# 16th PEACe

## Protein Expression in Animal Cells Conference

**September 24 to 28, 2023 • Sitges, Spain**

**www.peace-conference.org**

# CONFERENCE PROGRAM

# PROGRAM OVERVIEW

| September 25 | September 26 | September 27 | September 28 |
|---|---|---|---|
| 8:30-09:50<br>Expression systems 1 | 08:30-09:15<br>Invited Talk<br>Núria Montserrat<br>*Institute for Bioengineering of Catalonia, Spain* | 08:30-09:30<br>Bioprocess intensification 1 | 8:30-09:30<br>Molecular control and design for product quality 1 |
| 10:30-11:30<br>Expression systems 2 | 09:15-10:15<br>Protein engineering 1 | 10:00-11:25<br>Bioprocess intensification 2 | 10:00-11:15<br>Molecular control and design for product quality 2 |
| 11:30-12:30<br>PEACe keynote lecture<br>Sarah Dunn<br>*AstraZeneca, UK* | 10:45-11:40<br>Protein engineering 2 | 11:30-12:30<br>PEACe keynote lecture<br>Gavin Wright<br>*University of York, UK* | 11:15-11:45<br>Awards & Close |
| 13:30-14:30<br>General assembly | 12:05-13:00<br>Cell-based vaccines and viral particles production 1 | 14:00-15:45<br>Industrial workshops and panel<br>Mark Stockdale<br>*Asimov, USA*<br>Claes Gustaffson<br>*ATUM, USA*<br>Richard Altman<br>*Thermo Fisher Scientific, USA* | |
| 14:30-15:45<br>Cell engineering and genome editing 1 | 14:00-15:25<br>Cell-based vaccines and viral particles production 2 | | |
| 16:15-17:20<br>Cell engineering and genome editing 2<br>Sebastian Püngel<br>*Böhringer Ingelheim, Germany* | 18:00-22:00<br>Tour of Sitges<br>Guided walking tour of Sitges' highlights and special activity followed by a free night to enjoy the city or the hotel. | 15:45-16:15<br>Student-industry panel and networking | |
| 17:45-19:30<br>Posters session and cocktail<br>*Odd-numbered posters* | | 16:45-18:30<br>Posters session and cocktail<br>*Even-numbered posters* | |
| | | 19:30-00:00<br>Gala dinner | |

**September 24**

18:00-20:00
Welcome reception

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# PEACe 2023 Partners

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Hochschule Biberach
Biberach, Germany

# General Information

## Venue

**Meliá Sitges**
Carrer de Joan Salvat Papasseit, 38,
08870 Sitges, Barcelona, Spain
Tel.: +34 938 11 08 11

## Registration

All participants should register at the registration desk. The registration desk will be open at the Conference venue during the following hours:

| | |
|---|---|
| Sunday, September 24 | 16:00 - 19:00 |
| Monday, September 25 | 07:00 - 19:00 |
| Tuesday, September 26 | 08:00 - 15:00 |
| Wednesday, September 27 | 08:00 - 18:00 |
| Thursday, September 28 | 08:00 - 11:00 |

## Name Badge

Please wear your name badge at all times. This will ensure your access to the conference room and activities.

## Certifcate of Attendance

An official Certificate of Attendance will be available on demand (PDF).

## Disclaimer

The PEACe 2023 secretariat and organizers cannot assume liability for personal accidents, loss of or damage to private property of participants, and accompagnying persons, either during or directly arising from the PEACe 2023 Conference. Participants should make their own arrangement with respect to health and travel insurance.

## Security & Safety

Please do not leave bags and luggage unattended at any time, whether inside or outside session rooms.

# Monday, September 25, 2023

*Topic 1 : Expression systems*

Chairs :

Yves Durocher
Section Head of Mammalian Cell Expression
National Research Council, Canada

René Hubert
Director of Expression and Cell Engineering
Amgen, USA

08:30-09:50

Invited speaker

**Haruki Hasegawa** *(Amgen, USA)* 08:40-09:10
Spontaneous crystallization of multiple proteins in a cell: How many different protein crystals can we produce simultaneously?

Contributed oral presentations

**Robyn Emmins** *(GSK, UK)* 09:10-09:30
«Leaping-In» to a new CHO expression system

**Antonio Roldao** *(UBET, Portugal)* 09:30-09:50
Leveraging single-cell transcriptomics to tailor insect cells for production of AAV and influenza VLPs

BREAK

10:30-11:30

Invited speaker

**Simon Joubert** *(National Research Council, Canada)* 10:30-11:00
Process for generation of high-producing CHO cell lines for manufacturing of biologics using the CHO2353 tm cell line

Contributed oral presentation

**Pol Pérez Rubio** *(Universitat Autonoma de Barcelona, Spain)* 11:00-11:20
Cellular membrane-associated negative charges modulate polyplex internalization in HEK293 cell cultures
Jesús Lavado García, Laura Cervera Gracia, Francesc Gòdia

Flash presentation

**Maria Toth** *(University for Natural Resources and Life Sciences, Austria)* 11:20-11:25
The T7 inducible system for recombinant protein production in mammalian cells
Manuel Reithofer, Reingard Grabherr, Astrid Dürauer

PEACe keynote lecture 1

Keynote speaker:

Sarah Dunn
Director of Cell Line Development
AstraZeneca, United Kingdom

11:30-12:30

Use of CHO pools for rapid production of antibodies for clinical trials

LUNCH

13:30-14:30

PEIS General Assembly

*Topic 2: Cell engineering and genome editing*

Kerstin Otte
Full Professor of Pharmaceutical Biotechnology
Hochschule Biberach, Germany

Lise Marie Grav
Assistant Professor of Bioengineering
Danmarks Tekniske Universitet, Denmark

14:30-15:45

Invited speaker

**Sebastian Püngel** *(Boehringer Ingelheim, Germany)* 14:35-15:05
The power of transposases: How an ancient cellular mechanism revolutionized modern biopharmaceutical development

Contributed oral presentation

**Bjørn Voldborg** *(Danmarks Tekniske Universitet, Denmark)* 15:05-15:25
CHO Cell Lines for screening and production of recombinant proteins with optimal N-glycosylation

Flash presentations

**Mina Ghahremanzamaneh** *(University College Dublin, Ireland)* 15:25-15:35
GalMAX: Model-inspired glycoengineering for biopharmaceutical quality assurance
Aoife Harbison, Itzcoatl Gómez-Aquino, Alfonso Blanco, Sara Carillo, Jonathan Bones, Ioscani Jimenez del Val

**Jessica Willmott** *(GSK, United Kingdom)* 15:35-15:45
Utilising digital droplet PCR to aid Cell Line Selection of Chinese Hamster Ovary cell lines expressing complex modality antibodies

BREAK

16:15-17:20

Contributed oral presentations

**Nicole Borth** *(University of Natural Resources and Life Sciences, Austria)* 16:15-16:35

New approaches to understand population homo/heterogeneity and phenotypic behavior of suspension grown CHO host and producer cells

**Michael Betenbaugh** *(John Hopkins University, USA)* 16:35-16:55

Reconstruction of cysteine biosynthetic pathway in Chinese Hamster Ovary cells provides substrate flexibility and robust oxidative stress response

Yiqun Chen

**Pablo Di Gusto** *(UC San Diego, USA)* 16:55-17:15

Towards a whole cell network reconstruction for CHO cells

Athanasios Antonakoudis, Santiago Benavides Lopez, Pierrick Craveur, Alexander Sutherland, Norma A. Valdez-Cruz, Anne Richelle, Nathan Lewis

BREAK

17:45-19:30

Poster session (odd numbers)

# Tuesday, September 26, 2023

*Invited Talk*

Speaker:

Núria Montserrat
Group Leader in Pluripotency for Organ Regeneration
Institute for Bioengineering of Catalonia, Spain

08:30-09:15

How to engineer human pluripotent stem cells: generating human organoids to understand human development and disease

*Topic 3: Protein engineering*

Chairs:

Carlos Bosques
Senior Vice-President
Food Allergy Science Initiative, USA

René Hubert
Director of Expression and Cell Engineering
Amgen, USA

09:15-10:15

Invited speaker

**Umesh Muchhal** *(IGM Biosciences, USA)* 09:20-09:50
Multivalent IgM antibodies – New class of therapeutics with differentiated potency and safety profile

Contributed oral presentation

**Peter Tessier** *(University of Michigan, USA)* 09:50-10:10
Bispecific antibody shuttles targeting CD98hc mediate efficient and long-lived brain delivery of IgGs

Flash presentation

**Elisabeth Gludovacz** *(University of Natural Resources and Life Sciences, Austria)* 10:10-10:15
Heparin-binding motif mutations of human diamine oxidase allow the development of a first-in-class histamine-degrading biopharmaceutical
Kornelia Schuetzenberger, Marlene Resch, Katharina Tillmann, Karin Petroczi, Markus Schosserer, Sigrid Vondra, Serhii Vakal, Gerald Klanert, Jürgen Pollheimer, Tiina A. Salminen, Bernd Jilma, Nicole Borth, Thomas Boehm

BREAK

10:45-11:40

Invited speaker

**Rumana Rashid** *(Xencor, USA)* 10:45-11:15

Design and application of XmAb®: A heterodimeric Fc-containing bispecific antibody platform for immunotherapy

Contributed oral presentation

**David James** *(University of Sheffield, United Kingdom)* 11:15-11:35

Protein-specific signal peptides for mammalian vector engineering

BREAK

*Topic 4: Cell-based vaccines and viral particles production*

Chair:

Laura Cervera Gracia
Postdoctoral researcher
Universitat Autònoma de Barcelona, Spain

12:05-13:00

Invited speaker

**Yvonne Genzel** *(Max Planck Institute for Dynamics of Complex Technical Systems, Germany)* 12:10-12:40

Intensified cell-based virus production: A process development challenge for multiple cell-virus combinations!

Tilia Zinnecker, Sven Göbel, Udo Reichl

Contributed oral presentations

**Zalma Sanchez** *(Université de Montréal and National Research Council, Canada)* 12:40-13:00

Generation of multivalent enveloped VLPs based on the SARS-CoV-2 spike protein in engineered CHO cells

Sergio P. Alpuche-Lazcano, Matthew Stuible, Yves Durocher

LUNCH

14:00-15:25

Invited speaker

**Eduard Ayuso** *(DINAMIQS, Switzerland)* 14:00-14:30

Optimization and scaling up AAV manufacturing

Contributed oral presentation

**Donald Jarvis** *(University of Wyoming, USA)* 14:30-14:50
An Sf-rhabdovirus Risk Assessment
Ajay Maghodia, Mark Menghini, Donald Jarvis

**Marc Garcia Trujillo** *(Universitat Autònoma de Barcelona, Spain)* 14:50-15:10
Functionalizing enveloped nanoparticles via click chemistry: Optimization and comparison between HIV-1 gag-based virus-like particles and extracellular vesicles
Jesús Lavado García, Arnau Boix i Besora, Laura Cervera Gracia, Francesc Gòdia

Flash presentation

**Konstantina Tzimou** *(DTU, Denmark)* 15:10-15:15
Evaluation of AAV gene toxicity in mammalian cell culture for more efficient rAAV production
Jesús Lavado García, Lise Marie Grav, Lars Keld Nielsen

**Maren Schubert** *(TU Braunschweig, Germany)* 15:15-15:20
Production of virus-like-particles in a baculovirus-free insect cell expression system for antibody development

BREAK

18:00-22:00
WALKING TOUR AND SPECIAL ACTIVITY

# Wednesday, September 27, 2023

*Topic 5: Bioprocess intensification*

Chair:

Michael Butler
Principal Investigator
NIBRT, Ireland

08:30-09:30

Invited speaker

**Olivier Henry** *(Polytechnique Montréal, Canada)* 08:35-09:05
Process development and intensification for the production of SARS-COV-2 spike protein in CHO fed-batch cultures
Sebastian Juan Reyes, Phuong Lan Pham, Yves Durocher

Contributed oral presentation

**John Raven** *(Fujifilm, United Kingdom)* 09:05-09:25
Cell line and upstream process development for high density perfusion processes: Strategies for large scale high quality continuous upstream processes

Flash presentation

**Hoon Min Lee** *(University of Science and Technology, South Korea)* 09:25-09:30
Effects of autophagy-inhibiting chemicals on sialylation of Fc-fusion glycoprotein in recombinant CHO cell culture
Jong-Ho Park, Gyun Min Lee, Yeon-Gu Kim

BREAK

10:00-11:25

Invited speaker

**Venkata Tayi** *(Merck & Co., Inc., USA)* 10:00-10:30
Benefits and considerations for intensification of biologics manufacturing process to meet product demand

Contributed oral presentations

**Jesús Lavado Garcia** *(DTU, Denmark)* 10:30-10:50
The role of extracellular vesicles in the modulation of the cell density effect

**Cyril Boucher** *(Merck, Switzerland)* 10:50-11:10
Toward the implementation of integrated continuous bioprocessing for Mab manufacturing. From bench scale to 2kL-clinical manufacturing production-scale
Jean-Marc Bielser, Xavier Le Saout, Pierre Moretti, Jonathan Souquet

Flash presentations

**Dennis Karthaus** *(Sartorius, Germany)* 11:10-11:15

See the wood for the trees: A holistic route to an optimized AAV production process

Kathrin Teschner, Vera Ortseifen, Niklas Krämer, Pia Brinkert, Franziska Sundermann, Alyssa Vetter, Mareike Schulz, Tim Steffens, Sandra Klausing

**Christiana-Kondylo Sideri** *(Dublin City University, Ireland)* 11:15-11:20

Label-free quantitative proteomics analysis of CHO-DP12 and CHO-K1 under ER stress conditions

Michael Henry, Esen Efeoglu, Paula Meleady

PEACe keynote lecture 2

Keynote speaker:

Gavin Wright
Full Professor of Microbial Biochemistry
University of York, United Kingdom

11:30-12:30

Investigating the molecular basis of cellular recognition using large panels of recombinant proteins expressed in mammalian cells: A case study using the human immune system

Jarrod Shilts

LUNCH

14:00-15:45

Industrial workshops

**Mark Stockdale** *(Asimov, USA)* 14:00-14:20

**Claes Gustafsson** *(ATUM, USA)* 14:20-14:40

**Richard Altman** *(Thermo Fisher Scientific, USA)* 14:40-15:00

**Panel on new technologies** 15:00-15:45

15:45-16:15

Student-industry networking panel

BREAK

16:45-18:30

Poster session (even numbers)

19:30-00:00

GALA DINNER

# Thursday, September 28, 2023

*Topic 6: Molecular control and design for product quality*

Chairs:

Nathan Lewis
Professor, Departments of Pediatrics and Bioengineering
University of California - San Diego, USA

Carlos Bosques
Senior Vice-President
Food Allergy Science Initiative, USA

08:30-09:30

Invited speaker

**Gestur Vidarsson** *(Sanquin, The Netherlands)* 08:35-09:05
IgG glycoengineering in cells: Natural switches of Fc-receptor and complement-mediated activities

Contributed oral presentation

**Alina Ferdman** *(Asimov, USA)* 09:05-09:25
Predicting antibody production with a dynamic genetic construct simulator
Georgian Tutuianu, Alec A.K. Nielsen

Flash presentation

**Catherine Forest-Nault** *(Polytechnique Montréal, Canada)* 09:25-09:30
A low-temperature SPR-based assay for monoclonal antibody galactosylation and fucosylation assessment using FcγRII A/B
Jimmy Gaudreault, Yves Durocher, Gregory De Crescenzo

BREAK

10:00-11:15

Invited speaker

**Anne Trappe** *(Health Products Regulatory Authority, Ireland)* 10:00-10:30
A regulatory update on guidance for quality of biopharmaceuticals

Contributed oral presentation

**Nathan Lewis** *(UC San Diego, USA)* 10:30-10:50
Deploying systems and synthetic biology to engineer and manufacture better glycoproteins and subunit vaccines in CHO

**Anne B. Tolstrup** *(AbtBioConsult ApS, Denmark)* 10:50-11:10
Bispecifics - Different formats bring different treatment opportunities but also different CMC challenges

11:15-11:45

**Awards & Close**

# Poster Presentations

## Odd-numbered posters

01 **Andy Díaz Maneh**, (*Cubiq Foods - Universitat Autònoma de Barcelona, Spain*)
Development of a chemically defined culture media through a statistical design of experiments (DoE) methodology for the culture of duck primary cells
Pedro Muñoz, Andreu Camacho, Natalia Garcia-Aranda, Natasa Ilic, Yuliana Enciso, Javier Fuenmayor, Jesus Lavado-García, Francesc Gòdia, Mario Notari, Raquel Revilla

03 **Antonio Roldao**, (*iBET - Instituto de Biologia Experimental e Tecnologica, Portugal*)
Multi-stage bioreactor process for continuous production of influenza VLPs using IC-BEVS

05 **James Flynn**, (*NIBRT, Ireland*)
Utilising Dielectric Capacitance Measurements for Earlier Detection of Cell Health Decline in Bioprocesses
Laura Breen, Nils Bunte, Michael Butler

07 **Mina Ghahremanzamaneh**, (*University College Dublin, Ireland*)
GalMAX: Model-inspired glycoengineering for biopharmaceutical quality assurance
Aoife Harbison, Itzcoatl Gómez-Aquino, Alfonso Blanco, Sara Carillo, Jonathan Bones, Ioscani Jimenez del Val

09 **Nicolas Marx**, (*University of Natural Resources and Life Sciences, Austria*)
Identification of transgene integration sites, their structure and epigenetic status with Cas9-targeted nanopore sequencing in CHO cells
Krishna Motheramgari, Nicole Borth, Nicolas Marx

11 **Federico De Marco**, (*Austrian Centre of Industrial Biotechnology, Austria*)
Investigating the dark matter of the genome: A large-scale deletion screen defines the essentiality of unannotated genomic regions in Chinese Hamster Ovary cells

13 **Dubhe Beatriz Bulté Ocaña**, (*Technical University of Denmark, Denmark*)
Optimizing MAb production through cell lines development based on a CRISPR-Mad7-Targeted Integration approach in CHO cells
Lise Marie Grav, Alexandra Baer, Peter Ravn

15 **Caterina Ruggeri**, (*BOKU, Austria*)
RNA-seq meta-analysis of lncRNAs in CHO cells
Markus Riedl, Nicolas Marx, Krishna Motheramgari, Nicole Borth

17 **Victor Jimenez Lancho**, (*Acib GmbH, Austria*)
Sequential Activation of Multiple Gene Copies Facilitates Adaptation of CHO Cells to Increased Productivity
Peter Eisenhut

19 **Maja Papez**, (*Austrian Centre of Industrial Biotechnology, Austria*)
SLAM-seq reveals early transcriptomic response mechanisms upon glutamine deprivation in Chinese hamster ovary cells

21 **Stephen Bevan**, (*GSK, United Kingdom*)
Streamlining and automating clonal cell line selection
Yuen-Ting Chim, Robyn Emmins

23 **Manuel Reithofer**, (*Institute of Molecular Biotechnology, Department of Biotechnology, BOKU Vienna, Austria*)
The BacMam platform as efficient tool for stable cell line development
Maria Toth, Christopher Tauer, Reingard Grabherr

25 **Giulia Scapin**, (*Technical University of Denmark , Denmark*)
Transcriptional Response To Recombinant Protein Production In Isogenic Multi-Copy CHO Cells

27 **Jessica Willmott**, (*GSK, United Kingdom*)
Utilising digital droplet PCR to aid Cell Line Selection of Chinese Hamster Ovary cell lines expressing complex modality antibodies
Holly Corrigall, Robyn Emmins

35 **Emilia McLaughlin**, (*kyron.bio, France*)
Development of a next-generation bioproduction CHO cell line for optimized glycosylation of therapeutics
Marina Lochhead, Bjørn Voldborg

29 **Jan Küchler**, (*Max Planck Institute for Dynamics of complex technical systems, Germany*)
Absolute quantification of viral proteins during single-round replication in vaccine producer cells
Patricia Opitz, Ingo Jordan, Yvonne Genzel, Dirk Benndorf, Udo Reichl

31 **Elianet Lorenzo**, (*Universitat Autònoma de Barcelona, Spain*)
Development of functionalized HIV-1 Gag GFP Virus Like Particles with T22 peptide to selectively target CXCR 4+ cells
Marc García, Ugutz Unzueta, Francesc Gòdia, Laura Cervera Gracia

33 **Molly Robinson**, (*GSK, United Kingdom*)
Beacon Spotlight Assays - A toolbox of reagents to screen complex modalities during single cell cloning
Holly Corrigall, Robyn Emmins

# Even-numbered posters

02 **Hoon-Min Lee**, (*University of Science and Technology (UST), South Korea*)
Effects of Autophagy-inhibiting Chemicals on Sialylation of Fc-fusion Glycoprotein in Recombinant CHO Cell Culture
Jong-Ho Park, Gyun Min Lee, Yeon-Gu Kim

04 **Christiana-Kondylo Sideri**, (*National Institute for Cellular Biotechnology, Dublin City University, Ireland*)
Label-Free Quantitative Proteomics Analysis of CHO-DP12 and CHO-K1 under ER Stress Conditions
Michael Henry, Esen Efeoglu, Paula Meleady

06 **Dennis Karthaus**, (*Sartorius Xell GmbH, Germany*)
See the wood for the trees: A holistic route to an optimized AAV production process
Kathrin Teschner, Vera Ortseifen, Niklas Krämer, Pia Brinkert, Franziska Sundermann, Alyssa Vetter, Mareike Schulz, Tim Steffens, Sandra Klausing

08 **Chillel Jawara**, (*University of Sheffield, United Kingdom*)
Chemical Filtering: A Post-Transfection Directed Evolution Strategy to Improve Productivity of Difficult-to-Express Antibodies in CHO Cell Lines
Sarah Dunn, David James

10 **Mohamed Hussein**, (*acib / University of Natural Resources and Life Sciences (BoKu), Austria*)
In silico design of CMV promoter binding oligonucleotides and their impact on inhibition of gene expression in Chinese hamster ovary (CHO) cells
Maja Papez, Martina Baumann, Heena Dhiman, Sybille Galosy, Nicole Borth

12 **Ece Cagdas**, (*Technical University of Denmark, Denmark*)
Optimization of cytokine production in mammalian cells

14 **Sung Wook Shin**, (*Department of Molecular Science and Technology, Ajou University, South Korea*)
Quantitative Gene Expression Control Using the ROSE LP Platform: A Reproducible and Sustainable Transcriptional Regulation for CHO Cell Engineering
Honggi Min, Jiwon Kim, Jae Seong Lee

16 **Marzia Rahimi**, (*Technical University of Denmark, Denmark*)
Screening Vector Elements for Improved Antibody Production in CHO Cell Lines
Anna Christina Adams, Lise Marie Grav, Jesús Lavado García

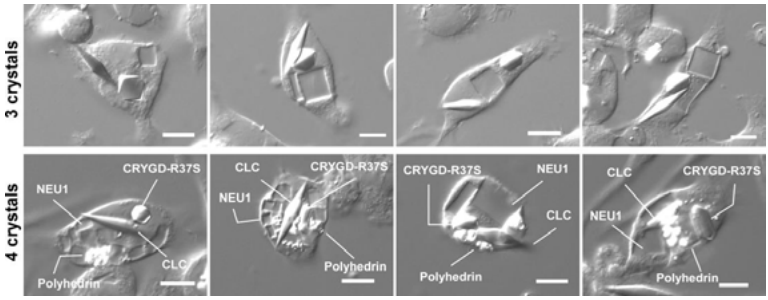
# Abstract Book

**Spontaneous crystallization of multiple proteins in a cell: How many different protein crystals can we produce simultaneously?**

Haruki Hasegawa

Discovery Protein Science, Amgen Inc. South San Francisco, CA, U.S.A

Intracellular protein crystallization occurs in many branches of life, yet the underlying cellular processes remain largely uncharacterized. This is partly caused by the scarcity of easily accessible recombinant model proteins that are necessary for in-depth study of intracellular phase separation events. Such limitation alone warrants a search for diverse classes of model proteins from literature and through the screening a library of proteins. Furthermore, to assess the potential values of "cell-made" protein crystals and cellular platforms that produce them, intracellular crystallization phenomena should also be understood using a broad spectrum of model proteins. After individually validating crystallization and subcellular crystal localization of human immunoglobulins and various cellular, viral, bacterial, or fungal proteins using recombinant mammalian cell expression systems, I demonstrate up to four independent crystallization events can take place simultaneously in a single cell. Notably, these proteins find their ways to crystallize in a highly crowded cellular milieu, while retaining each protein's characteristic crystal morphology even when other protein crystals are produced in close proximity. This study (1) presents an assortment of reproducible model protein tools for studying intracellular protein crystallization events, (2) demonstrates surprising phenotypic plasticity and biosynthetic capacity of mammalian cells that can be harnessed to achieve higher recombinant protein expression, and (3) paves a way toward establishing methods that can control the induction, quality, size, and yield of intracellular recombinant protein crystals.



