

Integrated Computational and Experimental Analysis of Recombinant Trastuzumab in ExpiCHO-S™ Cells

Saranraj Sankar ¹, Priya Rajan ^{1,*}

¹ Department of Bioinformatics, School of Life Sciences, Vels Institute of Science Technology and Advanced Studies, Chennai 600117, Tamil Nadu, India

* Correspondence: priya.sls@vistas.ac.in (P.R.);

Received: 16.03.2026; Accepted: 4.07.2026; Published: 15.10.2026

Abstract: In this work, a computational and experimental methodology for the design, expression, purification, and first biochemical characterization of recombinant trastuzumab is described. HADDOCK was used for molecular docking in order to examine the interaction between trastuzumab and the HER2 target. The anticipated binding orientation, interface interactions, and energetic contributions within the antibody-antigen complex were all revealed by the docking analysis. Stable complex formation was indicated by the top-ranked docking clusters' constant electrostatic and van der Waals contacts, good HADDOCK scores, and low intermolecular RMSD values. These results support the formation and propagation of a mammalian expression construct expressing trastuzumab in *Escherichia coli*. Plasmid isolation and colony screening were used to find positive transformants. The verified construct was subsequently transiently transfected into ExpiCHO-S™ cells for antibody expression, with maintained cell viability reported during the expression period. Protein A affinity chromatography was used to purify secreted trastuzumab, and size-exclusion high-performance liquid chromatography (SEC-HPLC) was used to evaluate its purity profile. A prominent monomeric signal with little discernible aggregation was shown by the chromatographic analysis. SDS-PAGE was used to further investigate the fractions that corresponded to the primary SEC peak in both reducing and non-reducing conditions. Bands compatible with intact IgG and the anticipated heavy and light chains following reduction were shown by electrophoretic analysis. Overall, the study shows that it is possible to produce and assess recombinant trastuzumab by combining computational modeling with mammalian expression and biochemical characterization techniques.

Keywords: trastuzumab; docking; protein A purification; SEC-HPLC; SDS–PAGE.

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1. Introduction

Breast cancer continues to be one of the most common cancers in the world and a major contributor to women's malignancy-related morbidity and mortality. Disease heterogeneity and treatment resistance remain key clinical issues despite tremendous progress in early diagnosis and therapeutic approaches. As a result, one of the top priorities in oncology research today is developing and improving tailored treatments that enhance precision and patient outcomes [1]. The human epidermal growth factor receptor 2 (HER2), which is excessively expressed in 15–20% of instances of breast cancer and linked to aggressive disease development and an adverse outcome, is the precise target of the humanized monoclonal antibody trastuzumab [2]. Trastuzumab improves patient survival and clinical outcomes by binding to the extracellular

domain of the HER2 receptor, which suppresses downstream tumor signaling pathways and enhances antibody-dependent cellular cytotoxicity [3]. Because of its clinical significance, trastuzumab's design and manufacturing must be optimized to guarantee both its therapeutic efficacy and worldwide accessibility. Monoclonal antibody development has accelerated due in large part to recent developments in computational biology and bioinformatics. Accurate identification and confirmation of antibody and antigen sequences are made possible by sequence analysis employing curated protein databases like UniProt and homology search methods like BLAST [4]. The prediction of molecular interactions and binding affinity between trastuzumab and the HER2 receptor is made easier by protein–protein docking tools like HADDOCK, which offer information on structural stability and functional performance [5]. Furthermore, by matching nucleotide sequences with host-specific codon usage preferences, codon optimization methods are used to improve translational efficiency and increase gene expression [6]. The proper assembly and coordinated production of antibody heavy and light chains in mammalian systems are further guaranteed by synthetic gene creation and vector design optimization. When combined, these computational techniques greatly simplify antibody engineering, lower the expense of experiments, and improve therapeutic potential [7]. The yield, quality, and biological activity of monoclonal antibodies are all significantly influenced by the choice of a suitable mammalian expression method. Because mammalian cells can carry out intricate post-translational alterations, such as appropriate folding and glycosylation, they are favored for the manufacture of therapeutic antibodies [8]. With its excellent protein output, scalability, and appropriateness for industrial biomanufacturing, the ExpiCHO-S cell line has become one of the most effective platforms for recombinant protein expression [9]. Through coordinated heavy and light chain manufacturing, this approach facilitates downstream processing, guarantees precise antibody assembly, and maintains steady gene expression. Recent developments in genetic engineering and logical vector design have further boosted co-expression of trastuzumab heavy and light chains in ExpiCHO-S cells, leading to greater functional activity, optimal glycosylation profiles, and increased secretion efficiency [10]. In addition to improving therapeutic efficacy, these advancements shorten production schedules, facilitating quicker clinical translation and better trastuzumab accessibility for patients across the globe [11]. The current study aims to enhance trastuzumab synthesis in ExpiCHO-S cells, with an emphasis on high yield, purity, and functional integrity. The exact genetic linkage and coordinated production of trastuzumab heavy and light chains within this expression system constitute a key tactic. Through the integration of sophisticated mammalian expression methods with bioinformatics-guided antibody design, this work seeks to create a reliable and expandable trastuzumab production platform.

2. Materials and Methods

2.1. Chemicals, kits, and reagents.

Tris-EDTA (TE) buffer and chemically competent *Escherichia coli* cells (One Shot® TOP10) were acquired from Invitrogen. Broth made with Luria-Bertani (LB) and Luria-Bertani HiMedia supplied the HiVeg agar (Miller formulation). The analytical-grade substances ampicillin, potassium chloride, sodium hydroxide (NaOH), sodium dodecyl sulfate (SDS), agarose, bromophenol blue, trypan blue, and 1 × phosphate-buffered saline (PBS) was employed without additional purification. OptiPRO™ serum-free medium (SFM) for DNA reagent complexation, ExpiFectamine™ CHO Enhancer, ExpiCHO™ Feed, ExpiCHO-S™

cells, ExpiCHO™ Expression Medium, and ExpiFectamine™ CHO reagent were purchased from Gibco and utilized in accordance with the manufacturer's instructions. The QIAprep Spin Miniprep Kit, which was acquired from Qiagen, was used to isolate and purify plasmid DNA.

2.2. Instruments and consumables.

An inverted microscope from Lawrence & Mayo was used to analyze the morphology and confluency of the cells. To do cell counting and viability studies, a Neubauer enhanced hemocytometer that was purchased from Marienfeld was used. An Eppendorf New Brunswick™ S41i CO₂ incubator shaker was used to sustain suspension cultures. Thermo Fisher Scientific provided the NanoDrop™ Lite spectrophotometer for measuring DNA and protein concentrations and the Multiskan™ SkyHigh microplate spectrophotometer for absorbance-based assays. Tarsons supplied the vent-cap Erlenmeyer shaker flasks that were used for suspension culture.

2.3. Protein-protein docking.

The UniProt database (<https://www.uniprot.org/>) provided the amino acid sequence of the human epidermal growth factor receptor 2 (HER2), the protein that targets breast cancer [12], and the DrugBank database (<https://go.drugbank.com/>) provided the sequence of the therapeutic monoclonal antibody trastuzumab. The accession number for Trastuzumab in the DrugBank database is DB00072 (<https://go.drugbank.com/drugs/DB00072>). The target protein HER2 was retrieved with the UniProt ID P04626 (www.uniprot.org/uniprotkb/P04626/entry). The Reverse Translate tool was used to convert protein sequences into nucleotide sequences according to codon use tables (www.bioinformatics.org/sms2/rev_trans.html) [13], and the ExPASy Translate tool was used to verify the accuracy of the sequences (<https://web.expasy.org/translate/>).

The HADDOCK 2.4 online server (<https://rascar.science.uu.nl/haddock2.4/>), a well-known protein-protein docking system to forecast binding behaviors and complex conformation, was used to carry out molecular docking between trastuzumab and HER2 [14]. The antibody and receptor structures were separated into distinct coordinate files and subjected to structural refinement prior to docking. To prevent interference during docking calculations, water molecules, ions, ligands, and other non-essential heteroatoms were eliminated. The Biovia Discovery Studio software was used to address incomplete residues and missing side chains. The interaction between the extracellular domain of HER2 and the complementarity-determining regions (CDRs) of trastuzumab was the main focus of docking. The computations were performed using the default force-field settings provided by HADDOCK. This approach made it possible to assess the molecular interactions that underlie trastuzumab's HER2 receptor binding efficacy and selectivity.

