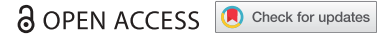


REPORT



Rapid production of a chimeric antibody-antigen fusion protein based on 2A-peptide cleavage and green fluorescent protein expression in CHO cells

Hans Van der Weken , Eric Cox *, and Bert Devriendt *

Laboratory of Immunology, Faculty of Veterinary Medicine, Ugent, Ghent, Belgium

ABSTRACT

To enable large-scale antibody production, the creation of a stable, high producer cell line is essential. This process often takes longer than 6 months using standard limited dilution techniques and is very labor intensive. The use of a tri-cistronic vector expressing green fluorescent protein (GFP) and both antibody chains, separated by a GT2A peptide sequence, allows expression of all proteins under a single promoter in equimolar ratios. By combining the advantages of 2A peptide cleavage and single cell sorting, a chimeric antibody-antigen fusion protein that contained the variable domains of mouse IgG with a porcine IgA constant domain fused to the FedF antigen could be produced in CHO-K1 cells. After transfection, a strong correlation was found between antibody production and GFP expression ($r = 0.69$) using image analysis of formed monolayer patches. This enables the rapid selection of GFP-positive clones using automated image analysis for the selection of high producer clones. This vector design allowed the rapid selection of high producer clones within a time-frame of 4 weeks after transfection. The highest producing clone had a specific antibody productivity of 2.32 pg/cell/day. Concentrations of 34 mg/L were obtained using shake-flask batch culture. The produced recombinant antibody showed stable expression, binding and minimal degradation. In the future, this antibody will be assessed for its effectiveness as an oral vaccine antigen.

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Introduction

Systemic vaccines often fail to induce an effective mucosal immune response, characterized by the induction of pathogen-specific secretory immunoglobulin A (SIgA).¹ Oral vaccines are much more effective in achieving mucosal immunity and have the added benefit of being easy and safe to administer.² One of the main drawbacks of oral vaccination is the poor uptake by the intestinal epithelium and the ensuing delivery to the underlying gut-associated lymphoid tissue.³ Selective targeting of vaccine antigens to a transport protein on the intestinal epithelium might solve this problem.⁴ Recently, we showed that antibody-mediated delivery of antigens towards aminopeptidase N (APN), a membrane receptor expressed on enterocytes and involved in epithelial transcytosis, triggered systemic and mucosal antibody responses in a piglet model.^{5,6} However, in these experiments, porcine APN-specific rabbit or mouse IgG were used, which resulted in rabbit or mouse IgG-specific immune responses upon oral vaccination in piglets. The presence of these antibodies might affect the efficacy of APN targeting in a prime-boost vaccination regime. To minimize these responses, a recombinant porcine APN-specific chimeric mouse-porcine IgA antibody, linked with a clinically relevant antigen, was designed. By replacing the mouse IgG constant domains with porcine IgA, minimal immune response and increased antibody stability is expected.⁷⁻⁹

Most recombinant antibodies are produced in Chinese hamster ovary (CHO) cells due to their capacity for correct folding, assembly and glycosylation, leading to improved production. The creation of a stable, high producer cell line is

essential to support the high demand for antibody production.¹⁰ Antibodies are complex molecules consisting of both heavy and light chain polypeptides. Moreover, the ratio of both chains affects the final production of the complete antibody.^{11,12} Efficient co-expression of the heavy and light chain is therefore one of the most important aspects in monoclonal antibody production. This co-expression can generally be achieved by either co-transfecting two separate vectors, each encoding a single antibody chain, or by transfecting a single vector encoding both chains.¹³ Expression on separate vectors often results in a poor balance of light and heavy chain expression levels, leading to reduced antibody production. Multiple studies have shown that expressing both chains from a single vector significantly improves the expression ratio.^{14,15} Co-expression on a single vector can be achieved by either using two separate promoters, an internal ribosome entry site (IRES) or “self-cleaving” 2A peptides.¹⁶ The use of an IRES-element often leads to reduced protein expression of downstream genes, ranging from 6 to 100%, making this system unpredictable.¹⁷⁻¹⁹

Self-cleaving 2A peptides are short, highly conserved sequences of 18–22 amino acids derived from viruses, such as foot-and-mouth disease virus (F2A), equine rhinitis A virus (E2A), porcine teschovirus-1 (P2A) and thosa asigna virus (T2A). They mediate “cleavage” of polypeptides during translation by steric hindrance, resulting in ribosomes skipping the formation of a glycyl-propyl (G-P) peptide bond at the C-terminus of the 2A peptide.^{20,21} After successful skipping, the 2A peptide remains bound to the

CONTACT Bert Devriendt  b.devriendt@ugent.be  Laboratory of Immunology, Faculty of Veterinary Medicine, Ugent, Belgium

*These authors contributed equally to this work.

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upstream protein and often a furin cleavage site is inserted to remove the remaining peptides. The use of 2A peptide cleavage mostly leads to higher expression levels compared to IRES-based expression,¹³ but can also lead to generation of aggregates due to incorrect cleavage and folding.¹⁶ Efficiency of correct cleavage and antibody production is highly dependent on the cell line used and 2A peptide sequence. T2A peptide cleavage in addition to a GSG sequence (GT2A) showed the highest cleavage efficiency and antibody expression levels in CHO cells.²⁰

Another major bottleneck in the production of recombinant antibodies is the selection of stable transfected cells with high expression. By using a 2A peptide sequence to link GFP expression to protein production, the screening time and effort could be significantly improved. Co-expression of fluorochromes with a protein of interest using 2A peptide sequences has been done before, but only for localization purposes and not for screening of antibody production levels.²² Here, we report on the use of 2A peptide cleavage for the simultaneous expression of both the heavy and light antibody chains, in combination with GFP to simplify and improve the screening of high producer antibody clones.

