An efficient platform for screening expression and crystallization of glycoproteins produced in human cells

Jeffrey E Lee[1,3], Marnie L Fusco[1,3] & Erica Ollmann Saphire[1,2]

[1]Department of Immunology and Microbial Science, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA. [2]The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA. [3]These authors contributed equally to this work. Correspondence should be addressed to E.O.S. (erica@scripps.edu).

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Glycoproteins are involved in diverse biological processes ranging from extracellular contact and recognition to intracellular signaling. Crystal structures of glycoproteins would yield tremendous insight into these processes. But glycoprotein structural analysis has been hindered by difficulties in expressing milligram quantities of stable, homogeneous protein and determining which modifications will yield samples amenable to crystallization. We describe a platform, which we have proven to be effective for rapidly screening expression and crystallization of a challenging glycoprotein target. In this protocol, multiple glycoprotein ectodomain constructs are produced in parallel by transient expression of adherent human embryonic kidney (HEK) 293T cells and are subsequently screened for crystals in microscale quantities by free interface diffusion. As a result, recombinant proteins are produced and processed in a native, mammalian environment, and crystallization screening can be accomplished with as little as 65 μg of protein. Moreover, large numbers of constructs can be generated, screened and scaled up for expression and crystallization, with results obtained in 4 weeks.

INTRODUCTION

Glycoproteins are thought to make up about 50% of all human and human pathogen proteins, but relatively few of these structures have been solved to date. Structural biology of glycoproteins is hindered by challenges in achieving high-level expression of soluble, stable and homogenous protein capable of forming well-diffracting crystals. Although attachment and processing of glycans is often a prerequisite for correct protein folding and stability, the chemical and conformational heterogeneity of complex-type glycans displayed on proteins produced in mammalian cells generally inhibits the formation of a well-ordered crystal lattice[1]. Iterative rounds of construct redesign or site-directed mutagenesis of N- or O-linked glycosylation sequons coupled with enzymatic deglycosylation are usually necessary to identify a protein construct suitable for crystallization. Chinese hamster ovary cells[2], yeast[3] and insect cell-based systems[4–7] are often used for glycoprotein expression. However, these systems are not ideal for rapid construct screening as initial setup of each stable cell line can be time consuming. Moreover, processing of glycans in yeast, insect and fruit fly cell lines differs from that which occurs in human glycan biosynthetic pathways, possibly influencing protein folding, stability, activity, secretion and immunogenicity[8–11]. Recent developments using transient transfection protocols of adherent HEK 293T cells have shown greater promise for higher throughput and overall yields of mammalian-expressed glycoproteins[12–14]. Many structural genomics centers that focus on human proteins now use the HEK293 cell line for expression screening and production[13], and this cell type is approved by the US Food and Drug Administration for clinical and commercial production of macromolecular therapeutics.

We describe a procedure for screening crystallization using transient expression in adherent HEK293T cells, one-step affinity purification and microfluidic free interface diffusion. This platform allows cloning, expression and crystallization to be performed in approximately 4 weeks, considerably faster than with other current protocols (**Fig. 1**). This protocol is now in standard use for all mammalian-expressed glycoproteins in our laboratory[15] and by several other groups[12,16]. Its utility is proven by our ability to screen 130 different constructs of the *Zaire ebolavirus* glycoprotein in complex with seven different antibodies for successful structural determination[15].

Considerations for test-scale expression system

The main considerations in the selection of a vector for expression of mammalian glycoproteins are:

(i) the plasmid should have a high copy number in *Escherichia coli* to produce milligram quantities of DNA required for large-scale transient transfections;
(ii) the promoter should be strong in mammalian cells;
(iii) the plasmid should encode an optimized secretion signal to ensure proper targeting and co- and post-translational processing of the cloned fusion protein; and
(iv) the plasmid should encode a high-affinity purification tag to bind low yields of the resulting secreted protein from large volumes of conditioned media, and also serve as a high-affinity epitope tag for protein detection.

Although many commercial vectors are available for expression of human proteins (reviewed in ref. 13), we have found the pDISPLAY expression system to be of particular utility. The pDISPLAY plasmid has a vector-encoded, C-terminal transmembrane domain and an immunoglobulin kappa (Ig κ) chain leader sequence, which targets the protein through the endoplasmic reticulum (ER) and Golgi networks for display on the cell surface[17]. In general, the ER provides efficient quality control and proteins that are secreted tend to be correctly folded[18–20]. We usually insert a stop codon before the vector-encoded transmembrane anchor to allow soluble protein to be released into the cultured media.

A strong human cytomegalovirus protomer ensures overexpression of the protein of interest, and the presence of a hemagglutinin (HA) tag allows the secreted protein to be selectively purified by immunoaffinity chromatography and detected by western blot.

Adherent HEK293T cells (**Fig. 2**) are easy to maintain, able to express and properly process recombinant human proteins[12,16] and are highly suitable for use in both average-sized laboratories and high-throughput structural genomic centers. HEK293T cells are widely available and are easily cultured using Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (1× GlutaMAX) and 5% (vol/vol) fetal bovine serum (FBS) in a 37 °C incubator with 5% CO_2. Exhaustive protocols on tissue culture and expression are available[21–25]. Test expression trials are performed in 6-well (9.5 cm^2 surface area) or in 24-well (1.9 cm^2 surface area) cell culture plates. High-level expression in small volumes is best obtained when the cloned gene of interest is codon-optimized, cells are transfected between 50 and 80% confluency and when a commercial reagent such as FuGENE HD or GeneJuice is used for transfection. The detection and analysis of dilute amounts of protein secreted into conditioned media are performed by western blot or ELISA analysis using antibodies directed to both linear and conformational epitopes. The use of an antibody against a linear epitope allows detection of total protein expressed, regardless of quality, whereas use of an antibody against a conformational epitope (if available) distinguishes properly folded from misfolded proteins. In our experience, although the vast majority of antibodies against conformational epitopes work well in western blots, binding of some antibodies of unusual conformational sensitivity may be disrupted by SDS-PAGE of their cognate antigens. In this case, non-denaturing immunoblots using native gels, ELISAs or dot blots with the target's natural ligand (if available) are alternate methods for the detection of proper folding.

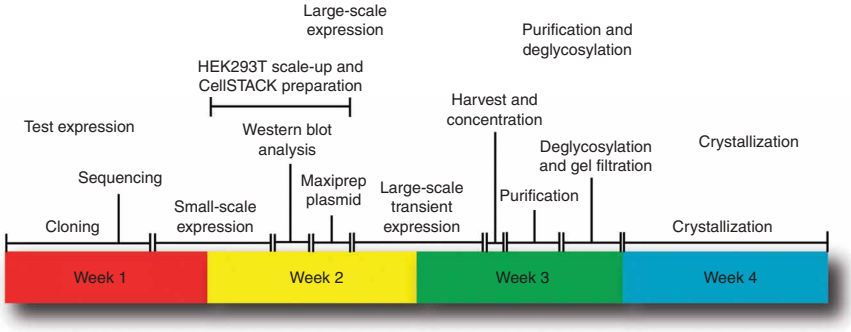

Figure 1 | Workflow for the HEK293T-microfluidic crystallization screening platform. Using this system, constructs can be screened for expression and crystallization and scaled up for structural studies in a matter of 4 weeks.

