growth rate (LB 0.248h<sup>-1</sup>, Riesenberg 0.356h<sup>-1</sup> and Terrific broth 0.332h<sup>-1</sup>) to culture the recombinant *E. coli* cells being a good option for scale up the rHA expression. During the fermentation, a series of culture samples were taken for monitoring the process; particularly, optical density (absorbance at 600 nm), as a biomass yield indicator. Besides, the rHAp yield was estimated by SDS-PAGE and bands densitometry. Figure 4 shows the optical density measured during the process plotted against time, while the rHAp yield during the process can be observed in figure 5. The product concentration obtained at the end of the process was 1.2 g of rHAp from 1L of bacterial culture, as estimated through SDS-PAGE image densitometry assessment. The processing of the obtained biomass evidenced the protein expressed is present in the form of insoluble inclusion bodies. Refolding process experiments were conducted in order to solubilize the expressed protein; After protein solubilization, ELISA tests demonstrated the presence of soluble refolded rHA protein. Figure 7 shows the protein profiles of the samples taken throughout the semi-purification process, from the harvest to the solubilization of the rHAp.

### 3.2. Immunological assay

All of the samples tested through ELISA showed detectable immunoreactivity. The highest titer was given by the solubilized rHAp, followed by the refolded protein, the PX'Therapeutics' batch, and finally, the inactivated IAV used as positive control came from a Mexican pharmaceutical company (Laboratorio Avi-Mex S.A. de CV.). The ELISA showed that the hemagglutinin produced in this work had a similar immunoreactivity as a commercial veterinary-grade hemagglutinin. The results can be seen in Figure 8.

## 4. Discussion

Using the biotechnological platform previously described, a recombinant modified Influenza A/H5N1 HA1 domain, suitable for subunitary vaccine production was prepared safely, without need of eukaryotic components, in a short time, and at high yield. An overall performance of this approach can be seen on Table 1. Despite the theoretical differences between the rHA and the hemagglutinin as part of viral particles, the ELISA assays demonstrated that the former was recognized by anti-HA antibodies. Hemagglutination assays (not shown) carried out using HAp did not display hemagglutination activity. Similar results were obtained by other groups [27] with some hemagglutinins tested. This could be due to the lack of a structure able to keep several HA1 domain particles together, as it does the viral particle *in vivo*. However, HAp showed *in vitro* immunoreactivity, consistent with guidelines for vaccines in poultry.

One of the main advantages of the platform is that the gene encoding HAp can be modified or constructed *de novo* using genetic editing or chemical synthesis with slight adjustments to the fermentation and purification processes, to produce abundant quantities of relatively pure rHA in independence from live virus and cell culture or pathogen-free chicken eggs. This represents a considerable advantage as compared to current methods, which require as much as two eggs per dose [28] and no less than six months after the strain is identified and isolated [29]. The reliability and flexibility of the system was tested by adapting the platform for the production of rHA1 from the A/Egypt/1063/2010(H5N1) chicken isolate but can be

adapted for production of other IAV strains, which are currently undergoing evaluation. Given that bacterial expression system such as the one described in this article are commonplace within biotechnological production chains, it can also be adopted in case of a pandemic event, since most academic institutions and biotechnological, and pharmaceutical companies possess the equipment needed for the implementation of this platform.

It is important to realize that these subunit antigens for vaccines, although promising in its initial results, has yet to be tested in live animals. Several subunitarian influenza virus vaccines have been developed based on rHA, using mammalian, insect, or bacterial cells as expression systems, and testing shows very encouraging results both in humans and animals [30,31]. However, previous testing with baculovirus-dependent systems have shown that protection against IAV H5 HA induced a neutralizing antibody response in only 23% of individuals with a single dose of 90 µg and a maximum of 52% after two doses of 90 µg [2]; results that do not comply with guidelines for vaccines in case of pandemics or epidemics due to the high cost of production, high dosage required, and deficient capability to immunize the subject. At the same time, several adjuvants are in development, so reformulation of recombinant hemagglutinin-dependent vaccines with the inclusion of one of these adjuvants could increase their immunogenic capabilities and surpass the need for large doses [32,33]. Other reports evaluated the immunogenicity of partial rHA1 sequences produced in bacteria against immunogenicity of complete rHA produced in insect cells. The results of these studies show that non-glycosylated partial region from recombinant hemagglutinin elicited neutralizing titers only four times lower than the insect cells-derived HA, although both inhibited viral entry into host animal cells [30].

Even considering the remaining challenge research on alternative production platforms of recombinant vaccines against IAV, the subunitarian approach looks promising. The available platforms for chicken egg-dependent vaccine production do not have the wherewithal to either rapidly or broadly respond in case of a pandemic or epidemic outbreak [34,35]. Further, studies with our platform will include modification of the protein, testing with adjuvants, assessment in live poultry, and improvement of the purification methodology. Still, our methodology provides for a scalable cost-effective platform for the production of AIV vaccines, which can potentially be adapted for different variants as required, that we anticipate will be ready to meet the required standards to cover the needs in the case of a health crisis.

### **Competing interests**

The authors declare that they have no potential competing interests.

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### **Authors contributions**

ELT: laboratory work and preparation of draft; AKMM: assistance with laboratory work; LMRM: analyses of results and aid in manuscript preparation; HABS: design and overall supervision of the experiments and manuscript.

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ELT biotechnology engineer; AKMM: genomic biotechnologist; LMRM: Virologist and Bioprocess Engineer; HABS: biomedical translational scientist and entrepreneur.

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## Figures

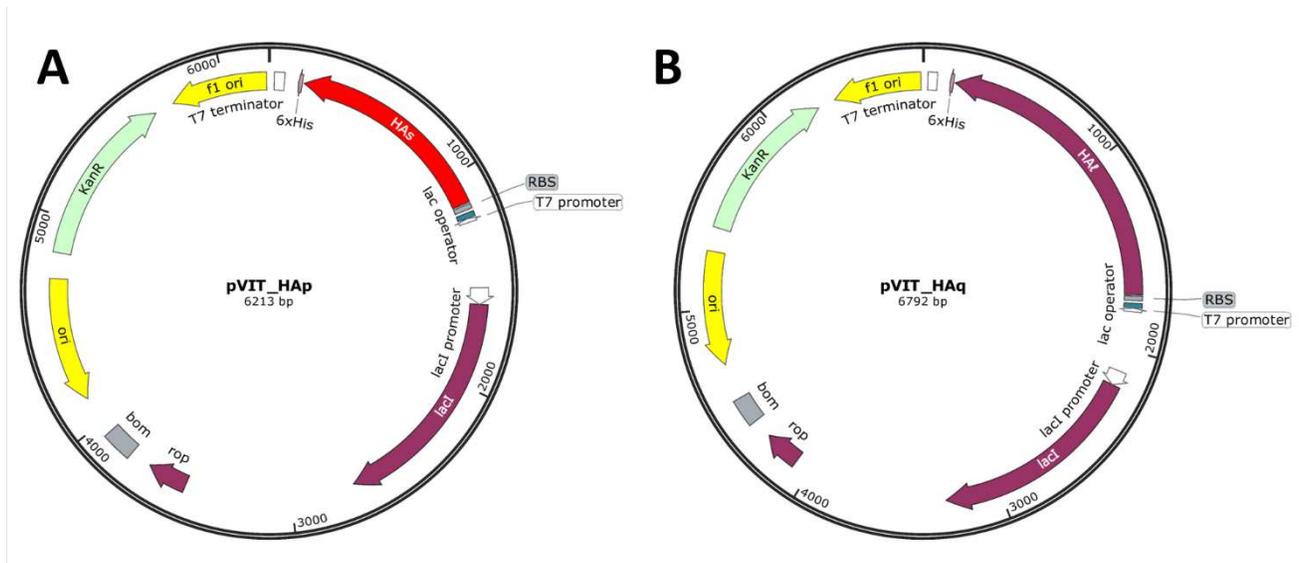


Figure 1. Expression plasmids for rHA. **A.** pVIT\_HAP plasmid map. The plasmid features the short hemagglutinin sequence, kanamycin resistance, a T7 promoter and lacI as a reporter. **B.** pVIT\_HAq plasmid map. The plasmid features the HAq hemagglutinin sequence, kanamycin resistance, a T7 promoter and lacI as a reporter.

