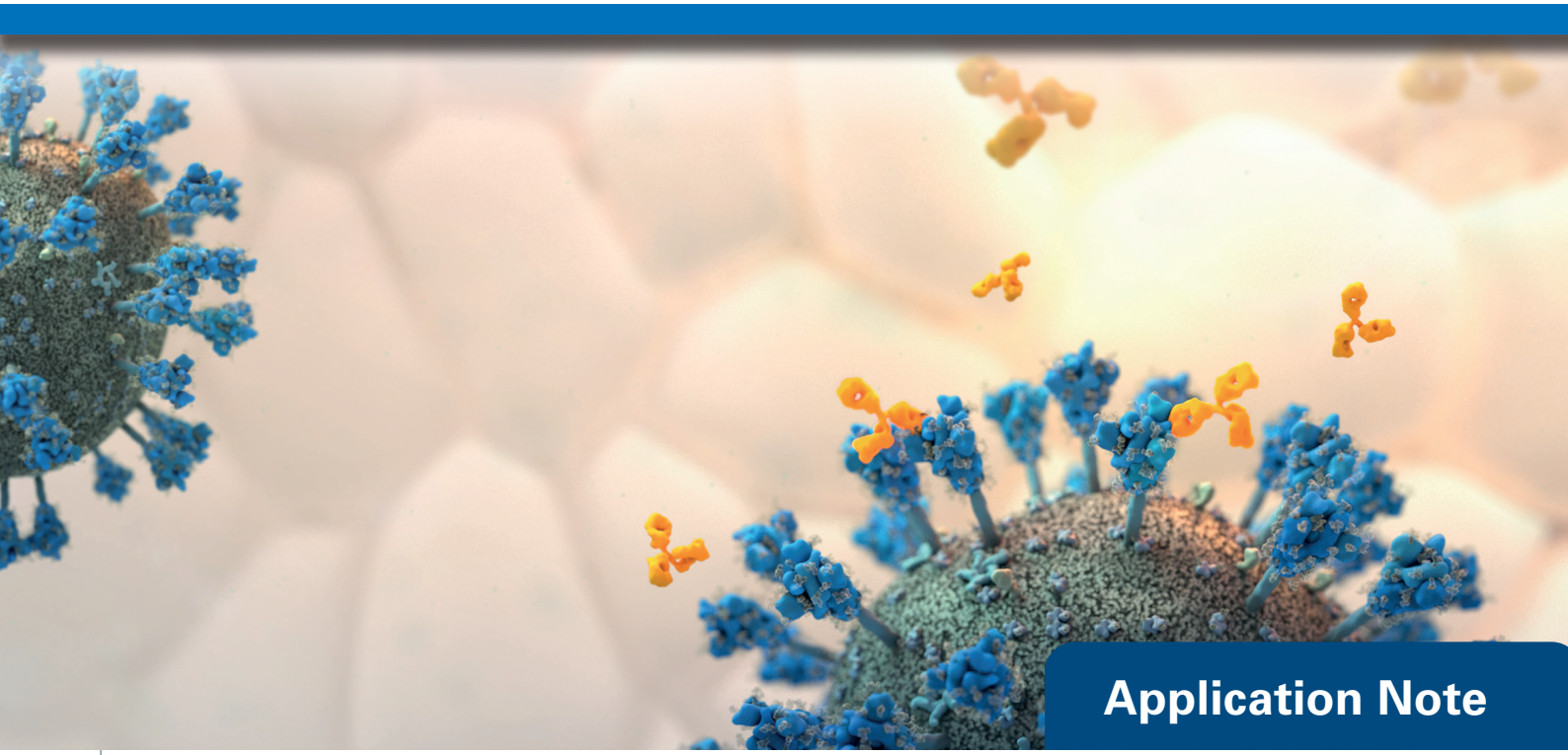

Application Note

SARS-CoV-2 antigens successfully produced with comparable quality from transient transfection and stable cell pool expression systems

Introduction

Since the global outbreak of the novel coronavirus (COVID-19) pandemic at the beginning of 2020, biomedical researchers, healthcare organizations, and pharmaceutical/biotech companies have collaborated and worked tirelessly to develop treatments and vaccines for SARS-CoV-2. With the first vaccines now gaining regulatory approval and becoming available, there remains a demand for accurate and effective rapid antigen tests to complement vaccines and standard diagnostic assays (e.g. polymerase chain reaction – PCR) as part of a robust exit strategy from this pandemic. Rapid antigen tests offer several advantages, including very short turnaround times (approx. 15-30 min) and reduced costs, which add capacity to stretched laboratories at their limit of PCR testing.