Considerations for cell culture scale-up and purification

A variety of containers, such as T225 cm^2 cell culture flasks, roller bottles (850 cm^2) or CellSTACKS (636 cm^2 per layer) can be used for HEK293T culture and expression. We favor the 10-layer CellSTACK system for larger-scale expression and 2- or 5-layer CellSTACKS for smaller scale exploratory studies (**Fig. 2**). CellSTACKS provide equivalent or larger surface area for cell attachment and growth than other vessels, yet package cell culture space effectively, thus requiring less incubator space and labor. Although disposable CellSTACKS are more costly than roller bottles, they can be made more cost-effective by washing and recycling the vessel.

Although we often use FuGENE HD in our laboratory for test transfections, the cost of this reagent for use in large-scale expression is prohibitively expensive. We have found that the use of traditional inexpensive transfection reagents, such as calcium phosphate (reagent of choice in our laboratory) or branched PEI[12,26,27], provides excellent transfection efficiencies and cost savings. Transfection is best performed when the cell population is in the late S phase of the cell cycle (exponential growth phase)[28], and in our hands this corresponds to ~70% cell confluency. We have also found that calcium phosphate–DNA precipitation is efficient when performed on ice for 20 min. Formation and solubility of calcium phosphate–DNA precipitate are highly sensitive to incubation temperature and timing, therefore any changes in incubation temperature will require a re-optimization of the timing and ratios of calcium, phosphate and DNA[28,29].

In our laboratory, glycoproteins are secreted into liter-scale volumes of conditioned media and are efficiently purified via a two-step procedure. First, large volumes of media are rapidly concentrated to more manageable amounts using a Centramate tangential flow filtration system. Then the recombinant protein is captured by affinity chromatography on an anti-HA column with high affinity and capacity. Bound proteins are gently eluted from anti-HA columns by competition with a synthetic HA peptide. Afterwards, the anti-HA affinity matrix can be regenerated multiple

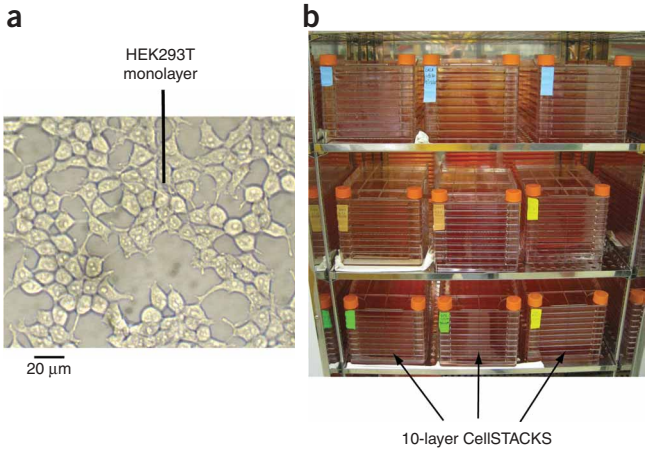

Figure 2 | Large-scale expression. (**a**) Adherent HEK293T cells grown to 70% confluency at 37 °C and 5% CO_2. This is the ideal cell density for transient transfection using either commercial or calcium phosphate transfection reagents. Healthy cells should adhere to the surface of the vessel and cluster together in a monolayer. (**b**) 10-layer Corning CellSTACKS shown in a Labline model 397 37 °C CO_2 incubator. Although a large incubator (68.5 cm × 161.3 cm × 72.5 cm) is shown here, the 33.5 cm × 20.5 cm footprint of the CellSTACK easily fits in standard CO_2 incubators as well. Each CellSTACK layer has 636 cm^2 surface area for cell attachment and the 10-layer vessel is capable of holding an average yield of 6.4 × 10^8 cells.

times without loss of performance. Alternatively, dilute concentrations of secreted protein may be loaded directly onto the affinity column without prior concentration, although this process is slower, or proteins may be fractionated by ammonium sulfate precipitation and reconstituted before affinity capture. Many other affinity purification tags such as biotin, glutathione *S*-transferase, myc-epitope, Flag and polyhistidine have also been developed. However, we prefer the HA tags for the ease of antibody-based purification and low cross-reactivity with host cell proteins. In our experience, low-yield proteins purified by metal affinity chromatography retain a number of host cell contaminants and require additional purification steps before crystallization.

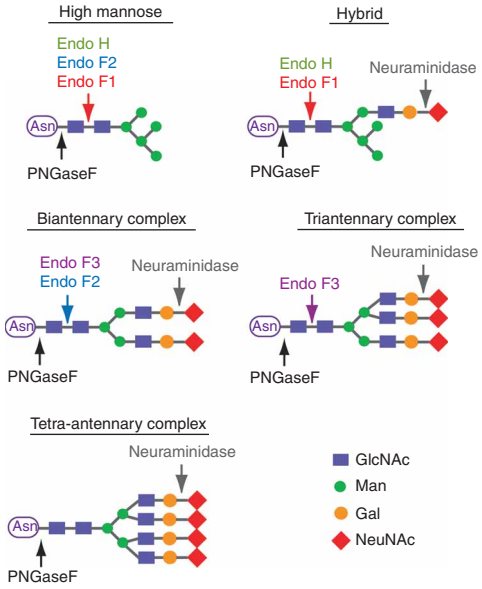

Figure 3 | Substrate specificities of selected glycosidases useful for large-scale deglycosylation. PNGaseF will cleave virtually all N-linked glycans, with the exception of those containing a fucose α(1–3)-linked saccharide to the inner most *N*-acetylglucosamine (GlcNAc). These fucose α(1–3)-linked glycans are commonly found in glycoproteins from plants, parasitic worms or insect cells. EndoH primarily cleaves within the chitobiose core in high mannose and some hybrid glycans. EndoF1 cleaves oligomannose and hybrid structures but not complex structures. EndoF2 and EndoF3 both cleave complex-type glycans. However, EndoF2 prefers biantennary complex-type oligosaccharides and to a lesser extent, oligomannose sugars, while EndoF3 cleaves both biantennary and triantennary glycans. Tetra-antennary oligosaccharides are not cleaved by the EndoF series of enzymes and require sequential hydrolysis down to a $Man_3GlcNAc_2$ structure. Neuraminidase is an exoglycosidase that removes terminal sialic acid residues from hybrid, biantennary, triantennary and tetra-antennary oligosaccharides. It is sometimes necessary to remove these negatively charged saccharides prior to deglycosylation with EndoH, EndoF or PNGaseF.

Strategies for the removal of N-linked glycans

N-linked glycans attached to proteins expressed in mammalian cells are usually complex, chemically and conformationally heterogeneous and frequently detrimental to the formation of well-ordered crystal lattices. Several strategies have been used to overcome the 'glycosylation problem'. Two popular methods involve using either an *N*-acetylglycosaminyltransferase I (GnT I)-deficient HEK293S cell line[30] or addition of glycan anabolic inhibitors such as swainsonine or kifunensine[16] to cell culture medium. Both strategies yield more homogeneous, high mannose-type oligosaccharide chains, which can be removed by EndoH deglycosylation[16].