**"Leaping-In" to a new CHO expression system**.

Robyn Emmins[1]

[1]Cell Line Development, GSK, UK

Since the Covid-19 Pandemic, there has been a marked shift in the field of Biologics Cell Line Development (CLD) with significant alignment in working practices and technologies employed; made possible by increased transparency resulting from a large number of publications detailing mechanisms to reduce the timelines required to progress therapeutics to patients.

In this presentation, we hope to contribute to this pre-competitive knowledge base by detailing how GSK has combined advances in laboratory automation and data analytics along with adoption of the “Leap-In” Transposase system, to enable timeline reduction to an Investigational New Drug (IND) application. These approaches have had immediate short-term timeline benefits in CLD but have also driven a strategic shift in the late-stage development and commercialisation plans for cell lines being developed in our laboratories today.

Data from proof of value studies related to the “Leap-In” Transposase system will be shared. Here we have been able to gain insights into the impact that vector design, codon optimisation and integration frequency can have within our proprietary cell line and media platform. In addition, we will share observations and optimisations underway to further augment our use of this platform, as we manage expectations around the development of cell lines for complex molecular modalities.

**Leveraging single-cell transcriptomics to tailor insect cells for production of AAV and influenza VLPs**

Nikolaus Virgolini[1], Marco Silvano[1], Ricardo Correia[1], Ryan Hagan[2], Paula M. Alves[1], Colin Clarke[2], Inês A. Isidro[1] and Antonio Roldão[1]

[1] iBET and ITQB-NOVA, Apartado 12, 2781-901 Oeiras, Portugal, [2] NIBRT and UCD, Foster Avenue, Mount Merrion, Blackrock, A94 X099 Dublin, Ireland

Major improvements to the insect cell - baculovirus expression vector system (IC-BEVS) remain limited by poor understanding of the underlying biological mechanisms driving host cell response to baculovirus infection and efficient expression of recombinant genes. Here, single-cell RNA-sequencing (scRNA-seq) of baculovirus-infected insect (Sf9 and High Five) cells producing AAV (as gene therapy vector) or influenza VLPs (as vaccine candidate) was implemented for the first time to shed some light into these mechanisms, providing key information for rational design of genetic engineering strategies to improve product titers and/or quality.

Using scRNA-seq an increase in cell heterogeneity as infection progresses was shown in both insect cell lines, as cells shifted towards clusters with higher expression of viral genes. Additionally, infected cells revealed varying transgene levels, highlighting limitations in recombinant gene expression using viral expression systems. This was further emphasized in the dual baculovirus, low MOI production process employed for AAV, where as little as 30 % of all cells at 24 hours post infection expressed both transgenes. Gene expression changes throughout infection and between different cell states were revealed using trajectory- and cluster-based approaches. Identified genes were associated to biological processes such as cell cycle and metabolic pathways, showing great promise to identify targets for assisting genetic and metabolic engineering in insect cells.

Overall, the increased knowledge on the underlying biological mechanisms of different IC-BEVS production processes herein attained, shows great promise to support advancements in insect cell-based biopharmaceutical production and encourages further use of transcriptomics in IC-BEVS.

**Process for generation of high-producing CHO cell lines for manufacturing of biologics using the CHO$^{2353}$ ™ cell line**

Simon Joubert, Matthew Stuible, Julie Guimond, Linda Lamoureux, Julien Leroy, Simon Lord-Dufour, Félix Malenfant, Sylvie Perret, Marjolaine Roy, François Vaillancourt, Phuong Lan Pham, Robert Voyer, Yves Durocher

National Research Council of Canada, Human Health Therapeutics Research Center, Montreal, Québec, Canada

Chinese hamster ovary (CHO) cells are the most widely used mammalian host for industrial-scale production of mAbs and other protein biologics. Selection of high-producing cell lines is a major bottleneck in the process of manufacturing a novel biologic and requires an extensive and lengthy screening campaign of several hundreds of clonally-derived cell lines. We have previously reported the development of an efficient cumate-inducible expression system. Here, we present a new GMP-banked parental cell line, amenable to both constitutive or cumate-inducible expression. We first present our process for selecting CHO pools and then cell lines using a semi-automated approach, where imaging analysis provides >99% probability that selected cell lines are single-cell derived. Following stable pool selection with MSX, fluorescently labeled cells are deposited at one cell per well in 384-well plates using a FACS and pictures of each well are taken to assess monoclonality. Hundreds of cell lines are then screened in 96-deepwell plate format to identify top producers, which then enter expression stability study in a 6-deepwell plate format (~20 mL). High-producing, stable cell lines are then tested in 1-5 L bioreactors. Screening more than 25 different operating conditions in 96-deepwell plate format, we identified the condition which is most predictable of good performance in our 6-deepwell format. We demonstrate good correlation of productivity and predictability between three different scales. Recent plasmid engineering efforts allowed to increase cell line productivity by 75%, and ~70% of selected cell lines show stable expression after at least 60 generations in culture. We also present how we have engineered our platform for the production of antibodies with reduced fucosylation, and recent development of a selection approach allowing to select more productive CHO minipools prior to single cell cloning. Using an intensified process, antibody productivity for a minipool reached 5.4 g/L. Finally, we present data supporting the use of stable cumate-inducible CHO pools for clinical development of trimeric SARS-CoV-2 spike subunit vaccine antigens.

**Cellular membrane-associated negative charges modulate polyplex internalization in HEK293 cell cultures**

Pol Pérez-Rubio[1], Jesús Lavado-García [1,2], Laura Cervera[1], Francesc Gòdia[1]

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Transient gene expression (TGE) is a versatile tool for heterologous gene expression that allows production in a short period of time. Some biopharmaceutical products rely exclusively upon TGE, such as the manufacturing of gene-therapy vectors like recombinant adeno-associated virus (rAVVs). A major limitation for a complete implementation of TGE at industrial scale is the cell density effect (CDE). The CDE is a biological phenomenon that limits the cell density at which cultures can be efficiently transfected and consequently limits production. Hence, the overall cost-effectiveness of the bioprocess is compromised. Transient transfection in HEK293 cell cultures at low and high cell density (HCD) is investigated to contribute to elucidate the mechanisms of the cell density effect. Particularly, the focus of the study is on the interactions between the cell membrane of HEK293 cells and DNA:PEI polyplexes that allow successful transfection. X-ray spectroscopy elemental analysis of polyplexes incubated in conditioned medium at high cell density revealed the appearance of sulfur interacting molecules interfering with transfection. Removal of this negatively charged molecules associated to extracellular vesicles (EVs) completely restored the capacity of transfection. Immunolabeling of sulphated cell membrane proteins coupled with polyplex fluorescent labeling was used for *in vivo* tracking of polyplex cell entrance pathways. Following polyplex contact to cells, a complete relocation of these sulphated proteins is observed allowing polyplex accommodation and internalization. The results of this work suggest that the CDE mechanism can be understood as a competitive inhibition between free and EV-associated negatively charged molecules and the ones present in the cell membrane.

**The T7 inducible system for recombinant protein production in mammalian cells**

Maria Toth, Manuel Reithofer, Reingard Grabherr, Astrid Dürauer

Department of Biotechnology, University of Natural Resources and Life Sciences Vienna, Austria

For the production of recombinant adeno-associated virus (AAV) in mammalian cells a triple transfection using three different plasmids is commonly used. One plasmid carries the two AAV genes *rep* and *cap*, responsible for DNA replication and capsid proteins, respectively. The second plasmid encodes adenoviral helper genes, namely DBP, E4 and VA RNA. The DNA encoding the therapeutic gene, to bepackaged into the viral particles is supplied by a third plasmid. Packaging cell lines, that stably express all AAV components have the advantage that, only one plasmid, encoding the target gene, must be transfected, which in turn increases production efficiency. However, the expression of cytotoxic proteins such as the AAV gene *rep* or adenovirus helper genes in stable cell lines requires a tight regulation of gene expression. While the Tet-ON system is widely used for protein production in mammalian cells, its dependence on antibiotic induction can create issues in large-scale manufacturing. We propose the use of the polymerase from the bacteriophage T7 as an alternative option. It employs the T7 promoter, not recognized by the mammalian host polymerase. The expression is activated by the introduction of a helper plasmid for the expression of the T7 RNA polymerase directed to the nucleus by a nuclear localization signal. The extensive knowledge about the T7 system in bacteria can be translated to mammalian cells, enabling the fine-tuning of expression through the use of various promoter variants. In our study, we investigate the feasibility of this inducible system in suspension HEK293 and adherent CHO K1 cells. Therefore, we designed and introduced different plasmids carrying GFP under the control of the T7 promoter. Basal and induced expression levels were assessed by flow cytometry. Our experiments demonstrate the inducibility of protein expression under the control of the T7 system. These results lay the foundation for the development of stable cell lines for the expression of toxic proteins in mammalian cells, thereby enhancing their stability and potentially contributing to viral vector production.

**Use of CHO pools for rapid production of antibodies for clinical trials**

Sarah Dunn, Cell Culture and Fermentation Sciences, BioPharmaceuticals Development, R&D, AstraZeneca, Cambridge, UK

The need for rapid development and production of antibody therapeutics was never more pressing than during the global COVID-19 pandemic. AstraZeneca successfully developed and manufactured AZD7442 (EVUSHELD)- two long-acting antibodies, targeting SARS-CoV-2, to provide prophylactic protection against COVID-19. The highly accelerated timelines challenged traditional cell line development processes and spurred the rapid adoption of novel approaches. Importantly, multiple health authorities endorsed the use of pools of CHO (Chinese hamster ovary) clones to provide material for toxicology and Phase 1 clinical trials, thereby, enabling these studies to be initiated within 3 months of antibody sequence selection. Use of well-characterized platform processes and comparability studies facilitated the transition from pools of CHO clones to clonally derived cell lines for pivotal studies and commercial manufacturing.

To advance other lifesaving molecules to patients as rapidly and safely as possible, it is desirable that we leverage the learnings and solutions implemented in the face of the pandemic and identify acceleration strategies that can be sustainably maintained. Challenges encountered and solutions implemented for COVID mAbs development will be discussed as well as new acceleration options for biologics development in the post-pandemic world.

**The Power of Transposases: How an Ancient Cellular Mechanism Revolutionized Modern Biopharmaceutical Development**

Sebastian Püngel [1]

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Since the early days of CHO cell line development, transgenes have been integrated randomly into the host cell genomes leading to highly variable integration pattern. As a result, time-consuming and labor-intensive clone screenings had to be accomplished to identify high-performing and genetically stable cell lines. In this talk, we will outline how the application of transposase-mediated transgene integration marks a paradigm change in the biopharmaceutical industry leading to substantially accelerated biologics development timelines in the future.

**CHO Cell Lines for screening and production of recombinant proteins with optimal N-glycosylation**

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The impact of N-glycosylation on the activity, specificity, stability, and immunogenicity of recombinant proteins is well established, but the possibility of controlling and designing the specific N-glycosylation has so far been limited to enzymatic treatment of the proteins, and tight control of media and bioprocess conditions. At the Technical University of Denmark (DTU) we have generated panel of glyco-engineered CHO cell lines (geCHO), each of which produce proteins with defined N-glycans. This panel can rapidly and easily produce different glyco variants of glycoproteins, to be used to investigate the effect of N-glycans in relevant assays, for easy screening of drug candidates to determine the optimal glyco form of the most potent, stable, and efficient candidate. After the optimal form is identified, the geCHO cell line can be used to generate material for pre-clinical and clinical studies, and eventually as production host for manufacturing. We have successfully applied the geCHO cell lines for production of a large number of glyco-proteins, from antibodies over fusion proteins, active enzymes and vaccine candidates to plasma proteins and protease inhibitors, all in µg to g scale.

We have applied the geCHO cell lines to the production of a recombinant form of alpha1-antitrypsin (AAT), with a bi-antennary non-fucosylated glycoprofile similar to the native AAT, which is required for the use of AAT as replacement therapy for AAT deficient patients. Using standard cell line development techniques on the geCHO cell line, we have generated an AAT production cell line producing more 1,4 g/L, shown to be stable over 60 generations. The geCHO panel has also been used to screen vaccine candidates against naturally occurring antibodies obtained from previously infected patients, revealing that the glycosylation on the vaccine candidate has a significant impact on the recognition of the antigen, suggesting that vaccines with specific glycosylation may have a superior potency.

The panel is now available for researchers, both in academia and industry, for research in the effects of glycosylation as well as the discovery and development of more potent and stable protein-based therapeutics.

**GalMAX: Model-inspired glycoengineering for biopharmaceutical quality assurance**

Mina Ghahremanzamaneh[1], Aoife harbison[1], Itzcoatl Gómez-Aquino[1], Alfonso Blanco[2], Sara Carillo[3], Jonathan Bones[1,3], Ioscani Jimenez del Val[1]

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N-galactosylation (β-1,4 galactosylation) is a significant source of heterogeneity in commercial monoclonal antibodies (mAb) and arises from metabolic and cellular machinery bottlenecks. In addition, levels of galactosylation are reported to impact mAb effector functions. Our GalMAX technology is a combination of two cell engineering strategies that enhances β-1,4 galactosylation by eliminating metabolite bottlenecks and cellular machinery bottlenecks. GalMax technology deployed to the mAb-producing CHO cell lines (DP12 and VRC01) with two genetic engineering events.

1. Knockout of core 1 β3-Gal-T-specific molecular chaperone (COSMC): In this stage, UDP-gal availability increased by aborting O-linked galactosylation which is the main sink of UDP-Gal. CRISPR-Cas9 technology was applied to eliminate cellular O-linked galactosylation by knocking out the COSMC. CRISPR-COSMC knockout (C-COSMC-KO) cells were enriched through lectin-aided fluorescence cell sorting (LA-FCS). Also, this COSMC-KO has been achieved using TALEN gene editing technology (T-COSMC-KO). 2. Overexpression of β4GalT: to increasing the level of expression of β4GalT, COSMC knockout cells (C-COSMC-KO and T-COSMC-KO) transfected with a plasmid containing the human β4-galactosyltransferase I gene to produce DP12 and VRC01 GalMAX cells. C-COSMC-KO, T-COSMC-KO and GalMAX variants of DP12 and VRC01 were cultured in batch and fed-batch mode for characterisation. LC-MS glycoprofiling was performed on the mAb product. In the batch and fed batch mode cultures, the DP12-GalMAX cells produced 96% and 92% galactosylated mAb glycoforms respectively (40% and 44% above parental cells). The VRC01-GalMAX cells yielded 98% and 95% galactosylated species (a 2.2-fold and 50% increase over parental cells). By yielding extremely high levels of β4-galactosylation, our model-inspired GalMAX strategy reduces product variability and has the potential to enhance the oncolytic activity of mAbs. GalMAX, therefore, has great potential to contribute to biopharmaceutical quality assurance. Additionally, this technology has this flexibility in utilizing either the TALEN or CRISPR strategy in the COSMC-KO stage. This offers benefits from both approaches depending on the demand for biopharmaceuticals.

**Utilising digital droplet PCR to aid Cell Line Selection of Chinese Hamster Ovary cell lines expressing complex modality antibodies.**

Jessica Willmott[1], Holly Corrigall[1] and Robyn Emmins[1].

[1]BioPharm Process Research, Drug Substance Development, GSK, United Kingdom.

Complex antibody modalities are becoming increasingly prevalent in the biopharmaceutical industry. These complex modalities, such as bi-specific antibodies, present unique challenges to the Chinese Hamster Ovary (CHO) expression system, namely due to the requirement to synthesise and correctly assemble the antibody into the desired heterodimer format. When the assembly of the desired antibody format is not achieved, product impurities such as halfbodies and homodimers, can arise. These impurities are then difficult to remove during purification processes and reduce the amount of the desired, functional antibody product.

Traditional cell line screening techniques in Cell Line Development (CLD) have focused on growth profiles and mAb titre produced by CHO cell pools post transfection. This strategy is highly successful at highlighting the highest producing pools expressing standard format antibodies, however for more complex molecules, such as bi-specific antibodies, these methods cannot distinguish correct heterodimer from other impurities. Opportunities now present themselves to develop novel screening tools to elucidate the quality of these complex antibodies at the earliest stages of cell line development.

Here we present the use of digital droplet PCR (ddPCR) to perform copy number analysis on pre-clonal CHO transfection pools, expressing bi-specific antibodies, to aid cell line selection. Using fluorescently tagged primer pairs, ddPCR can quantify the number of gene copies for each antibody chain within a cell population with high sensitivity. By monitoring the gene copy number of each antibody chain expressed in the CHO transfection pool we can predict the resulting product quality of the pool without the need for analytical assessment. By selecting transfection pools with high titre *and* desirable product quality to progress we can improve the quality of bi-specific expressing clonal cell lines generated in CLD.

**New approaches to understand population homo/heterogeneity and phenotypic behavior of suspension grown CHO host and producer cells**

Nicole Borth, Maja Papez, Giulia Borsi, Victor Jimenez Lancho, Markus Riedl, Caterina Ruggieri, Krishna Motheramgari, Heena Dhiman, Martina Baumann, Peter Eisenhut and Nicolas Marx

BOKU University Vienna and
acib, the Austrian Center of Industrial Biotechnology, Austria

Recombinant mammalian host cell lines, in particular CHO and HEK293 cells, are used for the industrial production of therapeutic proteins or gene therapy vectors. Despite their well-known genomic instability, the control mechanisms that enable cells to respond to changes in the environmental conditions or to stress are not yet fully understood, nor do we have a good understanding of the factors that lead to phenotypic shifts in long-term cultures. Identifying the genetic factors that govern cellular stress response or phenotypic drift can facilitate targeted genetic engineering to obtain production cell lines that demonstrate a higher stress tolerance and phenotypic stability. Therefore, we investigated two potential contributing factors: i) inherent diversity in transcriptomes as well as ii) cellular response to stress.

To understand the inherent diversity in transcriptomes within a population, we used two approaches: i) full-length coverage single-cell RNA sequencing (scRNA-seq) to investigate and compare cell-to-cell variability and the impact of standardized and homogenous culture conditions on the diversity of individual cell transcriptomes, and ii) a multi-study comparison of both coding and long-noncoding transcriptomes across different cell lines and subclones. For a dive into cellular stress response mechanisms, a so far unused technology that enables a kinetic understanding of changes in transcriptome was adapted for CHO. Slam-seq stands for thiol(SH)-linked alkylation for the metabolic sequencing of RNA and allows to differentiate between actively transcribed, nascent mRNA and total (previously present) mRNA in the sample, adding an additional, time-resolved layer to classic RNA-sequencing.

Our results show a surprising homogeneity in transcriptomes between cells in a given culture, a defined footprint of expressed genes across all CHO lineages and a time resolved response pattern to nutrient limitation.

