GenE-HumDi

UNIVERSITÄTS KLINIKUM FREIBURG

Joint GenE-HumDi COST Action and Medical Center – University of Freiburg Training School

Impact of GE delivery tools on target cells

September 9-11 2025 @Center for Translational Cell Research (ZTZ).
Breisacherstr. 115 (Ground Floor) - 79106 Freiburg – Germany

Local Organizers
Dr. Claudio Mussolino, Dr. Maria Silvia Roman Azcona

Trainers
Claudio Mussolino, Manuel Rhiel, Maria Silvia Roman Azcona, Carla Fuster Garcia (Medical Center - University of Freiburg, Germany), Duško Lainšček (National Institute of Chemistry, Slovenia), Manuel Goncalves (Leiden University Medical Center, The Netherlands), Hildegard Büning (Hannover Medical School, Germany), Marcello Maresca, Anna Lina Cavallo (AstraZeneca, Sweden)

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

Institute Transfusion Medicine & Gene Therapy

GenE-HumDi

UNIVERSITÄTS KLINIKUM FREIBURG

PROGRAMME

Day 1 – Tuesday, September 9th

Morning Lectures: Introduction to different delivery platforms

- 09:00–09:30 – Openings
- 09:30–10:15 – Delivery LNP – *Duško Lainšček*
- 10:15–11:00 – Cas9 immunological concerns – *Anna Lina Cavallo*
- 11:00–11:20 – Break
- 11:20–12:05 – Large capacity vectors – *Manuel Goncalves*
- 12:05–12:50 – AAVs – *Hildegard Büning*
- 13:00–14:00 – Lunch
- 14:00–18:00 – Lab activity

Day 2 – Wednesday, September 10

Morning Lectures: Tools for genome editing and safety issues

- 09:00–09:30 – Openings
- 09:30–10:15 – Genome editing tools – *Claudio Mussolino*
- 10:15–11:00 – Alternative Cas9 protein – *Marcello Maresca*
- 11:00–11:20 – Break
- 11:20–12:05 – Base editing and safety – *Manuel Rhiel*
- 12:05–12:50 – Epigenome editing – *Maria Silvia Roman Azcona*
- 13:00–14:00 – Lunch
- 14:00–18:00 – Lab activity

Day 3 – Thursday, September 11

Morning Lectures: Off-targeting issues

- 09:00–09:30 – Openings
- 09:30–11:40 – Discussion of lab activities
- 11:40–12:00 – Break
- 12:20–13:00 – Off targeting in genome editing – *Carla Fuster Garcia*
- 13:00–14:00 – Lunch/end of Training school

cost EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY

Funded by the European Union

Institute Transfusion Medicine & Gene Therapy

GenE-HumDi

UNIVERSITÄTS KLINIKUM FREIBURG

TRAINING SCHOOL REPORT

Impact of GE Delivery Tools on Target Cells

Dates: September 9–11, 2025
Location: Center for Translational Cell Research, Freiburg, Germany
Organized by: Claudio Mussolino, Maria Silvia Roman Azcona and Sibtain Heider.

---

1. Introduction

The *Training School on the Impact of GE Delivery Tools on Target Cells* was designed to provide an in-depth overview of delivery strategies for genome editing (GE) tools, a cornerstone for gene therapy and cellular engineering. The ability to efficiently and safely introduce genome editing reagents into diverse target cells remains one of the most critical challenges in advancing translational applications of CRISPR, base editing, and related technologies.

This three-day program combined theoretical lectures from international experts with complementary laboratory sessions. The lectures covered delivery vectors and nanoparticles, genome editing platforms, and safety and immunological aspects, while the hands-on sessions focused on evaluating delivery efficiency and editing outcomes using different approaches.

Participants had the opportunity to explore both conceptual frameworks and experimental techniques — from lipid nanoparticles and viral vectors to genome editing assessment in primary T cells. The interactive structure of the Training School fostered scientific exchange, critical thinking, and practical skill development.

---

2. Lecture Summaries

Day 1 – Tuesday, September 9

Theme: *Introduction to Different Delivery Platforms*

The first day introduced key technologies enabling the delivery of genome editing tools, with a focus on both nonviral and viral systems. Lectures covered lipid nanoparticles (LNPs), adenoviral and adeno-associated viral (AAV) vectors, and immune considerations associated with Cas9 delivery.