We favor an alternate approach that involves production of native glycoproteins without inhibitors and enzymatic deglycosylation of the resulting protein under native conditions using peptide *N*-glycosidase F (PNGaseF). Although deglycosylation with PNGaseF has been shown to increase aggregation in some proteins[31], not all PNGaseF-treated proteins aggregate, and PNGaseF is the most efficient glycosidase available. PNGaseF also has the broadest substrate specificities and cleaves between the asparagine side chain and the innermost *N*-acetylglucosamine (GlcNAc), thus removing the entire glycan moiety. Occasionally, some glycan attachment sites are buried or masked by the local protein structure and PNGaseF is unable to gain access. In such an event, the use of the EndoF series of glycosidases may be beneficial. The EndoF glycosidases have a unique glycan substrate preference (**Fig. 3**) and tend to be less sensitive to protein conformation, due to their cleavage between the two GlcNAc oligosaccharides in the chitobiose core, and may trim glycans for which PNGaseF treatment was unsuccessful. In some cases, sequential or simultaneous removal of terminal sugars from complex oligosaccharides may need to be removed using neuraminidase, β-galactosidase and *N*-acetylglucosaminidase before subsequent deglycosylation with EndoF or PNGaseF.

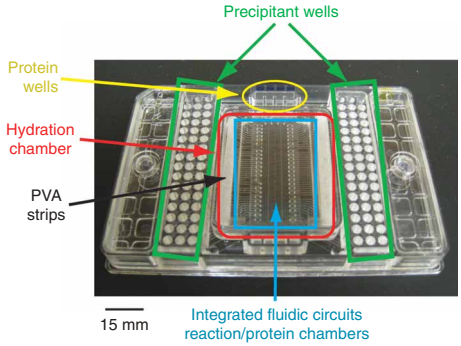

Figure 4 | Microfluidic crystallization chip. The Topaz 4.96 crystallization screening chip shown here contains 96 inlets for the crystallization reagent and inlets for four protein samples. Other chips are available with one or eight protein inlets. Integrated fluidic circuits and reaction chambers are embedded within the hydration chamber.

Considerations for crystallization

The use of robotic liquid handling systems has considerably reduced time and protein quantity required for crystallization screening. Indeed, presently available robots can accurately dispense ~100 nl of protein or reagent per drop. A recent, additional improvement in protein crystallization is the development of microfluidic circuits to dispense even smaller volumes of protein and reagents for crystallization. We recommend the use of the Fluidigm Topaz microfluidic free interface diffusion system, as outlined in the protocol below. The Topaz microfluidic screening chips (**Fig. 4**) contain interconnected miniaturized channels and elastomeric rubber valves that control the interface of protein sample and precipitant. Initially, diffusion of protein and precipitant sets a concentration gradient to allow the sampling of a wide

area of crystallization space. Subsequently, vapor permeability of the reaction chamber allows dehydration to further concentrate the sample, yielding results within 7 days. Quality hits can then be translated into standard 0.5–2.0 µl size hanging drop, vapor diffusion experiments. The system is quite expensive but, in our hands, has been a key determinant in the feasibility of structural analyses of some difficult targets. The Topaz system allows the screening of 96 different crystallization conditions with a mere 1.5 µl of protein, whereas standard laboratory liquid handling robotics will require almost seven times more protein.

This makes the Topaz system highly beneficial for crystallization screening of targets that are difficult to express or obtain. An increasing number of mammalian protein structures, including those of glycosylated proteins, have indeed been determined from initial crystallization hits obtained from microfluidic-based diffusion[32–39].

MATERIALS

REAGENTS

! CAUTION For all reagents, follow manufacturer's recommendations for storage of stock solutions and materials. Wear proper safety equipment, including laboratory coat, safety glasses and appropriate gloves, for all experiments.

- pDISPLAY expression vector (Invitrogen, cat. no. V660-20)
- *E. coli* XL-1 Blue competent cells (Stratagene, no. 200249)
- MiniPrep plasmid purification kit (Qiagen, cat. no. 27106)
- Adherent HEK293T cells (widely available) **! CAUTION** All cell cultures are considered biohazardous because of their potential to be infectious. Experimenters should wear proper protective clothing and work should be performed in an approved laminar flow hood using aseptic techniques. After completion of experiments, all surfaces, waste media, glassware, consumables and equipment should be disinfected according to institutional and governmental guidelines.
- DMEM (Gibco/Invitrogen, cat. no. 11965)
- 100× penicillin/streptomycin (pen/strep) (Gibco/Invitrogen, cat. no. 15140)
- FBS (Gibco/Invitrogen, cat. no. 10082)
- 100× GlutaMAX (Gibco/Invitrogen, cat. no. 35050)
- CO_2
- FuGENE HD transfection reagent (Roche, cat. no. 4709691) or
- GeneJuice transfection reagent (EMD Biosciences, cat. no. 70967)
- MiniProtean 10–15% gradient Tris-HCl polyacrylamide gel (Bio-Rad, cat. no. 161-1122)
- Blotting grade Blocker non-fat dry milk (Bio-Rad, cat. no. 170-6404)
- Mouse IgG anti-HA monoclonal primary antibody (clone 16B12) (Covance Research Products, cat. no. MMS-101P)
- Goat anti-mouse IgG F(ab′)2 alkaline phosphatase-conjugated antibody (Thermo/Pierce, cat. no. 31324)
- Goat anti-human IgG (H+L) alkaline phosphatase-conjugated antibody (Thermo/Pierce, cat. no. 31310)
- SIGMA *FAST* 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma, cat. no. B5655)
- Bovine serum albumin (BSA) (Sigma, cat. no. A2058)
- Goat anti-mouse IgG (H+L) horseradish peroxidase-conjugated antibody (Thermo/Pierce, cat. no. 31430)
- 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit (Thermo/Pierce, cat. no. 34021) **! CAUTION** Contents of this kit contain toxic or hazardous chemicals. TMB should not be exposed to direct light and should be treated as a mutagen. Wear proper personal safety equipment.
- H_2SO_4 (Sigma, cat. no. 435589) **! CAUTION** H_2SO_4 is a highly corrosive acid and will produce a highly exothermic reaction with water. Always dilute acid into water. Wear proper safety equipment including laboratory coat, gloves and safety glasses.
- MaxiPrep plasmid purification kit (Qiagen, cat. no. 12163)
- $CaCl_2$ (Sigma, cat. no. C5080)
- Bleach
- Sterile 1× PBS (Gibco/Invitrogen, cat. no. 14190)
- Anti-HA affinity matrix, clone 3F10 (Roche, cat. no. 1815016)
- Synthetic HA peptide (>95% purity, sequence: YPYDVPDYA)
- Tween-20 (Sigma, cat. no. P1379)
- Sodium azide, NaN_3 (Sigma, cat. no. S8032) **! CAUTION** Sodium azide is highly toxic by inhalation, ingestion or skin absorption. It should not be allowed to come into contact with heavy metals or their salts because it may react to form heavy metal azides, which are shock-sensitive explosives. Containers of sodium azide should be stored in secondary containers in a cool, dry secured storage separated from acids. Excess sodium azide and waste material containing this substance should be properly disposed in accordance with institutional, city, state and federal guidelines.
- Glycine (Sigma, cat. no. G8898)
- Pre-crystallization Test (PCT) (Hampton Research, cat. no. HR2-140)
- Peptide *N*-glycosidase (PNGaseF) (500,000 units/ml) (NEB, cat. no. P0704L)
- Endo-β-*N*-acetylglucosaminidase F1 (EMD/Calbiochem, cat. no. 324725)
- Endo-β-*N*-acetylglucosaminidase F2 (EMD/Calbiochem, cat. no. 324726)
- Endo-β-*N*-acetylglucosaminidase F3 (EMD/Calbiochem, cat. no. 324727)
- Hydration Fluid Concentrate (Fluidigm, part no. 89000005)
- 1× transfer buffer (see REAGENT SETUP)
- 3× SDS-PAGE non-reducing sample buffer (see REAGENT SETUP)
- 3× SDS-PAGE reducing sample buffer (see REAGENT SETUP)
- 2× HBS (see REAGENT SETUP)
- 1× PBS (see REAGENT SETUP)
- 1× PBS-Tween-20 (see REAGENT SETUP)
- HA peptide (see REAGENT SETUP)
- 1× PBS-BSA (see REAGENT SETUP)