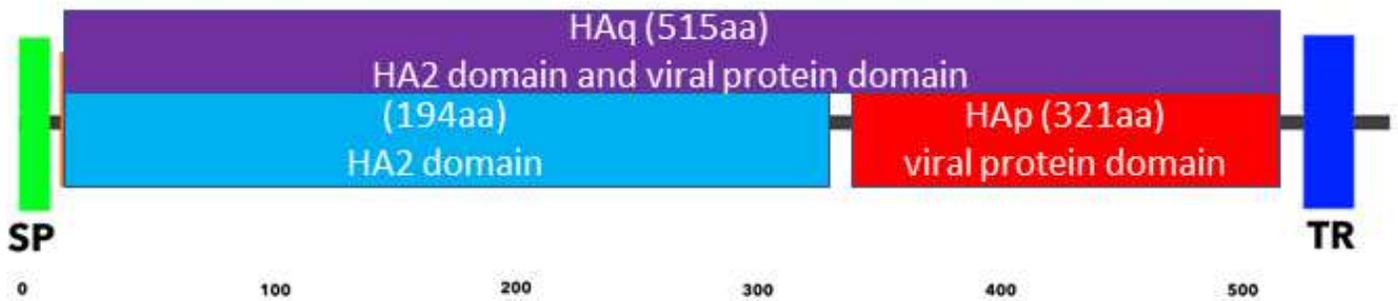
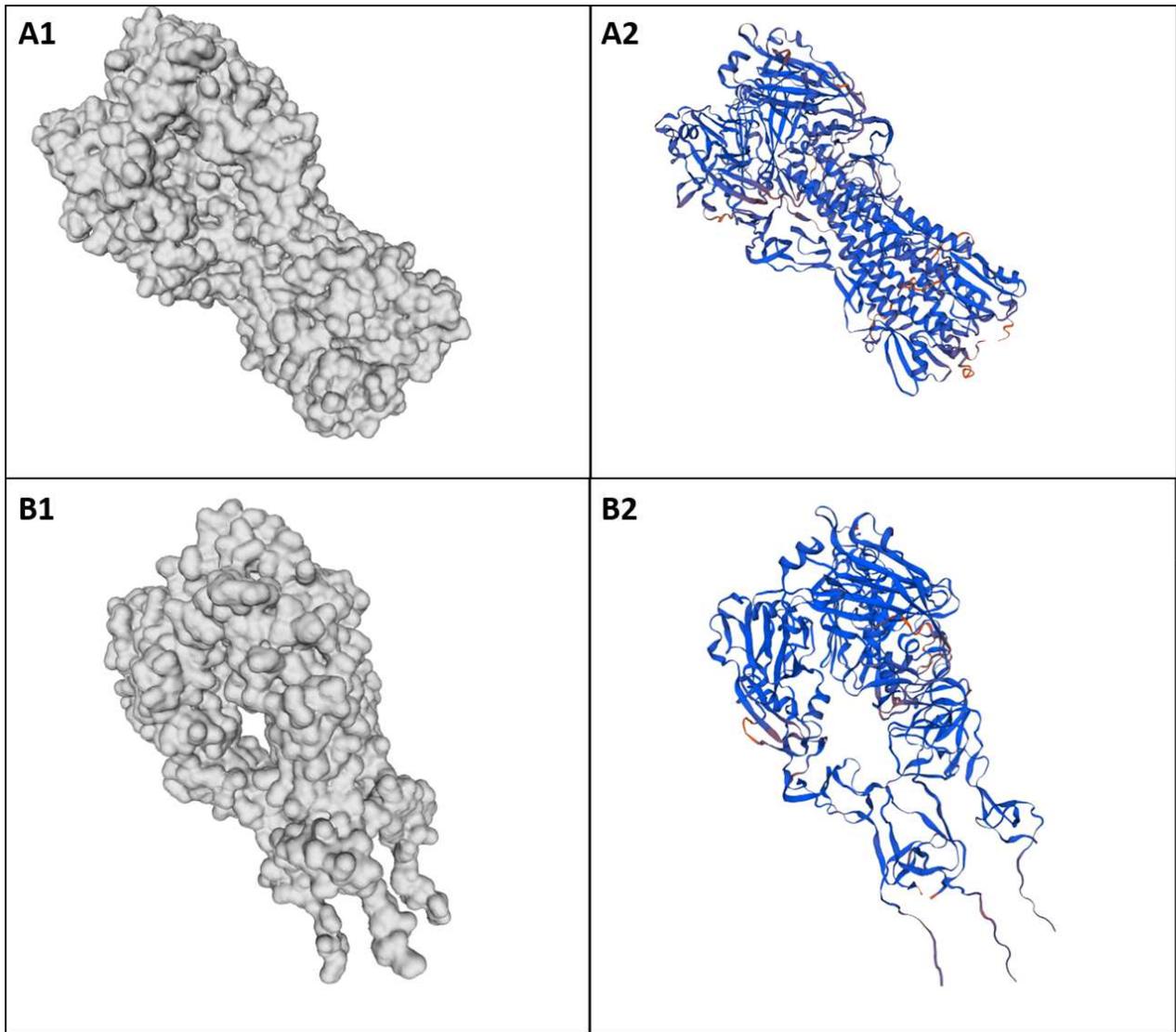


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*Figure 3. PDB Models for rHA structure. A. Three-dimensional inferred structure of HAq comprised both the viral protein domain and the HA2 domain. Modeled using SWISS-MODEL. B. Three-dimensional inferred structure of HAp contained only the viral protein domain. Modeled using SWISS-MODEL.*