Duško Lainšček – Delivery via Lipid Nanoparticles (LNPs)
Dr. Lainšček presented an overview of lipid-based delivery systems, emphasizing their increasing relevance as nonviral vectors for CRISPR-based applications. He described

COST EUROPEAN COOPERATION IN SCIENCE & TECHNOLOGY
Funded by the European Union
Institute Transfusion Medicine & Gene Therapy

various lipid formulations, including liposomes, lipid nanoemulsions, solid lipid nanoparticles, and nanostructured lipid carriers. The talk highlighted the modularity and tunability of LNPs, discussing the role of ionizable lipids, cholesterol, helper lipids, and PEG-lipids in determining efficiency and immunogenicity. Case studies included mRNA delivery for base editing and in vivo CAR-T generation. Dr. Lainšček also discussed advances in targeted LNPs using antibody conjugation and the use of machine learning to optimize lipid composition for cell-type specificity and endosomal escape.

Anna Lina Cavallo – Cas9 Immunological Concerns
Dr. Cavallo addressed one of the most critical challenges for therapeutic genome editing: the immune response to bacterial-derived nucleases such as Cas9. Her lecture reviewed the prevalence of pre-existing humoral and cellular immunity to *Streptococcus pyogenes* and *Staphylococcus aureus* Cas9 proteins, drawing from multiple clinical studies. She explained the mechanisms of innate and adaptive immune activation against Cas9 and described novel strategies to mitigate immunogenicity, including deimmunization of epitopes and chaperone-mediated degradation (FaDe-Cas9). The presentation also showcased preclinical data demonstrating reduced immune reactivity and comparable editing efficiency with engineered Cas9 variants, emphasizing the importance of patient screening and immune monitoring in therapeutic applications.

Manuel Gonçalves – Large-Capacity Adenoviral Vectors
Prof. Gonçalves discussed the challenges of delivering large or multicomponent genome editing systems, such as prime editors or transposon-based tools, which exceed the packaging capacity of most viral vectors. His talk focused on high-capacity adenoviral vectors (HC-AdVPs), which can accommodate payloads up to 36 kb and maintain episomal stability, minimizing genotoxicity. Using examples from his group’s work, he demonstrated applications in Duchenne muscular dystrophy (DMD) correction via prime editing, highlighting efficient editing in muscle and cardiomyocyte models. The lecture also compared adenoviral generations, discussing improvements in cytotoxicity profiles, retargeting strategies, and co-delivery of editing enzymes and repair templates.

Hildegard Büning – Adeno-Associated Virus (AAV) Vectors
Prof. Büning provided a comprehensive overview of AAV biology and its evolution as a therapeutic vector. She explained AAV structure, serotype diversity, and tissue tropism, as well as production strategies and genome packaging principles. The presentation highlighted the clinical success of AAV-based gene therapies (e.g., *Zolgensma*, *Luxturna*, *Hemgenix*), alongside challenges such as limited packaging size, pre-existing immunity, and manufacturing scale-up. A key focus was on capsid engineering — through rational design and directed evolution — to improve cell targeting, reduce off-target transduction, and overcome barriers in specific tissues such as liver or skin. The talk concluded with examples of modified AAVs developed for keratinocyte and hepatocyte targeting, showcasing their potential in precision delivery.

Day 1 Summary

The first day provided participants with a strong foundation in delivery technologies, contrasting the flexibility and scalability of LNPs with the specificity and transduction efficiency of viral vectors. Discussion sessions emphasized trade-offs between payload capacity, immunogenicity, and targeting precision — setting the stage for subsequent lectures on genome editing tools and safety aspects.

Day 2 – Wednesday, September 10

Theme: *Genome Editing Tools and Safety Issues*

The second day transitioned from delivery systems to the editing tools themselves. Experts presented the current landscape of genome editing nucleases, from classical ZFNs and TALENs to CRISPR-based systems, and explored innovative variants that improve precision and safety. The lectures emphasized engineering strategies to minimize off-target effects and the emerging potential of base and epigenome editors.

Claudio Mussolino – Genome Editing Tools: Principles and Applications
Dr. Mussolino opened the session by providing a historical and conceptual overview of genome editing technologies. He compared the main nuclease classes — Zinc Finger Nucleases (ZFNs), TALENs, and CRISPR/Cas systems — highlighting differences in design flexibility, cost, and scalability. His lecture focused on how genome editing exploits cellular DNA repair pathways such as non-homologous end joining (NHEJ) and homology-directed repair (HDR). Emphasis was placed on the importance of optimizing target design, PAM selection, and delivery format (DNA, mRNA, or RNP) to balance efficiency and safety. The session concluded with a discussion of preclinical models for primary T-cell editing and strategies to evaluate off-target activity using next-generation sequencing methods such as GUIDE-Seq and CAST-Seq.