Results

GFP expression levels correlate with antibody production levels by CHO cells

A tri-cistronic vector was designed expressing GFP and a recombinant antibody, separated by furin and a GT2A peptide sequence (Figure 1). CHO-K1 cells were transfected with this vector and were selected with G418 to produce stable transfections. Five days after transfection, clear GFP expression could be observed in some cells, but with a high variation in intensity (Figure 2(a)). After 9 days of selection, the 10% highest GFP-expressing cells were single-cell sorted into 96-well plates and grown further until colonies formed (Figure 2(b)). In total, from 460 GFP^{high} single cells, 50 colonies formed, of which 26 showed clear GFP expression and formed a monolayer patch. High

variability in colony size, growth kinetics and GFP expression levels were observed between colonies (Figure 2(c)). Twelve days after sorting, 23 colonies showed 100% confluency (Figure 2(d)) and were further expanded into a 24-well plate. After 4 days, the recombinant antibody production level in the supernatant of each clone was assessed using an anti-porcine IgA sandwich ELISA and compared to their respective GFP expression using image analysis. A strong correlation between antibody production and GFP expression for all 23 selected clones was found using Pearson correlation ($r = 0.55$; $p = 0.0064$) (Figure 2(e)). Colonies not showing significant antibody production showed either very low GFP expression or were found to be slow growing cells or colonies with heterogenous GFP expression levels (Figure 2(c)). Because the number of developed colonies was very low (10.8%) and half of them showed no clear GFP expression after colony development, a second experiment was performed using 12 days of selection before single cell sorting. A similar antibody harboring a single mutation (G99D) in the heavy chain variable domain, preventing it from binding APN, was used. After single cell sorting the GFP^{high} population (Figure 2(b)), 137 colonies were formed (29.8%), of which 96 (70.1%) showed clear GFP expression and formed monolayer patches, indicating that a longer selection procedure improved stable expression of GFP. After collection of supernatant, a very strong correlation was found between antibody production and GFP expression ($r = 0.69$; $p = 5.79 \times 10^{-15}$) (Figure 2(f), Left). Colonies not showing significant antibody production were again found to be slow growing cells or colonies with low or heterogenous GFP expression. Correlation increased further ($r = 0.78$; $p = 1.92 \times 10^{-20}$) 12 days post-sort, when cells reached 100% confluency (data not shown). Correlation between antibody production and GFP expression, determined by flow cytometry instead of image analysis, gave a much higher correlation coefficient ($r = 0.86$; $p = 4.84 \times 10^{-29}$) (Figure 2(f), Right), indicating that this method is more precise. The best producing clone was selected, grown further and adapted to suspension culture to enable large-scale recombinant antibody production and purification. Concentrations of 34 mg/L were obtained using shake-flask batch culture. Both antibody production and

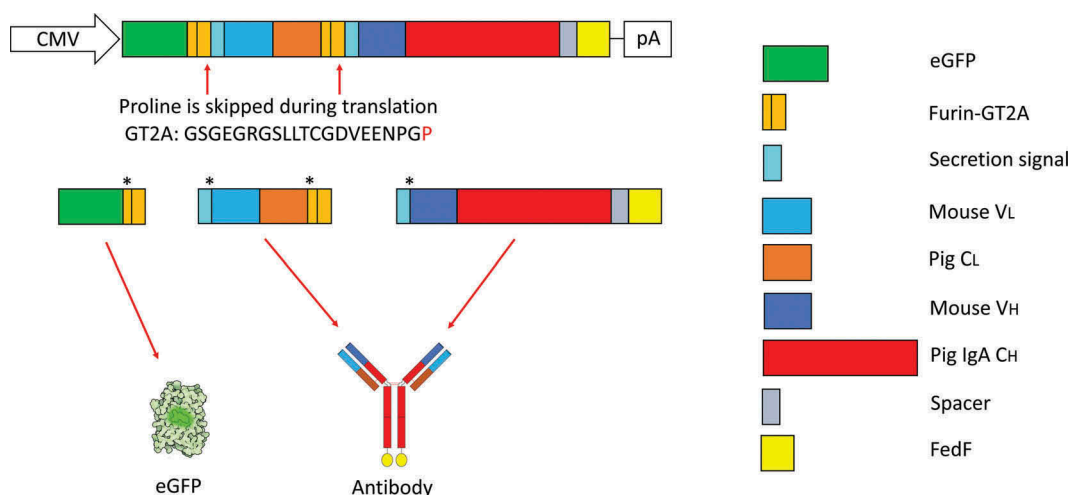


Figure 1. Schematic representation of the vector expressing eGFP and a recombinant antibody consisting of mouse variable domains, porcine IgA constant domains and the FedF antigen. Furin-GT2A: Furin recognition site, followed by a GSG + T2A-peptide sequence; VL: Mouse variable domain light chain; CL: Porcine constant domain light chain; VH: Mouse variable domain heavy chain; CH: Porcine constant domain IgA heavy chain; Spacer: (G₄S)₃-flexible linker; FedF: Tip adhesin of F18 + enterotoxigenic E. coli; pA: Polyadenylation signal. *: Post-translational cleavage of secretion signal and furin recognition site.