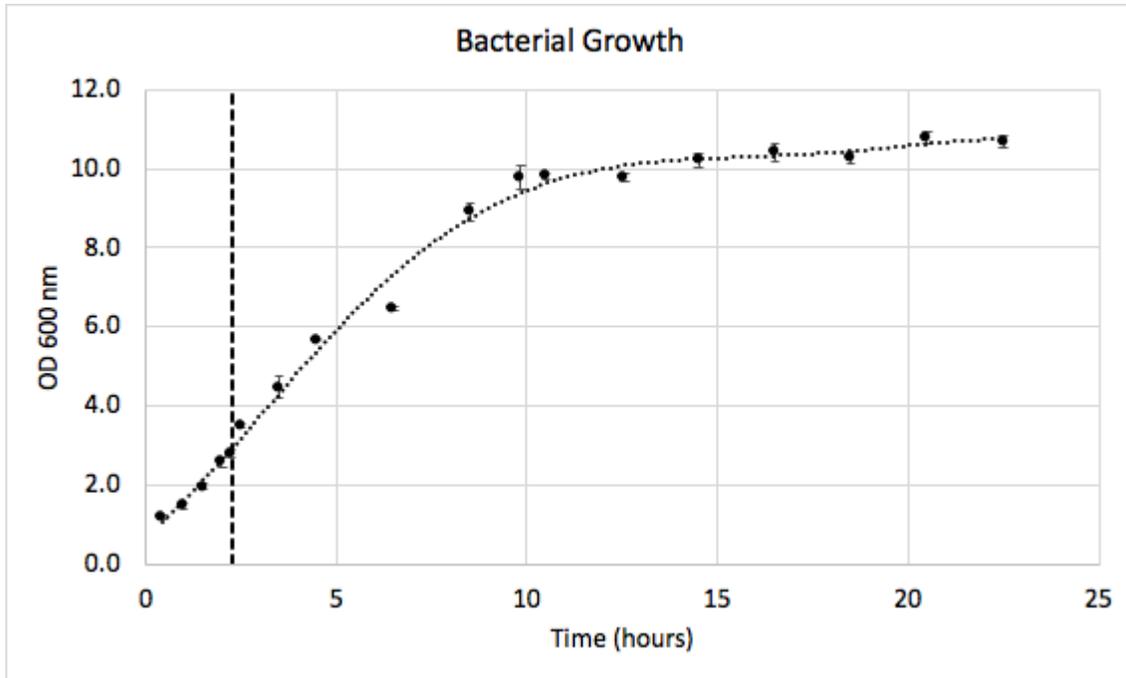


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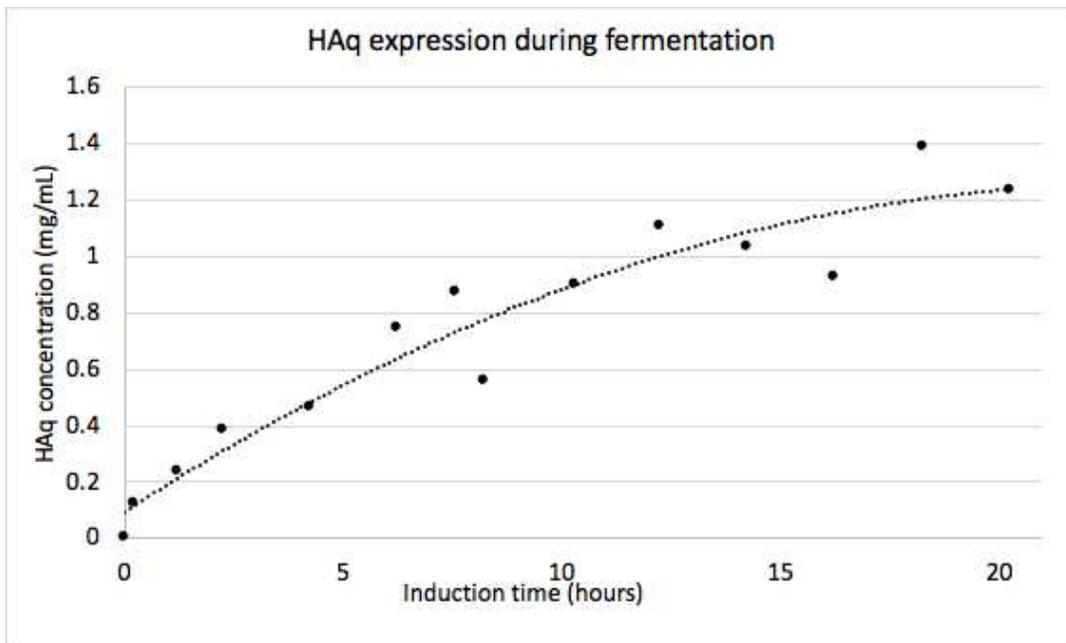


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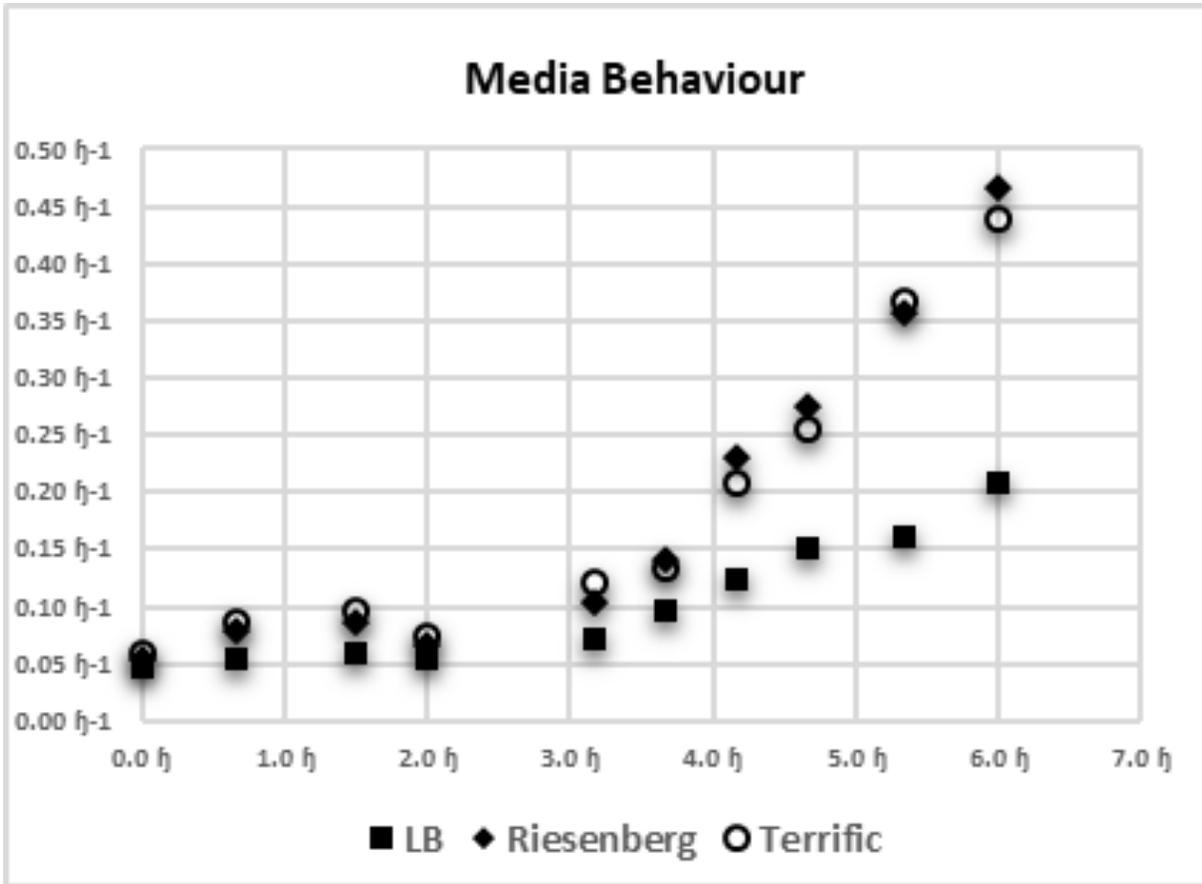


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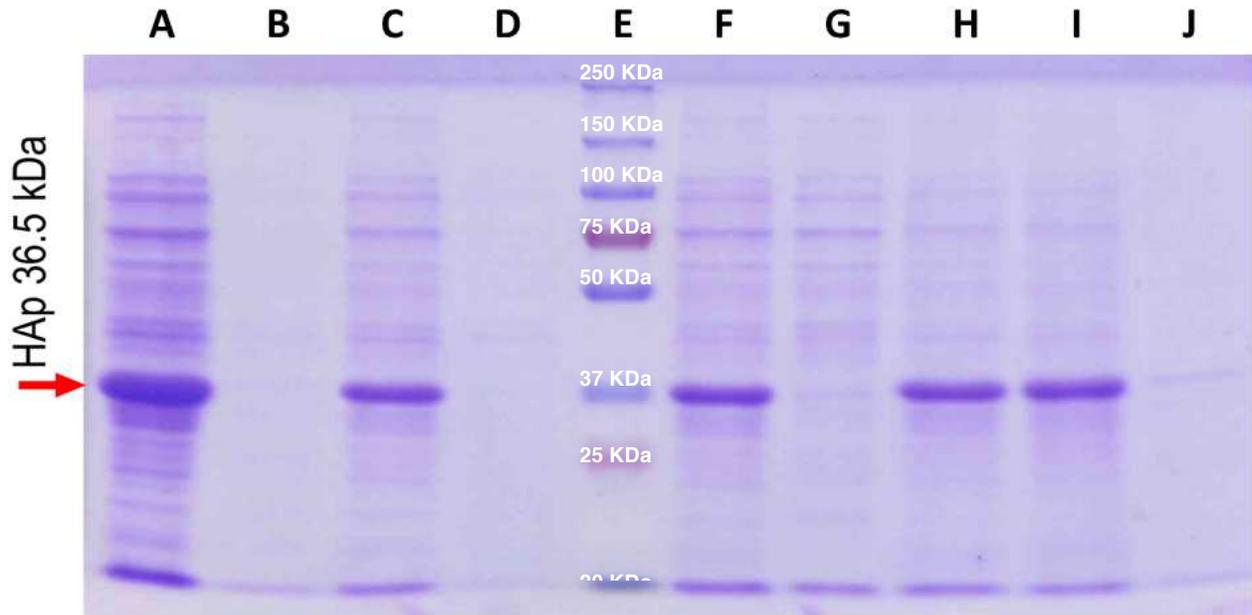