The SARS-CoV-2 virus is surrounded by an envelope of proteins and other components. Of these, the spike (S) protein has been extensively studied as central to the virus' cell entry mechanism – and has therefore been targeted for immunoassay development. The S protein is a glycoprotein of 180-220 kDa that exists as a trimer, containing the functional S1 subunit with a receptor binding domain (RBD) that interacts with the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell membrane (Figure 1). As such, RBD is a protein of interest for developing rapid antigen tests, as it is directly involved in the infection process. In addition, the low molecular weight (~26 kDa) and simplicity of RBD, as well as higher expected yield and lower cost for antigen production compared with larger proteins such as S1 or full-length spike glycoprotein (SGP) make it an attractive antigen for development.

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Methods:
Recombinant protein expression
Affinitiy purification
Transient gene expression in HEK
Stable cell pool generation in CHO
SARS-CoV-2 IgG ELISA
ACE-2 binding assay

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Large quantities of SARS-CoV-2 proteins and peptides, such as SGP, S1 and RBD, are needed to fulfill the global requirement for suitable antigens needed for developing sensitive in-vitro diagnostic (IVD) kits and for basic research.

Antigen production systems

Methods for producing viral diagnostic antigens by recombinant systems are varied, with their respective advantages and disadvantages. Proteins expressed in *E. coli*, Yeast, and insect expression systems often suffer from missing glycosylation, incorrect folding and post-translational modifications (PTMs), and incompatibility. In contrast, mammalian expression systems benefit from correct glycosylation and folding, but often involve complicated and expensive processes that can limit their use in large-scale industrial production. There are two key mammalian expression methods used for recombinant protein and antigen production:

Transient transfection

Transiently transfected cells express the foreign gene but do not integrate it into their genome, so the new gene will not be replicated. Human embryonic kidney (HEK) cells are commonly used for transient expression, and the short timelines of this technique have made it a popular system for SARS-CoV-2 antigen expression. HEK expression systems are also ideal for comparing different variants of the same proteins, such as novel variants of SARS-CoV-2 emerging globally. However, capacity and batch size are often limited with transient expression, making it more suitable for basic research.

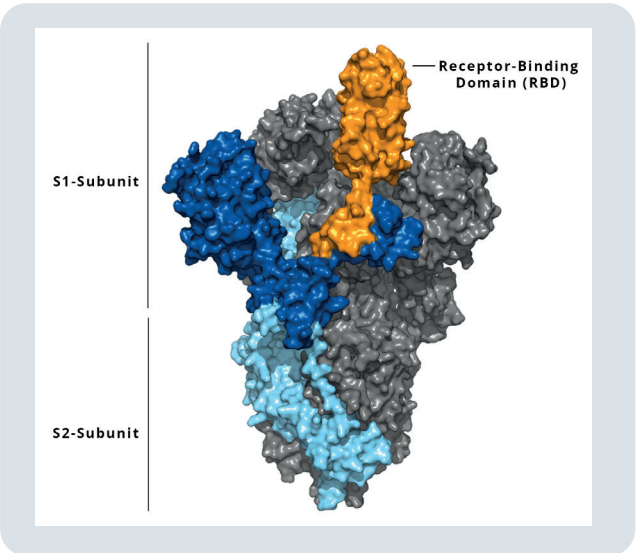

Figure 1: Structure of the trimeric SARS-CoV-2 Spike (S) glycoprotein, with one of the three protomers shown in color. The functional S1-subunit is depicted in dark blue, with the receptor-binding-domain highlighted in orange. The S2-subunit is depicted in light blue.

The rapid turnaround provides pharma research labs with fast answers to preliminary questions, enabling them to progress towards development goals sooner.