2.4. Plasmid vector design.

Further categorization: VectorBuilder, an online tool, was used to design the plasmid vectors (<https://en.vectorbuilder.com/products-services/service/online-vector-design.html>) [15]. The improved heavy chain and light chain of the Trastuzumab monoclonal antibody are linked by a 15-amino-acid synthetic peptide sequence, namely Glycine and serine, using the formula ((G4S)3) (Gly-Gly-Gly-Gly-Ser)₃. The Linker Sequence - GGC GGT GGA GGC TCA GGA GGG GGC GGA TCT GGC GGA GGA GGT TCC GGC GGA GGG GGC TCA.

The flexible glycine-serine linker [G₄S]₃ was used to join the optimized heavy and light chains of the trastuzumab monoclonal antibody in order to promote appropriate folding and functional assembly. The human cytomegalovirus (CMV) immediate-early promoter for high-level expression was present in the mammalian expression vector pcDNA3.1(+), which led to its selection. The linked gene cassette was placed into the vector's multiple cloning site, and a Kozak consensus sequence {GCCACC} was added upstream of the start codon to improve translation initiation [16]. Using the VectorBuilder codon optimization program, codon optimization was carried out to increase expression efficiency in CHO-S cells (<https://en.vectorbuilder.com/tool/codon-optimization.html>) [17]. The vector contains an ampicillin resistance gene for bacterial selection [18], a pUC replication origin for high-copy propagation in *E. coli* [19], and an SV40 late polyadenylation signal for appropriate transcription termination and mRNA processing [20]. BiotechDesk Private Limited was the source of the final synthetic plasmid construct.

2.5. Preparation of plasmid.

Following the manufacturer's instructions, the lyophilized plasmid vector containing the coupled trastuzumab heavy and light chains (4 µg) was reconstituted. In short, the vial was centrifuged at 8,000 rpm for 1 minute at 4°C after being allowed to acclimate to ambient temperature for 15 minutes. To guarantee that the plasmid DNA was completely resuspended, 40 µL of 1× Tris-EDTA (TE) buffer was then added to the vial and vortexed for one minute.

2.6. Preparation of bacterial culture medium.

In order to make Luria-Bertani (LB) broth, 5g of LB broth powder was dissolved in 100mL of distilled water. To prepare the solid medium, 5 g of LB powder was dissolved in 100 mL of distilled water and HiVeg agar. Both media were autoclaved for one hour at 121°C and 15 psi of pressure to sterilize them. A final concentration of 100 µg/mL of ampicillin was added to the LB agar medium after it had been sterilized and allowed to cool to about 50°C. After that, sterile Petri dishes were filled with melted agar, which was left to set at room temperature. Ampicillin was added to 50 mL of sterile LB broth in a flask, allowed to cool to room temperature, and then diluted to a final concentration of 100 µg/mL before use.

2.7. Transformation.

The chemically competent *Escherichia coli* cells from One Shot® TOP10 were taken out of storage at -80°C and allowed to gradually thaw on ice. To guarantee even distribution and prevent cell damage, 50 µL of competent cells ($\approx 1 \times 10^9$ cfu/µg) were combined with 1 µL of plasmid DNA (100 to 500 ng) and gently swirled by flipping the tube. To promote DNA-cell membrane contact, the mixture was incubated on ice for 40 minutes. The cells were subjected to heat shock by being placed in a dry bath that had been prepared to 42°C for 1.5 minutes. The cells were then immediately incubated on ice for 2 minutes in order to stabilize the cell membranes. Following the addition of 800 µL of LB broth supplemented with ampicillin (100 µg/mL), the cells were shaken and incubated for 1.5 hours at 37 °C to promote recovery and the production of the antibiotic resistance marker. Following recovery, LB agar plates containing ampicillin (100 µg/mL) were covered with a 5 µL aliquot of the converted cell suspension. Colonies grown from effectively converted cells were able to grow on the plates after they were incubated for the entire night at 37°C [21].

2.8. Quick plasmid isolation using alkaline lysis method.

For alkaline lysis, a bacterial colony was taken from LB agar plates containing ampicillin and put into a 1.5 mL microcentrifuge tube. After adding 15 μ L of lysis solution that included 0.1 to 0.2 N NaOH and 1% (w/v) SDS, the cells were carefully pipetted or briefly vortexed to resuspend them completely. To guarantee full cell lysis, the suspension was incubated for 10 minutes at 70 °C. For the purpose of stabilizing DNA and acting as a tracking dye, 6 μ L of a 1:1 combination of potassium chloride (KCl) and bromophenol blue was added to the lysate. To encourage DNA precipitation, the samples were gently mixed and incubated on ice for five minutes. The cellular debris was pelleted after centrifugation at 12,000 rpm for 5 min, and 7 μ L of clear supernatant was meticulously collected. Agarose gel electrophoresis was used to examine the plasmid DNA-containing supernatant and verify that the recombinant plasmid was present. The gel's band intensity was used to identify positive clones. To establish pure cultures for further study, we streaked a subset of positive colonies onto new LB agar plates treated with ampicillin and cultured them for 12-16 h at 37°C [22].

2.9. Plasmid isolation and purification.

Plasmid DNA encoding trastuzumab was obtained using the QIAprep Spin Miniprep Kit (Qiagen) per the manufacturer's instructions. The *E. coli* culture (5 mL) was harvested overnight at 25°C by centrifugation at 8,000 rpm for 3 minutes. After resuspending the bacterial pellet in 250 μ L of Buffer P1 containing RNase A, 250 μ L of Buffer P2 was added, and the mixture was gently mixed by inversion until total lysis was accomplished. In order to avoid plasmid DNA degradation, the lysis step was kept to ≤ 5 minutes. To pellet cellular debris, 350 μ L of Buffer N3 was then added, mixed right away by inversion, and centrifuged for 10 minutes at 13,000 rpm. For 30 to 60 seconds, the cleared supernatant was centrifuged after being moved to a QIAprep spin column. The column underwent centrifugation procedures to eliminate impurities after being successively cleaned with 0.5 mL of Buffer PB and 0.75 mL of Buffer PE. To get rid of any remaining ethanol, a final centrifugation phase lasting one minute was carried out. 50 μ L of Buffer EB (or nuclease-free water) was used to elute the plasmid DNA following a 1-minute incubation period and a 1-minute centrifugation at 13,000 rpm. A NanoDrop™ Lite spectrophotometer (Thermo Fisher Scientific) was used to measure the concentration and purity of the recovered plasmid DNA. The purified plasmid was then kept for use in further investigations at -20°C.

2.10. Restriction digestion.

The recombinant trastuzumab plasmid DNA was restriction digested for downstream analysis and construct verification. Each reaction was made with plasmid DNA, the proper restriction buffer, restriction enzymes, and nuclease-free water in a total volume of 20 μ L. To accomplish double digestion, plasmid DNA was first digested with EcoRV at 37°C for 50 minutes. This was followed by the sequential addition of BstBI and additional incubation at 65°C for 70 minutes. The digested products were separated on a 0.8% agarose gel using a DNA ladder for fragment size estimation and electrophoretic conditions of 120 V for one hour. To verify effective digestion and plasmid integrity, banding patterns obtained during electrophoresis were compared with the anticipated restriction profile of the trastuzumab construct. To ensure repeatability and precise build validation, we carried out all reactions and gel analyses under standard operating procedures.