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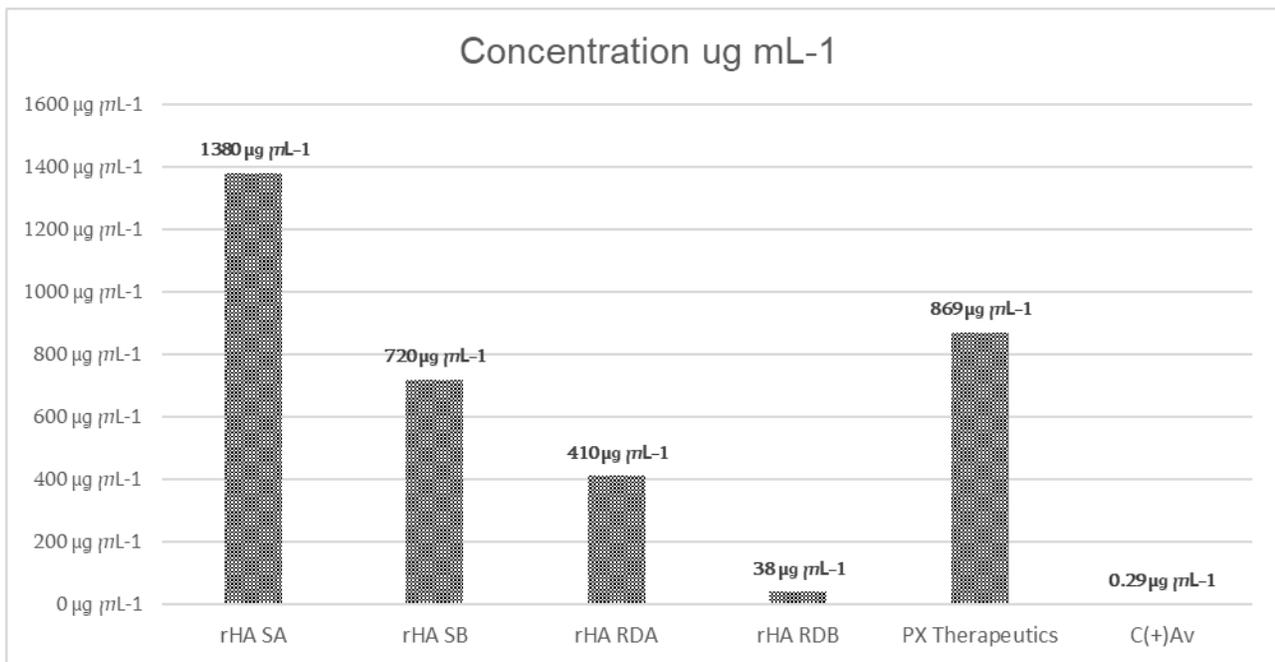


Figure 8. Concentrations of recombinant Hemagglutinin (rHA) in different preparations determined by ELISA. rHA SA: recombinant Hemagglutinin Solubilized batch A; rHA SB: recombinant Hemagglutinin Solubilized batch B; rHA RDA: recombinant Hemagglutinin Refolded by Dilution batch A; recombinant Hemagglutinin Refolded by Dilution batch B; C(+)-Av: Positive Control from Pharmaceutical company.



## Tables

Table 1. Production system performance for vaccine manufacture. The next table explore the main characteristics of use for several technologies for vaccine manufacture. The gold standard keeps running for production on embryo's egg technology, almost 100 years old technology from the first half of XXth century.

Production System Performance	Viral Culture (Inactivated, Live Attenuated)		Recombinant Hemagglutinin Expression (rHA)	
	Embryo	Cell	Bacterial	Animal
	Eggs	Culture	(i.e. <i>Escherichia coli</i> )	(i.e. Insect cell)
Albumin Allergenic Reactions	+	+	-	-
Accidental Infection Risk	+	+	-	-
Requires Big Facilities	+	-	-	-
Expensive Raw Materials	+	+	-	+
Major Costs Associated to Production (More than \$1USD cost per doses)	+	+	-	+
Major Time-Frames Associated to Production (More than 2 Months for production)	+	-	-	-

Table 2. Aminoacidic sequence of the coding sequences designed for recombinant hemagglutinin production. a) Short hemagglutinin variant designed for expression in *E. coli*. Signal peptide, HA2 domain and the transmembrane region are absent in this artificial sequence. b) Long hemagglutinin variant designed for expression in *E. coli*. Signal peptide and the transmembrane region are absent in this artificial sequence. c) Complete hemagglutinin sequence reference. Underlined is the signal peptide, italics indicate the HA1 domain, bold indicates the HA2 domain and normal text indicates the transmembrane domain. Features were identified using the SMART tool (Schultz, J., Milpetz, F., Bork, P., & Ponting, C. P. (1998). SMART, a simple modular architecture research tool: identification of signaling domains. *Proceedings of the National Academy of Sciences*, 95(11), 5857-5864.)

a) HAp
DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFNPVSE WSYIVEKTNPANDLCYPGNFNNYEELKHLLSRINRFEKIKIIPKSSWPDHEASLGVSSACPYQGGSSFYRNVVWLK KNNTYPTIKESYHNTNQEDLLVLWGIHHPNDEEEQTRIKYKNPTTYISVGTSTLNQRLVLPKIATRISKVNGQSGRVEFF WTILKSNDTINFESNGNFIAPENAYKIVKKGDSTIMKSELEYGNCSTKCQTPVGAINSSMPFHNIHPLTIGECPKYVK SNRLVLATGLRNSP
b) HAq
DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFNPVSE WSYIVEKTNPANDLCYPGNFNNYEELKHLLSRINRFEKIKIIPKSSWPDHEASLGVSSACPYQGGSSFYRNVVWLK KNNTYPTIKESYHNTNQEDLLVLWGIHHPNDEEEQTRIKYKNPTTYISVGTSTLNQRLVLPKIATRISKVNGQSGRVEFF WTILKSNDTINFESNGNFIAPENAYKIVKKGDSTIMKSELEYGNCSTKCQTPVGAINSSMPFHNIHPLTIGECPKYVK SNRLVLATGLRNSPQGEGRKKRGLFGAIAAGFIEGGWQGMVDGWYGYHHSNEQGSYAADRESTQKAIDGVTNK VNSIIDKMNTQFEAVGREFNLEKRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQL RDNAKELGNGCFEFYHRCNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGTYQ
c) Hemagglutinin sequence reported for the Influenza A/chicken/Egypt/1063/2010(H5N1) strain
<u>MEKIVLLLAIIVSLVKS</u> <i>DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGN PMCDEFNPVSE</i> <i>WSYIVEKTNPANDLCYPGNFNNYEELKHLLSRINRFEKIKIIPKSSWPDHEASLGVSSACPYQGGSSFYRN VVWLKKNNTYPTIKESYHNTNQEDLLVLWGIHHPNDEEEQTRIKYKNPTTYISVGTSTLNQRLVLPKIATRISKVNGQSGRVEF FWTILKSNDTINFESNGNFIAPENAYKIVKKGDSTIMKSELEYGNCSTKCQTPVGAINSSMPFHNIHPLTIGECPKYVKS</i> <b>NRL VLATGLRNSPQGEGRKKRGLFGAIAAGFIEGGWQGMVDGWYGYHHSNEQGSYAADRESTQKAIDGVTNK VNSIIDKMNTQFEAVGREFNLEKRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKV RLQLRDNAKELGNGCFEFYHRCNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGTYQ</b> <i>ILSIYSTVAS SLALAIIVAGLFLWMCNSGSLQCRICI</i>

Table 3. Physicochemical properties of the primers.