The InVivo Expression System for Transient Transfection: InVEST is a highly optimized system for transient gene expression, providing up to 80-fold increase in production compared with standard systems. With yields of up to 2000 mg per batch, InVEST meets the needs of organizations that require high quality recombinant proteins at high yields, in short timeframes. With a workflow of 4-6 weeks from DNA preparation, InVEST is a flexible HEK transient expression system that can produce commercial antigens at scale for time-sensitive applications such as SARS-CoV-2.

Stable cell pools

In stably transfected cells, the foreign gene is integrated into the genome and is replicated. Descendants of these cells will therefore also express the new gene, resulting in a stably transfected cell line. InVivo predominantly uses Chinese hamster ovary (CHO) cell lines to generate stable cell pools, and the key benefit of this approach is the ease of upscaling and larger batch sizes, which is particularly advantageous for pharma companies manufacturing IVD kits. However, the timeline for stable cell line development is longer than transient transfection, so is primarily used for scaling up established systems.

A unique stable CHO cell pool platform has been developed that combines the benefits of large batch sizes and easy upscaling with a shortened timeline, while maintaining good antigen or antibody titer. This large-scale expression system can produce gram amounts of material in a reduced time frame for industrial applications and applied research.

This study used two different strategies – transient HEK and stable pool CHO – to successfully produce SARS-CoV-2 antigens. Binding activity of antigens from each method against ACE2 and antibodies from COVID-19 positive patient samples were compared. The comparable results of antigens produced from each cell line demonstrates how high-quality proteins can be developed regardless of expression strategy.

Materials and methods

Transient gene expression of recombinant proteins

Transient protein production in HEK cells was performed using InVivo's InVEST. This includes the proprietary suspension-adapted human cell line HEK-INV, the novel expression vector pINV, and unique customized media, all synergistically optimized for highly efficient production of recombinant proteins. The cells were transiently transfected with the gene harboring expression vector using a polycationic polymer. 1 L fed-batch productions in shake flasks were performed using established InVivo standard protocols.
For more detailed information about InVEST, please visit www.transient-transfection.com

Stable gene expression of recombinant proteins

Using the piggyBac transposon-mediated active gene transfer, suspension adapted CHO cells were co-transfected with the recombinant protein-encoding expression vector (pPB-mono; piggyBac donor plasmid) and the piggyBac helper plasmid (pINV-PBase) coding the transposase. Through a two-step selection procedure, stable cell pools were generated and titers were measured from the cell culture supernatant by bio-layer interferometry (BLI). 1 L fed-batch productions in shake flasks and large-scale productions in a bioreactor system (2–10 L) were performed using established InVivo standard protocols.

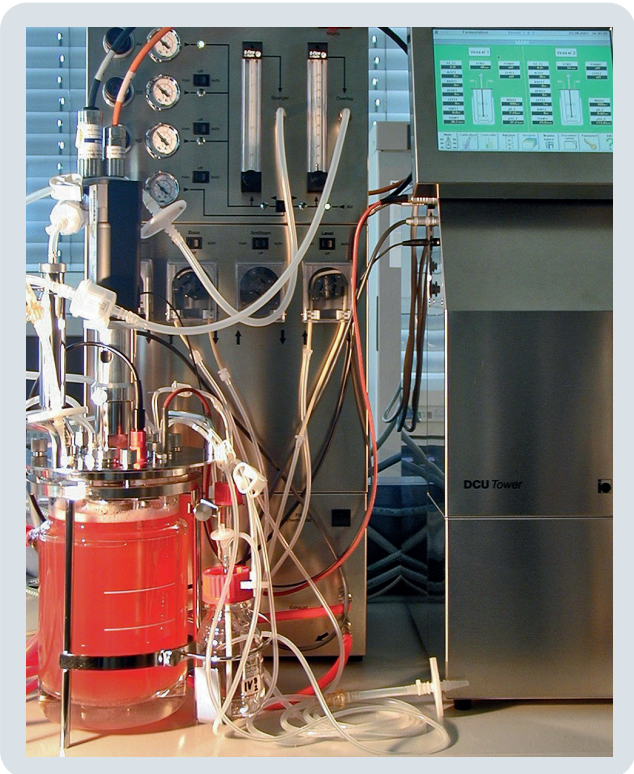

Figure 2: S1_RBD bioreactor production from stable CHO pool (InVivo BioTech Services GmbH)