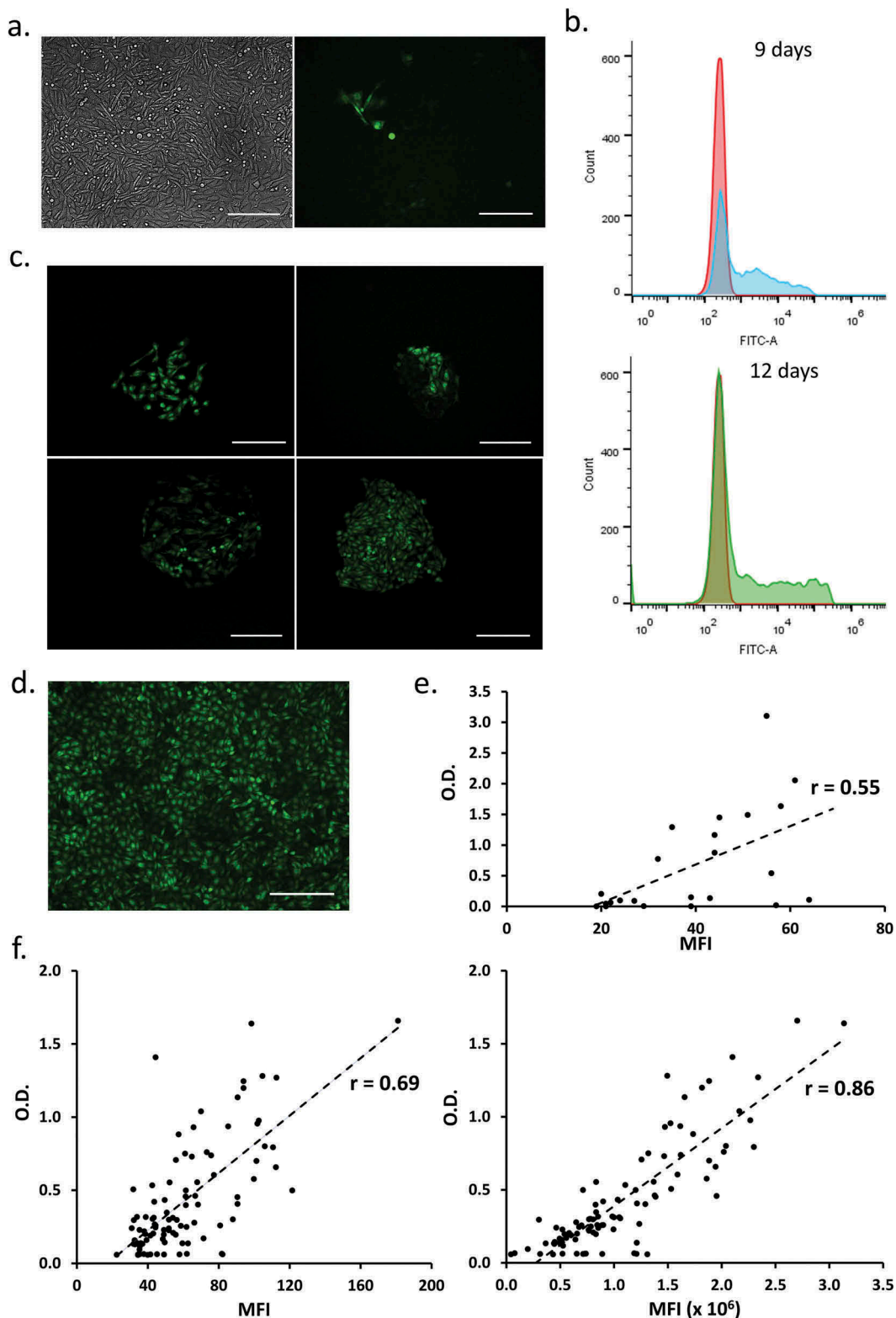


Figure 2. (a) Transmission and fluorescence microscopy images of CHO cells 5 days after transfection. (b) Variation in GFP expression levels from stable transfected cells, 9 and 12 days after transfection (blue and green, respectively), compared to non-transfected cells (red). (c) Variation in GFP expression and growth rate of developed colonies, 5 days after single cell sorting. (d) Colony development 12 days after single cell sorting. (e) Correlation between antibody production (O.D. in ELISA) and mean fluorescence intensity (MFI), determined by colony imaging ($r = 0.55$) for the first transfection. (f) Correlation between antibody production and MFI, determined by colony imaging (Left; $r = 0.69$) and flow cytometry (Right; $r = 0.86$) for the second transfection. Scale bars = 100 μm .

GFP expression levels remained stable after 4 months of culturing for the selected clones (Figure 3(a)). GFP expression levels also

remained correlated with antibody production over time ($r = 0.827$; $p = 0.084$) (Figure 3(b)).

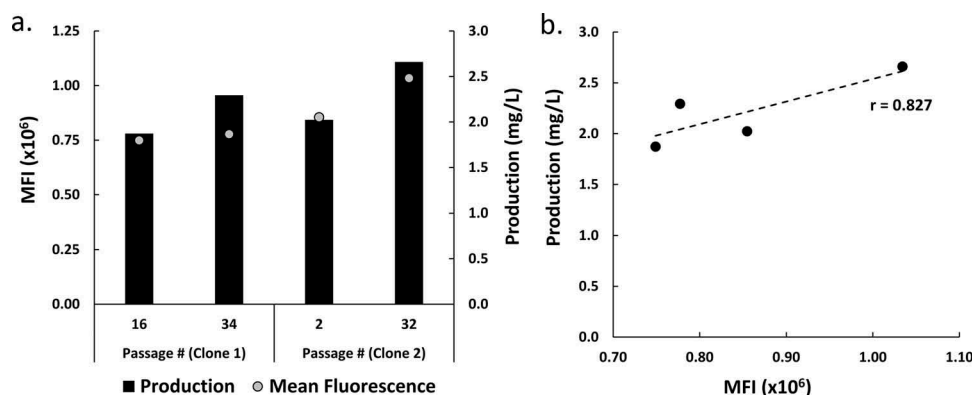


Figure 3. Stability study of GFP expression levels and antibody production over a period of 4 months, showing (a) stable GFP expression and antibody production over time and (b) correlation between GFP expression and antibody production over time.