**Reconstruction of cysteine biosynthetic pathway in Chinese Hamster Ovary cells provides substrate flexibility and robust oxidative stress response**

Yiqun Chen and Michael Betenbaugh

Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, Maryland USA

Cysteine is a critically important amino acid necessary for mammalian cell culture, playing key roles in nutrient supply, disulfide bond formation, and as a precursor to antioxidant molecules controlling cellular redox. Unfortunately, its low stability and solubility in solution make it especially problematic as an essential that must be added to Chinese hamster ovary and other mammalian cell cultures. Therefore, in this study, CHO cells have been engineered to include the capacity endogenously synthesize cysteine by overexpressing multiple enzymes, including cystathionine beta-synthase (CBS), cystathionine gamma-lyase (CTH) and glycine N-methyltransferase (GNMT) to reconstruct the reverse transsulfuration pathway and overcome a key metabolic bottleneck. Some limited cysteine biosynthesis was obtained by overexpressing CBS and CTH for converting homocysteine to cysteine but robust metabolic synthesis from methionine was only possibly after incorporating GNMT which likely represents a rate limiting step in cysteine biosynthesis pathway. CHO cells with the reconstructed pathway exhibit the strong capability to proliferate in cysteine-limited and cysteine-free batch and fed-batch cultures at levels comparable to wildtype cell line with ample cysteine supplementation, providing another selectable marker for CHO cell engineering. Incorporation led to the accumulation of the GNMT byproduct sarcosine, but its accumulation did not affect cell growth. Furthermore, pathway reconstruction maintained CHO cells' glutathione levels in cysteine-limited conditions, and greatly enhanced their survivability and ability to maintain redox homeostasis under oxidative stress induced by addition of menadione in cysteine-deficient conditions. Such engineered CHO cell lines can potentially substantially reduce or even eliminate the need to include cysteine in culture medium, which not only reduces the cost of mammalian media production but also promises to transform media design by solving the challenges posed by the low stability and solubility of cysteine and cystine in future mammalian biomanufacturing processes.

# Towards a Whole Cell Network Reconstruction for CHO Cells

Pablo Di Giusto[1], Athanasios Antonakoudis[2], Santiago Benavides Lopez[3], Pierrick Craveur[4], Alexander Sutherland[5], Adriana Valdez[3], Anne Richelle[6], Nathan E. Lewis [1,7]

[1]Department of Pediatrics, University of California San Diego, U.S.A; [2]Sartorius Corporate Research, Royston, UK; [3]Instituto de Investigaciones Biomédicas, UNAM, México; [4]Sartorius Corporate Research, Aubagne, France; [5]Sartorius Corporate Research, Umea, Sweden; [6]Sartorius Corporate Research, Brussels, Belgium; [7]Department of Bioengineering, University of California San Diego, U.S.A

Chinese hamster ovary (CHO) cells are pivotal in the biotherapeutic protein production industry. The increasing demand for recombinant therapeutic proteins underscores the necessity to enhance efficiency and yield, a process that remains largely empirical. Genome-scale models hold the potential to radically transform bioprocess and cell line engineering workflows due to their capacity to predict and comprehend whole-cell metabolism in silico. However, their predictive capacity is currently limited. This limitation is due to the absence of comprehensive knowledgebases that integrate complex molecular processes, extending beyond just cell metabolism, all of which collectively influence growth and protein yields. Here we aim to establish a well-curated knowledgebase of the molecular processes active in CHO cells, including metabolism, transcription/translation, and protein secretion. Using network reconstruction techniques, we first reconciled and refined all the existing CHO metabolic network reconstructions. Moreover, we were able to add more than 3000 reactions from human reconstructions by mapping the human genes associated to those reactions to CHO orthologs. By further addition and manual curation of subsequent reactions to fill the gaps generated in such reconciliation we developed a network comprising 10778 reactions, 8089 metabolites and 3232 genes. This resource also includes three-dimensional structural data of metabolites and proteins that enables the user perform protein-protein and protein-metabolite interaction analysis. In the next phase of this project, we will expand this knowledgebase to incorporate pathways relevant to gene expression, protein synthesis, and secretion. The result of this project will yield a high-quality map of all associated pathways with CHO gene associations. This knowledgebase will serve as a comprehensive tool for researchers and biotech companies alike, offering a framework for analyzing and interpreting data and constructing computational models to guide cell engineering and process optimization. This work represents a significant step forward in the biotherapeutic protein production field, paving the way for more efficient and targeted use of CHO cells.

**Development of a chemically defined culture media through a statistical design of experiments (DoE) methodology for the culture of duck primary cells**

Díaz-Maneh, A. [1, 2], Muñoz, P. [2], Camacho, A. [2], García-Aranda, N. [2], Ilic, N. [2], Enciso, Y. [2], Fuenmayor, J. [2], Lavado-García, J. [3], Gòdia, F. [1], Notari, M. [2] and Revilla, R. [2]

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[2]CUBIQ FOODS, [3]Technical University of Denmark

Abstract:

The development of optimized culture media plays a critical role in achieving high productivity and reproducibility in cell culture systems. Chemically defined culture media offer numerous advantages over media containing animal-derived serum, including enhanced control over culture conditions, improved reproducibility, and reduced risk of contamination. Even though wide effort has been made in the development of chemically defined media, only a few cost-effective media have been developed for avian cells. To address these limitations, we employed a statistical design of experiments (DoE) approach that allows for efficient and systematic exploration of multiple factors affecting cell culture performance.

In this study, we follow a Plackett-Burman methodology to make a wide screening of hormones, homeostatic molecules and growth factors on cells previously adapted to low-serum conditions to determine the most significant in terms of cell proliferation. Then, we carried out a response-surface methodology (Box-Behnken) of the compounds identified as beneficial during the screening process to maximize the synergies between them. Finally, the rational combination of the different optimal conditions determined using DoEs allowed us to validate the capacity of the culture media to sustain the growth of the duck cells both in 2D and in microcarrier cell culture.

The methodology presented here offers a systematic and efficient approach to identify critical compounds and optimize their concentrations in the media. Finally, valuable insights for the improvement and development of cell culture media not only for duck primary cells but also for other cell lines are identified.

**Multi-stage bioreactor process for continuous production of influenza VLPs using IC-BEVS**

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Insect cells are excellent hosts to produce recombinant proteins for many different biomedical applications, in particular when seconded by the baculovirus expression vector system (BEVS). However, the lytic nature of IC-BEVS processes limits its transition from traditional batch-based bioprocessing to more intensified and sustainable continuous bioprocessing.

In this study, a continuous multi-stage bioreactor process was established to produce influenza hemagglutinin (HA) displaying virus like particles (VLPs) using IC-BEVS. A set-up consisting of one cell growth bioreactor feeding non-infected insect Sf9 cells to three parallel production bioreactors operated at different residence times (RT) (18, 36, and 54 hours) was implemented and successfully run for approx. 3 weeks. Cell growth kinetics and viability varied across RT. Production of HA-VLPs was confirmed throughout the period of continuous operation, with a cyclic oscillatory-like profile of extracellular HA titer being observed in all production bioreactors; higher RTs were consistently associated with increased expression of HA-VLPs. Notably, no decrease in infectious baculovirus particles was observed in either of the production bioreactors.

Overall, this work demonstrates the first successful implementation of a continuous two-stage bioreactor setup for influenza HA-VLPs production using IC-BEVS at laboratory scale.

**Utilising Dielectric Capacitance Measurements for Earlier Detection of Cell Health Decline in Bioprocesses**

James Flynn[1], Laura Breen[1], Nils Bunte[1], Michael Butler[1]*

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In a bioprocess, cell viability is routinely monitored by the integrity of the outer membrane using dye exclusion, typically trypan blue. However, at the point when the cells turn blue, apoptosis is in a late stage and host cell proteins are released into the media. Early detection of the onset of apoptosis has the advantage of allowing an opportunity to reverse the metabolic decline of the cells and limit host cell protein release. In this study we used dielectric measurements to enable early detection of the demise of the cell population prior to membrane damage. At 1 L scale we showed that earlier inflection points are observed in bio-capacitance measurements, which share a signal equivalence to viable cell biomass. This earlier indication enables earlier intervention in bioprocess control up to 24 hours before trypan blue exclusion assay highlights cell health decline. Subsequently, we look at scaling down the analysis of bio capacitance from 1 L bioreactor scale to small scale cell culture, using Aber Pico Probes. The feasibility of reducing the scale from 1 L to 25 ml proved successful, presenting a means of screening research experiment conditions at a higher throughput for faster bio-capacitance experiment design.

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**GalMAX: Model-inspired glycoengineering for biopharmaceutical quality assurance**

Mina Ghahremanzamaneh[1], Aoife harbison[1], Itzcoatl Gómez-Aquino[1], Alfonso Blanco[2], Sara Carillo[3], Jonathan Bones[1,3], Ioscani Jimenez del Val[1]

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N-galactosylation (β-1,4 galactosylation) is a significant source of heterogeneity in commercial monoclonal antibodies (mAb) and arises from metabolic and cellular machinery bottlenecks. In addition, levels of galactosylation are reported to impact mAb effector functions. Our GalMAX technology is a combination of two cell engineering strategies that enhances β-1,4 galactosylation by eliminating metabolite bottlenecks and cellular machinery bottlenecks. GalMax technology deployed to the mAb-producing CHO cell lines (DP12 and VRC01) with two genetic engineering events.

1. Knockout of core 1 β3-Gal-T-specific molecular chaperone (COSMC): In this stage, UDP-gal availability increased by aborting O-linked galactosylation which is the main sink of UDP-Gal. CRISPR-Cas9 technology was applied to eliminate cellular O-linked galactosylation by knocking out the COSMC. CRISPR-COSMC knockout (C-COSMC-KO) cells were enriched through lectin-aided fluorescence cell sorting (LA-FCS). Also, this COSMC-KO has been achieved using TALEN gene editing technology (T-COSMC-KO). 2. Overexpression of β4GalT: to increasing the level of expression of β4GalT, COSMC knockout cells (C-COSMC-KO and T-COSMC-KO) transfected with a plasmid containing the human β4-galactosyltransferase I gene to produce DP12 and VRC01 GalMAX cells. C-COSMC-KO, T-COSMC-KO and GalMAX variants of DP12 and VRC01 were cultured in batch and fed-batch mode for characterisation. LC-MS glycoprofiling was performed on the mAb product. In the batch and fed batch mode cultures, the DP12-GalMAX cells produced 96% and 92% galactosylated mAb glycoforms respectively (40% and 44% above parental cells). The VRC01-GalMAX cells yielded 98% and 95% galactosylated species (a 2.2-fold and 50% increase over parental cells). By yielding extremely high levels of β4-galactosylation, our model-inspired GalMAX strategy reduces product variability and has the potential to enhance the oncolytic activity of mAbs. GalMAX, therefore, has great potential to contribute to biopharmaceutical quality assurance. Additionally, this technology has this flexibility in utilizing either the TALEN or CRISPR strategy in the COSMC-KO stage. This offers benefits from both approaches depending on the demand for biopharmaceuticals.

**Identification of transgene integration sites, their structure and epigenetic status with Cas9-targeted nanopore sequencing in CHO cells**

Klaus Leitner[1], Krishna Motheramgari[1], Nicole Borth[1,2] and Nicolas Marx[2]

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The initial step of using recombinant cell lines for protein production is the insertion of transgenes into the host genome. The integration site, the number of integrated copies as well as the epigenetic environment greatly influence the transgene expression level. In order to characterize transgene integrations in their entirety, we have adopted a fast and accurate method to detect the site of integration and the complete transgene structure in CHO cells using Cas9 targeted nanopore sequencing (nCATS). nCATS allows targeted, directional sequencing of long DNA fragments (>20 kbp) and simultaneously provides information about the DNA methylation status of the sequenced genomic region.

For nCATS applications, dephosphorylated high molecular weight genomic DNA is subjected to CRISPR/Cas9 mediated cleavage with crRNAs targeting genomic loci of interest. The generation of newly phosphorylated DNA ends at the induced double strand breaks allows site-specific, directional ligation of nanopore sequencing adapters and thus enriched sequencing of targeted sites. For proof of concept, we performed nCATS by targeting the Fut8 promoter in a CHO-K1 cell line after epigenetic modulation. Next, identification of transgene integration sites and their structure was performed in two recombinant CHO-K1 cell lines and results were compared to TLA-sequencing.

Enriched sequencing of the endogenous Fut8 promoter resulted in high on-target coverage and correct identification of the targeted region. Additionally, the DNA methylation status could be accurately assessed in cell lines that were previously subjected to targeted DNA methylation. For the identification of transgene integration sites, nCats was designed so that reads were sequenced from the integrated transgenic region into the neighboring chromatin, which allowed to precisely determine transgene integration sites. Interestingly, in contrast to TLA sequencing, different combinations of genome-transgene junction sites and a third integration site was detected by nCATS. The conformation of the integrated sequences were disclosed identifying plasmid concatemers of up to 21 kb in length, which could not be resolved previously.

Conclusively, nCats offers unprecedented accuracy to determine complex integration sites in CHO cells and simultaneously allows the characterization of their epigenetic context.

**INVESTIGATING THE DARK MATTER OF THE GENOME:**
**A LARGE-SCALE DELETION SCREEN DEFINES THE ESSENTIALITY OF UNANNOTATED GENOMIC REGIONS IN CHINESE HAMSTER OVARY CELLS**

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The biopharmaceutical sector relies heavily on Chinese Hamster Ovary (CHO) cells as a fundamental tool for studying several biological processes, and as a preferred host for the production of biotherapeutics. In parallel, decades of improvement in CHO cell genome assembly have become an indispensable tool for designing advanced genetic engineering strategies. Nevertheless, at present, we lack of large systematic studies in CHO that can precisely investigate the non-coding genome, regulatory elements, and poorly annotated genomic regions that can altogether be referred as the "dark matter" of the genome. In this regard, CRISPR screens are the most powerful resource that can bridge the gap between genotype and phenotype for these unknown and non-coding regions in suspension-cultivated production cell lines.

In this study, we introduce a genome-wide CRISPR-Cas9 screening platform for CHO cells with 112,272 paired guide RNAs targeting 14,034 genomic regions. Using this platform, we conducted a negative screen that actively selects dying cells to unravel the regions essential for cell survival under standard growing conditions. Our paired guide RNA-based perturbation approach hinges on the full deletion of intermediary genomic sequences up to 150 kb, thus overcoming the intrinsic limitations of introducing traditional frameshift mutations. This work revealed, with high confidence, a number of regions essential for CHO cell viability that currently lack of any annotation in the most recent PICRH genome assembly. The hits identified among the top-scoring regions whose paired guide RNAs showed the greatest depletion during the screen will then be individually analyzed, characterised, and validated.

Our study sheds a novel light on a substantial part of the mammalian genome which is traditionally difficult to investigate. Our results, in the context of the high value of CHO expression systems, represent an improvement toward a streamlined cell line development platform.

**Optimizing MAb production through cell lines development based on a CRISPR-Mad7-Targeted Integration approach in CHO cells**

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Although most processes for CHO cell line development are still based on a random or semi-random transgene integration, they lead to high phenotypic heterogeneity that can cause loss of expression. Previously, we reported how our targeted integration (TI) strategy, based on a combination of CRISPR-Cas9 and Recombinant Mediated Cassette Exchange (RMCE) tools, has shown an efficient and stable transgene integration with predictable and consistent transgene expression [1]. However, there are some challenges related to this strategy that we aimed to address in this work such as Intellectual Property issues concerning to the use of Cas9, lower productivity associated to a limited transgene copy number and the efficiency of RMCE which is inversely related to the size of the cassette to be integrated. In this way, we successfully generated CHO-S and CHO-DG44 master cell lines (MCLs) using a CRISPR-Mad7 system. Landing-pads (LPs) containing fluorescence markers flanked by Lox sites for cassette exchange were site-specifically integrated into known transcriptional hotspots. On the other hand, in order to maximize monoclonal antibody (MAb) expression from a single gene copy, Rituximab-producing cell lines were generated from the MCLs by RMCE using different targeted-expression cassette designs. Promoters of different strength were used to drive the expression of light chain (LC) and heavy chain (HC), and also T2A peptide was tested in order to reduce the cassette size, with alternate positions of LC and HC. All clones from both MCLs targeted with the different cassette designs were able to properly produce MAb. Overall, this study shows the relevance of different promoter strength combinations between LC and HC as well as the alternative use of T2A peptide, and how these elements are optimally used in expression cassette design to improve the performance of MAb-producing cell lines generated by TI. Additionally, we demonstrated that CRISPR-Mad7-based system can be an advantageous method for commercial MCLs generation [2].

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**RNA-seq meta-analysis of lncRNAs in CHO cells**

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Long non-coding RNAs (lncRNAs) are a relatively new class of RNAs involved in many regulatory functions, ranging from modulation of gene expression to modification of chromatin states of their target genes in mammalian cell models. The investigation of lncRNA function in Chinese hamster ovary (CHO) cells faces several challenges: lncRNAs are expressed in a lower amount compared to mRNAs, they are not well conserved across species, and they are often located in complex genomic locations. Moreover, for CHO cells in particular, comprehensive studies that could help mapping lncRNAs functions genome-wide are missing and we found that the lncRNA reference annotation is often not reliable. Consequently, our attempts to overexpress promising lncRNA candidates in CHO cells was futile. To overcome these bottlenecks and to provide a comprehensive overview of lncRNA conservation in CHO cells, we collected 15 RNA-seq datasets available from NCBI or from previous in-house studies to initially improve the reference annotation by performing a transcriptome-based assembly. Our RNA-seq samples include different host cell lines, producer and non-producer cell lines, and cell lines exposed to various culture conditions. Therefore, we next aimed at using the available metadata to perform differential expression analysis and identify lncRNAs classes and candidates that can be used as engineering targets. Principal component analysis (PCA) showed that samples from the same cell line (CHO-S, CHO-K1, or CHO-DXB11), although originating from different studies, cluster together. Generally, 1/5 of the annotated lncRNAs are expressed in all cell lines (1060) with DXB11 being the cell line that express the highest number of lncRNAs (up to 2125). Additionally, when looking at one host cell line (e.g. CHO-K1), the specific culture condition explains most of the variance within a sample set. Next, we evaluated the influence of different parameters (e.g. presence of glutamine in the media, growth rate, productivity, etc.) on the number of expressed lncRNAs and their expression level and assessed the differences between cell lines and samples. This in-depth differential expression analysis will help identifying lncRNAs that can be considered markers for the cell phenotypes of interest and/or targets of cell engineering to improve cell culture performances.

**Sequential Activation of Multiple Gene Copies Facilitates Adaptation of CHO Cells to Increased Productivity**

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The contribution of multiple gene copies to the expression of recombinant proteins is considered a disruptive and stressful event during development of high producing mammalian cell lines for production of therapeutic proteins. Classically, gene copy numbers of the recombinant product gene are randomly amplified to eventually achieve higher productivities. Rapid increases in productivity does not allow cells to adapt their transcriptome for efficient production. Hence, we aimed at utilizing a molecular toolbox that enables a stepwise increase of gene copy numbers. Four gene copies encoding for fusion proteins of fluorescent proteins, Green and Blue fluorescent proteins (GFP and BFP), fused to a fragment crystallizable region (Fc) are stably integrated into the CHO genome. Importantly, three of these genes are initially not expressed due to genetic repressor elements ("Rep") that sit between the gene's promoter and coding sequence. CRISPR/Cas9-mediated deletion of the individual Reps enables specific activation of the corresponding gene. For comparison, the same plasmid without the Reps, thus all four gene copies active from the beginning, was used as control. Utilizing quantitative real-time PCR, flow cytometry and Octet® measurements to quantify titers allowed us to dissect the contributions of each activated gene copy to the transcriptional, translational and secretory load. Our findings demonstrate that this molecular toolbox is well suited to increase production loads in a stepwise manner. We found that cell lines generated with successively activated gene copies achieved higher productivities and messenger RNA (mRNA) levels coding for the Fc region compared to cell lines that were obtained by integration of four actively expressed gene copies, suggesting that stepwise activation of production load on the cells helps CHO cells to adapt to increasing production challenges. In summary, our study showcases how our molecular toolbox can be employed to study the individual contribution of gene copy numbers and to eventually generate CHO production cell lines in a controlled and rational manner.

**SLAM-seq reveals early transcriptomic response mechanisms upon glutamine deprivation in Chinese hamster ovary cells**

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During biopharmaceutical production processes, mammalian cell cultures frequently experience environmental perturbations. To compensate, cells typically preserve metabolic resources by decreasing growth and/or productivity, therefore impacting the bioprocess and/or bioproduct quality. Thus, knowing the genetic factors that govern cellular stress response(s) can facilitate targeted genetic engineering to obtain production cell lines that demonstrate a higher stress tolerance. Here, we simulated nutrient deprivation stress by transferring Chinese Hamster Ovary (CHO) cells into a glutamine-free medium and investigated transcriptional dynamics using thiol(SH)-linked alkylation for the metabolic sequencing of RNA (SLAM-seq) along with standard RNA-seq of stressed and unstressed cells. In SLAM-seq, cells were labelled with 4-Thiouridine promptly after perturbation, followed by a step of alkylation and sequencing. The method allows differentiation between actively transcribed, nascent mRNA and total, previously present mRNA in the sample, adding an additional, time-resolved layer to classic RNA-sequencing. Early transcriptomic changes in the first hours after glutamine deprivation are reflected in the enrichment of Gene Ontology terms involved in Organic Substance Metabolic Process and Cellular Response to Stress. The cells tackle amino acid limitation by *Atf4* overexpression, a master metabolic regulator, leading to subsequent activation of its targets, namely *Asns*, *Slc7a11* and *Trib3*. These transcriptional events indicate a metabolic shift, which leads to the procurement of alternative nutrient sources and the diminishment of energetically costly processes, such as cholesterol metabolism. As the differential expression in *Atf4* was active for less than 24 h, it would likely have been missed in standard experimental designs from RNA-seq. Our results describe the successful establishment and application of SLAM-seq in CHO cells on the example of industrially relevant stress and therefore facilitate its future use in other scenarios where dynamic transcriptome profiling in CHO cells is essential.