2.11. Culture, thawing, and maintenance of ExpiCHO-S™ cells.

We purchased ExpiCHO-S™ cells from Thermo Fisher Scientific in India. A clonal offspring of the CHO-S lineage, the ExpiCHO-STM cell line was created especially for high-density suspension culture in ExpiCHO™ Expression Medium. Following the ExpiCHO™ Expression System technique, frozen cells were provided in a ready-to-use state and thawed straight into the specified culture medium. Cryovials were taken out of the liquid nitrogen storage (-19°C) and quickly thawed in a water bath at 37°C for 1-2 minutes while being gently stirred in order to recover the cells. Each vial's contents were aseptically transferred into a sterile 125 mL polycarbonate Erlenmeyer shake flask that held 30 mL of ExpiCHO™ Expression Medium that had been warmed up beforehand. The cultures were cultured on an orbital shaker set at 125 rpm (19 mm orbital diameter) at 37°C in a humidified incubator with 8% CO₂. On the third day after thawing, cell density and vitality were measured; viability was generally more than 90%. In order to sustain exponential growth, cultures were regularly observed and passed through. When the viable cell density was between 4 and 6 × 10⁶ cells/mL, subculturing was carried out. ExpiCHO™ cultures were seeded into brand-new, pre-warmed ExpiCHO™ Expression Medium at the recommended cell density for normal maintenance and transfection preparation. Viable cell counts were used to determine the necessary volume of cell suspension, which was then aseptically transferred into sterile shaking flasks. Until the required cell density was reached, cultures were kept at 37°C, ≥80% relative humidity, and 8% CO₂ while being continuously shaken at 125 rpm.

2.12. Transfection of ExpiCHO-S™ cells.

ExpiCHO-S™ cells were grown to their ideal densities before transfection in order to use the ExpiCHO-S™ expression system for transient transfection. The cultures were kept at 4 to 6 × 10⁶ viable cells/mL. To allow for overnight growth, they were passaged to a density of 3 to 4 × 10⁶ viable cells/mL on the day before transfection (Day 1). Viable cell density and viability were measured on the day of transfection (Day 0), and cultures generally reached 7 to 10 × 10⁶ viable cells/mL with viabilities of 95–99%. To achieve a final density of 6 × 10⁶ viable cells/mL, cells were diluted with freshly heated ExpiCHO™ Expression Medium and gently mixed to guarantee a consistent suspension. ExpiFectamine CHO was used to produce transfection components in accordance with the manufacturer's instructions. To create a complex, plasmid DNA was diluted in cooled OptiPRO medium, and ExpiFectamine™ CHO reagent was diluted individually in chilled OptiPRO™ medium before being mixed. After being incubated for one to five minutes at room temperature, the DNA–reagent mixtures were carefully stirred and introduced via droplets to the cell cultures. To guarantee effective mixing, gas exchange, and maximum transfection effectiveness, transfected cells were cultured at 37 °C in a humidified chamber with 8% CO₂ on a rotating shaker set at 125 rpm.

2.13. Harvest.

Cell cultures were taken when their viability fell to around 65%. To separate cells and detritus from the released protein-containing supernatant, the culture suspension was spun using a cooling centrifuge set to 4,000 to 5,000 × g for 30 minutes at 4°C. Before being sent to downstream analysis, the collected supernatant was further purified by filtering it through a membrane with a hole size of 0.22 µm.

2.14. Immunoaffinity purification of trastuzumab using Protein A magnetic beads.

To achieve homogeneity, Protein A magnetic beads were first thoroughly resuspended by pipetting several times or by gently inverting and rotating them for two minutes. A 1.5 mL microcentrifuge tube was filled with an aliquot of 25–50 μ L of beads, which were then cleaned with 400 μ L of binding/wash buffer. A magnetic separator was used to gather the beads after they had been gently mixed, and the supernatant was disposed of. To guarantee correct equilibration, this cleaning procedure was carried out twice. In order to facilitate antibody binding, 400 μ L of binding/wash buffer was gently rotated while trastuzumab (5–50 μ g/mL) and the equilibrated Protein A magnetic beads were incubated for 30 minutes at room temperature. After incubation, the beads were separated magnetically, and the antibody-containing supernatant was extracted. After that, 400 μ L of binding/wash buffer was used to wash the bead–antibody complexes four times in order to exclude proteins that were not precisely bound. In order to elute bound trastuzumab, non-denaturing conditions were used. After separating the bead-antibody complexes magnetically and washing them once with 400 μ L of binding/wash buffer, they were gently rotated for five minutes and then collected using a magnetic separator. The beads were gently rotated while incubated with 25–50 μ L of elution buffer for 5 minutes in order to elute trastuzumab. Following magnetic separation, the eluted fractions were gathered and kept for later analysis at -80°C . The purified trastuzumab was analyzed by SDS-PAGE under reducing and non-reducing conditions.

2.15. ELISA.

The FineTest® Human Trastuzumab ELISA Kit, which is based on the indirect ELISA principle, was used to estimate recombinant trastuzumab quantitatively. Samples were prepared by centrifugation to obtain clear supernatants; cell lysates were generated using lysis buffer containing PMSF followed by sonication and clarification by centrifugation; lyophilized standards were reconstituted and serially diluted, and the HRP-conjugated detection antibody was prepared in accordance with the manufacturer's instructions. After further washing, TMB substrate solution was added, and color development was allowed for 10 to 20 minutes in the dark. Trastuzumab concentration was quantitatively assessed by measuring absorbance at 450 nm using a microplate reader after stopping the reaction with stop solution.

2.16. Size-exclusion chromatography with high-performance liquid chromatography.

Purified trastuzumab samples were run through size-exclusion chromatography combined with high-performance liquid chromatography (SEC-HPLC) before being subjected to SDS–PAGE analysis. Before SDS-PAGE analysis, purified trastuzumab samples were examined using SEC-HPLC to determine molecular homogeneity, purity, and aggregation state. Recombinant trastuzumab purity and molecular homogeneity were assessed using size-exclusion high-performance liquid chromatography (SEC-HPLC) analysis utilizing an Agilent 1260 Infinity LC system fitted with a TSKgel G3000SWxl column. 2.0 μ L of the purified sample was injected for analysis after it was prepared in PBS (pH 7.2) at a concentration of 13.65 mg/mL. Using 0.1 mol/L sodium sulfate in 0.118 mol/L phosphate buffer (pH 6.7 ± 0.3) as the mobile phase and UV detection at 280 nm, chromatographic separation was performed. Each analysis had a total run length of 25 minutes, and chromatograms were integrated using a linear baseline correction approach with noise suppression within the retention time window of 8.0–20.0 minutes. For analysis, peaks with an area threshold of at least 0.05% of the entire

chromatographic area were taken into consideration. A minimum of three replicate injections were used to evaluate method repeatability, with acceptance criteria of $\leq 1.0\%$ RSD for retention time and $\leq 2.0\%$ RSD for peak area. A size-exclusion column equilibrated with phosphate-buffered saline (PBS, pH 7.2) as the mobile phase was used for the separation process. UV absorbance at 280 nm was used to track protein elution [23].

2.17. SDS-PAGE.

The HPLC-selected trastuzumab fraction's molecular integrity and purity were assessed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [24]. A 30% acrylamide solution was used to create a 12% resolving gel. 20 μ g of pure antibody was loaded per lane under both reducing (with dithiothreitol, DTT) and non-reducing conditions, and the protein content was assessed using the Bradford assay. The tracking dye was bromophenol blue, and the protein size was estimated using a pre-stained molecular weight marker (10 μ L). Standard procedures for electrophoresis were followed, with 30 to 40 minutes of room-temperature gel staining with Coomassie Brilliant Blue. Destaining was done for 6–12 hours until the protein bands were clearly visible. The purity and integrity of the purified trastuzumab were validated by the presence of separate bands that matched the anticipated molecular weights of the heavy and light chains.

3. Results and Discussion

3.1. Molecular docking of trastuzumab with HER2.

The HADDOCK 2.4 server was used to perform protein–protein docking between trastuzumab and the HER2 receptor in order to determine the most stable binding conformations and clarify the molecular basis of their interaction. Several docked complexes were obtained using HADDOCK clustering analysis [25], and they were ranked according to the HADDOCK score, cluster size, RMSD, and energetic contributions such as van der Waals and electrostatic interactions and the results were tabulated in Table 1.

Table 1. Calculated HADDOCK scores and related parameters of trastuzumab and HER2.