Purification and quality analysis of the recombinant antibodies

The produced recombinant antibody was purified by cross-linking mouse anti-porcine IgA antibodies on a Protein G column and their stability and functionality were assessed. The purified antibody was first analyzed with SDS-PAGE and Western blot (Figure 4). Under reducing conditions, bands around 25 (light chain) and 75 (heavy chain-FedF) kDa for the purified α APN-IgA-FedF (Lane 1) and bands around 25 (light chain) and 50 (heavy chain) kDa for porcine serum IgA (Lane 2) were revealed. No fusion products composed of light and heavy chains were observed (100 kDa expected), proving efficient cleavage of the furin-GT2A peptide sequence. Some minor degradation (5.3%) of the IgA heavy chain was observed, resulting in a band around 50 kDa.

Next, the functionality of the purified recombinant antibody was assessed by flow cytometry and immunohistochemistry. The

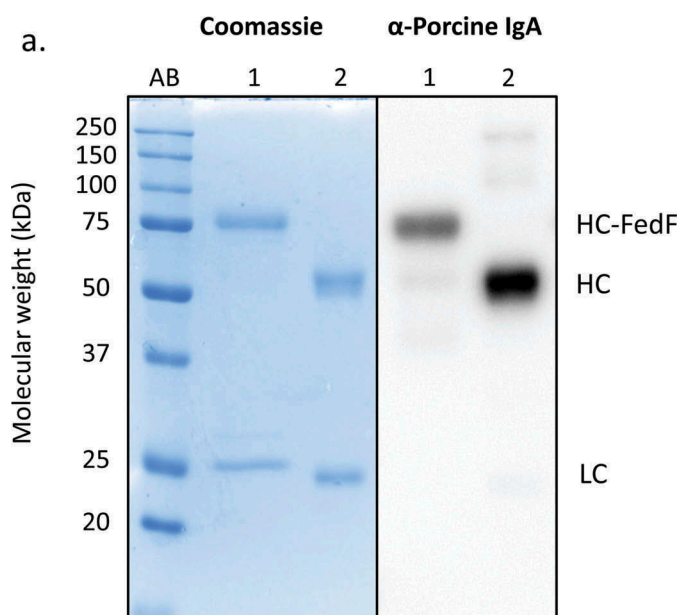


Figure 4. SDS-PAGE and Western blot of purified recombinant antibody (Lane 1: IgA-FedF) or porcine serum IgA (Lane 2) under reducing conditions. AB: Precision Plus Protein All Blue Standard, LC: Light chain, HC: Porcine IgA heavy chain, HC-FedF: porcine IgA heavy chain, linked to FedF.

purified recombinant antibodies were able to bind to an APN-expressing epithelial cell line (IPEC-J2-APN) (Figure 5(a)), as well as to intestinal epithelial cells of the pig ileum (Figure 5(c)). The fused FedF antigen was also detected after binding to the IPEC-J2-APN cell line (Figure 5(b)).

Discussion

The fastest and easiest way to produce a stable culture of antibody-producing cells is by using a mixed population of transfected cells. Since this results in an undefined and genetically heterogeneous cell population of high and low producer cells, this is only recommended for the initial screening steps of the produced antibody. Much higher antibody concentrations can be obtained by generating single cell clones, resulting in a defined and homogeneous cell system. This process of screening stable transfected cells for high producer clones via the traditional limited dilution technique is very time consuming and labor intensive and often takes between 6 to 12 months.^{12,23} Considerable efforts have been made to improve the selection process of high producer cells.^{24,25} Co-transfection with a GFP expressing plasmid is often performed to screen for successful transfections, but this cannot be directly correlated with antibody production. The use of an IRES-based approach, where a reporter protein is put under the same promoter as the expressed antibody allows for better selection. Approaches based on fluorescence-activated cell sorting (FACS) using fluorochromes as reporter proteins has allowed the rapid selection of strong producing clones due to the correlation between the fluorochrome and antibody production.²⁶⁻²⁸ However, in IRES-based expression vectors the expression of downstream genes is often lower, resulting in lower yields when producing multimeric proteins, such as antibodies.^{18,19} Since it has been shown that an excess of light compared to heavy chain results in higher production and reduced aggregation, this could also be considered an advantage for antibody production.¹⁴

To express all subunits in equimolar ratios, 2A peptide cleavage can be used, allowing even the use of tri- and quad-cistronic vectors.²⁹ The much shorter length of the 2A peptides (54–66 bp), compared to the IRES-sequence (500–600 bp), can be an advantage, especially when working with viral transfer