**Streamlining and automating clonal cell line selection**

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An ever-broadening array of assets and complex modalities are now being progressed through the GSK portfolio each bringing unique challenges to Cell Line Development (CLD) and our workflows. In recent years, BioPharm Process Research has invested heavily in a wide-reaching Next-Generation Automated Cell Line Development Capability project to, not only increase capacity by creating redundancy in key pieces of equipment such as the PhenomeX Beacon and Sartorius ambr automated bioreactor systems; but to also improve and streamline our workflows and decision making to best equip ourselves for the challenges that the diversified portfolio presents. The outputs of these efforts have had a significant positive impact on meeting ambitious timelines and delivering cell lines suitable for clinical trial commercial manufacture.

Integral to this capability project has been the investment in new technologies, a deliberate focus on our data infrastructure and ability to analyse and act efficiently upon the thousands of data points collected during the Cell Line Development workflow. As we continue our journey to further streamline the identification of high performing cell lines through data-driven predictions and smart triaging early in the CLD process, here we present some of these developments and the benefits they have had on portfolio progression internally within GSK.

**The BacMam platform as efficient tool for stable cell line development**

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Efficient recombinant protein production often requires the use of stable animal cell lines. However, these involve very cumbersome procedures and are difficult to obtain. Moreover, they rely either on very inefficient transfection processes using membrane permeating transfection reagents or on lenti-/retroviral transduction with the necessity of higher biosafety level laboratories. Therefore, we propose the use of baculoviruses for efficient gene delivery and stable cell line generation. These viruses offer several advantages over conventional approaches, as there are: (i) no need of high biosafety level laboratories, (ii) efficient transduction of various cell types, (iii) efficient integration of large DNA fragments into host cell genomes and others.

In our study, we compare the transient transfection efficiency of different transfection agents and baculoviruses for transient protein production in different cell lines as well as the potential of the different strategies for the generation of stable cell lines. We show that in transient experiments the baculovirus achieves similar or even higher gene delivery efficiencies as the best performing membrane permeating transfection agents. In addition, the protein production per individual cell, measured by GFP fluorescence, was also comparable or higher in baculovirus transduced cells. Of note, the virus does not stress the cells or leads to enhanced cell death, as obrserved with higher doses of the various agents. Moreover, experiments addressing the stable cell line generation displayed a higher probability of genome integration in baculovirus infected cells as compared to the different transfection agents used in this study.

Overall, the baculovirus platform offers advantages over standard transfection reagents not only for transient but also for stable transfection procedures. Additionally, the abundance of commercially available vectors and systems allow simple cassette generation for the production of large proteins like antibodies and the generation of stable cell lines producing those efficiently.

**Transcriptional Response To Recombinant Protein Production In Isogenic Multi-Copy CHO Cells**

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Despite significant advancements in CHO bioprocessing, challenges remain in the field of cell line engineering[1]. The development of CRISPR/Cas genome editing tools and the availability of genome sequencing data have allowed precise rational engineering of CHO cells[2]. However, cell productivity remains a complex trait to elucidate. Among different approaches, a holistic analysis of the cellular response to protein production through transcriptomic studies may facilitate our understanding and help to find new engineering targets. To fit this purpose, we generated a panel of CHO cells by multi-copy targeted integration where one, two or four copies of our genes of interest (GOI) - Erythropoietin (EPO) or Etanercept (ETN) - were integrated into specific genomic sites. Surprisingly, by increasing the gene dosage, we observed a bottleneck at the transcript level rather than solely at the protein level. Using RNA-seq, we investigated both common and recombinant protein-specific patterns of differential gene expression. The choice of transgene integration site influenced the overall transcriptome in nearby regions, emphasizing the need to reconsider transgene expression cassette design. Common transcriptional responses to increased protein production involved upregulation of genes related to ER, protein export pathways, and specific branches of the unfolded protein response (UPR), while genes associated with lysosomal and cell cycle pathways were downregulated. Notably, cells producing ETN exhibited upregulation of genes involved in innate immune responses against viruses. Our investigation provides valuable insights useful to identify engineering strategies aimed at alleviating the identified bottleneck in multi-copy isogenic cell lines.

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**Utilising digital droplet PCR to aid Cell Line Selection of Chinese Hamster Ovary cell lines expressing complex modality antibodies.**

Jessica Willmott[1], Holly Corrigall[1] and Robyn Emmins[1].

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Complex antibody modalities are becoming increasingly prevalent in the biopharmaceutical industry. These complex modalities, such as bi-specific antibodies, present unique challenges to the Chinese Hamster Ovary (CHO) expression system, namely due to the requirement to synthesise and correctly assemble the antibody into the desired heterodimer format. When the assembly of the desired antibody format is not achieved, product impurities such as halfbodies and homodimers, can arise. These impurities are then difficult to remove during purification processes and reduce the amount of the desired, functional antibody product.

Traditional cell line screening techniques in Cell Line Development (CLD) have focused on growth profiles and mAb titre produced by CHO cell pools post transfection. This strategy is highly successful at highlighting the highest producing pools expressing standard format antibodies, however for more complex molecules, such as bi-specific antibodies, these methods cannot distinguish correct heterodimer from other impurities. Opportunities now present themselves to develop novel screening tools to elucidate the quality of these complex antibodies at the earliest stages of cell line development.

Here we present the use of digital droplet PCR (ddPCR) to perform copy number analysis on pre-clonal CHO transfection pools, expressing bi-specific antibodies, to aid cell line selection. Using fluorescently tagged primer pairs, ddPCR can quantify the number of gene copies for each antibody chain within a cell population with high sensitivity. By monitoring the gene copy number of each antibody chain expressed in the CHO transfection pool we can predict the resulting product quality of the pool without the need for analytical assessment. By selecting transfection pools with high titre *and* desirable product quality to progress we can improve the quality of bi-specific expressing clonal cell lines generated in CLD.

**Development of a next-generation bioproduction CHO cell line for optimized glycosylation of therapeutics**

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A key challenge in the pharmaceutical production of recombinant therapeutic proteins is inconsistent and variable N-glycosylation. Alterations in the N-glycosylation profile between batch-to-batch productions of recombinant therapeutic proteins can lead to changes in many therapeutic attributes including their activity, stability, immunogenicity, dosage requirements, and pharmacokinetic properties. N-glycosylation varies in both the type of N-glycan attached at a specific site on the protein (microheterogeneity) and the number of predicted N-glycosylation sites that are occupied (macroheterogeneity). Macroheterogeneity has become an increasing concern in pharmaceutical development as it can significantly impact the biological function of therapeutic proteins. For example, complete N-glycosylation of the 2 predicted N-glycan sites on IFN-γ, used to treat multiple sclerosis, is required for its proper therapeutic function, including stability of the therapeutic *in vivo* (1). However when produced in Chinese Hamster Ovary (CHO) cells, the predominant bioproduction cell line used in the pharmaceutical industry, there is substantial variability in site occupancy where only 50% of IFN-γ produced has both N-glycan sites occupied resulting in reduced efficacy of the therapeutic (2). As such, there is a need in the pharmaceutical space to develop a robust bioproduction CHO cell line that expresses therapeutic proteins with reliable and optimized N-glycosylation profiles.

At kyron.bio, we have engineered CHO cells with a unique set of exogenous genes to increase the number of N-glycosylation sites that are occupied on recombinant therapeutic proteins. This work aims to enhance control over the glycosylation of therapeutic proteins produced in CHO cells, allowing the biopharmaceutical industry to reliably enhance activity and extend the serum half-life of therapeutic proteins.

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**Absolute quantification of influenza A viral proteins during single-round replication in different host cells**

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Madin-Darby canine kidney (MDCK), avian AGE1.CR or human HEK293SF cells can be used as host cells for influenza vaccine production. In order to improve production yields, a detailed understanding of virus replication can be crucial. In this context, quantitative data regarding the dynamics of viral protein expression could add highly valuable information, and by that support optimization of cell culture-derived vaccine manufacturing.

Here, we present a mass-spectrometry based assay for the absolute quantification of five major influenza A virus (IAV) proteins, namely hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix protein 1 (M1) and non-structural protein 1 (NS1), for single-round replication in different suspension host cells.

All investigated influenza virus proteins were detected over a single replication cycle of IAV in MDCK, AGE1.CR and HEK293SF cells. For MDCK cells there was a lag-time with constant, but not increasing viral protein production during the first 4 hpi. In contrast, protein production increased consistently from the beginning for NP and NS1 in AGE.CR and HEK cells. In MDCK cells, M1 was predominantly produced with 2.8E+08 copies/cell at 12 hpi. There, NP (4.5E+07 copies/cell) and NS1 (1.5E+07 copies/cell) were less abundant, followed by HA (7.0E+06 copies/cell) and NA (7.4E+05 copies/cell). Differently to this, in AGE.CR cells, M1, NP and NS1 were produced at equimolar levels at 2-3E+07 copies/cell, whereas HA (3.5E+06 copies/cell) and NA (6.1E+05 copies/cell) were produced at relative ratios similar to those in MDCK cells. In HEK cells all measured IAV proteins were detected at 2 hpi. Here, viral protein production increased linearly and resulted in similar concentrations for NP (1.3E+09 copies/cell), M1 (8.4E+08 copies/cell) and NS1 (8.3E+08 copies/cell). Again, HA (2.6E+08 copies/cell) and NA (2.8E+08 copies/cell) were produced less abundantly.

In sum, absolute IAV protein copy numbers were quantified in different infected host cells providing important insights into viral protein dynamics. A comparison of the three cell lines revealed surprising differential expression profiles that may impact replication and morphogenesis of IAV. In AGE1.CR and HEK cells, NP and NS1 were produced at similar amounts as M1, and envelope proteins at lower absolute levels compared to MDCK.

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**Development of functionalized HIV-1 Gag GFP Virus Like Particles with T22 peptide to selectively target CXCR 4+ cells**

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Targeted delivery of drugs is a very promising tool in nanomedicine, especially in cancer medicine where targeted therapies for metastatic tumor cells are urgently demanded. In this context, the aim of this work was to produce HIV-1 Gag GFP Virus Like Particles (Gag-VLPs) functionalized with the T22 peptide directed to CXCR4 expressing cells. CXCR4 is a cell surface receptor marker associated with several severe human pathologies, including metastatic colorectal cancer. The Gag-VLPs were produced extracellularly in HEK293 suspension cells and purified by anion exchange chromatography with a HiScreen Capto Q ImpRes column. The resulted Gag-VLPs, were functionalized with the T22 peptide by two-steps click chemistry reactions. The T22-Gag-VLPs were analyzed by immunoblots with a specific anti-T22 and anti-Gag-VLPs antibodies and using SE-HPLC and CryoTEM. The interaction between the functionalized T22-Gag-VLPs and the receptor CXCR4 was confirmed by Biacore and Dynamic light scattering. The capacity of the functionalized nanoparticles to penetrate target cells has been studied in vitro by internalization experiments monitored by flow cytometry and confocal images . Given the urgent demand for targeting agents in the research, diagnosis, and treatment of CXCR4-linked diseases, including colorectal cancer, T22-Gag-VLPs appears to be a promising strategy for the intracellular delivery of therapeutics RNA, protein drugs and toxins.

**Beacon Spotlight Assays - A toolbox of reagents to screen complex modalities during single cell cloning**

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The PhenomeX Beacon is a highly sophisticated single cell cloning instrument, which utlises optofluidics to manipulate the movement and isolation of single cells into nanopens on microfluidic chips. The clonal cells are cultured within their nanopen and using a "Spotlight Assay" are assessed for their ability to produce mAb-based molecules.

The Spotlight reagent is passed through the chip fluidics, left for a short incubation time, and then flushed from the chips. Spotlight bound to mAb-based molecules has higher mass and diffuses slower than Spotlight alone. Therefore, after flushing, the fluorescence intensity remaining in each pen is indicative to the concentration of mAb-based molecule each cell line is producing. Clonal cell lines with optimal molecular expression can be readily identified and exported from the Beacon.

Two Spotlight reagents that PhenomeX manufacture are Spotlight HuFc and Spotlight Hu Kappa (Huκ). Spotlight HuFc binds to an epitope in the Fc region of a mAb molecule, whereas Spotlight Huκ binds to a Kappa light chain of a mAb molecule.

Therapeutic mAbs that possess certain mutations in their Fc region have been shown to demonstrate Spotlight HuFc binding incompatibility. If the mAb molecule also has a Lambda light chain, there is currently no opportunity to compensate for an inability to detect in-pen mAb via Spotlight HuFc with a Light Chain specific Spotlight reagent. Internally at GSK, "off-chip" immunoassays have been developed, that allow for an assessment of the binding ability of a mAb to the Spotlight reagents prior to Beacon cloning. The screen determines the most suitable Spotlight reagent to use or highlights a requirement to implement alternate clone screening strategies for that campaign.

PhenomeX have recently developed a Hu Lambda (Huλ) Spotlight reagent, which binds specifically to a Lambda light chain of a mAb molecule. Here we present an assessment of the new Spotlight Huλ reagent, alongside a comparison with the current Spotlight HuFc and Spotlight Huκ; using cell lines expressing one of ten different GSK mAb-based molecules. These ten molecules provided a range of different Fc mutations in combination with both Kappa and Lambda light chains. The Spotlight toolbox of reagents were evaluated both on the Beacon system and in the "off-chip" immunoassays to determine their binding ability to ten mAb-based molecule variants.

**Efficient transient expression of monoclonal antibody in CHO cells with an optimized vector.**

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Transient Gene Expression (TGE) is a tool widely used for the preliminary study of vector design or the need for fast expression of proteins. Due to the challenging production of Monoclonal antibodies (Mab), multiple strategies to improve their expression have been tested, starting with the optimization of vectors. The Mab structure is complex because requires the expression of two genes, which could be inserted in the same vector or separated vectors. The design most used in a single vector includes the heavy (HC) and light (LC) chains separated by an Internal Ribosome Entry Site (IRES) element, however, previous studies in our laboratory showed that there was a higher production of the antibody when the vector does not contain that element, and latter is replaced for a second promoter sequence independently (bi-Prom). However, the efficiency of expression of this design in a single vector has not been compared with two vectors separated and should be studied. The main aim was to compare and optimize the expression of the Mab using different configurations of vectors. Methodologically, for the TGE we transfected ExpiCHO cells with the following vectors: bi-Prom vector, co-transfection with separated vectors with the same optimized promoter in different ratios of HC: LC DNA (1:1 and 3:2 respectively) and bi-Prom vector with and additional HC vector, The expression of antibody was evaluated by ELISA into the culture supernatant, qPCR and western blot. The results showed that the transfection with the bi-Prom vector had a higher Mab production in the culture supernatant, compared to the co-transfections of the HC and LC vectors separated. Surprisingly, the LC expression in the supernatant was higher than HC expression, even though their intracellular expression was similar. The expression profile of bi-prom vector with an additional HC vector was like latter. In conclusion, the use of a bi-prom vector is a very effective strategy for the expression of MAb in TGE, especially when is required a fast expression of antibody for physical and chemical, and functional characterization purposes. In addition, the promoter used for the construction of the bi-prom vector is an optimized promoter that does not undergo silencing and is recommended for the construction of vectors for stable transfection.

**Fast screening of membrane protein production by transient expression in insect and mammalian cells**

Joop van den Heuvel[1], Jagan Mohan Kaipa[1], Ganna Krasnoselska[2] and Ray J. Owens [2]

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Membrane proteins are difficult to express and purify biomolecules. In this presentation, we compared small-scale production of six selected eukaryotic integral membrane proteins in insect and mammalian cell expression systems using different techniques for gene delivery. The target proteins were C-terminally fused to the green fluorescent marker protein GFP to enable sensitive monitoring. We showed that the choice of expression systems makes a considerable difference to the yield and quality of the six selected membrane proteins. Virus-free transient gene expression (TGE) in insect High Five cells combined with solubilization in dodecylmaltoside plus cholesteryl hemisuccinate generated the most homogeneous samples for all six targets. Further, affinity purification of the solubilized proteins using the Twin-Strep® tag improved protein quality in terms of yield and homogeneity compared to His-tag purification. TGE in High Five insect cells offers a fast and economically attractive alternative to established methods that require either baculovirus construction and infection of insect cells or transient expression in mammalian cells for the production of integral membrane proteins.

**Robust Production of Virus-like-Particles in CHO and HEK293 Cell Lines**

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Roche Diagnostics GmbH, Penzberg, Germany

For the development of infectious disease immunoassays, the presentation of natively folded antigen is essential. This is especially challenging for the development of early IgM detection, as the specifier has to be presented in high density. The use of virus-like-particles (VLPs) is ideal as they display the native epitopes in multiple copies on their surface. However, VLP production is challenging - even when using state-of-the-art mammalian expression systems:

The production of both Rubella-like-particles (RLPs) and Dengue virion particles requires the expression of precursor polyproteins. These need to be properly cleaved and processed by the host cell machinery into functional structural components. Once processed, the structural proteins need to assemble within the secretory pathway before the particles are secreted.

We compared transient gene expression in HEK293 cells with subsequent stable CHO-K1 as well as stable HEK293 cell line development for robust high-quality VLP production. Our stable CHO-K1 platform showed low expression levels and improper processing and assembly of VLPs. As a result, we developed and optimized a new stable HEK293 platform.

The resulting clonal HEK293 cell lines produced properly assembled and functional Rubella and Dengue VLPs of high yield and quality. This platform can also be transferred for intracellular viral capsid production, e.g. Hepatitis A antigen, by co-expression of the P3 proteinase which is required for the procession of the Hepatitis A P1-2A polyprotein.

We present the development of high-producing clonal HEK293 cell lines of pure, stable and robust VLPs that are already used in diagnostic assays to detect infectious diseases.

**Synthetic Genetic Parts and Engineering Systems for Biologics Production and Gene Therapy**

David C. James[1,2]

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[2]Syngensys Ltd., Sheffield, U.K.,

Synthetic biology offers a new paradigm for genetic vector design, enabling product-specific cell engineering based on combinatorial tuning of primary cellular synthetic processes such as transcription, translation and translocation. Our engineering design system utilises a unique platform of genome-scale mining and informatic tools to generate libraries of synthetic parts with user-defined functionality and that can boost biologic manufacturability, replacing “one-size-fits-all” vectorology with design of context specific genetic systems. This new approach is demonstrated for (i) cell and vector engineering underpinning biopharmaceutical and AAV production systems and (ii) vector engineering for gene and cell therapy.

**The T7 inducible system for recombinant protein production in mammalian cells**

Maria Toth, Manuel Reithofer, Reingard Grabherr, Astrid Dürauer

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For the production of recombinant adeno-associated virus (AAV) in mammalian cells a triple transfection using three different plasmids is commonly used. One plasmid carries the two AAV genes *rep* and *cap*, responsible for DNA replication and capsid proteins, respectively. The second plasmid encodes adenoviral helper genes, namely DBP, E4 and VA RNA. The DNA encoding the therapeutic gene, to bepackaged into the viral particles is supplied by a third plasmid. Packaging cell lines, that stably express all AAV components have the advantage that, only one plasmid, encoding the target gene, must be transfected, which in turn increases production efficiency. However, the expression of cytotoxic proteins such as the AAV gene *rep* or adenovirus helper genes in stable cell lines requires a tight regulation of gene expression. While the Tet-ON system is widely used for protein production in mammalian cells, its dependence on antibiotic induction can create issues in large-scale manufacturing. We propose the use of the polymerase from the bacteriophage T7 as an alternative option. It employs the T7 promoter, not recognized by the mammalian host polymerase. The expression is activated by the introduction of a helper plasmid for the expression of the T7 RNA polymerase directed to the nucleus by a nuclear localization signal. The extensive knowledge about the T7 system in bacteria can be translated to mammalian cells, enabling the fine-tuning of expression through the use of various promoter variants. In our study, we investigate the feasibility of this inducible system in suspension HEK293 and adherent CHO K1 cells. Therefore, we designed and introduced different plasmids carrying GFP under the control of the T7 promoter. Basal and induced expression levels were assessed by flow cytometry. Our experiments demonstrate the inducibility of protein expression under the control of the T7 system. These results lay the foundation for the development of stable cell lines for the expression of toxic proteins in mammalian cells, thereby enhancing their stability and potentially contributing to viral vector production.

**Application of Digital Tools to Support Cell Line Development**

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Biopharm Process Research (BPR), within the Drug Substance Development group at GSK, is responsible for understanding the CMC characteristics of the biopharmaceutical portfolio to deliver high quality, transformational medicines. This encompasses selecting by first intent, Phase III and Commercial ready cell lines, expressing developable, innovative molecules and de-risking these in the relevant aspects of the New Product Introduction (NPI) facility.

As part of selecting Commercial ready cell lines, several digital tools in BPR seek to identify these cell lines from early stages of the Cell Line Development process.

This poster presents some examples of digital approaches at different stages of development leveraging machine learning and modelling, which are currently being evaluated or used in BPR to aid Cell Line Development and Selection.