Recombinant protein purification

Recombinant proteins expressed in this study contain a C-terminal hexa-histidine-tag. Proteins were purified using standard procedures established at InVivo. Cell culture supernatant was cleared by centrifugation and media was exchanged with purification buffer using tangential flow filtration (TFF). Immobilized metal ion chromatography (IMAC) followed by size exclusion chromatography was carried out using Äkta chromatography systems. Proteins were dialyzed in 20 mM NaPP/ 300 mM NaCl, pH 7.2 and protein concentration adjusted to ≥ 1 mg/ mL.

All cell culture media and reagents are serum- and animal component-free and were handled in a regulated ISO 9001:2015 environment.

Determination of binding activities of SGP, S1 and RBD to SARS-CoV-2 antibodies in patient sera

The antigens (SGP, S1 and RBD) were used at concentrations of 2 µg/ mL for coating of immunoassay plates overnight. After blocking with 1% BSA, serum of 15 SARS-CoV-2 PCR positive tested donors was applied to the plates at a dilution of 1:100. As a negative control, a pool of 10 donor sera, taken before 2018 was used. After 30 min incubation at room temperature (RT), the wells were washed three times with 250 µL washing buffer. Subsequently, 25 ng/ mL anti-human-IgG conjugated to horseradish peroxidase (HRP) was applied to the wells and incubated for 15 min. After another washing step (described above), 100 µL tetramethylbenzidine (TMB, ELISA substrate) was applied to each well and incubated for 15 min at RT. The reaction was stopped with 0.4 M sulfuric acid. The absorbance was measured at 450 nm within the next 15 min using a microplate reader. The measured absorbances of the positive samples using the different coating antigens was correlated using the GraphPad Prism software.

ACE2 binding assay with HEK and CHO RBD

The RBD proteins were biotinylated using NHS-LC-Biotin reagent. Assay plates were coated with recombinant ACE2 protein overnight using a concentration of 2.5 µg/ mL. After blocking with 1% BSA, a serial, 2-fold dilution of the biotinylated antigens (1 µg/ mL - 0.001 µg/ mL) was generated and applied to the assay plates. Subsequently, the plates were incubated for 2 hours at 25°C and 650 rpm. The wells were washed three times with 250 µL washing buffer. Next, 10 ng/ mL streptavidin peroxidase conjugate was applied to the wells and incubated for 30 min at 25°C and 650 rpm. After another washing

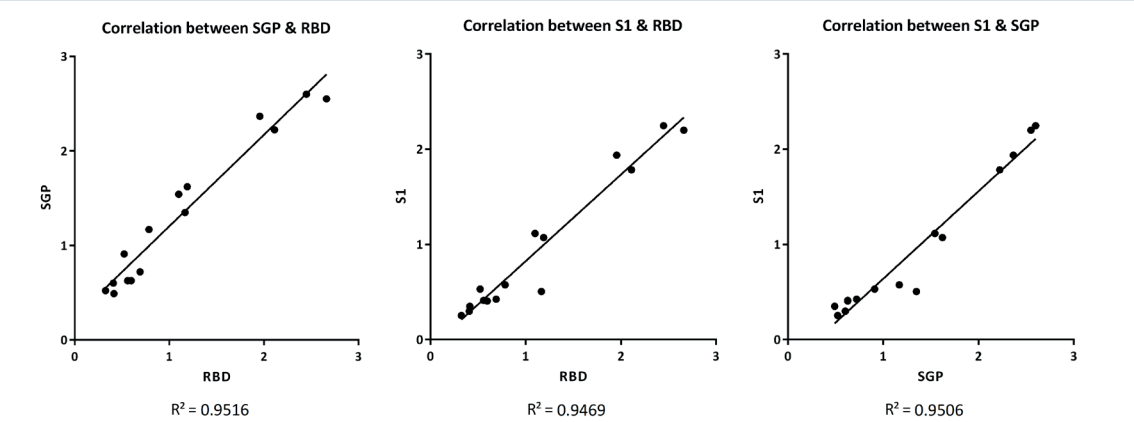

Figure 3: Comparison of the results from 15 SARS-CoV-2 IgG-positive serum samples on assay plates coated with SGP (Spike Glycoprotein), S1-Domain and RBD (Receptor Binding Domain) analyzed using the InVivo SARS-CoV-2 IgG ELISA setup.