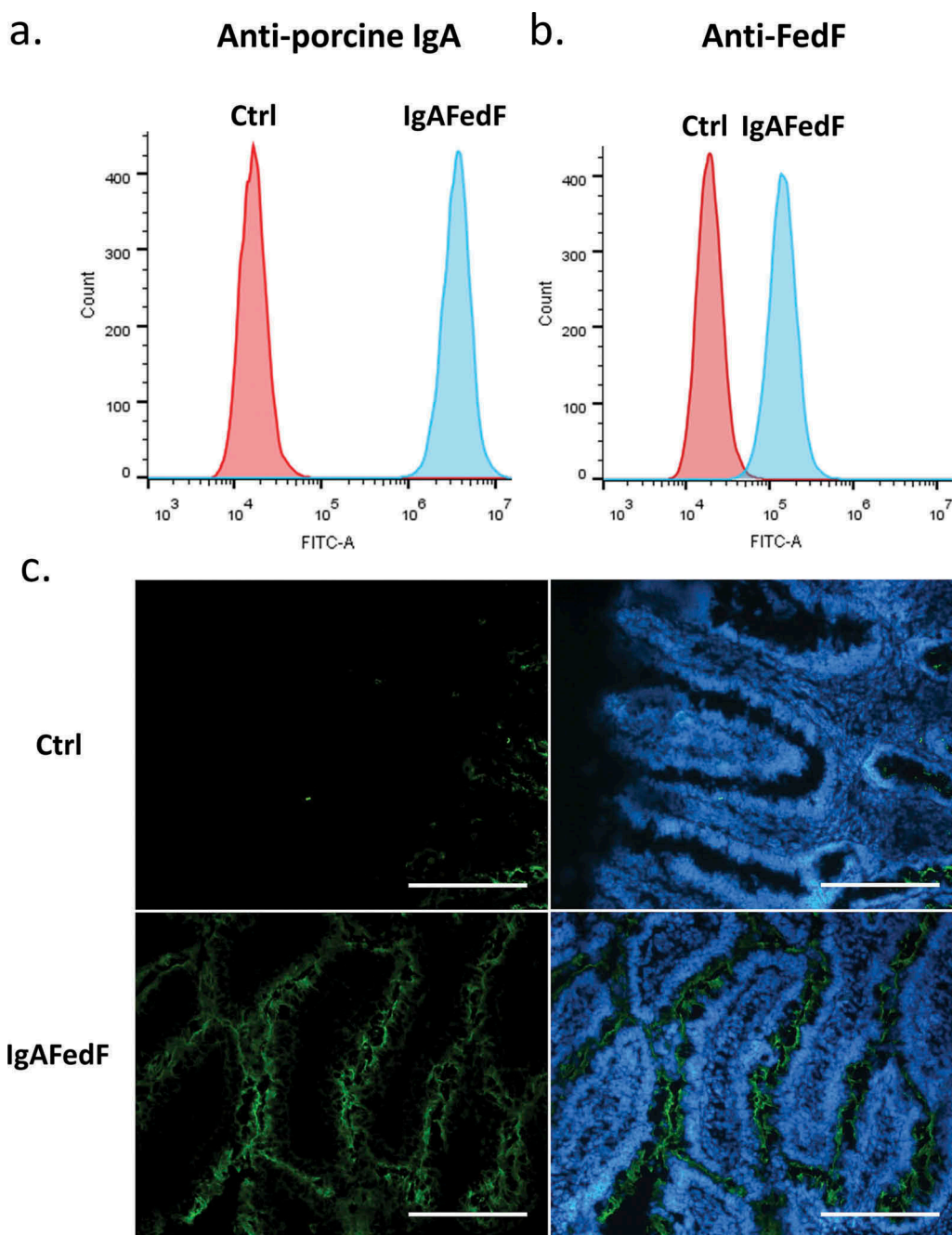


Figure 5. Binding analysis of purified antibody on the APN-expressing epithelial cell line IPEC-J2-APN using flow cytometry (a+b) and on small intestinal epithelial cells of porcine ileum (c). Detection of purified antibody was performed with FITC-conjugated anti-porcine IgA (a+c) or with FITC-conjugated anti-FedF (b). Nuclei were counterstained with Hoechst. Scale bar = 100 μ m.

vectors with a limited packaging capacity. One major drawback of 2A peptides compared to IRES is that inefficient cleavage results in the formation of fusion proteins and aggregates. This cleavage efficiency is highly dependent on the chosen cell line and the 2A peptide sequence, and can be improved by addition of a GSG sequence upstream of the 2A peptide. For our vector design, the GT2A sequence was chosen based on previous experiments performed by Chng et al. showing efficient cleavage and low (<10%) aggregate formation in CHO cells.²⁰

In our approach, as depicted in Figure 6, the advantages of single cell sorting were combined with 2A peptide cleavage by placing the genes encoding both antibody chains and GFP

under a single promoter, separated by a GT2A sequence. This vector design allowed the rapid selection of high producer clones for production of a chimeric antibody-antigen fusion protein. Within a time-frame of 4 weeks, a high producing clone with a specific antibody productivity of 2.32 pg/cell/day (pcd) had been selected. After adaptation of cells to suspension growth, production values of 34 mg/L could be obtained in a shake flask batch culture system. Since we are expressing a chimeric antibody-antigen fusion protein, lower expression compared to a regular antibody was expected, since protein folding might not be optimal. Therefore, it is difficult to compare specific productivity of selected clones with other