**Changes in the Chinese Hamster Ovary ubiquitinated proteome following a down-shift in culture temperature.**

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Since the 1980's, the Chinese Hamster Ovary (CHO) cell line has played a vital role in biopharmaceutical production, currently being the host cell line most used in biotherapeutics' production. These cells can undergo Endoplasmic Reticulum (ER) stress in response to environmental factors, causing enhanced unfolded protein response (UPR), which can potentially lead to bottlenecks in productivity. Ubiquitination is an essential part of the cell's ER stress response, and targets misfolded proteins for proteasomal degradation. Lowering of culture temperature is employed by the biopharmaceutical industry to enhance productivity of biotherapeutics, but the understanding of how the lower temperature causes these changes remains unclear. In this study, we characterised alterations to the whole cell proteome and the ubiquitinated proteome of a non-producer (CHO-K1) and an Erythropoietin producing CHO cell line (CHO SK15-EPO), following a reduction in culture temperature from 37°C to 31°C using High-Pressure Liquid Chromatography (HPLC)-tandem Mass Spectrometry (LC-MS/MS). Cells were grown in suspension at 37°C with a seeding density of 200,000 cells/mL. After 2 days, at the beginning of the exponential phase, cell culture temperature was shifted to 31°C and viability measured. Quantitative label-free LC-MS/MS proteomic analysis was performed on samples from day 4 and 7 at both 31°C and 37°C. Proteins were identified using Proteome Discoverer and exported to Progenesis QI for Proteomics for relative label-free quantitative analysis between experimental groups. Ubiquitinated peptide enrichment was performed via immunoprecipitation of diGly peptides using the PTMScan(R) Ubiquitin Remnant Motif (K-E-GG) Kit. As expected, the reduction in culture temperature results in a reduced growth rate, extended high culture viability and higher titre of EPO compared to the 37ºC culture. Both cell lines showed differences in protein expression and ubiquitinated peptide expression between temperatures at both days studied. Cells at 37°C showed greater expression of proteins related to protein folding and transport at both time points, while cells at 31°C show greater expression of UPR and caspase-mediated apoptosis related proteins. The level of ubiquitination present was highest in cells at day 4 at 31°C, with levels becoming more similar between both temperatures at day 7.

**Determination of the effect of trace metal variability on protein hydrolysate activity for BioPharma**

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The trace metal composition of serum-free media plays a crucial role in ensuring both the productivity and quality of recombinant proteins, including monoclonal antibodies. Protein hydrolysates (HL) as supplements significantly influence trace metals in producer cells' media, notably in cell lines like Chinese hamster ovary (CHO) cells. These HLs not only support optimal cell growth but also enhance the production of recombinant proteins. The challenge is that HLs are naturally heterogeneous in composition. Therefore, to be used as media supplements for potential therapies there is an increasing interest to develop the HLs as more defined and increase reproducibility. Within this work the trace metal content and uptake of HLs was determined by Inductively coupled mass spectrometry (ICP-MS). The effects of single metal salts at varying concentrations and combinations of metal salts when added directly to HLs was observed.

ICP-MS adeptly identified and quantified trace elements across varied HLs, showing bioactivity and accumulation characteristics. Sample preparation included acid digestion, dilution, and filtration. For individual metal salt studies, a 24 well-plate setup was employed, while metal-HL combinations were investigated within 50mL bioreactor tubes. ICP-MS unveiled distinct trace element variations in each HL, contributing to unique bioactivity profiles. Principal Component Analysis of the HLs' trace metal profiles demonstrated clustered separation by origin, indicating consistent uniqueness across batches. Cell uptake metal analysis revealed rapid non-linear accumulation. Single metal growth curves highlighted positive effects on cell growth, yet no individual metal outperformed HLs in enhancing IgG production. Combinations of metal salts failed to replicate HL effects, except for one notable combination.

In essence, employing ICP-MS to analyse trace elements in HLs enhances our comprehension of these elements' biological significance. To delve deeper into trace metal influences on CHO cells, we plan to identify the most potent bioactive combination. Additionally, this investigation will encompass two different media systems to assess whether distinct chemically defined setups impact HL performance divergently.

**How to engineer human pluripotent stem cells: generating human organoids to understand human development and disease**

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In recent years, considerable progress has been made in the development of faithful procedures for the differentiation of human pluripotent stem cells (hPSCs). An important step in this direction has also been the derivation of three-dimensional cell cultures that represent micrometer to centimeter size versions of human organs, the so-called organoids.

Here we will discuss current developments in the hPSCs-organoid field and emphasize the achievements and ongoing challenges of bringing together hPSC organoid differentiation, bioengineering and disease modelling with a particular focus on genetic and systemic disorders compromising kidney and heart. We will further discuss on how the convergence of stem cell biology and bioengineering now offers the possibility to provide physiologically relevant stimuli in a controlled fashion in these model systems resulting in the development of naturally inspired approaches to overcome major limitations of the organoid field.

**Multivalent IgM antibodies – New class of therapeutics with differentiated potency and safety profile**

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IgM antibodies offer unique structural advantages compared to commonly used IgG antibodies, including multivalent binding and availability of J-chain. We have developed recombinant IgMs using some of these features and protein engineering to enable generation of novel therapeutic options with differentiated potency and safety profiles. The greater avidity provided by ten binding arms of a pentameric IgM antibody can bind targets with significantly better affinity and selectivity, as well as allowing receptor clustering that is typically not feasible with bivalent binding provided by IgG antibodies. IgMs can bind multiple copies of a target simultaneously on the surface of tumor cells and create apoptotic signals that ultimately lead to death of cancer cells. Receptor agonists developed based on this platform have shown strong pre-clinical and promising clinical activity. Engineering of J-chain has enabled generation of IgM based T-cell engagers (TCE) by placing the T-cell targeting unit(s) on J-chain and using the multivalent binding of IgM core to target tumors in a very specific manner. Tumor specific antigens with much lower expression profile can be targeted efficiently because of IgM's increased avidity. The unique architecture of this IgM TCE platform may allow for a more physiological T-cell activation with a more favorable safety profile and pronounced activity. Our engineered IgM platform is being developed for both oncology and autoimmune indications. The facile engineering of J-chain is also being used to develop IgM therapeutics that can deliver cytokines to tumor milieu for greater effectiveness of existing activity and a favorable safety profile. IgM therapeutics based on all three modalities described above are being evaluated in clinic to assess their potential for better clinical responses. We have developed methods and technologies to efficiently produce engineered IgM antibodies at scale, overcoming a major challenge for development of this novel platform with yet untapped potential.

**Bispecific antibody shuttles targeting CD98hc mediate efficient and long-lived brain delivery of IgGs**

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The modest ability of antibodies to penetrate the blood-brain barrier (BBB) severely limits their use in diagnostic, imaging, and therapeutic applications. One promising strategy for brain delivery of IgGs is to use a bispecific BBB shuttle, which involves fusing an IgG to a second affinity ligand that engages a cerebrovascular endothelial target and facilitates transport across the BBB. Nearly all prior efforts have focused on the transferrin receptor (TfR-1) as the prototypical endothelial target despite inherent delivery and safety challenges. Here we report bispecific antibody shuttles that engage CD98hc, the heavy chain of the large neutral amino acid transporter (LAT1), and efficiently transport IgGs into the mouse brain parenchyma. Our work builds on a pioneering study that demonstrated the potential of CD98hc-mediated brain shuttling of antibody fragments (Zuchero et al., *Neuron*, 2016) and addresses several unique and unanswered questions. First, we have sought to deliver intact IgGs to the mouse brain using a 2x1 CD98hc shuttle that maintains the native structure of the brain-targeted IgG. Second, we have characterized the pharmacokinetics of untargeted IgGs shuttled into the brain via CD98hc and TfR-1. TfR-1 shuttles achieve earlier and higher peak brain concentrations, whereas CD98hc shuttles demonstrate slower decline in their brain concentrations, resulting in >100-fold higher blood:brain ratios at 7 days than those for their TfR-1 counterparts. Third, we have characterized the parenchymal engagement and cellular uptake of untargeted 2x1 CD98hc and TfR-1 shuttles. While TfR-1 targeting mediates internalization into various brain cell types for non-targeted IgGs, untargeted IgG/CD98hc shuttles remain associated with the cerebrovasculature. Fourth, we have incorporated IgGs that bind cell-type specific surface proteins on neurons, astrocytes, or oligodendrocytes into the 2x1 CD98hc shuttle, and shown that they display a profound temporal redistribution from initial localization at the cerebrovasculature to final localization at each brain cell type. Finally, we have demonstrated parenchymal delivery and receptor activation of a CD98hc-shuttled TrkB agonist IgG and are currently evaluating the neuroprotective activity of this agonist IgG in various acute and chronic neurological disorders.

**Heparin-binding motif mutations of human diamine oxidase allow the development of a first-in-class histamine-degrading biopharmaceutical**

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Excessive histamine concentrations in plasma and tissues cause unpleasant to life-threatening symptoms in mast cell activation syndrome, mastocytosis or anaphylaxis. Anti-histamines are often insufficiently efficacious. Human diamine oxidase (hDAO) can rapidly degrade histamine and therefore represents a promising new treatment strategy for conditions with pathological histamine concentrations.

Recombinant hDAO (rhDAO) is cleared from the circulation in rats and mice within less than five minutes due to heparan sulfate proteoglycan-mediated cellular internalization. This is highly unfavorable for a therapeutic protein that is intended to degrade plasma histamine. After replacement of positively charged amino acids of the heparin-binding motif with polar serine or threonine residues binding to heparin and heparan sulfate was strongly reduced. The double mutant rhDAO-R568S/R571T showed minimal cellular uptake. The short α-distribution half-life of the wildtype protein was eliminated, resulting in a significantly reduced clearance and a 6-hours half-life in rodents. The ability to degrade histamine was not impaired.

To improve the manufacturability, lower the immunogenicity and enhance the safety of rhDAO as a biopharmaceutical the unpaired cysteine 123 was mutated. Aggregate formation was thereby strongly reduced, without affecting the enzymatic activity or the in vivo plasma half-life.

The successful decrease in plasma clearance of rhDAO by mutations of the heparin-binding motif with unchanged histamine-degrading activity represents the first step towards the development of rhDAO as a first-in-class biopharmaceutical to effectively treat diseases characterized by excessive histamine concentrations in plasma and tissues.

**Design and Application of XmAb®: A Heterodimeric Fc-containing Bispecific Antibody Platform for Immunotherapy.**

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Xencor's XmAb® bispecific platform has enabled clinical development of anti-cancer drugs of at least three different classes: anti-CD3 x anti-(tumor antigen) bispecifics capable of recruiting T cells to tumor cells, bispecifics that simultaneously target two different immune checkpoint receptors or an immune checkpoint receptor and a costimulatory receptor, and immunomodulatory cytokines. The platform contains a heterodimeric Fc region, which empowers both bispecificity and altered valencies, while retaining the favorable developability profile of a monoclonal antibody. We have developed the platform by engineering isoelectric point differences into the Fc region, which facilitated straightforward purification of the heterodimeric species. The purification solution was later combined with a novel set of Fc substitutions capable of achieving heterodimer yields over 95% with little change in thermostability. Finally, we present manufacturing data reinforcing the robustness of the heterodimeric Fc platform at GMP scale.

## Protein-Specific Signal Peptides for Mammalian Vector Engineering

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Expression of recombinant proteins in mammalian cell factories relies on synthetic assemblies of genetic parts to optimally control flux through the product biosynthetic pathway. In comparison to other genetic part-types, there is a relative paucity of characterised signal peptide components, particularly for mammalian cell contexts. We describe a toolkit of signal peptide elements, created using bioinformatics-led and synthetic design approaches, that can be utilised to enhance production of biopharmaceutical proteins in Chinese Hamster Ovary cell factories. We demonstrate, for the first time in a mammalian cell context, that machine learning can be used to predict how discrete signal peptide elements will perform when utilised to drive ER translocation of specific single chain protein products. For more complex molecular formats, such as multichain monoclonal antibodies, we describe how a combination of *in silico* and targeted design rule-based *in vitro* testing can be employed to rapidly identify product-specific signal peptide solutions from minimal screening/design spaces. The utility of this technology is validated by deriving vector designs that increase product titres $\geq$ 1.8x compared to standard industry systems, for a range of products, including a difficult-to-express monoclonal antibody. The availability of a vastly expanded toolbox of characterised signal peptide parts, combined with streamlined *in silico/in vitro* testing processes will permit efficient expression vector re-design to maximise titres of both simple and complex protein products.

**Intensified cell-based virus production: a process development challenge for multiple cell-virus combinations!**

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Tilia Zinnecker, Max Planck Institute for Dynamics of Complex Technical Systems, Bioprocess Engineering Group, Magdeburg, Germany
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Key Words: Perfusion, high-cell-density, influenza virus, AMBR, screening

Development of cell culture-based viral vaccine antigen candidates use mostly adherent cells. Typically, maximal cell concentrations of about 2.0E06 cells/mL are infected to produce 1E07-1E08 infectious virus particles/mL in large scale manufacturing. Depending on the virus, cell-specific virus yields can be as low as 1-10 infectious virions/cell (Hepatitis C virus, flaviviruses) or up to 120 000 virions/cell (adenovirus). Host cells growing in suspension cultures are available, but typically a parallel cell line or even cell clone screening is not done easily. Improved cell culture media and new process technologies, such as automated single-cell cloning or parallel microbioreactor systems together with advanced perfusion systems allow for new approaches in viral vaccine development to be evaluated.

In this presentation, we report on process intensification via perfusion mode cultivations towards high-cell-density cultures for different suspension cell lines combined with various viruses, such as influenza A, Modified Vaccinia Ankara, yellow fever, Zika and vesicular stomatitis virus. Miscellaneous cultivation systems including shaker flasks, stirred tank and orbital shaken bioreactors were combined with several feeding strategies with a variety of perfusion devices, such as ATF (alternating tangential flow filtration), TFF (tangential flow filtration), TFDF (tangential flow depth filtration), acoustic filter and inclined settler. Maximum cell concentrations of 1.5E08 cells/mL and virus titers of 1E10 infectious virions/mL often with significantly improved cell-specific virus yields could be reached. The use of an AMBR15 system for cell line and clone screening will equally be discussed. Clearly, these process intensifications approaches could make a paradigm change for large scale manufacturing of next-generation vaccines.

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**Generation of multivalent enveloped VLPs based on the SARS-CoV-2 spike protein in engineered CHO cells.**

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Enveloped virus-like particles (eVLPs) are self-assembled nanostructures similar to viruses but that lack viral genetic material, rendering them non-infectious. In recent years, eVLPs have been increasingly well-regarded for vaccine development and cargo delivery. Herein, using endogenous retroviral-like particle (RVLP)-knockout CHO cells (CHO-C2), we generated multivalent eVLPs based on SARS-CoV-2 spike protein to incorporate distinct viral antigens such as influenza virus hemagglutinin (H1) and neuraminidase (N1).

Our eVLP production method is based on transient gene expression (TGE) in CHO-C2 cells with plasmids encoding the full-length spike (FL-S) protein and the desired H1 and N1 antigens. The self-assembled eVLPs are secreted into the culture media and purified by chromatography using a spike affinity resin. Western blot analysis and immuno-gold labeling transmission electron microscopy (TEM) were performed to confirm the presence of the co-expressed spike, H1 and N1 proteins on the FL-S eVLP surface. Preliminary *in vivo* data of bivalent FL-S / H1 and FL-S / H1 / N1 demonstrated that eVLPs induced S, H1 and N1 humoral and cellular responses.

Overall, FL-S eVLPs represent a novel platform that could potentially facilitate the manufacturing of bivalent SARS-CoV-2/Influenza vaccines.

## An Sf-rhabdovirus Risk Assessment

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The baculovirus-insect cell system is used to produce several licensed biologics, including vaccines and an immunomodulator. In addition, many companies are developing this system to manufacture other biologics, including rAAV vectors. Established cell lines derived from the caterpillar, *Spodoptera frugiperda* (Sf), are commonly used as the host component in this binary system. However, a landmark study from 2014 showed all Sf cell lines tested were contaminated with a novel rhabdovirus, now known as Sf-rhabdovirus (Ma et al., J. Virol. 88:6576). In this presentation, we identify the likely progenitor of cell line-associated Sf-rhabdoviruses as a contaminant of the caterpillar from which the original Sf cell line was derived. Interestingly, there is a significant difference in the matrix protein coding sequences of the Sf cell line- (Sf-RV) and caterpillar- (Sf-CAT-RV) associated viruses that might theoretically impact their infectivity and/or host ranges. Therefore, we investigated the infectivity and host ranges of both viruses. Our analysis of the susceptibility of mammalian cell lines to these viruses revealed Sf-CAT-RV, but not Sf-RV, can infect Vero, a kidney epithelial cell line derived from African green monkeys. Our further analysis of the susceptibility of severely immunocompromised (RAG2IL2R$^{-/-}$) mice to these two forms of the Sf-rhabdovirus suggested both could establish productive infections in these hosts, which persisted for about 2 weeks. However, these infections had no obvious adverse impacts on their health and were cleared. These results suggest neither Sf-RV nor Sf-CAT-RV, which could arise from the former by genetic reversion, are likely to be a serious threat to human health.

**Functionalizing Enveloped Nanoparticles via Click Chemistry: Optimization and Comparison between HIV-1 Gag-based Virus-Like Particles and Extracellular Vesicles**

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In recent years, enveloped nanoparticles such as extracellular vesicles (EVs) and virus-like particles (VLPs) have emerged as promising nanocarriers capable of transporting bioactive molecules for drug delivery and vaccination. Human Immunodeficiency Virus type-1 (HIV-1) Gag-based VLPs are produced by transient transfection in HEK293 cells overexpressing the Gag polyprotein of the HIV-1 virus fused in frame with enhanced Green Fluorescent Protein (eGFP) to distinguish VLPs from the coproduced EVs. A methodology based on a copper-free click chemistry approach, concretely the bio-orthogonal copper-free azide-alkyne cycloaddition reaction is proposed to functionalize both nanoparticles. Dibenzocyclooctyne-sulfo-N-hydroxysuccinimidyl ester (DBCO-sulfo-NHS), that can link any amine containing molecules present on the envelope of VLPs and EVs, is used as a bifunctional crosslinker to covalently link azide-containing molecules to nanoparticles' surface. Cy5-azide is used as reporter molecule to quantify nanoparticle functionalization, calculated as the average number of Cy5 molecules covalently linked per nanoparticle. The covalent union of Cy5 has been demonstrated by western blot. Reaction kinetics at different temperatures have been carried out to determine the optimal reaction conditions, achieving an average of 274 Cy5 molecules covalently linked per particle. A Design of Experiments (DoE) has been conducted to optimize nanoparticle concentration and the concentrations of both reagents, resulting in a 3.4-fold increase in nanoparticle functionalization. The difference in functionalization between VLPs and EVs has been studied using super-confocal microscopy, revealing a significantly lower functionalization rate in VLPs (4.8%) compared to virtually 100% in EVs. These findings demonstrate the potential of functionalizing VLPs and EVs through click chemistry and reveal valuable insights into membrane composition between Gag-VLPs and co-produced EVs, which could be used to design improved purification strategies to separate both nanoparticles.

**Evaluation of AAV gene toxicity in mammalian cell culture for more efficient rAAV production**

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Gene therapies have emerged as a top trend in modern medicine, holding significant potential for treating diseases at the genetic level. Among the various delivery systems available, adeno-associated virus (AAV) vectors have gained attention for in vivo gene therapy due to their favorable safety profile and high efficiency in gene delivery. However, one major challenge in the field of AAV-based gene therapies is the limited production yields of recombinant AAV vectors (rAAVs), preventing their manufacturing at large-scale. Moreover, the expression of AAV proteins in the producer cells can lead to cellular toxicity, stress and eventually apoptosis, impacting titers and the final yield of the produced rAAV [1].

AAV genes are organized in an overlapping manner within the AAV genome, maximizing the utilization of the limited genetic capacity. In this work, AAV gene sequences were engineered to isolate each gene product for individual evaluation. Every AAV gene was cloned into three different vectors with varying promoter strength to enable their study at different expression levels. Each of them was studied individually and in combination in HEK293 cells to assess their impact on cell culture, focusing on evaluating their toxicity over a period of three days.

The acquired quantitative data provide insight into the gene-specific and combinatorial toxicity of AAV genes. These results allow for a better characterization of AAV gene toxicity profiles and shed light on the expression levels at which each protein exerts its toxic effects, which was found to vary among the studied proteins.

Understanding of the toxicity profiles of AAV genes can guide the development of more efficient vectors for use in triple transfection, a common technique for generating rAAVs, and design of cell engineering strategies, offering the opportunity to enhance rAAV production yields.

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**Production of virus-like-particles in a baculovirus-free insect cell expression system for antibody development**

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For vaccination and diagnostics, the expression of virus-like-particles (VLPs) in high quality is essential. One of the most often used expression systems for VLPS is the baculovirus expression vector system (BEVS) due to its high yields. However, it is restricted in respect of adaption of the ratios of the required structural proteins and VLP quality might be hampered by low cell viability. Furthermore, purification of VLP is often challenging due to simultaneously produced baculoviral particles and proteins. Here, we show that our baculovirus-free High Five cell expresses VLPs in high yields and quality, avoiding the pitfalls of BEVS. Fluorescent SARS-CoV-2 VLPs were evaluated by Nanotracking analyisis (NTA), ELISA, cytometer and microscope (TEM, cLM, Figure 1A) confirming ~145 nm diameter, ACE2 binding and the typical "Corona" aura. We used this fluorescent VLPs to develop a high-throughput assay to screen our anti-SARS-CoV-2 antibody candidates for inhibition of the binding to ACE2 positive cells in cytometer (Figure 1B). Furthermore, transferability of the system for the production of other VLPs like Hantavirus and Influenza was tested and confirmed by TEM. Finally, we compared yields archived by BEVS to our baculovirus-free system, underlining the great potential of our flexible plasmid-based method.