step (described above), 100 µL TMB was added to the wells. The reaction was stopped with 0.4 M sulfuric acid. The absorbance was measured at 450 nm within the next 15 min using a microplate reader. Dose-response curves and half maximal effective concentration (EC50) calculations were made using the GraphPad Prism Software.

Results and discussion

Determining SGP, S1 and RBD binding activities

A SARS-CoV-2 IgG ELISA results showed high antibody binding activities for full-length SGP, S1 subunit and RBD, and correlation between each of the antigens was high (SGP vs RBD: R2 = 0.9516; S1 vs RBD: R2 = 0.9469; S1 vs SGP: R2 = 0.9506 [Figure 3]). The SGP antigen showed slightly higher sensitivity due to its length and greater number of epitopes for antibody binding. However, given the good correlation between RBD and SGP, using RBD alone is deemed suitable for the same applications, and is in some cases preferable due to its lower cost and more straightforward expression.

The high correlation between the three antigens is beneficial to pharma companies and researchers, as it provides a broader choice of antigens with confidence that results will be comparable to other materials. Due to its direct involvement in infection process, the second part of this study focuses on the RBD antigen.

Comparing the quality of HEK and CHO RBD antigen production

Transient transfection (HEK) and stable cell pool (CHO) expression systems have not traditionally intersected, with researchers favoring HEK transient expression for its short turnaround times and industrial labs favoring stable CHO pools for easy scale-up and large batch sizes. However, the results of the binding activity comparison between RBD antigens produced from the proprietary InVEST HEK transient expression system (RBD-HEK) and InVivo's stable CHO pool platform (RBD-CHO) showed a nearly perfect correlation (Figure 4). The results indicate that putative differences in the glycosylation pattern of the antigens will not affect the SARS-CoV-2 IgG ELISA analysis results. Figure 5 shows dose response curves for RBD-HEK and RBD-CHO binding to ACE2, indicating similar binding activities of antigens produced from the two different expression systems.

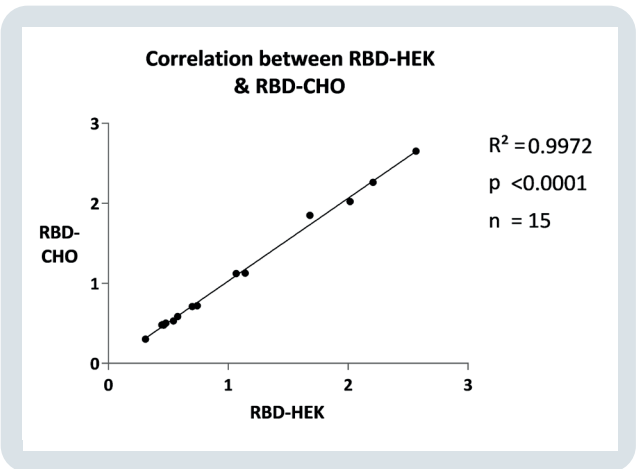

Figure 4: Comparison of the binding activity of RBD-HEK and RBD-CHO coated onto immunoassay plates in the InVivo SARS-CoV-2 IgG ELISA setup.

The comparable results of transient HEK and stable CHO pool expression systems in this study are important for several reasons. In basic research, some variability in the transient expression approach is beneficial, as researchers require a degree of flexibility to change the length of the SARS-CoV-2 antigen amino acid sequence or to introduce mutations, for example. Additionally, the results from