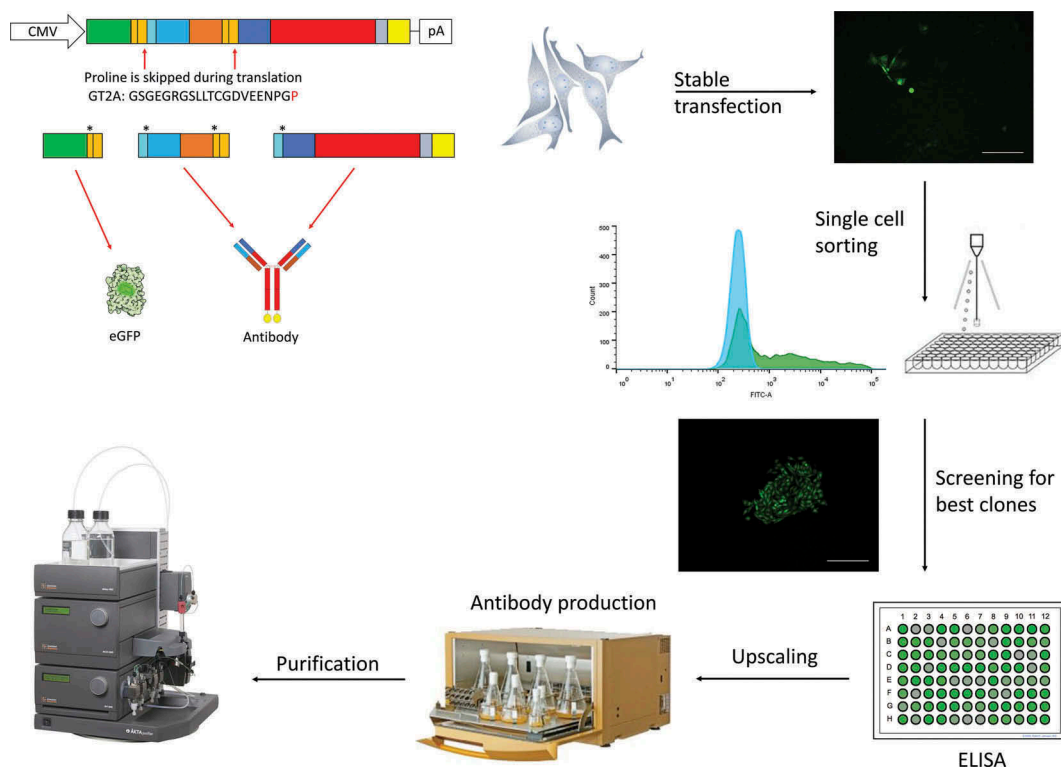


Figure 6. Antibody production workflow: from vector construction to antibody production and purification.

advanced selection techniques since the produced antibodies are not similar.

Stable expression and binding of the target antigen was confirmed for our chimeric antibody-antigen fusion protein, indicating it is feasible to construct antibodies in this manner. Only minimal degradation was observed after purification. This chimeric mouse-porcine antibody will potentially decrease porcine anti-mouse immune responses after immunization as previously described.⁷ Some GFP impurities were observed in the cell culture supernatant. We suspect these GFP impurities are caused by dead cells due to loss of membrane integrity, since GFP did not contain a secretion signal in our vector. In our approach, no GFP impurities were detected after porcine IgA-specific purification of the recombinant IgA antibodies. Therefore, a purification step, such as size-exclusion chromatography or affinity chromatography, should be added to obtain a highly pure product for use in clinical applications.

In total, 122 GFP-expressing colonies were found after single cell sorting of the GFP^{high} population. Most of these colonies showed a strong correlation between antibody production and GFP expression after image analysis of the formed monolayer patches. Some colonies with heterogenous expression of GFP were detected. These showed no or very low antibody production after further colony development. Correlation of antibody production and GFP expression was much higher when determining GFP expression using flow cytometry, but was not necessary for determining best producer clones. Selection based on image analysis could already be performed 5 days after single cell sorting, allowing faster selection of high producer clones. Using automated image

analysis, work load can be significantly reduced since automated selection of high GFP expressing clones can be performed for further expansion.^{30,31} Flow cytometric analysis does require a sufficient number of cells, which extends the time necessary to do the analysis. These cells can also not be used for further colony development, increasing selection time.

Our results indicate that the selection of GFP^{high}-expressing cells using FACS allows much faster selection of strong antibody-producing CHO cells compared to standard limited dilution techniques. In total, high-producing cells could be selected in as little as 4 weeks. Compared to other advanced screening techniques, high-producing clones could be obtained in half the time. For this experiment, a broad selection cutoff was made of the top 10% GFP^{high} cells. More stringent conditions, e.g., single cell sorting of top 1% GFP^{high} CHO cells, could be used to further improve the selection process. Additionally, the use of multiple sequential selection steps could be used to select for even higher antibody-producing cells. This will, however, result in an increased selection time, potentially doubling the selection process time.

In most systems, as well as in our own, the expression of the selection marker is put under control of a separate promoter. Generally, the SV40 promoter is chosen due to its high stability and low propensity for gene silencing.³² Since placing the selection marker under a separate, more stable promoter than the gene of interest could be a disadvantage, it would be interesting to put both genes under a single promoter. A further improvement to our approach would be to construct a quad-cistronic vector, in which the expression of the genes encoding the selection marker, eGFP and the antibody

heavy + light chains are all controlled by a single promoter. Indeed, constructing both antibody chains and a selection marker in a tri-cistronic vector under the control of a single promoter improved the selection of stable high antibody-producing cells.¹⁴ Moreover, the use of 2A peptide cleavage allows the design and construction of quad-cistronic expression vectors.²⁹ Further research is required to investigate whether this type of system would be feasible. Combining an IRES-based approach with 2A peptide cleavage could also be very interesting if the use of quad-cistronic vectors proves difficult. Since the selection marker is preferred to be expressed in lower quantities, this can be placed behind an IRES-sequence downstream of the 2A-construct.

In conclusion, our approach showed that it is feasible to select stable transfected cells producing functional chimeric antibody-antigen fusion proteins in a relative short timeframe. In the future, this construct will be assessed for its effectiveness as an oral vaccine strategy.

Material & methods

Vector construction

A sequence coding for a recombinant chimeric mouse-porcine IgA-FedF fusion antibody, separated by furin + GT2A peptide sequences was synthesized by Genscript and cloned into the pcDNA3.1-N-eGFP vector at the KpnI restriction site, resulting in a tri-cistronic vector encoding 3 separate polypeptides under the control of a single CMV promoter. A (G₄S)₃-flexible linker was placed between the heavy chain and the FedF antigen to improve stability and reduce steric hindrance. A furin recognition site upstream of each GT2A sequence results in post-translational cleavage of this fragment from the final protein (Figure 1). The variable light and heavy chain sequences were derived from an anti-pig APN hybridoma cell line (IMM013) as described by Baert et al.⁶ The porcine constant light (AAA03520.1) and IgA heavy (AAA65943.1) sequences were derived from the GenBank genetic sequence database. For the antigen, the 15th to 165th amino acid of the F18 fimbrial adhesin FedF (4B4P) was used as previously described by Tiels et al.³³