Clusters	HADDOCK Score	Cluster size	RMSD	Van der Waals energy	Electrostatic energy	Z-score
1	-46.2 ± 0.9	43	6.7 ± 0.1	-32.0 ± 5.3	-81.8 ± 29.3	-0.1
2	-53.0 ± 6.9	13	5.5 ± 0.3	-33.6 ± 3.5	-79.8 ± 31.6	-1.3
3	-52.0 ± 3.5	11	4.3 ± 0.7	-26.0 ± 1.8	-129.1 ± 20.4	-1.2
4	-47.8 ± 5.0	10	7.3 ± 0.3	-24.0 ± 4.0	-137.6 ± 31.7	-0.4
5	-50.0 ± 7.2	8	3.1 ± 0.3	-17.3 ± 5.1	-172.4 ± 47.1	-0.8
6	-45.7 ± 5.0	7	3.4 ± 0.5	-14.8 ± 4.3	-177.9 ± 39.1	-0.0
7	-44.0 ± 3.1	7	8.8 ± 0.1	-34.4 ± 3.0	-68.5 ± 8.1	0.3
8	-41.7 ± 9.9	5	6.9 ± 0.3	-21.5 ± 4.7	-118.4 ± 49.2	0.7
11	-33.0 ± 4.1	4	8.6 ± 0.1	-28.1 ± 2.5	-58.0 ± 24.5	2.2
12	-41.3 ± 8.3	4	6.2 ± 0.1	-19.8 ± 5.8	-113.9 ± 35.0	0.7

With the lowest HADDOCK score (-53.0 ± 6.9) and a substantially negative Z-score (-1.3), Cluster 2 was the most advantageous docking solution among the created clusters. This indicates great statistical significance in comparison to the entire docking population. With a low RMSD value of 5.5 ± 0.3 Å and a moderate cluster size of 13 structures, this cluster showed good topological convergence and binding integrity.

Favorable van der Waals (-33.6 ± 3.5) and electrostatic energy contributions (-79.8 ± 31.6) maintained the association, demonstrating the importance of both long-range electrostatic

interactions and hydrophobic contacts in complex formation. A stable and distinct binding mode was indicated by Cluster 3's comparatively high HADDOCK score (-52.0 ± 3.5), Z-score of -1.2 , and lower RMSD (4.3 ± 0.7 Å). This cluster was notable for having stronger electrostatic interactions (-129.1 ± 20.4), indicating that charge-driven interactions play a major role in trastuzumab binding at particular HER2 epitopes. The decreased cluster size in comparison to Cluster 2, however, indicates a slightly lower level of conformational sampling. Despite having a less favorable Z-score (-0.8) and relatively weaker van der Waals contacts, Cluster 5 had the lowest RMSD (3.1 ± 0.3 Å), implying a very consistent docking orientation. Despite being structurally stable, this cluster's lower energetic favourability caused it to rank lower overall.

Conversely, clusters with positive Z-scores (Clusters 8, 11, and 12) showed less favorable interaction energies, higher RMSD values, and weaker HADDOCK scores, all of which showed less-than-ideal binding conformations. Therefore, it is less plausible that these clusters reflect biologically significant interactions between trastuzumab and HER2. Trastuzumab's robust and specific binding to HER2 is generally confirmed by the docking study, which is in line with its well-established clinical effectiveness in treating HER2-positive breast cancer.

The thermodynamic stability of the antibody–receptor complex is shown by the predominance of negative Z-scores and HADDOCK scores among the top-ranked clusters. Crucially, the balance between van der Waals and electrostatic contributions highlights how cooperative the molecular contacts controlling trastuzumab recognition of HER2 are. A scatter plot showing the correlation between the proportion of common connections (FCC) and the HADDOCK score for docked Trastuzumab-HER2 complexes (Figure 1A). Single docking solutions are represented by individual dots, while cluster representatives are indicated by colored symbols with error bars showing standard deviations within each cluster. More favorable and structurally consistent binding mechanisms are indicated by lower HADDOCK scores in conjunction with higher FCC values.

Electrostatic interaction energy for Trastuzumab-HER2 docking clusters plotted against interface root mean square deviation (i-RMSD) as shown in Figure 1B. Error bars are displayed for cluster centers, and each point represents a distinct docking stance. Stable and energetically advantageous antibody–antigen interactions are represented by clusters with lower electrostatic energies at lower i-RMSD values. Correlation graphic showing the van der Waals interaction energy for docked Trastuzumab-HER2 complexes as a function of i-RMSD (Figure 1C). Standard deviations for cluster averages are shown. At the binding interface, optimum packing and advantageous hydrophobic interactions are reflected in clusters with lower i-RMSD values and stronger negative van der Waals energies. The distribution of electrostatic interaction energies among HADDOCK clusters is displayed in a box-and-whisker graphic (Figure 1D). Outliers, median values, and interquartile ranges are displayed.

Stronger long-range electrostatic contributions to the stability of Trastuzumab-HER2 binding are indicated by clusters with lower median electrostatic energies. Van der Waals interaction energies for various HADDOCK clusters are represented using a box-and-whisker method (Figure 1E). Different interface packing efficiencies and short-range interactions that contribute to complex stabilization are reflected in variations in median values and dispersion. The trastuzumab–HER2 binding interface is computationally represented by the docking study, which also highlights possible interaction patterns and energetic contributions.

Experimental research is needed to verify binding affinity, biological activity, and therapeutic efficacy, even though these results support the predicted complex.

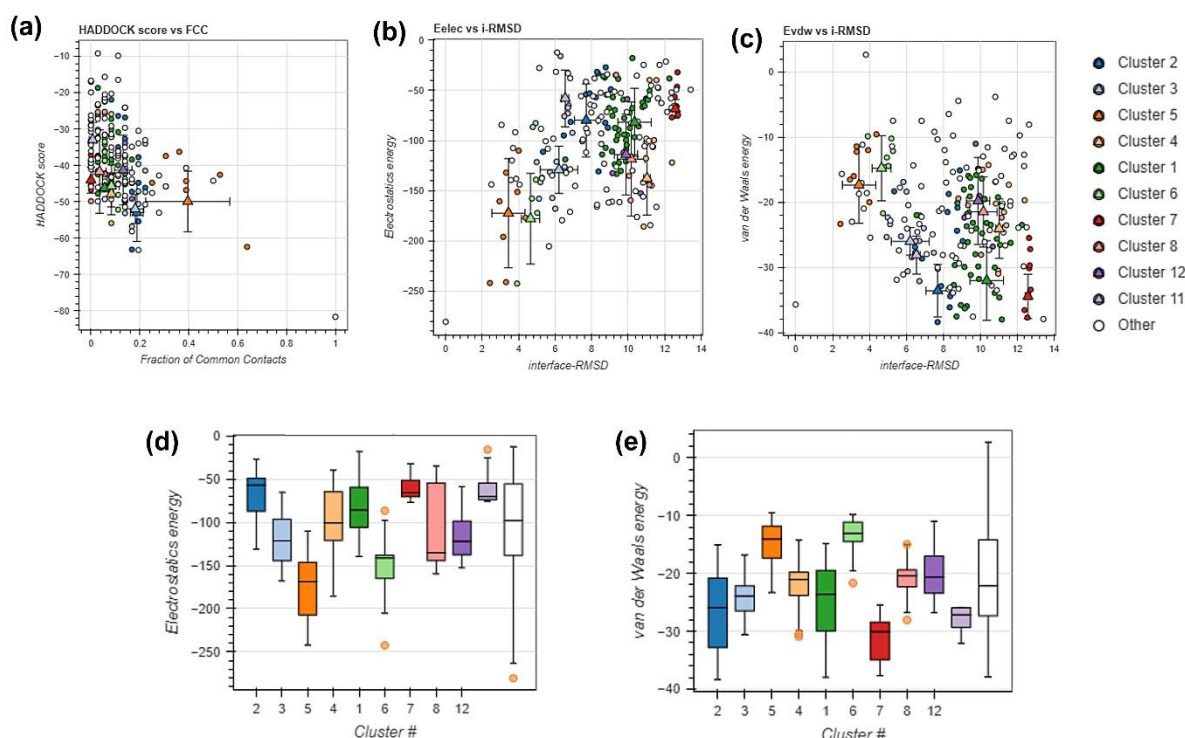


Figure 1. (a) HADDOCK score versus fraction of common contacts (FCC); (b) Electrostatic energy versus interface RMSD; (c) Van der Waals energy versus interface RMSD; Cluster-wise distribution of (d) electrostatic interaction energies, and (e) van der Waals interaction energies.