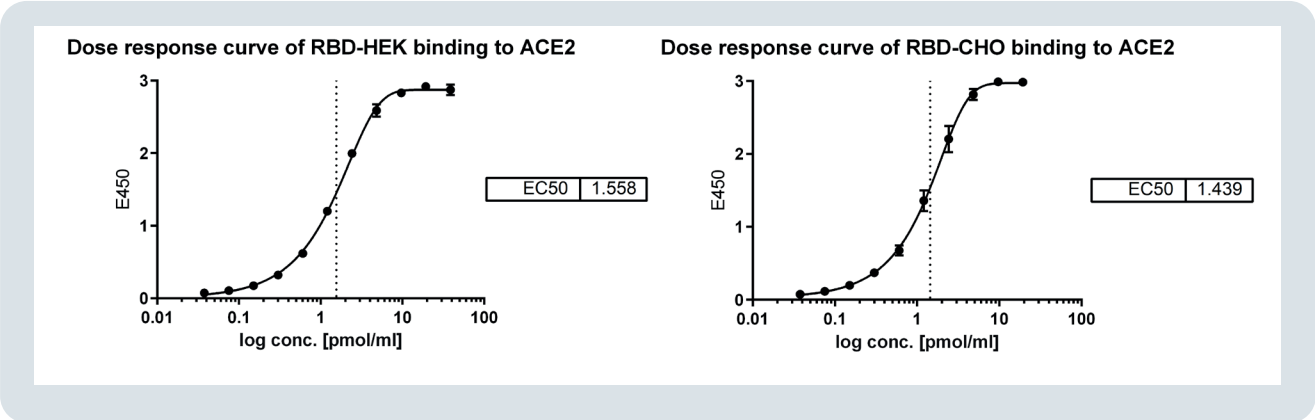

Figure 5: The binding of the SARS-CoV-2 receptor binding domains RBD-HEK and RBD-CHO to the ACE2 was analysed via ELISA. Dose response curves showing RBD-HEK (left) and RBD-CHO (right) binding to ACE2. The calculated EC50 (half maximal effective concentration) values are indicated by a dashed line.

the comparison show how the expression system can be easily chosen according to user needs, such as batch size and speed, without concern over which will provide better antigen quality.

Traditionally, running two strategies in parallel has not been a common approach for recombinant protein or antigen production. Nevertheless, the results of this study show that it can be very advantageous to run both transient transfection (HEK) and stable pool lines (CHO) at the same time. Materials are of comparable quality and there is no loss of sensitivity or reactivity.

Conclusion

- To mitigate the rising need for IVDs as a result of the COVID-19 pandemic, diverse SARS-CoV-2 proteins including SGP, S1 and RBD, are being produced in large-scale quantities for the development of highly sensitive IVD kits, as well as for basic research.
- Researchers and manufacturers can expect the same results from binding assays from full-length SGP antigens and recombinant S1 and RBD antigens.
- The comparable material quality from transient HEK and stable pool CHO methods provides freedom to choose an expression system depending on specific needs: HEK is suitable for faster production at a smaller scale, and CHO for larger batch sizes.
- In situations such as the COVID-19 pandemic, the flexibility this offers is extremely advantageous. Biopharma companies can optimize pipelines for their candidate/biomarker rapidly, with the option to change expression systems to accommodate changing needs of the company, without compromising quality of materials.
- InVivo's stable CHO cell lines complement the proprietary InVEST transient HEK manufacturing platform, to meet the growing demand for long-term bulk quantities in the gram scale, with short timelines and no delays.

About InVivo BioTech Services

InVivo BioTech Services GmbH is the leading European contract manufacturing organization in mammalian cell culture for production of monoclonal antibodies and recombinant proteins for research & development, in vitro diagnostics and pre-clinical studies. With more than 20 years of experience, the company has consistently grown over time and exhibits today approximately 2500 square meters (~ 26,900 square feet) of laboratory and operational space in the Berlin greater area. In 2017, Bruker Daltonics integrated InVivo to gain a reliable partner for the production of in-vitro diagnostic kits, biological standards and chemical matrices. For more information, please visit www.invivo.de

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For more information about the SARS-CoV-2 antigens available from InVivo BioTech Services, please visit
https://www.invivo.de/cell-line-development/sars-cov-2-antigens/

For more information about InVivo's optimized transient HEK cell based expression system InVEST, please visit
www.transient-transfection.com

For more information about InVivo's optimized stable pool CHO concept, please visit
https://www.invivo.de/cell-line-development/stable-cell-line-development/

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