Cell lines

CHO-K1 cells were cultured in Ham's F12 nutrient mixture (Gibco), supplemented with 10% fetal calf serum (FCS, Integro), 2 mM L-glutamine (Gibco), 100 U/ml penicillin, 100 µg/ml streptomycin (P/S) and 1 mM sodium pyruvate (Gibco) at 37°C, 5% CO₂. After transfection, 1 mg/ml Geneticin® (G418, Life technologies) was added for initial selection of stable transfectants. When stable clones were obtained, the concentration of G418 was lowered to 0.15 mg/ml for maintenance and stability analysis. Cells were subcultured every 3–4 days, when 100% confluency was reached. The highest producing clone was adapted to suspension culture growth as described by Costa et al.³⁴ The suspension medium (EX-CELL ACF CHO; Sigma) was supplemented with 6 mM L-glutamine, 5% FCS, 1% P/S and 0.15 mg/ml G418. Suspension-adapted cells were seeded at a density of 5.0×10^5 cells/ml and grown in 200 ml medium

in 500 ml shaker flasks at 37°C and 150 rpm without CO₂. When the maximal cell density was reached (3.0×10^6 cells/ml), the cells were first expanded to 400 ml and then 2 L medium in 1 and 5 L shaker flasks, respectively for recombinant antibody production at a seeding density of 5.0×10^5 cells/ml. When the cells reached their maximal density (3.0×10^6 cells/ml) in the 5 L shaker flasks, the temperature was shifted to 30°C for optimal antibody production.¹⁰ The cell culture supernatant was collected after 10 days when the cell viability dropped below 90%.

A porcine intestinal epithelial cell line (IPEC-J2) was stably transfected with pAPN.⁵ These cells were cultured in Dulbecco's modified Eagle's (DMEM)/F12 (Gibco), supplemented with 10% FCS, 1% recombinant human insulin, transferrin and sodium selenite (ITS, Sigma), 5 ng/ml recombinant human epidermal growth factor (Invitrogen), 4 mM L-glutamine (Gibco), P/S and 0.15 mg/ml G418 at 37°C and 5% CO₂.

Stable transfection

Stable transfections of CHO cells with the expression plasmid were performed using the jetPRIME transfection reagent (polyplus) following the manufacturer's instructions. The cells (5.0×10^4) were seeded in a 24-well plate, grown to 60–80% confluence (1 day) and transfected with 0.5 µg plasmid DNA expressing eGFP and the recombinant antibody. After 4 hours, medium was replaced with selective medium (1 mg/ml G418). After 5 days, the cells were expanded to 6-well plates and further selected for 9 to 12 days before single cell sorting.

Single cell sorting and selection of best producing clones

Nine or 12 days after stable transfection, single cell sorting was performed using a FACSAria III cell sorter (BD Biosciences). All transfected cells were detached using trypsin buffer (0.25% trypsin + 1 mM EDTA) and resuspended in PBS + 1% FCS. Propidium iodide (0.5 µg/ml; Sigma) was added to stain dead cells. Doublets and dead cells were excluded, and the 10% highest GFP expressing cells were selected and single-cell sorted into five 96-well plates (460 wells in total). These cells were then further cultured until colonies formed. Medium was replaced every 4 days. After 12 days, most colonies had grown to 100% confluence and were expanded to 24-well plates at a concentration of 1.0×10^5 cells/well. Supernatant was subsequently collected after 4 days to measure total antibody secretion. Cells were collected, counted and analyzed using flow cytometry (Cytoflex) and mean GFP expression was measured.

To match antibody production levels with GFP expression levels, live cell imaging of transfected and sorted CHO cells was performed with an Olympus IX81 inverted fluorescence microscope (Olympus). Exposure time for eGFP was 200 ms after excitation. Five days after transfection and 5 or 12 days after sorting, both transmission and fluorescence images were taken to observe colony formation and GFP fluorescence. The fluorescent images of formed monolayer patches were processed with ImageJ software to determine the mean fluorescence intensity (MFI) for each colony.

Analysis of GFP expression stability

Stability of GFP expression was analyzed over time using flow cytometry. Selected clones were maintained in culture for over 4 months. Cells were subcultured every 3–4 days, when 100% confluency was reached. GFP expression was measured for each selected clone at specific time-points. Dead cells were excluded using Sytox blue staining (5 nM; Molecular probes). A total of 10,000 viable, single cells were measured for each clone (Cytoflex, Beckman Coulter). At specific time-points, cell culture supernatant was collected, and recombinant IgA antibody concentration was determined using an IgA-specific sandwich ELISA. Selected clones retained their GFP expression and antibody production for a period of 4 months.

Sandwich ELISA

Polystyrene 96-well plates (Polysorb; Nunc-Immuno) were coated with polyclonal anti-porcine IgA (H + L) (A100-102A; Bethyl) in PBS (pH 7.4) at a concentration of 10 µg/ml. After incubation at room temperature (RT) for 2 hours, the wells were washed three times with PBST (PBS + 0.1% Tween-20), followed by incubation with 250 µl blocking buffer (3% bovine serum albumin (BSA) in PBST) overnight at 4°C. After washing 3 times with PBST, 100 µl of supernatant (1/4 dilution) was added and incubated for 2 hours at RT. Supernatant from non-transfected CHO cells was added as negative control. A serial dilution of porcine reference serum with known concentration of IgA (RS10-107-4; Bethyl) was added to determine antibody concentrations. After incubation, wells were washed 3 times with PBST and 100 µl polyclonal anti-pig IgA-HRP (A100-102P; Bethyl) was added in PBST + 1% BSA (1:10,000 dilution) for 1 hour at 37°C. Wells were washed 3 times with PBST, after which 50 µl ABTS (Roche) was added for 30 minutes at 37°C. The absorbance was measured at 405 nm with a Spectra Fluor microplate reader (Tecan). Specific antibody productivity qab (pg/cell/day) was calculated with the following formula:

$$qab = \frac{ab}{N} * \frac{\ln\left(\frac{Cn}{C0}\right)}{Cn - C0}$$

where ab is the amount of produced antibody in pg, C0 and Cn are the initial and final number of cells in each well and N was the incubation time (days) until supernatant was collected.