3.2. Construction of trastuzumab vector.

In order to ensure proper transcriptional and translational coordination, the Trastuzumab HC (469 amino acids; ~1407 bp) and LC (233 amino acids; ~699 bp) were linked via a short 48 bp flexible linker sequence. This approach allowed for balanced expression of both chains, which is essential for effective antibody assembly and secretion in mammalian expression systems [26]. The Trastuzumab expression vector was successfully constructed to enable the coordinated expression of full-length humanized monoclonal antibody heavy chain (HC) and light chain (LC) from a single plasmid backbone. With a total size of ~7508 bp, the finished plasmid contained every crucial regulatory element needed for high-level expression in eukaryotic cells.

These included polyadenylation signals, transcriptional regulatory regions, strong promoter and enhancer sequences, and antibiotic resistance markers for bacterial selection. A high-copy origin of replication was also present in the plasmid backbone, which allowed for effective amplification and propagation in *E. coli*. The vector map (Figure 2) shows the construct's general architecture. Taken together, these results confirm the successful creation of a stable, well-constructed Trastuzumab expression vector suitable for downstream expression in mammalian host systems.



Twelve colonies were chosen at random from the transformation plate and subjected to rapid (quick) plasmid DNA isolation. DNA bands (Figure 3C) matching the anticipated size of the Trastuzumab expression vector were found when the separated plasmids were subjected to 1% agarose gel electrophoresis. The recombinant plasmid's stable maintenance in *E. coli* and successful cloning were verified by this screening phase.

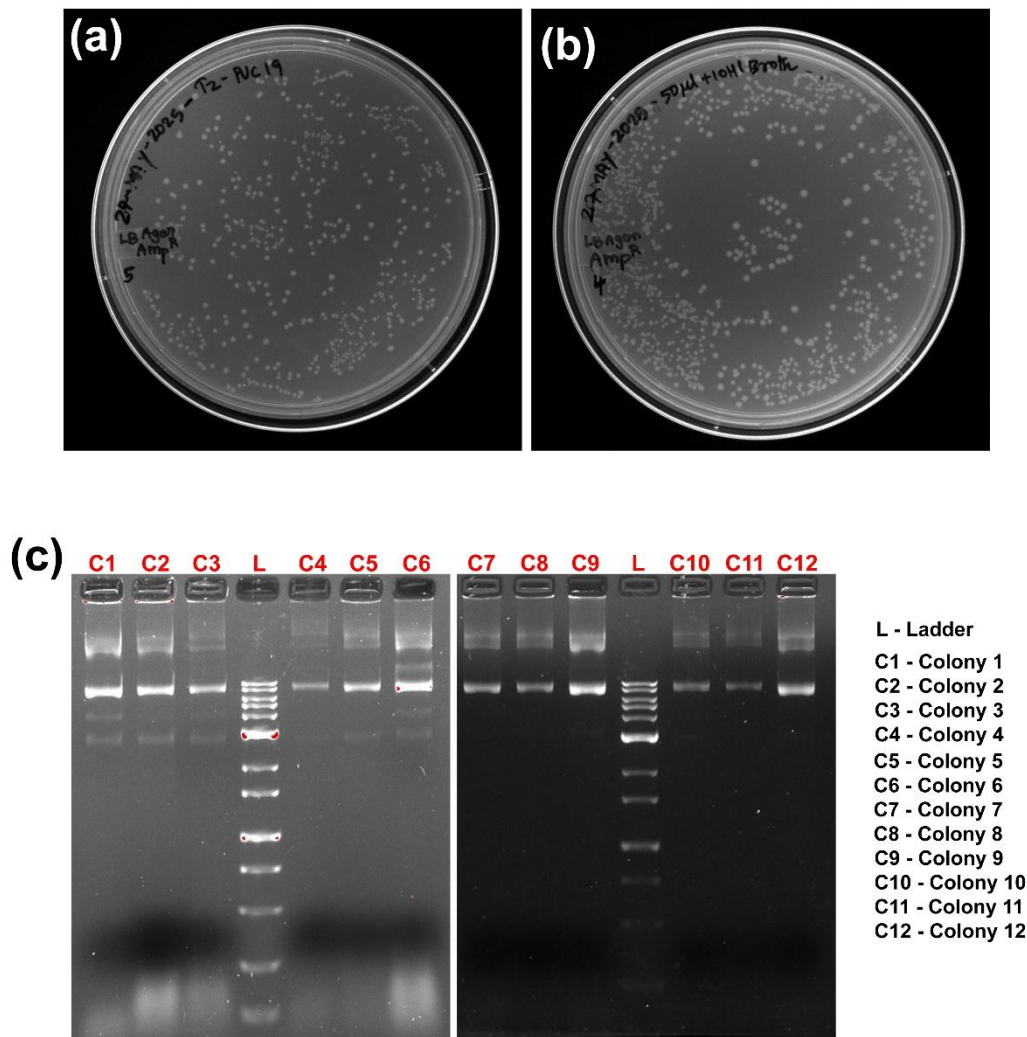


Figure 3. The well-isolated colonies formed on the plates of (a) Control; (b) Trastuzumab transformed cells; (c) The DNA band separation of twelve colonies observed in 1% agarose gel electrophoresis.

3.4. Plasmid isolation and screening of positive clones.

After the initial agarose gel analysis, colonies 1, 6, 9, and 12 were determined to be positive clones and chosen for additional study based on the caliber and strength of plasmid bands seen. To verify clonal purity and plasmid stability, these colonies were streaked onto brand-new LB agar plates containing ampicillin. Successful plasmid retention and antibiotic resistance were demonstrated by the robust development of these transformed colonies on selective LB agar plates; Figures 4A to 4D display the representative outcomes. The QIAprep Spin Miniprep Kit was then used to isolate high-quality plasmid DNA from overnight cultures of the four chosen colonies in accordance with the manufacturer's instructions. Agarose gel electrophoresis verified that the isolation process produced intact plasmid DNA with little degradation [28]. The recombinant Trastuzumab expression vector was efficiently lysed, purified, and recovered, as evidenced by the clear and distinct plasmid bands seen in all four colonies (Figure 4E). A NanoDrop Lite spectrophotometer was used to evaluate the extracted plasmids both quantitatively and qualitatively [29]. For all four colonies, the A260/280 ratios were consistently 1.90, indicating minimal protein contamination and good plasmid purity. The results showed effective plasmid amplification across the chosen clones, with plasmid concentrations of 1396.4 ng/μL for colony 1, 1329.7 ng/μL for colony 6, 1098.3 ng/μL for colony 9, and 911.1 ng/μL for colony 12. The highest plasmid concentrations were found in

colonies 1 and 6, indicating superior plasmid copy number and/or growth performance. All of these findings support the recombinant Trastuzumab expression plasmid's effective transformation, steady proliferation, and high-yield isolation.