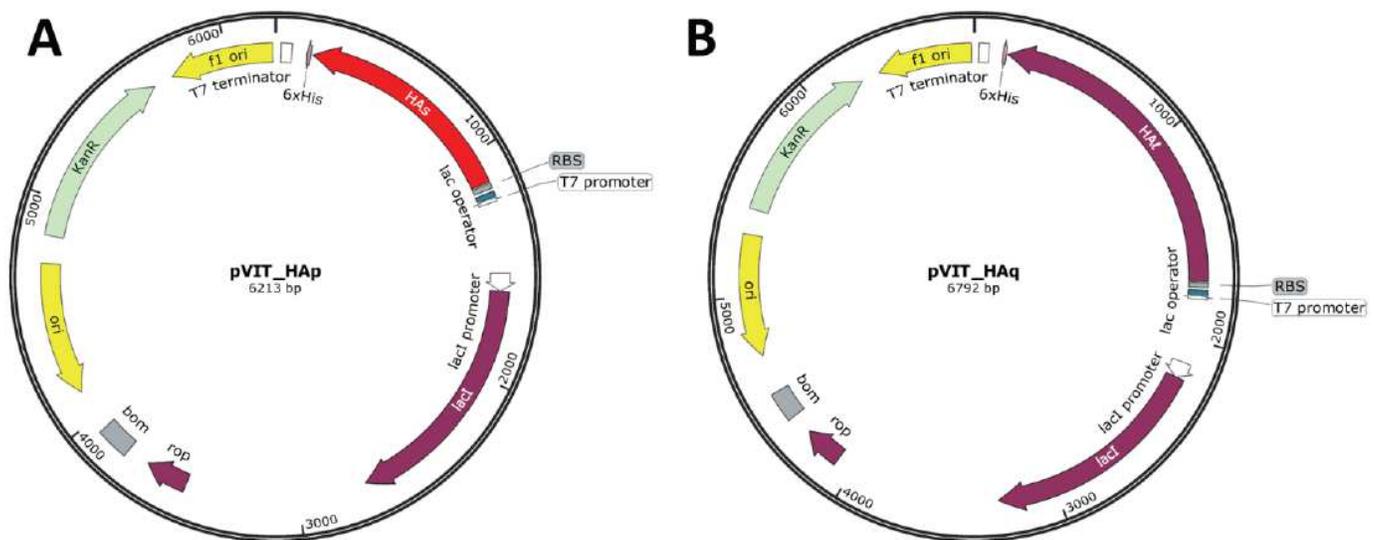
HAp-F				
5'-CTG ATT CAT ATG GAT CAG ATT TGC ATT GGC TAT C-3'				
GC content	Melting temperature	Hairpin $\Delta G$	Hairpin $T_m$	Self-dimer $\Delta G$
38.2%	59°C	-3.1	41°C	-7.82 kcal/mol
HAb-R				
5'-ATT CCG CTC GAG TTA TTA CTG CGG ACT ATT ACG CAG ACC GGT-3'				
GC content	Melting temperature	Hairpin $\Delta G$	Hairpin $T_m$	Self-dimer $\Delta G$
50%	67.9°C	-5.76	64.6°C	12.43 kcal/mol

Table 4. Bioinformatically inferred physicochemical properties of HAp and HAq. MW: Molecular weight. pI: Isoelectric point. hydrophobicity.

HAp, length = 322 aminoacids					
10	20	30	40	50	60
DQICIGYHAN NSTEQVDTIM EKNVTVTHAQ DILEKTHNGK LCDLDGVKPL ILRDCSVAGW					
70	80	90	100	110	120
LLGNPMCDEF PNVSEWSYIV EKTNPANDLC YPGNFNNYEE LKHLLSRINR FEKIKIIPKS					
130	140	150	160	170	180
SWPDHEASLG VSSACPYQGG SSFYRNVVWL IKKNNTYPTI KESYHNTNQE DLLVLWGIHH					
190	200	210	220	230	240
PNDEEEQTRI YKNPTTYISV GTSTLNQRLV PKIATRSKVN GQSGRVEFFW TILKSNDTIN					
250	260	270	280	290	300
FESNGNFIAP ENAYKIVKKG DSTIMKSELE YGNCSTKCQT PVGAINSSMP FHNIHPLTIG					
310	320				
ECPKYVKSNR LVLATGLRNS PQ					
MW	pI	Instability index	Aliphatic index	GRAVY	
36359.05	6.86	32.70 (stable)	78.66	-0.528	

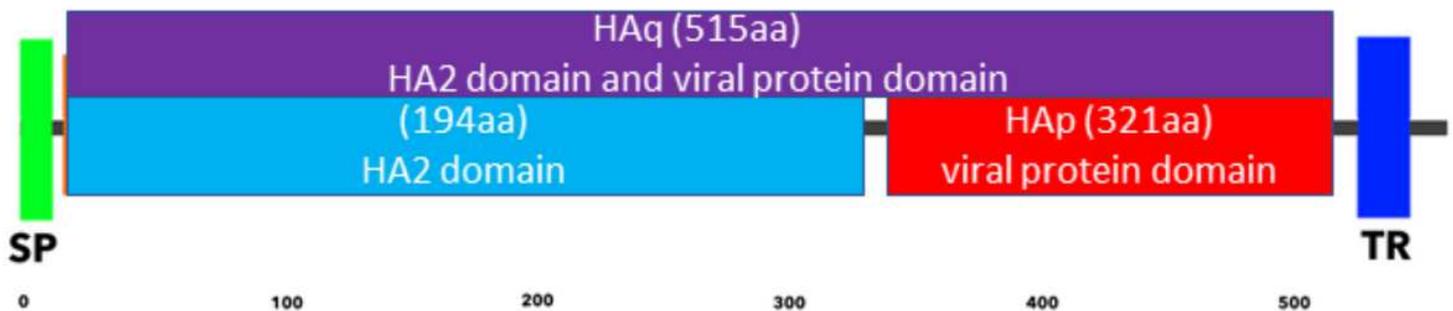
HAq, length = 515 aminoacids					
10	20	30	40	50	60
DQICIGYHAN NSTEQVDTIM EKNVTVTHAQ DILEKTHNGK LCDLDGVKPL ILRDCSVAGW					
70	80	90	100	110	120
LLGNPMCDEF PNVSEWSYIV EKTNPANDLC YPGNFNNYEE LKHLLSRINR FEKIKIIPKS					
130	140	150	160	170	180
SWPDHEASLG VSSACPYQGG SSFYRNVVWL IKKNNTYPTI KESYHNTNQE DLLVLWGIHH					
190	200	210	220	230	240
PNDEEEQTRI YKNPTTYISV GTSTLNQRLV PKIATRSKVN GQSGRVEFFW TILKSNDTIN					
250	260	270	280	290	300
FESNGNFIAP ENAYKIVKKG DSTIMKSELE YGNCSTKCQT PVGAINSSMP FHNIHPLTIG					
310	320	330	340	350	360
ECPKYVKSNR LVLATGLRNS PQGEGRRKKR GLFGAIAGFI EGGWQGMVDG WYGYHHSNEQ					
370	380	390	400	410	420
GSGYAADRES TQKAIDGVTN KVNSIIDKMN TQFEAVGREF NNLEKRIENL NKKMEDGFLD					
430	440	450	460	470	480
VWTYNAELLV LMENERTLDF HDSNVKNLYD KVRLQLRDNA KELGNGCFEF YHRCNECME					
490	500	510			
SVRNGTYDYP QYSEEARLKR EEISGVKLES IGTYQ					
MW	pI	Instability index	Aliphatic index	GRAVY	
58602.71	6.02	36.72 (stable)	73.22	-0.657	