Antibody purification

A mouse anti-porcine IgA antibody (in-house) was crosslinked on a HiTrap Protein G column (GE Healthcare) using dimethyl pimelimidate (Thermo Fisher). Non-crosslinked antibodies were removed after washing with PBS and elution buffer (1 M glycine, 3 M NaCl, pH 2.7). Next, supernatants containing the recombinant antibody or porcine serum were run over the column overnight at 4°C at a flow rate of 1 ml/min. After a washing step to remove non-specific binding, elution buffer was added, and the eluate was collected. After dialysis, a bicinchoninic assay (BCA) was performed following the manufacturer's recommendations (Pierce BCA protein assay kit) to determine eluted antibody concentration. Purity and stability of purified antibody was subsequently tested.

SDS-PAGE and western blotting

The stability and purity of the purified recombinant antibody was determined using Coomassie staining and western blot. Purified antibody (2 µg) or pig serum IgA (2 µg) was mixed with loading buffer (x5) containing β-mercaptoethanol (Gibco), boiled for 10 minutes at 95°C and separated on a 12% polyacrylamide gel at 120V in SDS-PAGE buffer for 2 hours using the Powerpac 300 system (Bio-rad). Coomassie staining was performed with Coomassie staining solution (1 g Coomassie Brilliant Blue (Bio-rad), 10% glacial acetic acid, 50% methanol in ddH₂O) and destained overnight (30% glacial acetic acid, 10% methanol in ddH₂O). Western blotting was performed by transferring the antibodies to PVDF membranes (Life technologies) using the PowerPac 300 system for 2 hours at 80V. Membranes were blocked overnight at 4°C with 5% skimmed milk in PBS with 0.2% Tween-80. IgA heavy chains were detected with an HRP-conjugated anti-porcine IgA (A100-102P, Bethyl). Washing between each step was done with PBST (PBS + 0.1% Tween-20). Detection was performed using pierce ECL western blotting substrate (Thermo Fisher) and the chemidoc MP imaging system (Bio-rad).

FCM binding assay

For APN binding analysis, flow cytometry was performed using the IPEC-J2-APN cell line. Cells were grown until 90% confluence and detached with StemPro accutase (Gibco). Detached cells (3.0×10^5) were transferred to a conical bottomed 96-well microtiter plate (Gibco) in 200 µl culture medium and centrifuged for 3 min at 350 g and 4°C. The cells were incubated with 10 µg/ml purified recombinant antibodies or medium containing 1% pig reference serum (RS10-107-4, Bethyl) and incubated for 30 minutes on ice. Staining of bound antibodies was performed with a fluorescein isothiocyanate (FITC)-conjugated anti-porcine IgA antibody (1:100 dilution) (A100-102F, Bethyl) (30 minutes on ice). The antibody-linked FedF fragment was detected using 50 µg/ml FITC-conjugated porcine anti-FedF polyclonal antibody (in-house) (30 minutes on ice). Dead cells were excluded using Sytox blue staining (5 nM; Molecular probes). A total of 10,000 viable, single cells were measured for each condition (Cytoflex, Beckman Coulter).

Immunohistochemistry

Cryosections of porcine ileum (3 week-old piglet) were made and fixed for 10 minutes in acetone at -20°C. Next, the cryosections (12 µm) were washed with PBS and blocked with 10% goat serum (diluted in PBS), followed by incubation with 10 µg/ml APN-binding recombinant antibody or PBS containing 1% pig reference serum for 1 hour at 37°C in a humidified chamber. After washing with PBS, FITC-conjugated anti-porcine IgA (1:100) was added for IgA detection. A nuclear counterstain with Hoechst (10 µg/ml) was performed for 5 minutes. After additional washing, the slides were mounted with DABCO. Images were acquired with a fluorescent microscope (Leica).

Statistical analysis

The correlation between the MFI of each colony and the measured optical density (OD) values in the pig IgA ELISA was determined by Pearson correlation using SPSS Statistics 25. P-values were calculated by two-tailed student's t-test. $P < 0.05$ was considered significant.

Abbreviations

APN	Aminopeptidase N
BSA	Bovine serum albumin
CHO	Chinese hamster ovary
CMV	Cytomegalovirus
E2A	2A peptide derived from equine rhinitis virus
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced GFP
ELISA	Enzyme-linked immunosorbent assay
ETEC	Enterotoxigenic <i>Escherichia coli</i>
F2A	2A peptide derived from foot-and-mouth disease virus
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FedF	Adhesin of F18+ ETEC
G418	Geneticin [®]
GFP	Green fluorescent protein
GSG	Glycine-Serine-Glycine amino acid sequence
GT2A	T2A with a GSG linker
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IPEC-J2	Intestinal porcine enterocyte cells isolated from the jejunum
IPEC-J2-APN	APN-expressing IPEC-J2 cell line
IRES	Internal ribosome entry site
MFI	Mean fluorescence intensity
P2A	2A peptide derived from porcine teschovirus-1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIgA	Secretory IgA
T2A	2A peptide derived from <i>Thosea asigna</i> virus

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No potential conflict of interest was reported by the authors.

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ORCID

Hans Van der Weken  <http://orcid.org/0000-0002-3104-9355>

Eric Cox  <http://orcid.org/0000-0003-4281-2990>

Bert Devriendt  <http://orcid.org/0000-0002-3222-8769>

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