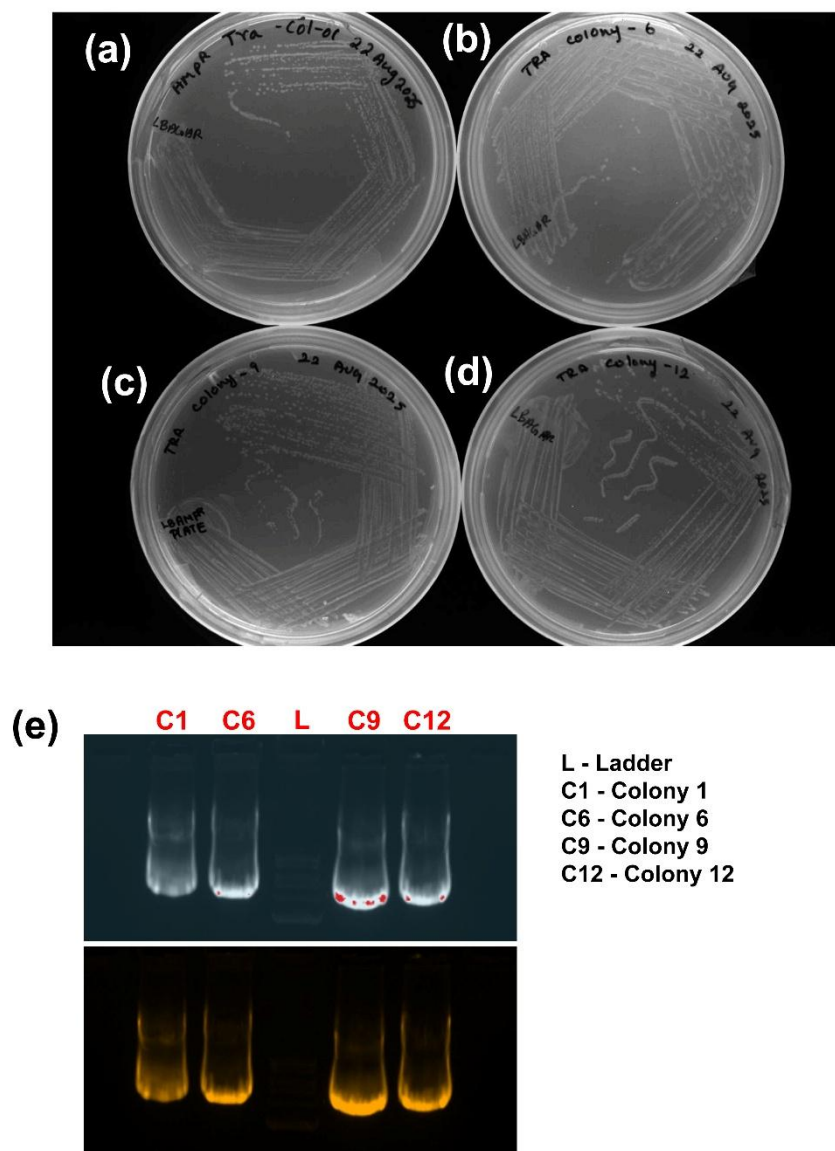


Figure 4. Robust development of the transformed colonies on LB agar plates: (a) Colony 1; (b) Colony 6; (c) Colony 9; (d) Colony 12; (e) Clear and distinct plasmid bands seen in all four colonies.

3.5. Restriction digestion.

The presence of the trastuzumab insert in the recombinant plasmid isolated from Colony 1 was verified by restriction digestion analysis. The construct was made up of a 2182 bp insert and a 5377 bp vector backbone, totaling 7559 bp. The insert was separated from the plasmid backbone by sequential double digestion using EcoRV and BstBI. The digested product's agarose gel electrophoresis revealed two separate DNA fragments that matched the anticipated sizes of roughly 5377 bp and 2182 bp. The released trastuzumab insert was represented by the smaller band, and the plasmid backbone by the larger band. The banding pattern in Figure 5 showed that the insert was successfully removed from the recombinant plasmid and agreed with the theoretical restriction map. No significant unexpected bands or abnormal digestion products were observed, indicating effective enzyme activity and no major

plasmid rearrangements. These results provide molecular evidence that the expression vector contains the correctly sized insert and confirm successful production of the recombinant trastuzumab plasmid.

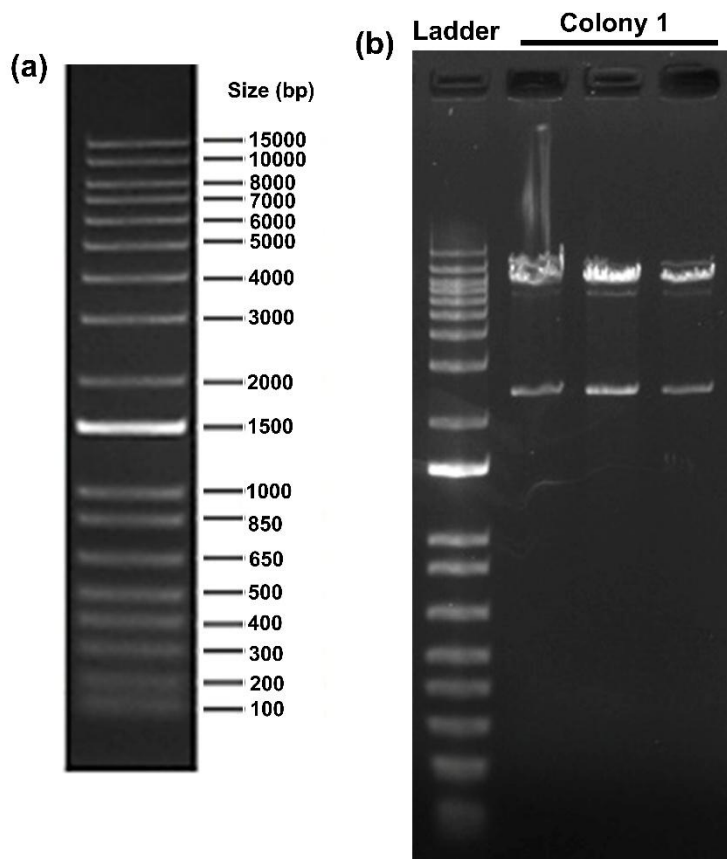


Figure 5. (a) DNA ladder (15,000 bp) loaded at 0.5 µg/lane and resolved in 1× TAE buffer for molecular size estimation; (b) Agarose gel electrophoresis of the recombinant trastuzumab plasmid from Colony 1 following double digestion with EcoRV and BstBI.

3.6. ExpiCHO-STM cells morphology following trastuzumab transfection.

To evaluate the health, vitality, and adaptability of ExpiCHO-STM cells after transient expression of the Trastuzumab construct. The results are shown in Table 2 and Figure 6 A. Two separate 250 mL cultures of ExpiCHO-S™ cells were used to produce recombinant trastuzumab. While retaining high viability (96%), the viable cell density (VCD) in Flask 1 rose from 6.0×10^2 cells/mL on Day 0 to a maximum of 9.6×10^1 cells/mL on Day 1. Similarly, Flask 2 had 96% viability and a peak VCD of 8.7×10^2 cells/mL on Day 1. Cell density rapidly decreased after the exponential growth phase, and on Day 12, when VCDs were 3.2×10^2 and 3.9×10^1 cells/mL and viabilities were 69% and 61% for Flasks 1 and 2, respectively, harvesting was carried out. The similar growth kinetics and viability profiles seen in both biological replicates confirm the successful temporary expression of recombinant trastuzumab in the ExpiCHO-S™ system and show consistent culture performance. Most cells maintained a normal spherical morphology with smooth, well-defined borders and a consistent size distribution, according to microscopic analysis with a 40× objective lens. Most cells were in suspension with no aggregation, confirming ideal growth conditions and successful preservation of shear-sensitive CHO cultures throughout the prolonged expression period. A significant percentage of cells survived the expected viability drop during late-stage transient expression, with little evidence of widespread cell lysis or debris. The existence of surviving,

morphologically stable cells at Day 14 indicates that the ExpiCHO-S™ system successfully supported prolonged recombinant antibody synthesis and that Trastuzumab expression did not cause severe cytotoxic stress [30, 31]. These findings support the downstream gathering and purification of secreted trastuzumab from the culture supernatant and are in line with the documented resilience of ExpiCHO-based systems for high-yield monoclonal antibody production.

Table 2. Growth kinetics and viability profile of ExpiCHO-S™ cells during transient expression of recombinant trastuzumab in two independent 250 mL culture flasks.