EQUIPMENT

- 6-well culture plates; surface area: 9.5 cm^2 (Corning, cat. no. 3516)
- 24-well culture plates; surface area: 1.9 cm^2 (Corning, cat. no. 3526)
- Immobilon-P PVDF membrane (Millipore, cat. no. IPVH07850)
- Costar 96-well microtiter plates (Corning, cat. no. 3690)
- 10-layer CellSTACK; surface area: 6,360 cm^2 (Corning, cat. no. 3312)
- 5-layer CellSTACK; surface area: 3,180 cm^2 (Corning, cat. no. 3311)
- 2-layer CellSTACK; surface area: 1,272 cm^2 (Corning, cat. no. 3310)
- 0.22 µm vacuum filter (Corning, cat. no. 431097)
- Amicon Ultra centrifugal concentrators (Millipore)
- Topaz 1.96 screening chip (Fluidigm, part no. TPZ-M-1.96)
- Topaz 4.96 screening chip (Fluidigm, part no. TPZ-M-4.96)
- OptiMix-1, 2, 3, PEG 96 deep-well block sparse matrix screens (Fluidigm)
- Other deep-well crystallization sparse matrix screens (Hampton Research, Qiagen, Molecular Dimensions, Emerald Biosystems or those developed in-house)
- Water bath
- Laminar flow hood
- CO_2 incubator
- Centrifuge
- Microcentrifuge
- Electrophoresis system
- Western blot transfer apparatus
- Microplate reader
- Centramate tangential flow filtration system (Pall Corporation)
- 8-channel P20 micropipettor
- Topaz Nanoflex IFC Controller (Fluidigm)
- AutoInspeX II workstation (Fluidigm)
- Vibration-free incubator (for crystallization)

REAGENT SETUP

1× transfer buffer For 1 l of aqueous solution: 3.0 g of Tris base, 14.4 g of glycine and 200 ml of methanol; bring volume up to 1 l with ddH_2O. Store at room temperature (RT; 22 °C).

3× SDS-PAGE non-reducing sample buffer For 10 ml of solution: 0.6 g of SDS, 3 ml of glycerol, 1.8 of ml 1.0 of Tris-HCl pH 6.8 and 1 mg of bromophenol blue; bring volume up to 10 ml with ddH_2O. Store at RT.

3× SDS-PAGE reducing sample buffer For 10 ml of solution: 0.6 g of SDS, 3 ml of glycerol, 1.8 ml of 1.0 Tris-HCl pH 6.8, 1 mg of bromophenol blue and 1 ml of 2-mercaptoethanol; bring volume up to 10 ml with ddH_2O. Aliquot into 1 ml fractions and store at −20 °C until use.

2× HBS For 1 l of aqueous solution: 10.0 g of HEPES, 16.0 of g NaCl, 0.74 g of KCl and 0.40 g of $Na_2HPO_4.7H_2O$. Adjust pH of solution to 7.1 and fill to 1.0 l with ddH_2O. Sterile filter, aliquot into 50-ml fractions and immediately

store at −20 °C. ▲ CRITICAL 2× HBS should be either made fresh or thawed from frozen aliquots on the day of transfection.

1× PBS For 1 l of aqueous solution: 8.0 g of NaCl, 0.2 g of KCl, 1.4 g of Na_2HPO_4 (anhydrous) and 0.24 g of KH_2PO_4. Adjust pH of solution to 7.4 and fill to 1.0 l with ddH_2O. Store at RT.

1× PBS-Tween-20 For 1 l of aqueous solution: 8.0 g of NaCl, 0.2 g of KCl, 1.4 g of Na_2HPO_4 (anhydrous), 0.24 g of KH_2PO_4 and 1 ml of Tween-20. Adjust pH of solution to 7.4 and fill to 1.0 l with ddH_2O. Store at RT.

HA peptide For 100 ml aqueous solution: dissolve 100 mg of peptide into a final volume of 100 ml 1× PBS. Aliquot into 10 ml fractions and store at −20 °C until use.

1× PBS-BSA For 100 ml of aqueous solution: dissolve 0.8 g of NaCl, 0.02 g of KCl, 0.14 g of Na_2HPO_4 (anhydrous), 0.02 g of KH_2PO_4 and 3 g of BSA into a final volume of 100 ml of ddH_2O. Store at 4 °C.

EQUIPMENT SETUP

Preparing CellSTACKs for re-use Disinfect the CellSTACK by soaking overnight in 1.5 l of 10% (vol/vol) bleach. HEK293T cells should detach. Rinse the CellSTACK copiously with sterile filtered deionized H_2O, followed by 1× sterile PBS. Store the CellSTACK in 1.0 l of sterile 1× PBS supplemented with 10× pen/strep until use. The CellSTACK layers should appear transparent after cleaning and, in our hands, can be re-used effectively two additional times.

PROCEDURE

Cloning and sequencing ● TIMING 5 d

1| Clone gene of interest into pDISPLAY or another appropriate expression vector using standard methods. Amplify and purify vector (Qiagen MiniPrep kit is helpful for purification).

Small-scale expression ● TIMING 4 d

2| Add 2 × 10^6 HEK293T cells to a six-well culture plate with 2 ml of DMEM, 1× pen/strep, 1× GlutaMAX and 5% (vol/vol) FBS. Incubate at 37 °C with 5% CO_2 for 4 h to allow cell attachment. CO_2 is necessary to activate the bicarbonate buffer in DMEM. For higher throughput, add 4 × 10^5 HEK293T cells to a 24-well cell culture plates with 1 ml of DMEM, 1× pen/strep, 1× GlutaMAX and 5% (vol/vol) FBS.