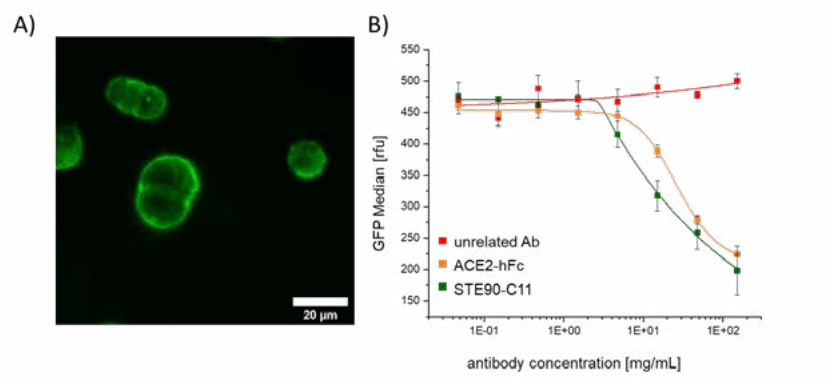


*Figure 1: A) Fluorescent SARS-CoV-2 VLPs binding on the surface of ACE2 positive Expi293 cells. B) Fluorescent signal measured in cytometer of fluorescent SARS-CoV-2 VLPs binding to ACE2 positive Expi293 cells in presence of an unrelated antibody (red), soluble ACE2-mFc (orange) and our anti-SARS-CoV-2 antibody STE90-C11 (green). (Jaron, M. et al „Baculovirus-Free SARS-CoV-2 Virus-like Particle Production in Insect Cells for Rapid Neutralization Assessment" Viruses 2022)*

**Process development and intensification for the production of SARS-COV-2 spike protein in CHO fed-batch cultures**

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Exploratory data analysis on early process development datasets can allow to understand how measured variables and process conditions relate to each other and how they impact protein production. In this investigation, batchwise principal component analysis was carried out so as to identify key variables in the production of SARS-CoV-2 spike protein by stable CHO cell pools across different reactor systems (Multifors and BioFlo-1L). Once key features were determined, XGBoost predictive models were built to forecast endpoint titers. It was found that extending culture longevity and reducing peak lactate accumulation were paramount to improving process outcomes, and that oxygen requirements were closely related to protein production. With this gained knowledge, further process development was performed in 1.8 L benchtop stirred-tank bioreactors (DASGIP parallel bioreactor system). The objective was to improve process conditions such that key variables would be kept in the ranges previously determined to yield high protein production. Consequently, DO set points and air caps were chosen on the basis of improving culture longevity. Once hydrodynamic conditions were tuned to an optimal zone, various dynamic feeding strategies were developed and compared to static bolus feeding. Feeding based on oxygen uptake rate, biocapacitance signal and cell counts were assessed. With the biocapacitance-based strategy, improved integral of viable cell concentration (1.4-fold), culture longevity (1.93-fold increase in endpoint viability) and protein yield (2.8-fold) were achieved when compared to the unoptimized process. It was also determined that dosage is a critical parameter given that slow continuous feeding increased longevity when compared to repeated bolus additions. This study emphasizes the importance of exploratory data analysis as it can be used to focus efforts on strategies that improve key metrics impacting protein production. It also highlights the benefits of designing feeding strategies around metabolically relevant signals and dosing them in such a way that nutrient concentration variation is diminished.

**Cell line and upstream process development for high density perfusion processes: Strategies for large scale high quality continuous upstream processes**

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Fed-batch processes remain the dominant culture mode for the clinical supply of biopharmaceuticals. Continuous biomanufacturing offers a paradigm shift due to the inherent advantage of higher productivity allowing the implementation of smaller process equipment which results in cost-effective, lean and agile manufacturing facilities. FUJIFILM Diosynth Biotechnologies have developed a connected and integrated process to support continuous manufacturing process development anywhere from DNA to product at large scale production. Moving to continuous manufacturing we must re-evaluate and leverage the correct platform technologies (host cell line, expression vector, cell line development process, cell culture media/feed and process control strategy) to rapidly identify the critical cellular performance criteria important for continuous manufacturing processes. Multiple mAb expressing Apollo™ X CHO DG44 cell lines were generated in a traditional fed-batch cell line screening process and evaluated in a scale down perfusion process to identify the key cellular performance criteria for high quality continuous manufacturing cell lines selection. Next, an exemplar cell line was selected to display process performance at multiple bioreactor scales. We have shown the application of both a new cell line development and continuous upstream production platform results in reliable and high quality continuous upstream biomanufacturing processes for multiple Apollo™ X CHO-DG44 derived cell lines and recombinant monoclonal antibody products. During perfusion cell line development all cell lines achieved >60x$10^6$ cells/mL irrespective of growth performance at fed batch, and the smaller scale reactor work was a good predictive tool for process development/cell line screening. In both small scale and large scale models a titre of 1.8g/L/day (> 40g/L cumulative) was achieved with comparable product quality. High product quality and titre yield provides promising data for the future of biopharmaceutical development. Given the current climate and need for large volume production of biopharmaceuticals, a system that can produce ~ 5 times the amount of yield of a fed-batch process with a smaller and easily deployable facility is highly desirable.

**Effects of Autophagy-inhibiting Chemicals on Sialylation of Fc-fusion Glycoprotein in Recombinant CHO Cell Culture**

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Cell death is one of the critical issues in recombinant Chinese hamster ovary (rCHO) cell culture because it affects the quality and productivity of therapeutic glycoproteins. Autophagy, known as type II of programmed cell death (PCD), is a catabolic pathway that recycles intracellular components by lysosomal degradation to use as substrates for biosynthesis and energy generation. In this study, we investigated the effects of autophagy inhibition on sialylation of Fc-fusion glycoprotein in rCHO cell culture. Three chemical inhibitors known to inhibit each different stage (SP600125, LY294002, and bafilomycin A1) were individually treated in two rCHO cell lines producing the same Fc-fusion glycoprotein derived from DG44 and DUKX-B11. The isoform distribution and sialylated *N*-glycan formation of Fc-fusion glycoprotein were analyzed by isoelectric focusing (IEF) gel analysis and anion exchange liquid chromatography. All chemical inhibitors significantly decreased the proportion of highly sialylated *N*-glycans of Fc-fusion glycoprotein in both cell lines. Given the high viability on day 1 of two cell lines treated with the three chemical inhibitors, we expect that sialylation damage was not due to sialidases released by cell membrane disruption but rather altered intracellular events. The expression of *N*-glycosylation-related genes and the amount of intracellular nucleotide sugars, which are important factors of the *N*-glycosylation pathway, were examined to elucidate the decreased sialylation by inhibiting autophagy. These results suggest that the decreased sialylation of Fc-fusion glycoprotein was not due to altered expression of *N*-glycosylation-related genes but to the limited availability of intracellular nucleotide sugars. Taken together, the results from this study indicate that autophagy inhibition has detrimental effects on sialylation of Fc-fusion glycoprotein by reducing the availability of intracellular nucleotide sugars.

**Benefits and considerations for intensification of biologics manufacturing process to meet product demand**

Venkata Tayi

Merck & Co., Inc., Rahway, New Jersey, USA

The demand for biologics to satisfy the unmet medical needs has been increasing over the last few decades. The processes to produce biologics from mammalian cells have evolved over time starting with simple batch process to well optimized fed-batch processes. While speed-to-clinic is the focus in the initial stage of drug development that naturally dictates the need to go with a simple process like fed-batch, the possibility of increasing demand for the product drives the necessity to develop a high productivity process. This results in the efforts to improve biologics manufacturing processes through process intensification. While bioprocess intensification through either intensified fed-batch or intensified continuous manufacturing process has benefits in terms of increased volumetric productivity, low cost-of-goods and reduced manufacturing scales, the factors like product quality comparability, operational complexity, process portability to different manufacturing sites will also influence the process selection. Bioprocess intensification results in product-producing cells at a state of very high cell densities which creates an environment of significant nutrient demand as well as a high level of inhibitory metabolites accumulation that ultimately dictates the productivity and quality of therapeutic proteins. A stoichiometrically well balanced cell culture media/feed system that supports high nutrient demand while reducing unnecessary metabolites accumulation will ultimately result in a best process with high productivity. Intensified continuous manufacturing has significant benefit over intensified fed-batch in terms of reduced metabolites accumulation while maintaining relatively high cell densities. This provides an overall advantage of at least 2-fold higher productivity and consistent product quality for intensified continuous manufacturing. However, scale-up feasibility for both processes will ultimately dictate the overall advantage of one process over the other. This presentation topic will cover the aspects of bioprocess intensification of cell culture processes with relevant examples.

**The role of extracellular vesicles in the modulation of the cell density effect**

Jesús Lavado-García[1,2], Pol Pérez-Rubio[1], Laia Bosch-Molist[1], Elianet Lorenzo-Romero[1], Laura Cervera[1], Francesc Gòdia[1]

[1] Universitat Autònoma de Barcelona (UAB), Barcelona, Spain
[2] The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark (DTU), Lyngby, Denmark.

One of the current challenges in the animal cell bioprocessing field is the so-called cell density effect (CDE). It is defined as the reduction in cell-specific productivity when transient gene expression is carried out at increasing densities. Although it has affected bioprocess intensification for many years, the molecular causes of the CDE remain unknown to date. When studying high cell density (HCD) cultures, one remarkable trait is the high production of extracellular vesicles (EVs), leading to a high EV concentration in the extracellular medium. In this work, we observed that EVs directly impacted transfection efficiency. Based on the study of how EVs interact with their surroundings at the extracellular and intracellular space, different strategies were implemented, restoring transfection efficiency at HCD.

Our results suggest that EVs directly influence transfection efficiency of cell cultures via both physicochemical and cellular interactions. Upon cryo-TEM and NTA characterization, EVs were observed to physically disrupt DNA:PEI polyplexes, altering their integrity, size and electrodensity. The resulting low-quality polyplexes significantly reduced transfection efficiency. Transfection was fully inhibited when polyplexes were exposed to conditioned medium from high cell density cultures and was completely restored after EV-depletion. Likewise, EVs also act as vehicles for cell-to-cell communication. EVs coming from HCD cultures triggered physiological changes in low cell density (LCD) cultures emulating the CDE at LCD. These physiological changes were studied with confocal microcopy and high throughput proteomics. After EV fractionation, all proteins carried in the EV fraction responsible for blocking transfection were identified, characterizing the signaling molecules triggering this physiological shift. Based on these results, UGCG enzyme was overexpressed to mitigate these effects, improving transfection efficiency by 15%.

These findings could shed some light on the molecular causes behind the CDE and allow the implementation of new bioprocessing strategies to overcome it, paving the way for the improvement and intensification of transfection-based bioprocesses.

**Toward the implementation of Integrated Continuous Bioprocessing for Mab Manufacturing. From bench scale to 2kL-Clinical Manufacturing production-scale.**

Cyril Boucher, Johan Chami, Jean-Marc Bielser, Xavier Le Saoût, Pierre Moretti, Jonathan Souquet
Merck KGaA - Switzerland

The challenges facing the pharmaceutical industry require a focus on developing more efficient and cost-effective production processes while ensuring compliance with regulatory requirements. Agility, versatility and constant technology innovation are primordial to accelerate first-in-human clinical studies.

At Merck KGaA, our ambition was to develop a solution for clinical manufacturing production at 2kL-scale allowing an increase in our day-to-day process productivity, decreasing our cost of goods while maintaining a reduced footprint. This solution based on new technology implementation was named COMPAC$^2$T™ *(Continuous Mode Purification And Cell Culture Technology)* and combined a modular upstream process (USP) based on single-use technology (SUT) with a continuous purification process.

Our journey started in 2016 by testing our vision at lab-scale and by challenging it at 200L-scale between 2017 and 2019. In 2020, our story was well-advanced after the conclusion our first GLP pre-clinical run at 200L-scale demonstrating the successful implementation of perfusion based on SUT and the development of an integrated continuous purification.

At this point, it was the moment to transform our vision into reality. Starting 2020, a huge effort on class-D suites revamping, process intensification and new technology implementation at 2kL-scale was performed. Beginning of 2022, a shake-down run was performed to fine-tune our process and solve the last technical issues. Finally, in October 2022, the first GMP end-to-end clinical manufacturing production was concluded in the generation of almost 94 Kg of Drug Substance in accordance with our quality specifications. This presentation will describe the journey from concept to implementation of an end-to-end continuous manufacturing process for clinical production.

## See the wood for the trees: A holistic route to an optimized AAV production process

D Karthaus[1], K Teschner[1], V Ortseifen[1], N Kraemer[1], P Brinkert, F Sundermann, A Vetter[1], M Schulz[1], T Steffens[1], S Klausing[1]

[1] Sartorius Xell GmbH, Germany

The promise to provide long lasting therapeutic effects, especially for monogenetic diseases, has resulted in over 2000 viral-based therapeutics projects in the pre-clinical and clinical pipelines. With an expected compound annual growth rate (CAGR) of more than 22 % from 2020 to 2030, gene therapy has become an important market for the biotechnology and pharmaceutical industry. The vast majority of viral vectors is presented by adeno associated virus (AAV) expressed by HEK293 as preferred host. Viral vector manufacturers are facing unique challenges to reduce time-to-market and cost of goods sold (COGS), as viral expression processes are still highly variable. Optimization must consider diverse factors like cell line, serotype, plasmids, cell culture media, transfection conditions and the cultivation process as well as complex analytics to characterize the final product.

In this work, we present a “Design Space” approach, addressing these key process variables. A media panel was tested with different cell lines revealing the impact of media formulation on cell growth and increased AAV titer. Design of experiments (DoE) was used to optimize process parameters, identifying stirring speed and pH as relevant factors in Ambr15®-based processes. Most processes currently use a transient transfection approach. As transfection is highly inefficient, and only a minor portion of plasmid DNA is taken up into cells, the choice of reagent and optimization of DNA:reagent ratio can lead to major improvements in titer. Using DoE, we determined optimal transfection conditions in different media. Furthermore, we identified components to enhance AAV production and optimized enhancer concentrations to increase AAV titers by more than three-fold. Enhancing capability was seen for single small molecules, supplement mixtures and feeds. Finally, an extensive analytical portfolio was applied to characterize AAV quantity and quality. Overall, our holistic approach facilitates, accelerates, and increases the chance of selecting the best media and processes for AAV production.

**Label-Free Quantitative Proteomics Analysis of CHO-DP12 and CHO-K1 under ER Stress Conditions**

Christiana-Kondylo Sideri[1,2], Michael Henry[1], Esen Efeoglu[1], Paula Meleady[1,2,3]

[1]National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland

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[3]SSPC, The Science Foundation Ireland Research Centre for Pharmaceuticals, Limerick, Ireland

Chinese Hamster Ovary (CHO) cells are the most commonly used host cell line used in the biopharmaceutical industry for the production of vaccines, monoclonal antibodies and recombinant proteins due to their ease of culture, high productivity, reduced apoptosis and their ability to replicate human-like post-translational modifications such as glycosylation. However, the lack of knowledge of mechanisms involved in protein production, folding and secretion still remain a bottleneck preventing improved production and quality. In CHO cell cultures, expression of high levels of recombinant biopharmaceuticals is linked to inducing endoplasmic reticulum (ER) stress, causing enhanced unfolded protein response (UPR) levels to maintain cell viability and productivity. This study aims to enhance our understanding of ER stress mechanisms in CHO cells. Mass spectrometry-based (MS) proteomics and subsequent gene ontology analysis of IgG producer (CHO DP-12) and non-producer (CHO-K1) CHO cells were investigated focusing on protein folding, UPR and ERAD. Both cell lines were treated with tunicamycin, which inhibits N-glycosylation of newly synthesised polypeptide chains in the ER, and with the proteasome inhibitor MG132, which can reduce the degradation of ubiquitin-conjugated proteins. Upon induction of ER stress, MS differential expression data between treated and control cells identified a series of upregulated and downregulated proteins, especially in ER related stress pathway and ubiquitin mediated degradation, which have not been associated with ER stress previously. These can be further studied to understand their involvement in the production of biotherapeutics.

# Investigating the molecular basis of cellular recognition using large panels of recombinant proteins expressed in mammalian cells: a case study using the human immune system

Jarrod Shilts[1,2] and Gavin J. Wright[1]

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Individual cells within metazoan organisms must communicate with one another to ensure that they function collectively as a coordinated biological system. Frequently, this intercellular communication is initiated by specific extracellular protein–protein interactions involving membrane-embedded cell surface receptor proteins that transduce signals to elicit an appropriate cellular response. Because of their accessibility, cell surface proteins are considered excellent drug targets but despite their therapeutic potential, identifying extracellular interactions has remained an underexplored area due to the biochemical challenges of manipulating membrane proteins and the typically fleeting nature of their interactions.

To ultimately gain knowledge of extracellular protein:protein interactions on a genome-wide scale, we have developed a scalable assay based upon detecting direct binary binding events between hundreds of different recombinant proteins expressed in mammalian cells. The assay can be applied to a range of different biological contexts and reliably and specifically detects interactions with monomeric half-lives of less than a second and with a negligible artifactual false positive rate.

To illustrate the scalability of this approach we have systematically mapped the interactions between 630 different receptor proteins that represent the cell surface receptor repertoire of human leukocytes. We identified 28 new interactions that were independently validated, resulting in a high-confidence view of the receptor wiring that connects human immune cells. This work provides a systematic perspective on the intercellular communication within the human immune system and may provide new opportunities for therapeutic intervention.

**Effects of Autophagy-inhibiting Chemicals on Sialylation of Fc-fusion Glycoprotein in Recombinant CHO Cell Culture**

Hoon-Min Lee[1, 2], Jong-Ho Park[2, 3], Gyun Min Lee[3], and Yeon-Gu Kim[1, 2]

[1]Department of Bioprocess Engineering, KRIBB School of Biotechnology, University of Science and Technology (UST), Korea
[2]Biotheapeutics Translational Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Korea
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**See the wood for the trees: A holistic route to an optimized AAV production process**

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## Chemical Filtering: A Post-Transfection Directed Evolution Strategy to Improve Productivity of Difficult-to-Express Antibodies in CHO Cell Lines

Author: Chillel Jawara, PhD student, Cell Line Development, Astrazeneca, Cambridge, UK
Astrazeneca supervisor: Sarah Dunn, Cell Line Development, Astrazeneca, Cambridge, UK
University of Sheffield supervisor: David C James, Department of Chemical and Biological Engineering, University of Sheffield

Difficult-to-express (DTE) proteins are characterized by low product yield, poor product quality and reduced culture viability. Determining an appropriate engineering strategy to improve productivity often requires a lengthy troubleshooting process and the results are likely to be cell line- and product-specific. This poster presents a small molecule-based strategy utilizing directed evolution principles to enrich cell pools expressing a DTE antibody for cells with improved productivity.

**In silico design of CMV promoter binding oligonucleotides and their impact on inhibition of gene expression in Chinese hamster ovary (CHO) cells**

Hussein M.K.[1,2], Maja Papež [1,2], Baumann M[1], Dhiman H[1,2], Galosy S[3], Borth N[2]

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The modulation of expression levels of endogenous genes or transgenes can be of great interest for diverse applications, e.g. to study genotype-phenotype relations via knock-down or overexpression of the gene of interest, or to fine-tune expression strength to study interconnections and synergies. During the last decades, several tools in the field of synthetic biology have been established for controlling gene expression in mammalian cells such as Chinese Hamster Ovary (CHO) cells, one of the most important cell system for basic research as well as for the production of biopharmaceuticals. These tools include, amongst others, the use of non-coding RNAs (siRNAs, miRNAs) interacting with mRNA to reduce translation, or the more recent method CRISPR in combination with repression or epigenome modifying domains to reduce or activate transcription.

Here we describe the use of non-coding RNAs (triplex-forming oligos – TFOs) that directly interact with DNA to form RNA-DNA-DNA triplexes. A panel of TFOs were designed to specifically bind to promoter regions and block expression of the downstream gene. As proof of concept, we used the viral cytomegalovirus (CMV) promoter/enhancer region, often used for driving strong expression in mammalian cells. Initial TFOs were designed and tested. TFOs achieved a decrease in CMV activity in a transient expression system. A point mutation within the CMV promoter was introduced to resolve a binding hindrance, thus enabling the design of longer TFOs (26–30 nts). Selected TFOs achieved a reduction in recombinant hCD4 expression of up to 85% in CHO-K1 cells.

Hussein, Mohamed K., et al. "In silico design of CMV promoter binding oligonucleotides and their impact on inhibition of gene expression in Chinese hamster ovary cells." Journal of Biotechnology 359 (2022): 185-193.

## Optimization of cytokine production in mammalian cells

Ece Cagdas[1], Lise M. Grav[1], Nathan E. Lewis [2], Lars K. Nielsen[3]

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Biopharmaceuticals are commonly manufactured using mammalian cells due to their ability to produce therapeutic proteins with human-like post-translational modifications. Among the mammalian cell systems, Chinese hamster ovary (CHO) cells are the most widely used for biopharmaceutical production due to their high productivity and suitability for large-scale manufacturing. However, optimal biopharmaceutical production requires suitable cell hosts with properly configured secretory machinery. Low yield production can occur if the secretory machinery does not match the target product, rendering the product economically unviable.

Cytokines that are of major therapeutic importance and play a crucial role in treating various diseases are often considered difficult to produce. They can be used to treat many diseases such as cancer, autoimmune diseases and viral infections. The human secretome study revealed that some cytokines are produced in lower levels (ex. GM-CSF and IL21) or cannot be produced (ex. IL15) in CHO cells. Previous studies have shown that certain cytokines are not produced efficiently in CHO cells but can be produced in the human embryonic kidney 293 (HEK293) cells. To determine the molecular basis for this difference, we will analyze RNAseq library to compare gene expression profiles of good and poor cytokine producing cells and tissues. We aim to identify secretory machinery genes that are critical for cytokine production. We have some cells are correlated with cytokine expression. These candidate genes will then be screened for their impact on cytokine-productivity using multiplex CRISPR activation in cytokine-producing cell lines. Ultimate goal is to develop high cytokine-producing CHO and HEK293 cell lines by addressing the specific requirements of each biopharmaceutical product through data-driven engineering.