S. No	Day	VCD (Million Cells/ml)	Viability (%)
FLASK 1: MAXI TITRE - 250mL			
1	-1	4	98.5
2	0	6	99
3	1	9.6	96
4	5	8.2	87
5	6	5.5	90
6	8	5.3	82
7	10	4.1	78
8	12	3.2	69
FLASK 2: MAXI TITRE - 250mL			
1	-1	4	98.5
2	0	6.2	97
3	1	8.7	96
4	5	8.2	88
5	6	6.4	89
6	8	5.7	79
7	10	4.8	72
8	12	3.9	61

3.7. ELISA.

Recombinant trastuzumab was quantified by ELISA using a four-parameter logistic (4PL) standard curve. The results were shown in Table 3 and Figure 6B. The results showed good linearity and assay performance with an R^2 value of 0.999. The absorbance at 450 nm increased consistently at standard concentrations between 0 and 100 ng/mL, and the low CV values demonstrated acceptable assay repeatability. TRA-10000F, TRA-20000F, and TRA-50000F produced signals above the upper limit of quantification among the tested samples, indicating concentrations above the assay's detection range and requiring further dilution for accurate assessment.

Table 3. Quantification of recombinant trastuzumab by indirect ELISA. Samples were analyzed using a 4-parameter logistic standard curve, and antibody concentrations were calculated after applying the respective dilution factors.

Samples	Avg. Concentration (ng/mL)	Dilution correction (ng/mL)	Yield (µg/mL)	Yield (mg/mL)	Yield (g/L)
TRA-10000F	> Max	-	-	-	-
TRA-20000F	> Max	-	-	-	-
TRA-50000F	> Max	-	-	-	-
TRA-100000F	96.25	9625000	9625	9.625	9.625

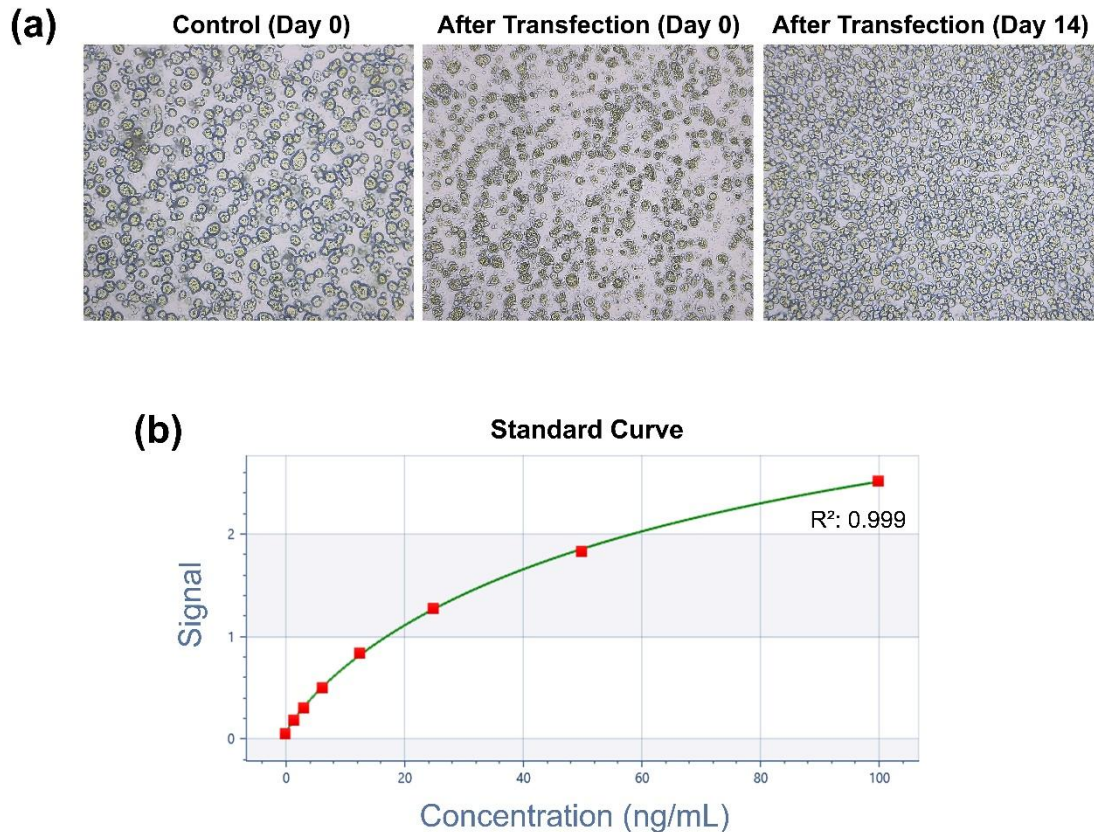


Figure 6. (a) Morphological alterations in the ExpiCHO-S™ cells post trastuzumab transfection on Day 0 and Day 14; (b) Quantification of recombinant trastuzumab by ELISA using a 4-parameter logistic standard curve.

TRA-100000F, on the other hand, produced an average concentration of 96.25 ng/mL and was within the calibration range. The final trastuzumab concentration was calculated to be roughly 9.6 µg/mL after the proper dilution factor was applied. These results show the sensitivity and dependability of the ELISA approach for antibody measurement, as well as the successful detection and quantification of recombinant trastuzumab.

3.8. Purification and SEC-HPLC analysis of recombinant trastuzumab.

The eluted Trastuzumab fractions were analyzed by size-exclusion chromatography coupled with high-performance liquid chromatography (SEC-HPLC) to assess molecular homogeneity, purity, and aggregation state after harvest, clarification, and affinity purification using Protein A magnetic beads. The results are given in Table 4 and Figure 7A. Protein species eluting from the size-exclusion column could be sensitively detected through SEC-HPLC analysis [32, 33], which measured UV absorbance at 280 nm. With a retention time of roughly 12.3 minutes, the chromatographic profile showed a dominant, symmetrical peak that made up about 95% of the entire peak area. This prominent peak is consistent with the expected molecular size of trastuzumab and corresponds to its monomeric form. Less than 5% of the entire area was occupied by small peaks that eluted early (8.7–10.2 minutes), which may indicate the presence of trace amounts of high-molecular-weight molecules or tiny contaminants. The recombinant trastuzumab generated in ExpiCHO-S™ cells exhibits exceptional purity and structural integrity, as seen by the high area percentage of the primary peak. Proteolytic cleavage during expression and processing is further confirmed by the lack of notable low-molecular-weight degradation products. Purified recombinant trastuzumab's

SEC-HPLC examination showed a dominating monomeric peak that accounted for 95% of the chromatographic area, demonstrating the antibody preparation's great molecular homogeneity. At retention times of roughly 9.3 and 10.2 minutes, minor aggregate species were found, which together accounted for 5% of the sample. The overall purity was found to be 95%, which is in line with SDS-PAGE results. Additionally, the endotoxin level was ≤ 0.1 EU/mg, indicating that the purified antibody was suitable for use in subsequent experiments.

Table 4. SEC-HPLC peak integration profile of recombinant trastuzumab showing retention time, peak characteristics, relative abundance, and peak assignment.

RT (min)	Height	Width (min)	Area	Area %	Assignment
8.7	0.9	0.9	17.5	0	Noise/void
9.3	0.6	0.7	21.7	1	High MW aggregate (~670 kDa)
10.2	2.9	1.6	124.4	4	Aggregate (~158 kDa)
12.3	122.4	3.4	3363.3	95	Monomer (~44 kDa)
Sum	—	—	3526.9	100	—

3.9. Selection of purified trastuzumab for SDS-PAGE analysis.

The purified Trastuzumab fractions corresponding to the primary monomeric peak were chosen for additional SDS-PAGE analysis under reducing and non-reducing conditions based on the SEC-HPLC results showing high molecular homogeneity and negligible aggregation. Only highly pure and structurally intact antibody preparations were sent for electrophoretic analysis thanks to the SEC-HPLC data, which acted as a crucial quality-control step. By using affinity purification, SEC-HPLC, and SDS-PAGE in a sequential analytical approach, antibody integrity and purity are robustly confirmed, enhancing the dependability of subsequent biochemical and functional evaluation of the recombinant trastuzumab.