# Figures



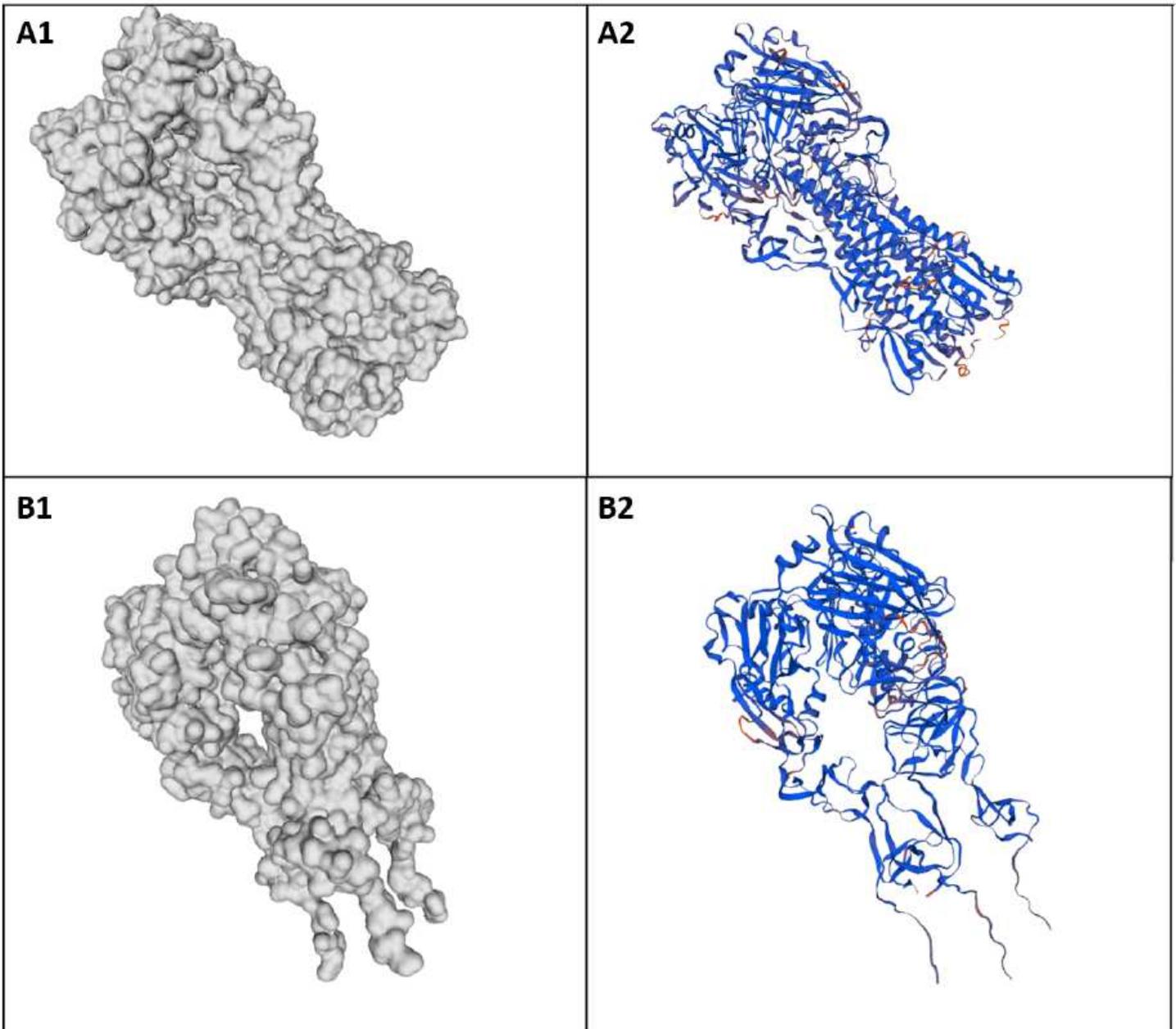
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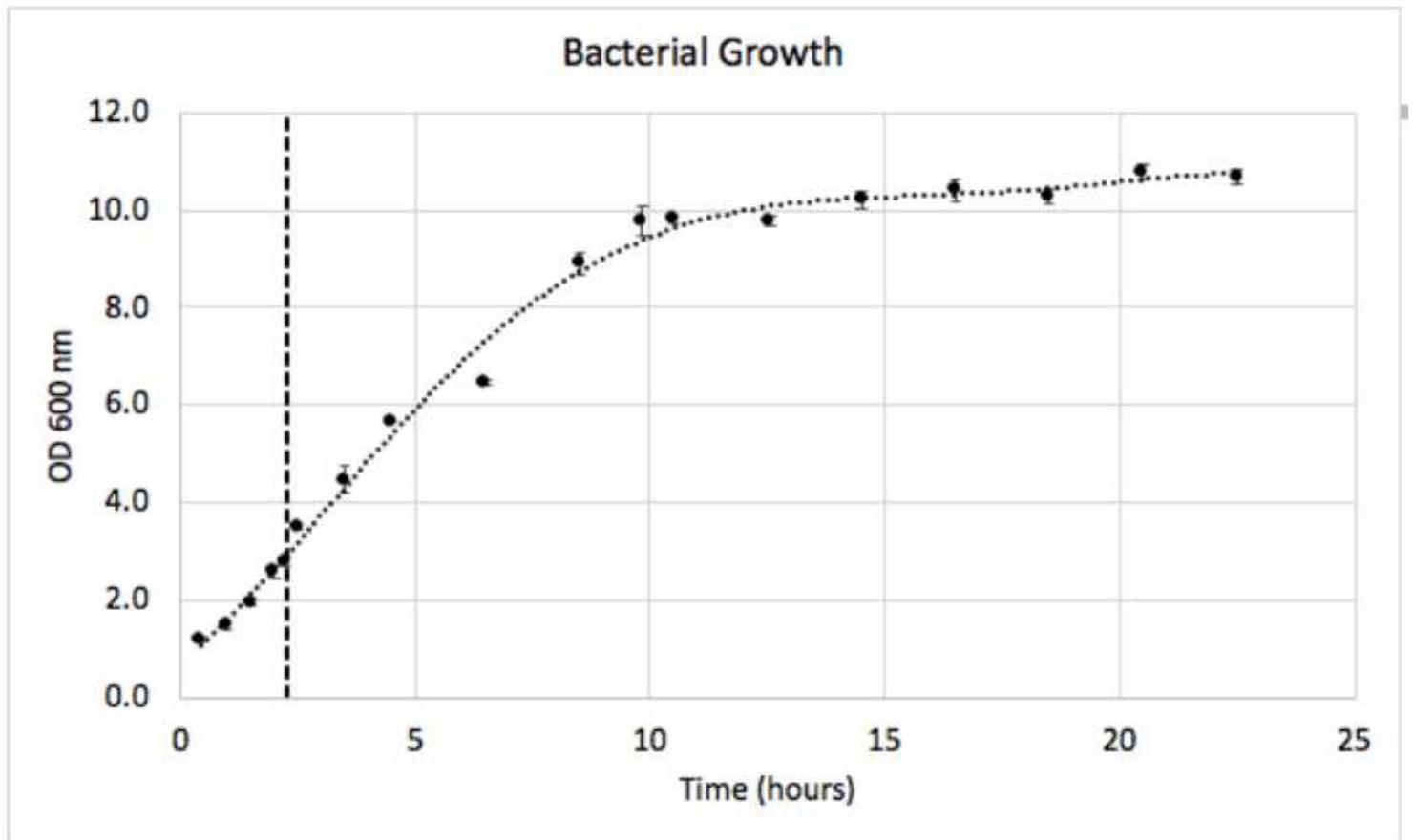
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**Figure 4**

Growth curve of the *E. coli* rHA producer during the fermentation in 1L bioreactor. The dashed vertical line indicates the addition of IPTG for the induction of HA expression. The dotted line represents a 5th order regression fit and the error bars represent the standard deviation of triplicates.