! CAUTION All cell cultures are considered biohazardous because of their potential to be infectious. Experimenters should wear proper protective clothing and work should be performed in an approved laminar flow hood using aseptic techniques. After completion of experiments, all surfaces, waste media, glassware, consumables and equipment should be disinfected according to institutional and governmental guidelines.

? TROUBLESHOOTING

3| Pipette 3 µl of FuGENE HD directly into 97 µl of serum-free DMEM.

▲ CRITICAL STEP Always dilute FuGENE HD into serum-free medium and do not allow undiluted reagent to come into contact with plastics. Undiluted FuGENE HD may be absorbed by the plastics.

4| Add 1 µg of Qiagen MiniPrep-purified DNA (from Step 1) to the FuGENE–DMEM mixture, gently mix and incubate for 30 min at RT.

▲ CRITICAL STEP Always use a greater volume (in microliters) of FuGENE HD reagent than mass of DNA in micrograms (we use a 3:1 ratio). To prevent DNA shearing, mix gently.

5| Pipette the FuGENE–DNA mixture dropwise onto 70% confluent HEK293T cells. Ensure even dispersal.

6| Incubate the cells at 37 °C with 5% CO_2 for 4 days. To monitor expression, carry out western blot analysis, as described in Steps 7–15. Alternatively, if antibody binding is disrupted by SDS-PAGE, perform sandwich ELISAs (**Box 1**) instead of western blot analysis.

Western blot analysis ● TIMING 8 h

7| Decant the supernatant and microcentrifuge at 16,000*g* for 20 min at RT. To detect expression, mix 10 µl aliquots of cell culture supernatant with 3× SDS-PAGE non-reducing sample buffer and separate at 200 V for 45 min on a 10–15% SDS-Tris-HCl polyacrylamide gel or another suitable polyacrylamide gel.

▲ CRITICAL STEP Do not denature sample by heating or adding reducing agent, as this will interfere with recognition by antibodies directed to conformational epitopes, preventing analysis of proper protein fold.

8| Activate the Immobilon-P membrane by soaking it for 2 min in 100% methanol followed by a 2 min soak in 1× transfer buffer.

9| Assemble western blot transfer apparatus, fill reservoir with 1× transfer buffer and transfer at 100 V for 1 h. Immerse apparatus in ice to avoid overheating.

10| Block the transferred membrane for at least 1 h in 5% (wt/vol) non-fat milk in 1× PBS-Tween-20 or overnight with 3% (wt/vol) non-fat milk in 1× PBS-Tween-20.

11| Incubate the membrane with primary antibody (1 µg ml^{-1}) with 3% (wt/vol) non-fat milk in 1× PBS-Tween-20 for 1 h.

12| Wash by rocking immunoblot in 25 ml of 1× PBS-Tween-20 for 10 min and decant. Repeat three times.

BOX 1 | PROTEIN ANALYSIS BY SANDWICH ELISA ● TIMING 1.5 d

1. Coat the wells of a Corning Costar 96-well microtiter plate with 50 µl of a capture probe (either a conformational antibody or ligand) at a concentration of 1–5 µg ml^{-1}. Cover the plate with Parafilm and incubate for 2 h at RT.
2. Remove the capture antibody solution and wash the plate three times with 1× PBS-Tween-20.
3. Block remaining protein-binding sites in microtiter plate wells for 1 h using 100 µl of 1× PBS-BSA.
4. Remove the blocking solution and rinse with 1× PBS-Tween-20.
5. Add 50 µl of the media containing the secreted protein and incubate overnight at 4 °C. For quantitative results, always compare signal of unknown sample to those from a standard curve and perform experiments in triplicate.
6. Remove the media and wash the plate five times with 1× PBS-Tween-20.
7. Add 50 µl of a mouse anti-HA antibody (1:1,000 dilution in 1× PBS-BSA) and incubate for 1 h at room temperature.
8. Wash the plate five times with 1× PBS-Tween-20.
9. Add 50 µl of a goat anti-mouse IgG horseradish-peroxidase-conjugated secondary antibody (1:2,500 dilution in 1× PBS-BSA) and incubate for 1 h at room temperature.
10. Wash each well 10 times with 1× PBS-Tween-20.
11. Add 50 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate solution from the Pierce TMB substrate kit to each well and incubate for 15–30 min at room temperature or until desired color develops.

! CAUTION TMB should be kept out of direct light, as it is photosensitive and should also be handled as a mutagen.

12. Add 50 µl of 2 M H_2SO_4 to stop the reaction.
13. Read the optical density at 450 nm.

13| Incubate with appropriate secondary antibody for 1 h (we use 1:1,000 dilution of alkaline phosphatase-conjugated antibody).

14| Wash with 25 ml of 1× PBS-Tween-20 for 10 min and decant. Repeat three times.

15| Dissolve one tablet of SIGMA *FAST* BCIP/NBT in 10 ml of deionized H_2O. Develop blot to desired intensity, rinse with deionized H_2O and let dry. Color development should occur quickly.

? TROUBLESHOOTING

CellSTACK preparation ● TIMING 5 d

16| Scale up growth of HEK293T to ~2.0 × 10^8 cells. Three T225 cm^2 flasks of HEK293T cells grown to 100% confluency contain ~2.0 × 10^8 cells.

17| Purify 1 mg of DNA for transfection using the Qiagen MaxiPrep plasmid purification kit. A 1 l overnight culture of *E. coli* XL-1 Blue should produce 2–3 mg of pDISPLAY vector.

18| Pre-warm all media to 37 °C prior to addition of cells. Add ~2.0 × 10^8 HEK293T cells into a 10-layer CellSTACK containing 1.2 l DMEM, 1× pen/strep, 1× GlutaMAX and 5% (vol/vol) FBS and incubate overnight at 37 °C with 5% CO_2 to allow for cell attachment and growth.

? TROUBLESHOOTING

Large-scale transient expression ● TIMING 4 d

19| Prepare the calcium phosphate–DNA mixture in laminar flow hood. Mix 1 mg of DNA (from Step 17), 6.8 ml of 2 M $CaCl_2$ and 60 ml of deionized H_2O on ice. All solutions should be sterile-filtered. If DNA is dilute (<0.5 mg ml^{-1}), appropriately decrease the amount of deionized water added.

20| Pipette this solution dropwise onto 67 ml of pre-chilled 2× HBS on ice. Vortex or mix well. 2× HBS should be made freshly or stored frozen to prevent pH drift.

21| Incubate on ice for 20 min. Solution should become cloudy from formation of a fine white precipitate.

▲ CRITICAL STEP Timing of the calcium phosphate–DNA mixture on ice is critical; do not exceed or drop below 20 min incubation.

22| Add calcium phosphate–DNA mixture to the media in the 10-layer CellSTACK. For optimal transfection, cells should be at ~70% confluency. Thoroughly mix with media, distribute evenly over cells and incubate in CO_2 incubator (5% CO_2) at 37 °C for 4 days.