**Quantitative Gene Expression Control Using the ROSE LP Platform: A Reproducible and Sustainable Transcriptional Regulation for CHO Cell Engineering**

Sung Wook Shin[1], Honggi Min[1], Jiwon Kim[1], Jae Seong Lee[1,2]*

[1]Department of Molecular Science and Technology, Ajou University, Suwon 16499, Republic of Korea, [2]Department of Applied Chemistry and Biological Engineering, Ajou University, Suwon 16499, Republic of Korea

Quantitative and sustainable regulation of gene expression in mammalian cells is invaluable for dynamic cell engineering. Compared to transiently delivered or randomly integrated inducible elements, targeted-integration (TI) can provide reproducible transcriptional fold induction and precise gain-of-function (GOF) phenotypes in cells. In this regard, we employed CRISPR/Cas9-mediated TI of four different combinations of doxycycline (dox)-inducible all-in-one payloads containing landing pad (LP) sequences, which allowed subsequent replacement of various effector genes via recombinase-mediated cassette exchange (RMCE), in two safe harbor loci in CHO cells. We demonstrated that integration site-dependent epigenetic silencing of TetOn3G actuator expression cassettes via promoter methylation contributed to an inducible gene expression performance. Of the eight combinations between integration sites and vector design, one optimized inducible expression cassette design, termed as the Robust Overexpression via Site-specific replacement of Effector (ROSE) LP, rendered homogenous and sustainable expression control of engineering target genes at the defined locus. The one-copy integrated stable ROSE LP cell line exhibited dynamic dose-dependent gene expression, showing an increase of ~350-fold in EGFP transcripts upon treatment with 1 μg/mL dox. Based on these findings, we evaluated whether our ROSE LP platform cell lines can be readily applied for stoichiometric functional gene studies and dynamic CHO cell engineering. Quantitative expression control of the monoclonal antibody heavy chain and effector genes resulted in a dose-dependent GOF phenotype (i.e., specific productivity, antibiotics resistance, etc.). Collectively, our data suggest that the ROSE LP platform can provide a robust, stable, and reusable transcription regulation system for diverse CHO cell engineering.

**Screening Vector Elements for Improved Antibody Production in CHO Cell Lines**

Marzia Rahimi, Anna Christina Adams, Jesús Lavado-García and Lise Marie Grav[1]

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Chinese hamster ovary (CHO) cells are widely recognized for their ability to produce monoclonal antibodies (mAbs) with human-like post-translational modifications. However, achieving high antibody yields in CHO cells requires careful optimization. Recent advances in gene manipulation and vector engineering have shown promise in enhancing antibody productivity. Nevertheless, each antibody requires individual tuning of all the different genetic elements due to their unique characteristics, leading to a costly and time-consuming optimization process.

In this study, we employed vector engineering techniques to screen genetic elements for the expression of six different antibodies and their impacts on productivity in CHO cells. Our expression vectors were carefully designed to evaluate alternative codons in variable and constant regions, signal peptides, lambda and kappa light chain isotypes, and specific mutations in the constant domain of the heavy chain. These engineered vectors were transiently expressed in CHO cells, assessing the influence of each genetic element on antibody productivity by measuring antibody titers.

Our findings demonstrate that modifications of each vector element yield different effects on different antibodies. Generally, optimizing codons within variable regions enhances antibody expression, although the degree of improvement varies among antibodies. Notably, certain antibodies exhibited remarkable increases of up to 10-fold, while others only showed a 2-fold increase. Similarly, the choice of signal peptide significantly impacted the expression levels of different antibodies, resulting in varied outcomes. Furthermore, substituting the kappa light chain isotype with lambda led to decreased expression for specific antibodies, while modifications to the codons of the constant heavy chain caused a notable decrease in antibody expression across all antibodies. Interestingly, incorporating or removing specific mutations in the constant region of the heavy chain did not produce significant changes in any antibody expression.

Our study underscores the importance of personalized customization and fine-tuning strategies to maximize the yield and productivity of individual antibodies in CHO cells. By highlighting the diverse impacts of vector engineering techniques, including codon optimization, signal peptide selection, and light chain isotype substitution, we emphasize the need for tailored approaches to achieve optimal results. These findings contribute to the advancement of CHO cell-based production systems and provide valuable insights for the optimization of therapeutic antibody manufacturing.

**Leap-in Transposases(R) - A New Paradigm of Cell Line Development**

Ferenc Boldog, Sowmya Rajendran, Maggie Lee, Molly Hunter, Candice Tallsalt, Nicolay Kulikov, Thomas Purcell, Mark Welch, Claes Gustafsson*, Oren Beske and Jeremy Minshull

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The generation of robust and stable cell lines for the commercial production of protein therapeutics is critical. Current methodologies to introduce recombinant genes into production strains relies on random integration, a method limited by poor integration rates, concatemer formation, transgene rearrangements and instability. To address these limitations and others, ATUM has developed the Leap-In Transposase(R) platform. This flexible and robust platform enables the precise and stable integration of genes of interest. This remains true with large and complex constructs with multiple open reading frames (ORF's) each under discrete expression control. Taken together, the Leap-In platform enables the robust generation of stable high expressing cell lines for routine and complex molecules such as bispecifics and multispecifics.

**Strategies to remove Single Cell Cloning from Genetic Knock Out Screens in Host Cell Line Engineering**

Jannis Marzluf[1,2], Jennifer Klein[1], Christoph Zehe[1] and Ann-Cathrin Leroux[1]

[1]Sartorius Stedim Cellca GmbH, Ulm, Germany,
[2]University of Ulm, Ulm, Germany

Chinese Hamster Ovary (CHO) cell lines are the most common used host for biopharmaceutical production. Novel difficult to express biomolecules, increasing productivity and quality as well as challenges in serving market demands define the need for constant improvements in the host cell lines used. Genetic engineering is used to optimize metabolic balances by adjusting gene expression levels or adverse proteins may be removed by knocking out coding genes to improve performance, productivity, quality and ease of downstream processing. While refined single gene knock out (KO) host cell lines can be easily generated by genetic engineering utilizing the CRISPR toolbox, throughput, timelines and screening of putative genetic targets still display significant challenges in host cell line engineering.

Here, we present a highly efficient workflow utilizing CRISPR gene editing to screen candidate genes for KO using stable CHO pools. We show that generated pools are genetically stable for long periods of time (>6 weeks) in single KO and up to 7x multiplexed KO approaches. These stable pools are able to capture the phenotype observed in monoclonal KO cells, reproducing previously confirmed performance effects on the bioprocess. Furthermore, the evaluation via KO pools can represent the heterogeneity observed in the host cell without the need to generate tens of KO clones for each candidate gene. This enables a gene target capacity increase of 250 % with the same workload and an additional timeline reduction of 9 weeks to 5 weeks when compared to our old process based on the generation and fed-batch evaluation of 10 to 12 KO clones for each addressed gene target.

We have successfully identified multiple genes for an enhanced bioprocess phenotype and the generation of refined host cell lines using this novel workflow.

**Study of the effects of anti-apoptotic genes in CHO cell cultures via targeted integration.**

David Catalán-Tatjer[1], Saravana Kumar Ganesan[1], Ivan Martínez-Monge[1], Jesús Lavado-García[1], Lise Marie Grav[2] and Lars Keld Nielsen[1, 3]

[1]The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Denmark, [2] DTU Bioengineering, Technical University of Denmark, Denmark, [3]Australian Institute for Bioengineering and Nanotechnology, University of Queensland, Australia

The overexpression of specific genes for the development of high producer cell lines can be performed using two different strategies: random and targeted integration. Random integration is the preferred method in the industry as, when properly coupled with high throughput screening, it is able to achieve the highest titers of protein ever recorded. However, the final phenotype may be due to the intended genetic modification or as a consequence of the screening process. To be able to discriminate between these two options, industrious efforts are needed to characterize the genome location, expression levels and epigenetic modifications. A solution to this problem is offered by targeted integration in which the location of the insertion and the copy number is shared in all subsequent cell progeny.

In this project, the effect of overexpressing anti-apoptotic genes via targeted integration to enhance productivity in CHO cells has been studied. Apoptosis is triggered in mammalian cells under stressful conditions, such as nutrient depletion, high osmolality or accumulation of toxic by-products. Several previously reported works use random integration as a method to generate anti-apoptotic-expressing cell lines, thus presenting a challenge to compare the effects of the different expressed genes.

Here, CRISPR/Cas9 and recombinase-mediated cassette exchange (RMCE) were used to develop isogenic EPO-producing cell lines expressing relevant anti-apoptotic genes as bcl-2, bcl-$x_L$ and mcl-1 to shed light on this topic. As anticipated by the literature, we observed a 2-fold increase in EPO specific productivity ($q_P$) when overexpressing bcl-$x_L$. Interestingly, the same increase was observed overexpressing bcl-2, contrary to published literature.

In conclusion, this work demonstrates that only via targeted integration can we compare the effect of different genes and accurately perform cell line engineering studies. In our case, to select the best genes to mitigate apoptotic effects in CHO cell cultures.

**Towards a comprehensive CRISPR-Cas9 deletion screen strategy using a paired guide RNA approach**

Ivy Rose Sebastian[1,2], Federico De Marco[1,2], Antonino Napoleone[1,2], Nina Bydlinski[1], Martina Baumann[1], Nicole Borth[2]

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CRISPR-Cas9 is a powerful and versatile genome editing tool that provides a potent strategy to gather in-depth genetic information and engineer cell lines with specific genotypic and phenotypic requirements. In our study, we exploit the efficiency and flexibility of the CRISPR system by employing a paired guide RNA (pgRNA) strategy to ensure a complete functional knockout of selected genes-of-interest within the Chinese Hamster Ovary (CHO) genome.

To test the functionality of our approach in a pooled format, we simulated a small-scale deletion, where selected pgRNA sequences covering a broad range of deletion sizes, spanning from 12 to 106 kbps, were co-transfected with a Cas9-encoding plasmid into a CHO-K1 cell line. In all cases, deletion PCR conducted on genomic DNA clearly showed that these deletions were introduced at their respective Cas9 targeted sites. Furthermore, mRNA expression surveyed by qRT-PCR also showed a reduction in gene expression, even at a pool level.

In conclusion, we successfully showed that the different design decisions employed for the pgRNA expression cassette resulted in precise and expected deletions. Overall, this study sets an essential baseline required for setting up functional genome-wide deletion screens and could be extended for similar applications within the larger scope of industrial biotechnology.

## Unravelling lentiviral transduction heterogeneity in difficult-to-transduce suspension cell lines for genome-wide CRISPR-Cas9 screening

**Antonino Napoleone[1], Ivy Rose Sebastian[1], Federico De Marco[1], Nicole Borth[2]**

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Uncovering gene functions on a broad scale using CRISPR-Cas9 screens has proved to be an efficient strategy due to its enormous potential for discovering new cell line engineering targets. The main strength of CRISPR-Cas9 is the programmability of the system through the delivery of engineered guide RNAs (gRNAs), that can be designed to target and edit specific genomic loci. These gRNA libraries are commonly packaged and delivered through lentiviral vectors, which enable stable and efficient integration into the host cell genome. Nevertheless, there are many critical factors, including a high rate of lentiviral-induced genetic recombination, as well as the susceptibility of target cells to infection, which, if neglected, could add noise to the final data output.

In this study, we selected two suspension-adapted mammalian cell lines, based on their well-known employment in recombinant protein production, namely, CHO-K1 (Chinese Hamster Ovary) and HEK293-6E (Human Embryonic Kidney) cells. We established an optimised protocol based on a 3rd generation lentiviral system to enhance the transduction efficiency to a level where our CRISPR-Cas9 library delivery becomes feasible and consistent at a genome-wide scale. Based on the susceptibility to lentiviral infection, which is largely determined by intrinsic cell line properties and the transduction methodology used, two distinct procedures—static transduction and spinoculation—were investigated. The variation in the integration profile was further assayed at a genomic level by droplet digital PCR (ddPCR), to simultaneously quantify both the lentiviral vector and the gRNAs promoter sequences across the two cell lines. Hence, with this method, we have not only shown that it is possible to ensure a single integration in our transduced population but have also revealed the underlying heterogeneity of lentiviral transduction at efficiencies greater than 30%.

Overall, we were able to show a cell line-dependent response to lentiviral integration and establish a consistent strategy to increase the transduction efficiency in difficult-to-transduce cell lines, demonstrating the importance of modulating the infection rate to limit the presence of multiple integrations, hence minimizing one of the main sources of noise that commonly bias the outcome of pooled CRISPR screens.

## Evaluation of AAV gene toxicity in mammalian cell culture for more efficient rAAV production

Konstantina Tzimou[1], Jesús Lavado-García[2], Lise Marie Grav[1], Lars Keld Nielsen[2]

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Gene therapies have emerged as a top trend in modern medicine, holding significant potential for treating diseases at the genetic level. Among the various delivery systems available, adeno-associated virus (AAV) vectors have gained attention for in vivo gene therapy due to their favorable safety profile and high efficiency in gene delivery. However, one major challenge in the field of AAV-based gene therapies is the limited production yields of recombinant AAV vectors (rAAVs), preventing their manufacturing at large-scale. Moreover, the expression of AAV proteins in the producer cells can lead to cellular toxicity, stress and eventually apoptosis, impacting titers and the final yield of the produced rAAV [1].

AAV genes are organized in an overlapping manner within the AAV genome, maximizing the utilization of the limited genetic capacity. In this work, AAV gene sequences were engineered to isolate each gene product for individual evaluation. Every AAV gene was cloned into three different vectors with varying promoter strength to enable their study at different expression levels. Each of them was studied individually and in combination in HEK293 cells to assess their impact on cell culture, focusing on evaluating their toxicity over a period of three days.

The acquired quantitative data provide insight into the gene-specific and combinatorial toxicity of AAV genes. These results allow for a better characterization of AAV gene toxicity profiles and shed light on the expression levels at which each protein exerts its toxic effects, which was found to vary among the studied proteins.

Understanding of the toxicity profiles of AAV genes can guide the development of more efficient vectors for use in triple transfection, a common technique for generating rAAVs, and design of cell engineering strategies, offering the opportunity to enhance rAAV production yields.

[1] Wang, D., Tai, P.W.L. & Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. Nat Rev Drug Discov 18, 358–378 (2019).

**Production of virus-like-particles in a baculovirus-free insect cell expression system for antibody development**

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For vaccination and diagnostics, the expression of virus-like-particles (VLPs) in high quality is essential. One of the most often used expression systems for VLPS is the baculovirus expression vector system (BEVS) due to its high yields. However, it is restricted in respect of adaption of the ratios of the required structural proteins and VLP quality might be hampered by low cell viability. Furthermore, purification of VLP is often challenging due to simultaneously produced baculoviral particles and proteins. Here, we show that our baculovirus-free High Five cell expresses VLPs in high yields and quality, avoiding the pitfalls of BEVS. Fluorescent SARS-CoV-2 VLPs were evaluated by Nanotracking analyisis (NTA), ELISA, cytometer and microscope (TEM, cLM, Figure 1A) confirming ~145 nm diameter, ACE2 binding and the typical "Corona" aura. We used this fluorescent VLPs to develop a high-throughput assay to screen our anti-SARS-CoV-2 antibody candidates for inhibition of the binding to ACE2 positive cells in cytometer (Figure 1B). Furthermore, transferability of the system for the production of other VLPs like Hantavirus and Influenza was tested and confirmed by TEM. Finally, we compared yields archived by BEVS to our baculovirus-free system, underlining the great potential of our flexible plasmid-based method.

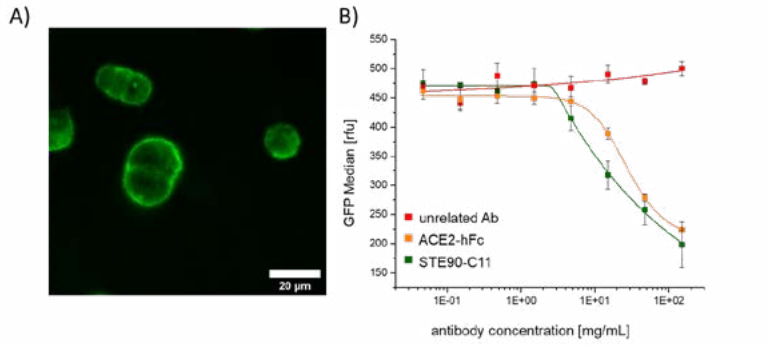


*Figure 1: A) Fluorescent SARS-CoV-2 VLPs binding on the surface of ACE2 positive Expi293 cells. B) Fluorescent signal measured in cytometer of fluorescent SARS-CoV-2 VLPs binding to ACE2 positive Expi293 cells in presence of an unrelated antibody (red), soluble ACE2-mFc (orange) and our anti-SARS-CoV-2 antibody STE90-C11 (green). (Jaron, M. et al „Baculovirus-Free SARS-CoV-2 Virus-like Particle Production in Insect Cells for Rapid Neutralization Assessment" Viruses 2022)*

**Alternative Antivenom Production through Mixing of CHO cell lines**

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Polyclonal antibodies continue to be used in many clinical applications including fighting infectious diseases. Snake antivenoms is an example where a mixture of antibodies is critical to neutralize a broad spectrum of toxins found in snake venom. Synthetic anti-venoms made of multiple humanized monoclonal antibodies (mAbs) produced by individual CHO cell lines would overcome the issue of xeno-immunogenicity of conventional antivenoms (derived from horses), while enabling the design of antivenoms targeting multiple snake species. Producing individual batches of mAbs and mixing them afterwards is associated with significant validation costs and single batch production of mAb cocktails would thus be attractive. We selected four snake toxin-specific antibodies against Sub-Saharan African snake species and generated stable anti-toxin producer clones through targeted integration in CHO cells, creating isogenic cell lines. In this way, we aim to reduce clonal variation, possibly resulting in cell lines behaving similarly in a mixture. Mixing cell lines showed reproducible cell line and antibody ratios across Batch and Fed-Batch scale. Perfusion data is currently underway. We tested protein A - purified product in an ELISA (Enzyme-Linked ImmunoSorbent Assay) analysis and the mixture bound to all four toxins, leading to the assumption of it being able to neutralize the toxins. These results suggest that it would be possible to mix and match cell lines to produce tailored antivenoms biosynthetically containing human antibodies without the use of horses as production host.

**Beyond exponential phase: Metabolic phenotypes in the stationary phase of CHO cell cultures**

Jerneja Štor[1,2], David Ruckerbauer[2,3], Diana Széliová[3], Sarah Sacco[4], Jamey Young[4], Nicole Borth[1], Jürgen Zanghellini[3]

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Chinese Hamster Ovary (CHO) cell lines are typically grown in rich media to ensure high titers of recombinant proteins. However, it has previously been shown that the excessive supply of amino acids (AA) in the media leads to the accumulation of intermediates of AA metabolism, with some of these negatively affecting cell growth, productivity and product quality. Here, we use carbon-13 metabolic flux analysis (13C-MFA) to identify differences in central carbon and AA metabolism between (i) exponential (exp.) and stationary (stat.) phases and (ii) between a producer (PC) and a non-producer (NPC) cell line. We performed fed-batches with a temperature shift for both PC and NPC and extensively sampled each in exp. and stat. phases to determine phenotypic behavior and nutrient exchange rates. For the 13C-MFA we used both [1,2-13C2]glucose and [U-13C5]glutamine as tracers. Comparing the cell lines in both phases, the PC generally showed moderately higher exchange rates for high-flux AAs and metabolites, with no considerable differences for low-flux AAs. Overall, major differences were observed between the exp. and stat. phases. Specifically, exchange rates significantly dropped for most measured metabolites and AAs, except for lactate which switched to consumption. On the intracellular level we observed high fluxes through the end reactions of glycolysis and through the entire TCA cycle; in terms of the cell lines, these fluxes were higher in the PC compared to the NPC, indicating that this behavior is a consequence of the antibody (mAb) production. When the cultures transitioned to the stationary phase, both cell lines maintained high fluxes through the TCA cycle, despite the aforementioned general decrease in exchange rates. Interestingly, we observed significant drops in the production of aspartate and glutamate and, at the same time, higher fluxes through catabolic reactions of several essential AAs. This indicates that under stressful conditions, i.e. limited availability of the typical carbon sources glucose and glutamine, the cells revert to alternative sources of carbon to produce TCA intermediates. We observed that the burden of mAb productivity is reflected in the central carbon metabolism and that the cells undergo major metabolic changes during stat. phase in order to adapt to the stress and energetic demands associated with mAb production.

**Development of a transient protein expression process with media and feeding strategy optimization**

Fabian Holenstein[1], Kevin Fischer[2], Theresa Stelzer[1], Gregg Baldeshwiler[1], Simone Popp[1]

[1]Department Novartis Institute for Biomedical Research, NIBR Biologics Center – Novartis Pharma AG, Basel, Switzerland, [2]Zürich University of Applied Sciences, Wädenswil, Switzerland

The aim of this work was to develop a new protocol for the transient transfection of HEK293-6E cells with the goal of producing antibodies in which the process is free of animal components while achieving at least the same level of quantity and quality as with the established protocol 1. Thereby cover our needs for the efficient preparation of therapeutic antibody candidates in the early in vitro and in vivo profiling phase. Together with testing of different feed systems, optimal cell densities during transfection and the DNA:PEI ratio, it was possible to develop a protocol that is free of animal components and achieved an equal or higher titer in 6 out of 8 tested antibody productions. Variations from these protocols are expected for CHO and other HEK-293 cells maintained in other laboratories under different culture conditions.

**Establishment of a membrane protein expression capability for affinity screening and structure-based drug design**

Jenny Gunnarsson[1], Melanie Dannemeyer[1], Carina Raynoschek[1], Arjan Snijder[1], William Lee[1], Robert Roth[1]

[1]Reagents and Assay Development, Discovery Sciences, AstraZeneca

Membrane proteins constitute a large fraction of the proteome in the cell and play an important role in many cellular functions such as signal transduction, cell adhesion and transportation. Membrane proteins are therefore a major category of drug targets for pharmaceuticals. However, due to their unique physical properties and requirement for association with cellular membranes, expression of membrane proteins is often challenging. Further, the development of methods to isolate the proteins in sufficient amounts and quality to enable characterization and structural studies is required.

We have recently established a new capability within AstraZeneca to develop, validate and streamline bioreagent production of difficult-to-produce membrane proteins for DNA-encoded library screening, biophysical mode of action studies, and to support cryo-EM structure-based drug design.

This work includes a wide range of tools from molecular biology, protein expression, purification, quantification, and characterization. We use novel approaches in construct design to modulate protein expression yields and functionality. Predominantly, insect and mammalian cells, using both plasmid-based and virus-based delivery methods, are preferred expression hosts. Additionally, cell line engineering has been applied to improve yields of membrane protein expression in human embryonic (HEK) cells. Expression protocol optimizations with a focus on transient culture conditions allowed us to further enhance expression yields.