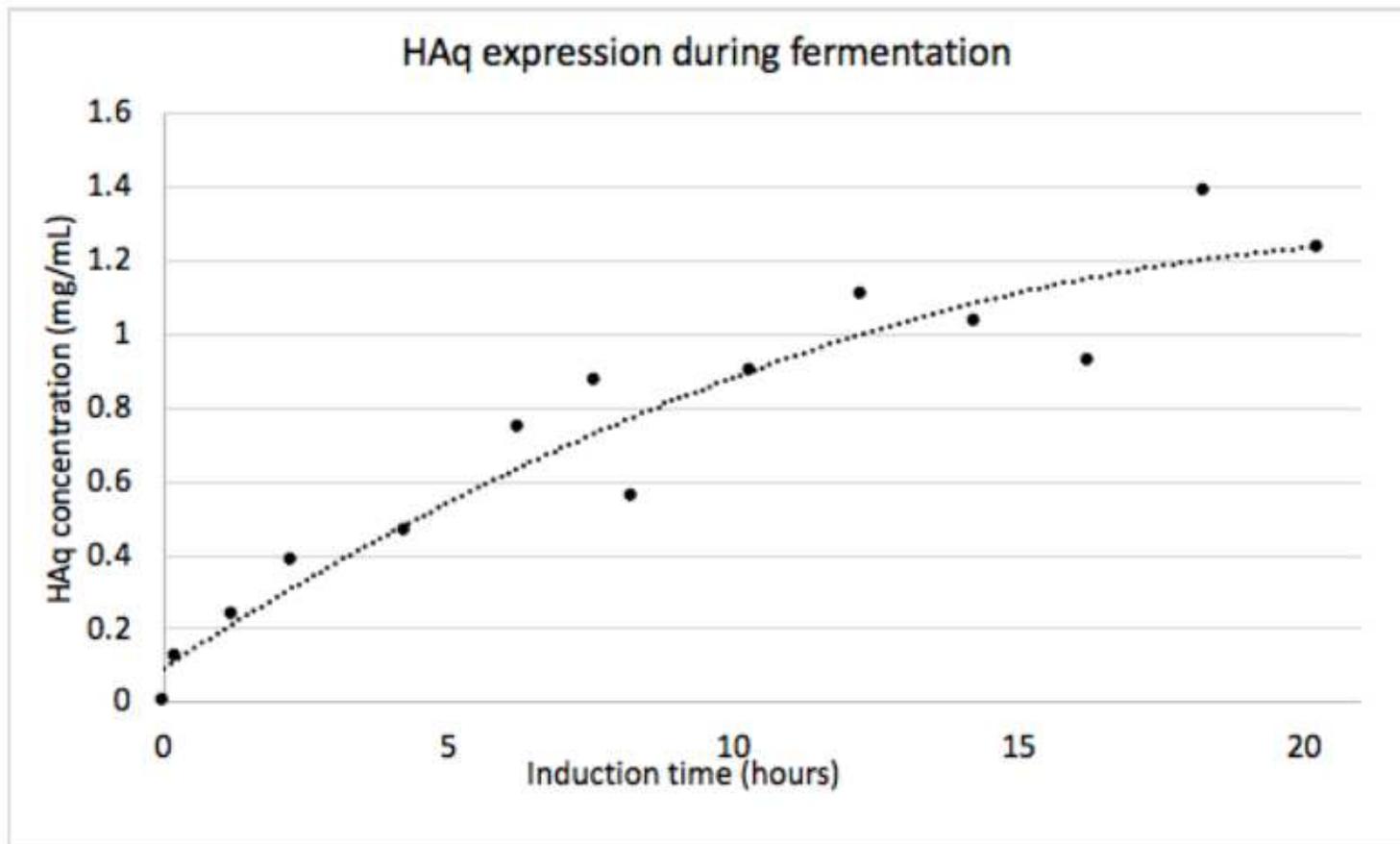
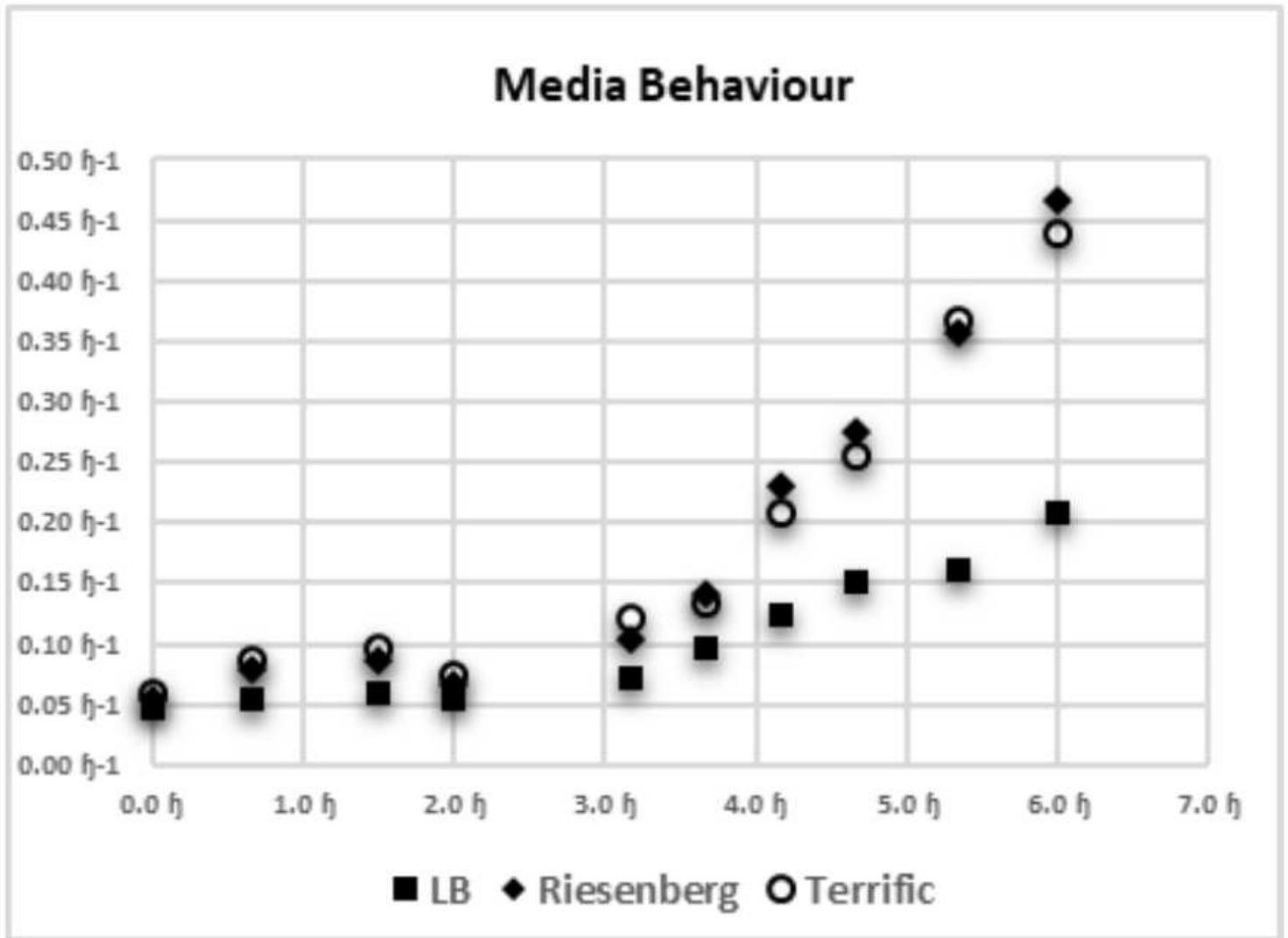