? TROUBLESHOOTING

Supernatant harvest and concentration ● TIMING 4 h

23| Harvest supernatant 4 days post-transfection. Centrifuge the supernatant at 10,000*g* for 20 min at RT and filter using a 0.22 μm vacuum filter apparatus. Note that the 10-layer CellSTACKS can be re-used, as described in EQUIPMENT SETUP.

24| Concentrate the supernatant to 150 ml using the Centramate tangential flow filtration system. Buffer-exchange the concentrated sample into PBS by adding 500 ml of 1× PBS and concentrating down to 150 ml, repeating this process five times. Recirculate the sample through the system for 30 min to recover additional sample from the membrane.

Purification ● TIMING 1 d

25| Pre-equilibrate a 1 ml anti-HA affinity column with 1× PBS and load sample by gravity at a flow rate of <1 ml min^{-1}.

26| Wash the column with 10 ml of 1× PBS (Optional step: Use 1× PBS-Tween-20 to remove BSA contaminants).

27| Wash the column with 30 ml of 1× PBS.

28| Dissolve the synthetic HA peptide in 1× PBS (1 mg ml^{-1}) and pre-warm to 37 °C.

29| Pipette 1 ml of HA peptide to the anti-HA column and allow peptide to enter the resin. Collect flow-through. Turn off column flow once peptide reaches the bed height and incubate entire column at 37 °C for 15 min.

30| Repeat Step 29 two additional times.

31| Pipette 1 ml of 1× PBS onto the column and flow into resin until the meniscus reaches the bed height. Collect the flow-through.

? TROUBLESHOOTING

32| Pool fractions according to SDS-PAGE analysis.

33| Repeat Steps 25–32 to reload the flow-through to capture additional protein.

34| Regenerate anti-HA column with 10 column volumes of 0.1 M glycine pH 2.2, and store at 4 °C in 1× PBS with 0.02% (wt/vol) NaN_3.

Deglycosylation ● TIMING 2 d

35| Perform a time course test deglycosylation reaction. Set up a series of digestions at RT and at 37 °C, as shown in the table below. Collect 10 μl aliquots at time points of 0, 2, 4, 6, 8, 24 and 48 h. To stop the reaction, immediately add 3× SDS-PAGE–reducing sample buffer to each aliquot and heat sample at 95 °C for 5 min.

Glycosidase	Units of glycosidase	Protein (μg)	Buffer	Final volume (μl)
PNGaseF	500	40	8 μl of 10× PBS pH 7.4	80
EndoF1	0.04	40	8 μl of 1 M sodium citrate pH 5.5	80
EndoF2	0.01	40	8 μl of 1 M sodium acetate pH 4.5	80
EndoF3	0.01	40	8 μl of 1 M sodium citrate pH 5.5	80

? TROUBLESHOOTING

36| Analyze the deglycosylation reaction by Coomassie-stained SDS-PAGE, anti-HA-probed immunoblot analysis or mass spectrometry. Deglycosylation should result in a molecular weight size shift of bands visualized by western blot (see the ANTICIPATED RESULTS). To identify whether the glycoprotein is completely deglycosylated, compare the molecular weight of the deglycosylated glycoprotein (obtained by mass spectrometry) to its theoretical protein-only molecular weight. Note that some deglycosylation reactions may not lead to complete removal of oligosaccharides, as glycan sites may be sterically hindered from enzyme access.

37| Select deglycosylation enzyme and reaction condition yielding the most homogeneous sample. Small-scale test reaction volumes and starting material are scaled up linearly for preparative use.

38| Separate deglycosylated glycoprotein from enzyme and cleaved glycans using size-exclusion chromatography prior to crystallization.

Microfluidic crystallization screening ● TIMING setup: 3 h; crystal growth: 7 d

39| Concentrate purified protein to 10–20 mg ml^{-1} using an Amicon Ultra-0.5 microcentrifugal concentrator. It may be helpful to determine ideal protein concentration for crystallization using the PCT kit from Hampton Research or another commercial

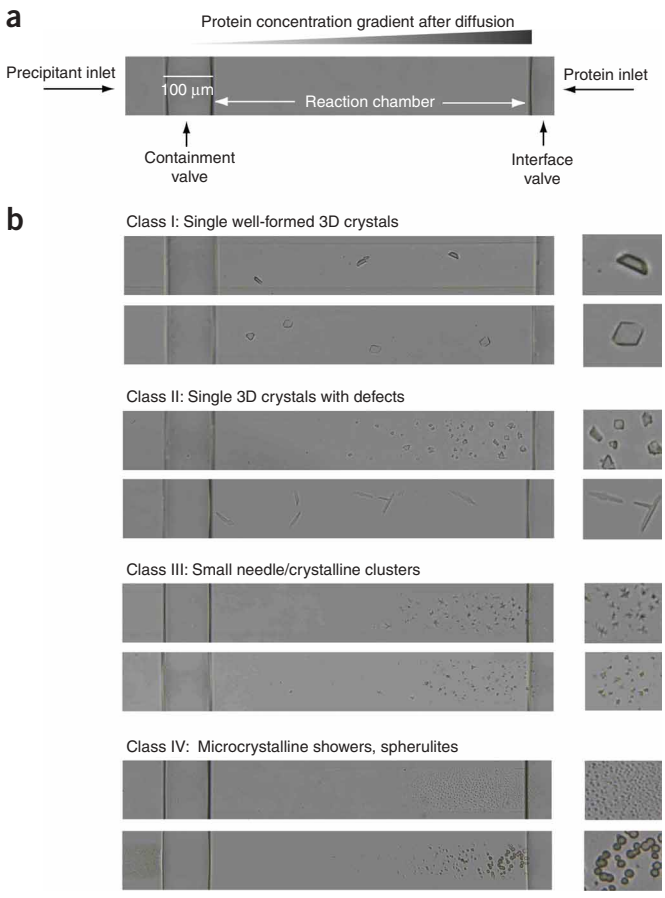

Figure 5 | Crystallization using microfluidic free interface diffusion. (**a**) In each reaction chamber of a Topaz crystallization chip, the release of the containment and interface valves allows protein and precipitant to enter from the right and left sides of the chamber, respectively. This creates a protein and precipitant concentration gradient in the chamber, which allows the sampling of a wide area of crystallization space. (**b**) Selected examples of ZEBOV GP-KZ52 crystals belonging to the four classes of hits obtained by free interface diffusion. Classes I and II are characterized by single three-dimensional crystals with no or minor visible defects, and are immediately suitable for translation to vapor diffusion experiments. Class III and IV hits are typically poor quality clusters or microcrystals and probably require further optimization prior to translation.

vendor. In general, initial concentration of protein needs to be higher for free interface diffusion experiments than that typically used for vapor diffusion experiments, due to the direct mixing of both precipitant and protein in the reaction chambers.

40| Pipette 750 µl appropriately diluted Hydration Fluid Concentrate onto each PVA strip inside the hydration chamber and close the chamber lid. For most screens, a 2:1 ratio of Hydration Fluid Concentrate to water is sufficient, whereas for other screens, such as OptiMix-3 (a high salt screen), a 1:1 dilution is better.

41| Run the Prep script on the Topaz IFC Controller to activate control valves and charge the hydrated chip.