Using this new capability, we have produced high-quality membrane protein reagents of several different protein classes such as ion channels and GPCR's which enabled DNA-encoded library affinity screening, the determination of high-resolution cryo-EM structures, and mode of action studies.

**Mammalian highthroughput expression platform (mHTX) for protein reagent generation and beyond**

Maja Firczuk, Angita Shrestha, Alex Zwetsloot, Gurdaman Singh, Michael Mullin and Kate Smith, GSK, UK

Purified recombinant proteins are essential reagents for many drug discovery projects. There are multiple fit for purpose criteria, such as purity, stability, correct oligomeric state as well as function dependent, case specific requirements. To meet these requirements the protein needs to be expressed at suitable level in correctly folded, non-aggregated state, with affinity tag accessible for purification. Thus, the design of expression construct is critical for success. Computational options can guide the design, but outcomes need to be tested experimentally. For this purpose at GSK we developed a mammalian highthroughput expression platform (mHTX). It allows design, generation, and expression tests of hundreds of constructs in parallel. This screen is then complemented by small-scale one step purification for multiple hits, which gives a good approximation of yield and purity.

In this talk I will introduce the mHTX process and the challenges encountered while developing robust, high-throughput and automation friendly methodologies. I will present examples demonstrating utility of the platform, how it was instrumental for delivering both secreted and membrane protein reagents, delivering a selection of constructs to be tested in X-ray crystallography or for identifying best tag combinations for assays. This high-throughput approach also enables us to assess feasibility of purifying novel, unprecedented protein targets, allowing to de-risk this aspect of launching a new drug discovery project.

The applications of mHTX platform go beyond identification of highly expressed constructs towards the optimization of cellular reagents for different applications. We are constantly working on expanding our workflows – optimizing automation and data capture solution, exploring high-throughput technologies for protein QC and expression and screening for protein complexes.

**Setting Up an Effective Protein Expression Workflow**

Nan Yang, Alex Kirkpatrick, Matt McKenna, and Rich Altman

Thermo Fisher Scientific

5781 Van Allen Way, Carlsbad, CA, 92008

Transient protein expression technology enables study of gene regulation and protein structure and function. Utilization of recombinant protein expression can also vary widely—from investigation of function in vivo to large-scale production for biotherapeutic drug discovery and structural studies. Using the right protein expression system for your specific application is critical to success. Consider protein solubility, functionality, purification speed, and yield when choosing an expression system. We offer a wide selection of superior mammalian, insect, yeast, bacterial, algal, and cell-free protein expression systems to suit your research needs.

**Understanding Cellular Limitations of HEK293**
**Characterization of genetic and epigenetic state**

Georg Smesnik, Nikolaus Virgolini, Astrid Dürauer and Nicole Borth

Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria

Human embryonic kidney cells (HEK293) have emerged as a good expression system for the production of difficult to express proteins and viral vectors, such as adeno-associated virus (AAV). As characteristic for other immortalized cell lines, HEK293 cells have also been reported to reveal genomic instability causing a continuously evolving and rearranging genome. Events such as chromosomal translocations or copy number alterations were observed through long-term cultivation and subcloning. In addition, changes to the epigenome are linked to stress response or adaptations and were recently shown during AAV production. This raises the question how these molecular mechanisms influence manufacturing processes. The objective of this study is to improve the understanding of the genetic and epigenetic variability of HEK293 cell lines in response to various culture conditions. Therefore, adherent HEK293 cells were adapted to suspension growth, using various commercially available serum-free media formulations. Upon adaptation, cells were phenotypically characterized and whole-genome and DNA-methylome analyses performed and compared to parental, adherently growing cells, as well as to other commercially available HEK293 suspension cell lines. Overall, this study generates a basis for further omics characterization and improves our understanding of cellular limitations and genomic and epigenomic instability of HEK293 cells, which could be leveraged to improve the system towards enhanced product quality and titers in manufacturing.

**A Low-temperature SPR-based Assay for Monoclonal Antibody Galactosylation and Fucosylation Assessment Using FcγRII A/B**

Catherine Forest-Nault[1], Jimmy Gaudreault[1], Yves Durocher[2] and Gregory De Crescenzo[1]

[1]Department of Chemical Engineering, Polytechnique Montreal, Canada,

[2]Human Health Therapeutics Research Centre, National Research Council of Canada, Canada

The N-glycosylation profile of monoclonal antibodies (mAbs) plays a crucial role as a Critical Quality Attribute (CQA) due to its influence on pharmacodynamic and pharmacokinetic properties, including binding to Fcγ receptors (FcγRs). Surface plasmon resonance (SPR)-based assays have been widely used to analyze the impact of N-glycosylation on the interaction between IgG1 and FcγRs. However, the 1:1 kinetic model often exhibits poor fit, leading to a wide range of thermodynamic and kinetic constants. In this study, we propose an enhanced SPR-based assay in combination with a different analysis approach. By utilizing a coiled-coil mediated tethering approach for FcγRs, we conducted binding experiments at different temperatures. Notably, performing the interactions at 10°C enhanced the kinetic dissimilarities between glycoforms of Trastuzumab (TZM), uncovering subtle differences that were not observed at the standard temperature of 25°C. We observed higher affinity of galactosylated TZM with FcγRIIA, while afucosylated TZM displayed increased affinity with FcγRIIB. The fast kinetics of these FcγRs can contribute to accelerate the analysis process and reduce material consumption, enabling more efficient real-time monitoring. To analyze the sensorgrams, we evaluated the area under the curve during the dissociation phase, employing an integral of a sum of exponential decays to describe a heterogeneous analyte model. This approach provided valuable insights into the binding dynamics and complemented traditional affinity-based studies. Overall, our study demonstrates the potential of enhanced SPR assays and glycoengineering approaches for investigating the impact of N-glycosylation on IgG1-FcγR interactions. The proposed data analysis techniques based on the shape of the SPR signal offer valuable insights into the behavior of glycoforms, facilitating the development of improved therapeutic antibodies. Our developed technology holds significant promise for real-time monitoring of mAb glycosylation during cell culture production and the rational design of novel mAb glycoforms.

**Biotinylation of recombinant proteins by co-expression with BirA in a range of different cell hosts**

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Biotin (Vitamin B7) is an essential coenzyme that is required by all organisms. Biotinylation is the process of attaching biotin to a protein which is carried out by biotin protein ligases (BPLs). Biotin binds Streptavidin and Avidin with very high affinity and this affinity is exploited in a variety of assay formats including biophysical approaches like surface plasmon resonance (SPR). Proteins can be specifically biotinylated using the natural specificity of the *E. coli* protein BirA. BirA specifically biotinylates a single lysine (K122) on the BCC subunit of acetyl-CoA carboxylase. Thus, adding this acceptor peptide sequence to a protein allows specific biotinylation using recombinant BirA. Biotinylation of recombinant proteins can be carried out by co-expressing the target protein along with BirA. To achieve this aim, we designed a series of constructs for intracellular co-expression in *E. coli*, intracellular and secreted co-expression in HEK/CHO and insect cells. Biotin is added to a final concentration of 4μM at the point of IPTG induction (for *E. coli*) or at the point of co-transfection/co-infection (for HEK/CHO & insect respectively). Biotinylation was confirmed across all expression systems using a combination of intact mass spectrometry and peptide mapping as part of the routine quality control package. The data shows that biotinylation of recombinant proteins can be carried out by co-expressing the target protein along with BirA. This can be done across all the expression systems we currently offer at Peak Proteins (*E. coli*, mammalian (HEK/CHO), insect) and we regularly use this technology to biotinylate proteins for our clients for a range of uses including compound screening and SPR. This offers significant advantages such as savings in time and material costs.

**Heparin-binding motif mutations of human diamine oxidase allow the development of a first-in-class histamine-degrading biopharmaceutical**

Elisabeth Gludovacz[1, 2], Kornelia Schuetzenberger[3], Marlene Resch[2], Katharina Tillmann[4], Karin Petroczi[2], Markus Schosserer[1], Sigrid Vondra[5], Serhii Vakal[6], Gerald Klanert[1], Jürgen Pollheimer[5], Tiina A. Salminen[6], Bernd Jilma[2], Nicole Borth[1], Thomas Boehm[2]

[1]Department of Biotechnology, University of Natural Resources and Life Sciences, Vienna, Austria, [2]Department of Clinical Pharmacology, Medical University of Vienna, Austria, [3]Center for Medical Physics and Biomedical Engineering, Medical University of Vienna, Austria, [4]Center for Biomedical Research, Medical University of Vienna, Austria, [5]Department of Obstetrics and Gynecology, Medical University of Vienna, Austria, [6]Structural Bioinformatics Laboratory, Biochemistry, Faculty of Science and Engineering, Åbo Akademi University, Turku, Finland

Excessive histamine concentrations in plasma and tissues cause unpleasant to life-threatening symptoms in mast cell activation syndrome, mastocytosis or anaphylaxis. Anti-histamines are often insufficiently efficacious. Human diamine oxidase (hDAO) can rapidly degrade histamine and therefore represents a promising new treatment strategy for conditions with pathological histamine concentrations.

Recombinant hDAO (rhDAO) is cleared from the circulation in rats and mice within less than five minutes due to heparan sulfate proteoglycan-mediated cellular internalization. This is highly unfavorable for a therapeutic protein that is intended to degrade plasma histamine. After replacement of positively charged amino acids of the heparin-binding motif with polar serine or threonine residues binding to heparin and heparan sulfate was strongly reduced. The double mutant rhDAO-R568S/R571T showed minimal cellular uptake. The short α-distribution half-life of the wildtype protein was eliminated, resulting in a significantly reduced clearance and a 6-hours half-life in rodents. The ability to degrade histamine was not impaired.

To improve the manufacturability, lower the immunogenicity and enhance the safety of rhDAO as a biopharmaceutical the unpaired cysteine 123 was mutated. Aggregate formation was thereby strongly reduced, without affecting the enzymatic activity or the in vivo plasma half-life.

The successful decrease in plasma clearance of rhDAO by mutations of the heparin-binding motif with unchanged histamine-degrading activity represents the first step towards the development of rhDAO as a first-in-class biopharmaceutical to effectively treat diseases characterized by excessive histamine concentrations in plasma and tissues.

**Production and purification of human peptide hormone fusion proteins**

Alexander Stadler[1], Davide D'Angelo[1], Nicolas Marx[1], Marko Poglitsch[2], Nicole Borth[1]

1. Department of Biotechnology, BOKU University of Natural Resources and Life Sciences Vienna, Austria.

2. Attoquant Diagnostics, Vienna, Austria

Background and novelity:

Peptide cascades play an important role in human physiology and follow complex regulatory and release kinetics. A more detailed study of these mechanisms and the development of novel analytical techniques requires the production peptide precursor molecules which is frequently hampered by the fact that final precursor peptide processing is affected by post-translational modifications specific to different protein expression systems. Here, we are developing a platform for the production and purification of diverse peptide hormones using preselected scaffolds and cleavage by a versatile protease. A controlled proteolytic release of target peptides from a fusion protein, can be used for diverse analytical and research applications, where a biologically active peptide hormone is constantly released over time.

Experimental approach:

Fusion proteins of different carrier scaffolds and hormone peptides, with a his-tag attached by a linker, were recombinantly expressed in Chinese hamster ovary cells. The fusion proteins were designed so that the bioactive peptide can be released by proteolytic cleavage after His-Trap purification in a controlled manner. The efficiency and stability of peptide release is dependent on the protease and its cleavage site, as well as its molecular environment defined by correct folding and also post-translational modifications.

Results and discussion

In our first experiments we focused on production and purification of the fusion proteins constructs to confirm the correct sequence and folding of the final peptides and that the purification tag does not have a negative influence on product formation. Therefore, different combinations of carrier proteins, linkers and peptides were transiently expressed in CHO cells. Expression was confirmed by Western Blot and the products were purified chromatographically utilizing a HisTrap. Additionally, the release of the final peptides from the carrier protein was confirmed by Western Blot and the correct structure of the peptides was analyzed by LC-MS/MS. With this approach we hope to provide a platform that can be used to establish controllable constant levels of short living peptides in future cell culture experiments, to better study their function.

**IgG glycoengineering in cells: Natural switches of Fc-receptor and complement-mediated activities.**

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Antibodies of the IgG class are the most abundant immunoglobulin type in humans and most mammals. It contains a single N-linked glycan in the Fc-region, required for its functional activities through IgG-Fc receptors (FcγR) on myeloid and NK cells, as well as complement mediated effector functions through the classical pathway. This N-linked glycan is highly variable, consisting of a biantennary glycan with a variable amount of galactose, low abundance but variable amount of sialic acid, variable amount of galactose, but is almost exclusively fucosylated. However, certain immune responses in humans evoke abundant afucosylated IgG responses which can be either transient (COVID-19), or long lived (malaria, alloimmune responses in pregnancy) lasting at least a decade. Meanwhile, newly formed immune responses are particularly rich in galactose, which subsides within weeks or months. IgG-galactosylation is also highly increased during pregnancy, but decreases with advancing age, especially during menopause and is associated with autoimmune flairs. Our research has shown how to generate glycovariants in cellular systems to probe their functionality. Of these variations, absence of fucosylation enhances binding and effector functions though FcγRIII, while galactosylation enhances IgG hexamerization and thereby binding to C1q resulting in subsequent complement activation. These changes are highly relevant in infectious diseases, autoimmunity, vaccine responses, and for cancer applications, where the right glycosylation pattern can determine both protective capacity, but also result in life-threatening pathologies in extreme cases.

## Predicting Antibody Production with a Dynamic Genetic Construct Simulator

Alina Ferdman, Georgian Tutuianu, Alec A.K. Nielsen
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The design of antibody expression vectors is currently a manual and imprecise process. Scientists typically use a fixed vector to express protein chains, which sacrifices production titers for simplicity. More advanced vector design requires analysis of multiple scientific publications to find genetic parts and relevant design rules, typically relying on intuition and mental models to make design decisions. Optimizing vector designs is challenging because the feedback loop from making a change to collecting experimental data to evaluate that change is extremely slow, and the factor space of possible improvements to explore comprises a combinatorial explosion of parameters.

To address these challenges and provide scientists with quantitative, objective, and accurate design tools, we developed a simulation platform to predict the dynamic behavior of a user-defined genetic construct. By modeling molecular and cellular processes, the platform outputs the expression trajectory of the final antibody protein complex. In addition to predicting a user-defined genetic construct's behavior, the platform is also able to automatically generate designs by algorithmically searching the genetic design landscape and using the simulator to evaluate design candidates. We apply this platform to optimize antibody production in Chinese hamster ovary cells.

Concretely, the simulation platform uses ordinary differential equations to biophysically model transcriptional flux, translational flux, mRNA concentration, protein concentration, protein complexation, ligand binding, protease cleavage, cis contextual effects, extracellular secretion, and copy number, among other functionalities. The model is parameterized using high-throughput experimental characterization of constitutive genetic parts. On a data set consisting of part combinations not seen during training, our model predicted relative expression of fluorescent proteins in expression vectors in CHO with an $R^2$ correlation coefficient of 0.9. Our simulation platform has the potential to improve the design and thereby increase the titer of antibody expression vectors by reducing the extent of time consuming experimental screens and shortening the design-build-test-learn feedback loop for genetic design optimization.

**A Low-temperature SPR-based Assay for Monoclonal Antibody Galactosylation and Fucosylation Assessment Using FcγRII A/B**

Catherine Forest-Nault[1], Jimmy Gaudreault[1], Yves Durocher[2] and Gregory De Crescenzo[1]

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The N-glycosylation profile of monoclonal antibodies (mAbs) plays a crucial role as a Critical Quality Attribute (CQA) due to its influence on pharmacodynamic and pharmacokinetic properties, including binding to Fcγ receptors (FcγRs). Surface plasmon resonance (SPR)-based assays have been widely used to analyze the impact of N-glycosylation on the interaction between IgG1 and FcγRs. However, the 1:1 kinetic model often exhibits poor fit, leading to a wide range of thermodynamic and kinetic constants. In this study, we propose an enhanced SPR-based assay in combination with a different analysis approach. By utilizing a coiled-coil mediated tethering approach for FcγRs, we conducted binding experiments at different temperatures. Notably, performing the interactions at 10°C enhanced the kinetic dissimilarities between glycoforms of Trastuzumab (TZM), uncovering subtle differences that were not observed at the standard temperature of 25°C. We observed higher affinity of galactosylated TZM with FcγRIIA, while afucosylated TZM displayed increased affinity with FcγRIIB. The fast kinetics of these FcγRs can contribute to accelerate the analysis process and reduce material consumption, enabling more efficient real-time monitoring. To analyze the sensorgrams, we evaluated the area under the curve during the dissociation phase, employing an integral of a sum of exponential decays to describe a heterogeneous analyte model. This approach provided valuable insights into the binding dynamics and complemented traditional affinity-based studies. Overall, our study demonstrates the potential of enhanced SPR assays and glycoengineering approaches for investigating the impact of N-glycosylation on IgG1-FcγR interactions. The proposed data analysis techniques based on the shape of the SPR signal offer valuable insights into the behavior of glycoforms, facilitating the development of improved therapeutic antibodies. Our developed technology holds significant promise for real-time monitoring of mAb glycosylation during cell culture production and the rational design of novel mAb glycoforms.

**A regulatory update on guidance for quality of biopharmaceuticals**

Anne Trappe[1]

[1]Health Products Regulatory Authority, Dublin, Ireland.

The ICH Q8/Q9/Q10 guidelines provide a comprehensive approach to pharmaceutical development, manufacturing, and post-approval activities, with a focus on quality by design (QbD), risk management, and continuous improvement.

The guidelines are considered essential for the development, manufacturing, and post-approval activities of biopharmaceutical products. The guidelines are used by regulatory authorities to assess the quality, safety, and efficacy of biopharmaceutical products and to ensure that they are consistent with regulatory standards.

They provide a common language and framework for regulatory authorities and industry stakeholders, which helps to facilitate communication and collaboration between these groups. Effective communication between these groups provides an opportunity for a risk-based approach to be applied to drug development and approval. A risk-based approach can provide flexibility by allowing companies to focus their efforts and resources on the areas of greatest risk to patient safety and product quality and in turn can help to streamline the drug development process and reduce unnecessary regulatory burden.

Regulatory flexibility, based on the ICH Q8/Q9/Q10, is essential to support innovation in the biopharmaceutical industry and ensure that new drugs can be developed and brought to market in a timely and efficient manner. It is important that this flexibility is balanced with appropriate regulatory oversight and a commitment to ensuring the safety, efficacy, and quality of pharmaceutical products.

**Deploying Systems and Synthetic Biology for the Optimization and Enhancement of Glycoprotein Therapeutics and Subunit Vaccines**

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Protein glycosylation is fundamentally important to most biological processes and critical to the safety and efficacy of most biopharmaceuticals. However, the diversity and complexity of glycosylation challenge efforts to identify the optimal glycoforms of drugs, let alone control the glycan structures and unravel how engineering efforts impact the host cells.

To enable rational glycoengineering of cells for drug manufacturing and elucidate how cell engineering strategies impact the host, we comprehensively studied the impact of glycoengineering on more than 180 CHO cell clones, wherein each has single or multiple glycosyltransferase genes knocked out. First, the clones were all glycoprofiled, and we developed a novel computational platform to rapidly study the changes in glycosylation across all mutants. Second, we quantified the impact of different glycosyltransferase knockouts on the bioprocessing phenotypes of the CHO cells (e.g., cell size, growth, viability, and metabolism). Third, we conducted a large-scale RNA-Seq study of the clones to study the molecular basis of the phenotypic changes. Finally, we used the resulting knowledge and a computational toolbox to build a refined panel of engineered cells, containing combinations of knockouts and knock-ins of 17 genes associated with glycosylation.

Using this geCHO BioDesign platform, we are able to screen for and identify optimal glycoforms of therapeutic proteins and identify how one can engineer mammalian cells and bioprocess conditions to obtain the desired glycosylation. We have deployed this platform to develop glycoengineered biosimilars, biobetters, and a substantially enhanced subunit vaccine for hepatitis C. Thus, we are now seeing big data glycomics, systems biology and synthetic glycobiology accelerate the design and control complex therapeutics for diverse disease indications.

## Bispecifics – different formats bring different treatment opportunities but also different CMC challenges.

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Bispecific molecules entering clinical trials and obtaining market authoritization have increased dramatically over the past few years. Amongst the 12 monoclonal antibodies and derivatives that were granted first approval in EU or US during 2022 alone, 4 (33%) were bi-specifics. For cancer treatment alone, hundreds of bispecifics are presently under pre-clinical development or going through clinical studies. To date, about 9 bispecifics have obtained market approval for therapeutics treatment of patients, primarily in various cancer indications. Here, T-cell engaging bi-specifics is a class of particular interest due to their high efficiency in engaging the immune system and typical low-dose requirements compared to mAbs.
With respect to structural design, multiple different formats are being tested by the many sponsors in the field. Some scaffolds have proven to be more successful than others, both with respect to efficiency in each indication, immunogenicity and with respect to CMC properties like manufacturability, titer and yield. For example, yield may be affected by purification methods that can be applied as well as by the molecule's ability to fold correctly and to avoid incorrect pairing in case of multichain formats. Product stability could also vary in comparison with mAbs that typically show long-term stability. Most bi-specifics do contain at least some IgG domains since these have proven beneficial in mitigating the CMC challenges mentioned above. Quality assessment of bi-specifics follow the same guideline principles as other biologics, but with enhanced focus on both the dual functional/binding properties of the molecules and on the structural quality aspects of the individual format.
In this talk trends of bi-specifics on the market or undergoing clinical development will be shown and advantages and drawbacks of the multiple formats will be discussed. The presentation will include case studies from different companies who have started clinical trials with differently designed molecules each adapted to hopefully enhance their desired mode-of-action.