3.10. SDS-PAGE analysis of purified trastuzumab under reducing and non-reducing conditions.

ExpiCHO-STM cell culture supernatant was used to purify the recombinant trastuzumab. The results were displayed in Table 5. Its structural integrity and purity were assessed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in both reducing (R) and non-reducing (NR) conditions (Figure 7B). The apparent molecular weights of the resolved protein bands were estimated using a pre-stained molecular weight protein marker (Lane M1) with a range of 10 to 250 kDa. The purified Trastuzumab sample migrated as a single, noticeable band with an apparent molecular weight of about 150 kDa under non-reducing conditions. The complete immunoglobulin G (IgG) molecule, in which the heavy and light chains are still covalently bound by interchain disulfide links, is represented by this band. This band's crisp, distinct appearance indicates high molecular homogeneity and suggests that native antibody assembly was maintained throughout handling, purification, and expression. Significantly, no other high-molecular-weight bands were detected above the 150 kDa range, indicating that there was no multimer formation or antibody aggregation. Likewise, the absence of low-molecular-weight bands shows less proteolytic cleavage or degradation [34]. The SEC-HPLC results, which showed a strong monomeric peak with minimal aggregation, are confirmed by our observations. Disulfide linkages within the antibody were broken during analysis under reducing conditions, which led to the anticipated separation of trastuzumab into its heavy and light chains. Two different bands were seen: the immunoglobulin heavy chain, which is represented by a primary band at around 50 kDa, and the light chain, which is represented by a second band at about 25 kDa. The ExpiCHO-STM expression system's proper

antibody folding, assembly, and post-translational processing are confirmed by the bands' distinct resolution and suitable molecular weights. The heavy and light chain bands' comparable intensities also imply that both chains are expressed in balance, which is necessary for effective IgG assembly. Highlighting the excellent purity attained by Protein A–based affinity purification, no extra bands suggestive of truncated products, misfolded species, or host cell protein contamination were seen.

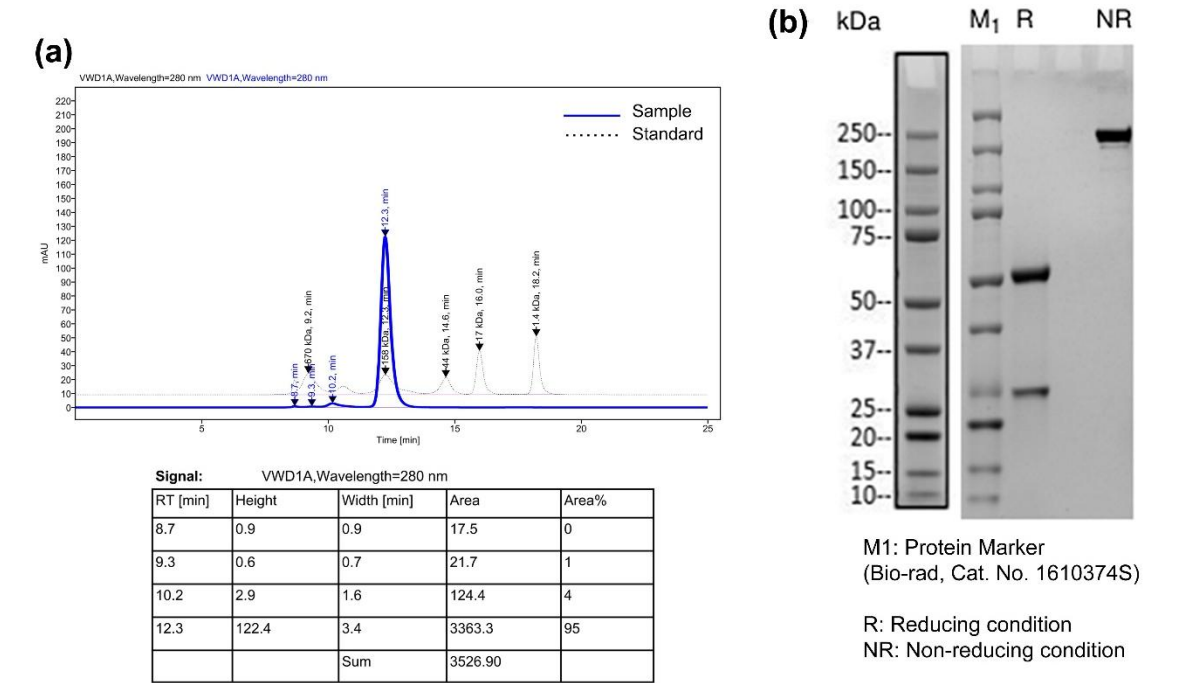


Figure 7. (a) SEC-HPLC analysis for protein selection based on size; **(b)** Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in both reducing (R) and non-reducing (NR) conditions.

The effective synthesis of structurally intact, full-length trastuzumab is strongly supported by the SDS–PAGE profile under both reducing and non-reducing conditions. While the emergence of distinct ~50 kDa and ~25 kDa bands under reducing conditions verifies adequate heavy and light chain synthesis and assembly, the existence of a single ~150 kDa band under non-reducing circumstances demonstrates good interchain disulfide bonding and native antibody shape. The SDS-PAGE results validate the high purity and structural integrity of the recombinant trastuzumab expressed in ExpiCHO-S™ cells, in conjunction with the SEC-HPLC data showing high molecular homogeneity and low aggregation. These outcomes support the purified antibody's suitability for additional downstream uses, such as therapeutic characterization, binding investigations, and functional tests.

Table 5. Electrophoretic characterization of trastuzumab by SDS–PAGE under reducing and non-reducing conditions.

Lane	Condition	Sample Description	Loading amount	Observed Bands (kDa)	Observations
M1	–	Protein Marker (Bio-Rad)	–	250, 130, 100, 70, 55, 25	Ref. Molecular weight ladder
2	R	Trastuzumab under reducing condition	2 µg	Heavy Chain ≈ 50 kDa Light Chain ≈ 25 kDa	Increased band density
4	NR	Trastuzumab under non-reducing condition	4 µg	Intact IgG ≈ 150 kDa	Strongest signal intensity

4. Conclusions

An integrated methodology for the computational evaluation, expression, purification, and biochemical characterization of recombinant trastuzumab is described in this work. A structural model of the anticipated trastuzumab–HER2 interaction was produced by HADDOCK-based molecular docking, which also found advantageous binding interfaces backed by docking scores, electrostatic interactions, and low RMSD values. Before being expressed in mammals, the trastuzumab expression construct was successfully created, multiplied in *E. coli*, and confirmed by plasmid separation and restriction analysis. Protein A affinity chromatography allowed for the effective purification of the recombinant antibody, and transient transfection of ExpiCHO-STM cells produced consistent cell growth and antibody production. A mostly monomeric antibody production with the anticipated molecular properties under reducing and non-reducing conditions was shown by subsequent SEC-HPLC and SDS-PAGE tests. Together, these results show that computational modeling, mammalian expression, and biochemical characterization techniques can be successfully combined for the synthesis and first testing of recombinant trastuzumab. Because of limited access to specialized analytical facilities, we did not perform advanced orthogonal analyses such as peptide mapping, glycosylation profiling, intact mass analysis, endotoxin testing, or host-cell protein assessment, although we characterized recombinant trastuzumab using SEC-HPLC and SDS-PAGE. To confirm product quality and molecular characteristics, future research will focus on thorough structural and biophysical characterization.

Author Contributions

Conceptualization: S.S. and P.R.; Methodology: S.S.; Software: S.S.; Validation: S.S. and P.R.; Formal analysis: S.S.; Investigation: P.R. and S.S.; Resources: P.R.; Data curation: S.S.; Writing-original draft preparation: S.S.; Writing-review and editing: S.S. and P.R.; Visualization: S.S.; Supervision: P.R.; Project administration: P.R. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

Funding

This research received no external funding.

Acknowledgments

The authors thank Biomaz Infosearch, Madurai, Tamil Nadu, for technical support.

Conflicts of Interest

The authors declare no conflict of interest.

Artificial Intelligence Disclosure

The online tool QuillBot, an AI-powered writing assistant, was used to check grammatical accuracy and language clarity in this manuscript. No AI-generated content was used in the conceptualization, analysis, or interpretation of the study results.

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