42| Using an eight-channel micropipettor, add 15 µl of crystallization sparse matrix screen (OptiMix-1,2,3, PEG or other commercial/in-house screens) into the appropriate precipitant well.

▲ **CRITICAL STEP** Perform this within 20 h of Steps 40–42 to avoid dehydration of the crystallization reagent in the precipitant well. Examine the chip under a light microscope to ensure the absence of bubbles at the bottom of reagent wells, which would interfere with proper dispensing of reagent into the microfluidic circuitry.

43| Pipette 1.5 µl of protein into the protein inlet (marked on the chip with blue square(s)).

44| Run the load script. Check under a light microscope that all chambers are filled.

? TROUBLESHOOTING

45| Record a time = 0 image scan (negative control) using the AutoInspeX II workstation.

46| Initiate diffusion using the FID Control RT script.

47| Store screening chip in a vibration-free incubator and monitor for crystal hits and growth over a 7-day period using the AutoInspeX II.

? TROUBLESHOOTING

48| Score the crystallization results (we use a class I–IV scale, **Fig. 5**). Select best crystallization hits (class I or II) for translation to traditional, 0.5- to 2.0-µl-size hanging drop vapor diffusion. Class III or IV crystals may require additional screening in Fluidigm 1.96 screening chips to improve crystal quality. Chances of successful translation increase when crystals of high quality (class I or II) are chosen. See manufacturer's translation guide for more detailed instructions.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2, 18	Cells do not attach	Cells are sick or dead	Check cell viability using Trypan blue staining
		Contamination with bacteria, yeast or fungi	Check cells for signs of contamination under the microscope Decontaminate all surfaces, media and equipment. Thaw new cells and start over
		Media without divalent ions, or wrong plate type used	Check media and tissue culture appropriate plates
	Cloudy medium	Contamination with bacteria, yeast or fungi	Check cells for contamination under the microscope; decontaminate
2–15	No/low test expression levels	Poor transient transfection	Try a higher ratio of transfection reagent to DNA
			Check that cells are ~70% confluent and have a low passage number before transfection
			Ensure a constant level of 5% CO_2 or proper buffering of bicarbonate-based buffer
			Try different transfection reagent
		Cells are dead/not viable	Check cell viability using Trypan blue staining
			Check for contamination
		Protein is not folded correctly	Check the cell pellet for insoluble, unsecreted protein
			Test expression of other construct variants
		Codon usage	Check the DNA sequence for the use of rare codons; if necessary, synthesize a version of the gene codon-optimized for expression in mammalian cells
		SDS is interfering with antibody binding in western blots	Analyze secreted proteins by one or more of: non-denaturing western blot using native gels, dot blot with ligand or sandwich ELISA
19–22	Low protein yield	Poor transient transfection	Make sure DNA is successfully precipitated with calcium phosphate. Calcium phosphate–DNA solution should turn cloudy with a fine white precipitate
			Ensure pH of 2× HBS is 7.1
			Ensure a constant level of 5% CO_2
			Optimize the amount of DNA; try increasing or decreasing the amount of DNA
			Check DNA purity; the A_{260}/A_{280} ratio should be 1.8 or greater
		Contamination with bacteria, yeast or fungi	Check cells under microscope for signs of contamination
		Suboptimal growth of cells	Transfection efficiencies may decrease if cells have been passaged for many generations. Start a new culture
25–31	BSA contamination after anti-HA purification	Insufficient wash	Increase volume of PBS-Tween-20 wash to 60 ml
			Increase Tween-20 in wash to 0.2% (vol/vol)

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Low protein yield allowing excess nonspecific binding of BSA	Use a smaller anti-HA column
			Optimize construct or codon usage for higher protein yields
	Protein does not bind to purification matrix	Acidic pH/poor buffering	Check pH of sample and buffer exchange into 1× PBS if necessary
		Loading of sample too rapid	Slow the flow rate (keep under 1 ml/min)
		Inaccessible purification tag	Check that purification tag is intact by performing a reduced and denatured western blot analysis; if accessibility is a problem, subclone tag to the opposite terminus or add a linker between the protein and tag
		Column was not properly regenerated prior to use	Regenerate column using 0.1 M glycine pH 2.0
31	Little or no protein is eluted	Protein is degraded	Add protease inhibitors and perform purification at 4 °C
		Protein retained on column	Elute any column-bound protein using 0.1 M glycine pH 2.0, collect eluant and detect the presence of protein by western blot; if protein is retained on column, optimize HA peptide elution protocol by incubating HA peptide for longer periods of time at 37 °C
		Protein has precipitated on column due to aggregation or instability	Test expression of other construct variants; try adding multimerization motifs to oilgomeric proteins, vary N- or C-terminal truncations, try fully glycosylated protein
35	No or incomplete deglycosylation	Glycan site is sterically hindered	Sequential or simultaneous deglycosylation using a cocktail of glycosidases; use neuraminidase to remove terminal sialic acid, followed by the EndoF series, EndoH or PNGaseF
			Point mutations to delete restricted glycan sites
			Express protein using GnTI-deficient HEK293T cells[30]. Either cleave glycans with EndoH or PNGaseF or leave intact for crystallization
			Add urea (0.5–2.0 M final concentration) to transiently destabilize protein and enhance enzyme access; after deglycosylation, dialyse to urea-free buffer
	Degradation of protein	Glycosidase contains contaminating proteases	Use recombinant glycosidases rather than those purified from natural sources
			Add protease inhibitors to deglycosylation reaction
			Look for contaminating proteases using a universal protease substrate like caesin
44	Some chambers are not filled	Air bubble in channels or in the reagent well	Dispense more reagent/protein in well and reload

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
47	Crystals disappear/deteriorate over time	Evaporation of the reaction chamber may lead to mechanical stresses on the crystal	Document images of each reaction chamber on a daily basis
47 and 48	No crystallization hits	Insufficient sampling of crystallization space	Use different sparse matrix screens to sample alternate regions of crystallization space
		Protein quality and conformational flexibility	Characterize the protein using DLS and/or native gels. Strive for homogeneity and monodispersity
			Create new protein variants using deuterium-exchange mass spectrometry (DXMS) to identify and eliminate flexible regions[40,41]
		Protein solution is undersaturated (especially if all wells are clear)	Increase protein concentration; use the Pre-crystallization Test (PCT; Hampton Research) or similar to ensure ideal concentration is being used for crystallization (note: concentration should be ~2–3× higher for free interface diffusion than vapor diffusion)
48	Abundance of salt crystal hits	Dehydration of reaction chamber	It is normal for the reaction chamber to dry out, allowing salt crystals to form over time; be skeptical of crystal hits that appear on day 7
			Ensure the PVA strips are evenly coated with hydration fluid and hydration chamber is tightly closed
		Crystal screens have high salt content as a precipitant	Use sparse-matrix screens that have lower salt concentrations or are primarily PEG-based
	Distinguishing salt from protein crystals		Look for salt crystal formation in the precipitant inlet side of reaction chamber or for identical crystals formed by different proteins in same precipitant conditions
			Set up control reaction with buffer (no protein)
			In situ diffraction of Topaz crystallization chip crystals at the synchrotron (prototype setup on BL8.3.1 at the Advanced Light Source, Berkeley, CA, USA) if crystals are large enough
	Trouble translating hits to vapor diffusion	Free interface diffusion and vapor diffusion feature different crystallization kinetics	Try different well-to-protein ratios
			Try different PEG to salt/additive ratios
			Optimize crystal hits in 1.96 chip to obtain higher quality hits prior to translation
			Try capillary counterdiffusion crystallization