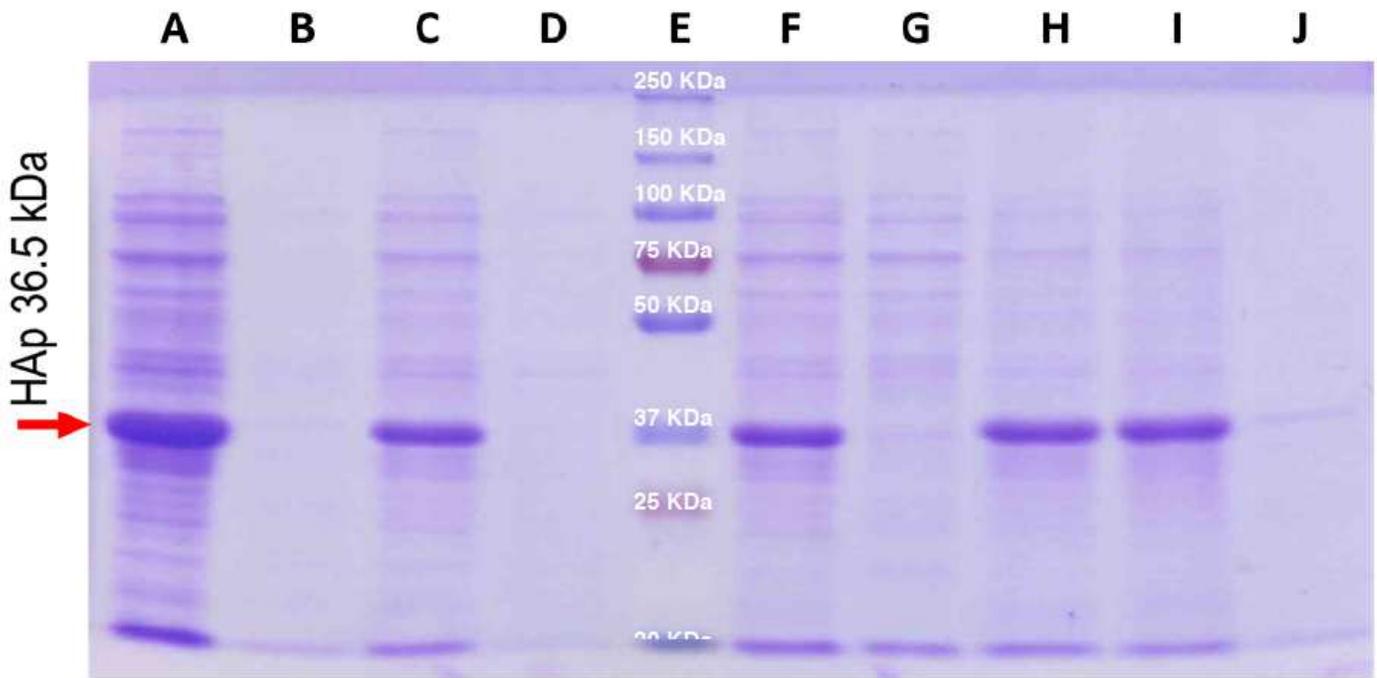
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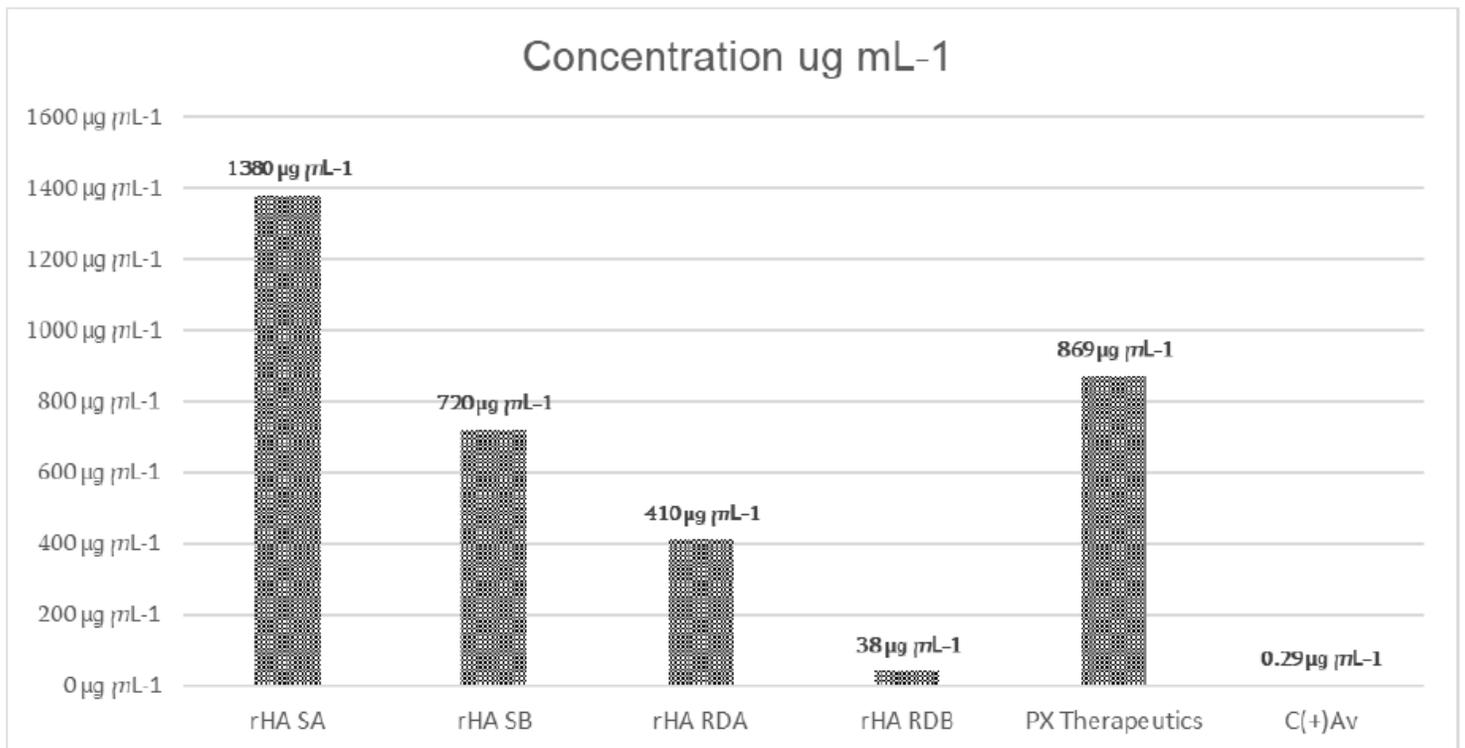
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