● TIMING

Step 1, Cloning and sequencing: 5 d
Steps 2–6, Small-scale expression: 4 d
Steps 7–15, Western blot analysis: 8 h
Steps 16–18, CellSTACK preparation: 5 d
Steps 19–22, Large-scale transient expression: 4 d

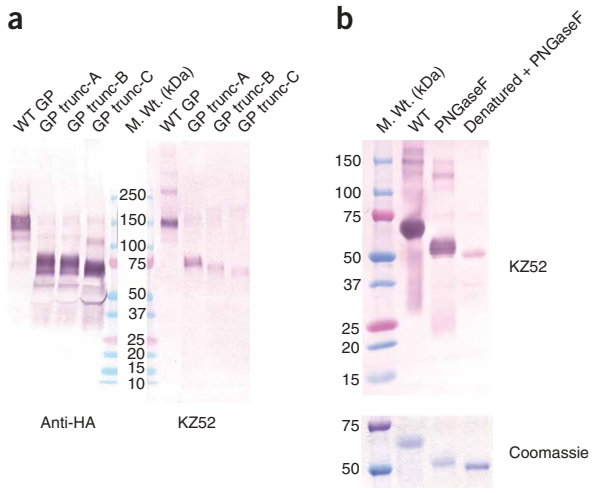

Figure 6 | Test expression and deglycosylation of glycoproteins. (**a**) Small-scale expression screening of selected *Zaire ebolavirus* GP truncation variants. Conditioned media containing EBOV GP variants was harvested 4 days post-transfection, and 10 µl of each culture supernatant was separated on a non-denaturing 10–15% gradient SDS-Tris-HCl polyacrylamide gel and probed with mAb-anti-HA or mAb-KZ52 (conformational). The KZ52 antibody[42] was isolated from a human survivor of a 1995 outbreak in Kikwit, Democratic Republic of the Congo (formerly Zaire) and recognizes an epitope bridging both the attachment and fusion subunits of *Zaire ebolavirus* GP[15]. All ZEBOV GP variants were overexpressed and secreted, as illustrated by the anti-HA probed immunoblot. However, only wild-type GP and GP trunc-A were recognized by KZ52, suggesting that this particular truncation has not significantly altered the native fold. (**b**) PNGaseF deglycosylation of HEK293T-expressed ZEBOV GP trunc-A. The extent of deglycosylation was monitored by both non-denaturing western blot using KZ52 as the primary antibody and non-reducing, Coomassie-stained SDS-PAGE analysis. As a positive control for complete deglycosylation, ZEBOV GP trunc-A was denatured at 95 °C for 5 min with 0.5% (wt/vol) SDS and treated with PNGaseF. Note that a significant fraction of denatured ZEBOV GP trunc-A is not recognized by the conformational antibody, KZ52, but a molecular weight size shift between the native and denatured deglycosylation reactions suggest incomplete deglycosylation at two N-linked sites. Analysis of the crystal structure confirms that local protein structure at these sites would limit enzyme access.

Steps 23 and 24, Supernatant harvest and concentration: 4 h
Steps 25–34, Purification: 1 d
Steps 35–38, Deglycosylation: 2 d
Steps 39–46, Microfluidic crystallization setup: 3 h
Steps 47–48, Microfluidic crystallization results: within 7 d
Box 1: Protein analysis by sandwich ELISA: 1.5 d

Note that the times indicated are estimates, and some steps may require additional time. For example, we utilize a general two-step purification composed of anti-HA and size-exclusion chromatography. However, some proteins will require additional purification steps to achieve homogeneity. In addition, enzymatic deglycosylation may lead to increased protein sample heterogeneity due to incomplete deglycosylation. This may require additional optimization of the glycosidase reaction conditions or screening of glycan site mutations. Note that the scale up of HEK293T cells for the CellSTACK preparation (prior to large-scale transient transfection) may be performed in parallel with the small-scale expression and western blot analysis steps.

ANTICIPATED RESULTS

Crystal structures of viral glycoproteins are difficult to achieve given the challenges to production, purification, crystallization and diffraction inherent in the heavily glycosylated, oligomeric, metastable and flexible nature of these proteins. The expression and crystallization platform described here was instrumental in the determination of the structure of the trimeric, prefusion *Z. ebolavirus* glycoprotein (ZEBOV GP)[15]. In the case of ZEBOV GP, the strategy described here allowed rapid expression screening for over 140 constructs containing various truncations and multiple combinations of mutations to N-X-S/T glycosylation sequons. Moreover, the HEK293T cell culture of the best constructs could be subsequently scaled up to quantities that allowed growth and screening of 50,000 crystals. It is anticipated that this platform will be a useful tool not just for viral glycoproteins but also for mammalian proteins in general.

Constructs selected for scale-up should be characterized by a single strong band at the proper molecular weight on both linear anti-HA and conformation-dependent antibody immunoblots, indicating adequate expression level, monodispersity and proper folding (**Fig. 6a**, for example). In our laboratory, large-scale expression in HEK293T cells routinely results in the production of ~2–10 mg of protein per liter of media. It is expected that glycans produced in HEK293T cells can be efficiently removed by PNGaseF (**Fig. 6b**). However, deglycosylation is often incomplete due to poor steric access of the attachment site by the local conformation of the protein. In the case of ZEBOV GP, resistant glycan sites were removed by site-directed mutagenesis of the N-X-S/T glycosylation sequons[15].

Microfluidic free interface diffusion crystallization (Topaz) uses only 6–7 µl of protein (10 mg ml^{-1}) to screen 384 sparse matrix conditions and yields results within 7 days. Protein crystals usually appear closer to the right-hand side of the chamber, which is the region of highest protein concentration. However, the concentration gradient formed in the horizontal axis of the chamber may sometimes permit crystallization in the middle of the chamber. Crystals closest to or contained within the precipitant inlet side (far left) are usually salt. Crystallization hits can be scored into four classes (I–IV; **Fig. 5**). Class I crystals appear perfect, with sharp edges and thickness in three dimensions. Class II crystals are similar to class I, but have minor defects, such as irregular, jagged or rounded edges or satellites. Class III crystals are small needle or crystalline clusters, whereas class IV crystals are showers of microcrystals or spherulites. In the case of ZEBOV GP and other projects in our group, the success

of translation is highly dependent on the quality of the crystal hit and number of unique hits obtained. Class I and II crystal hits have high translation percentages and can often be translated directly into similar conditions of the crystallization screen. Translation of class III and IV crystals tends to be difficult and likely requires additional optimization of pH, salts, PEG or additives using a 1.96 chip to yield more crystalline class I- and II-type crystals prior to translation.

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