Marcello Maresca – Alternative Cas9 Proteins and Next-Generation Editors
Dr. Maresca's lecture explored the growing diversity of CRISPR-associated proteins beyond SpCas9, including SaCas9, Cas12a (Cpf1), and Cas12b. He presented comparative data on their size, PAM compatibility, and potential for multiplexing and AAV packaging. The session also highlighted ongoing efforts to develop Cas variants with improved fidelity or reduced immunogenicity, and the use of nickases or catalytically inactive Cas9 (dCas9) for gene regulation applications. Real-world examples illustrated how smaller Cas orthologs enable editing in hard-to-transfect or primary cell types, expanding therapeutic possibilities for in vivo delivery.

Manuel Rhiel – Base Editing and Safety Considerations
Dr. Rhiel focused on base editing — a powerful approach for single-nucleotide conversion without inducing double-strand DNA breaks. He described the biochemical mechanisms of cytosine and adenine base editors (CBE and ABE) and their fusion architectures combining deaminases with Cas nickases. The lecture emphasized challenges in minimizing off-target deamination and RNA editing, along with ongoing efforts to refine editor components for therapeutic safety. Dr. Rhiel also introduced prime editing as a complementary, programmable system capable of small insertions, deletions, and corrections, highlighting preclinical success stories and safety-testing pipelines.

Maria Silvia Roman Azcona – Epigenome Editing and Functional Regulation
Dr. Roman Azcona closed the day with an introduction to epigenome editing as an emerging layer of gene regulation. Using catalytically inactive Cas9 (dCas9) fused to effector domains such as KRAB (repressors) or VP64/p300 (activators), researchers can modulate transcription without altering the DNA sequence. She demonstrated examples of gene silencing in mammalian cells and discussed how epigenetic modulation can complement traditional editing for therapeutic purposes. The talk concluded with an overview of safety issues, including reversibility, chromatin remodeling, and long-term epigenetic stability.

Day 2 Summary
This day provided a comprehensive view of the modern genome editing toolbox. Participants gained a strong understanding of the mechanisms behind different editors, how they integrate with delivery systems, and how safety and specificity must guide experimental design. The combination of technical lectures and follow-up lab work enabled participants to contextualize theoretical advances within practical, hands-on workflows.

Day 3 – Thursday, September 11

Theme: *Off-Target Effects and Evaluation of Results*

The final day was devoted to data analysis, interpretation, and safety aspects, with particular emphasis on off-target detection and mitigation strategies. It began with a collaborative discussion of laboratory findings, where participants compared GFP expression data, editing efficiencies, and sequencing results across different delivery conditions.

Discussion of Laboratory Results
Groups presented their experimental observations from the two-day practical activity. The analysis demonstrated clear differences in transfection efficiency between plasmid DNA and

mRNA delivery, with mRNA typically achieving faster expression and lower cytotoxicity. Electroporation parameters and recovery media were discussed as key determinants of viability. Flow-cytometry analysis of TRAC knockout T cells illustrated phenotypic validation through TCR loss, complemented by sequencing data for genotypic confirmation. Participants reflected on technical troubleshooting and the balance between editing efficiency and cell health.

Carla Fuster Garcia – Off-Targeting in Genome Editing
Dr. Fuster Garcia's lecture addressed one of the most critical safety concerns in therapeutic genome editing: unintended off-target cleavage. She reviewed the molecular origins of off-target activity, including mismatched guide-RNA binding and chromatin accessibility, and summarized available detection techniques such as GUIDE-Seq and CAST-Seq. Case studies demonstrated how different delivery methods (viral vs non-viral) and guide RNA chemistry influence specificity. Dr. Fuster Garcia also discussed algorithmic prediction tools and the use of high-fidelity Cas9 variants and truncated sgRNAs to minimize undesired edits. The lecture closed with a forward-looking perspective on integrating computational modeling and deep sequencing for risk assessment in preclinical pipelines.

Day 3 Summary
The final day consolidated the participants' learning by linking experimental data interpretation with theoretical safety considerations. Through both discussion and formal lectures, attendees deepened their understanding of off-target phenomena and of how delivery parameters affect editing precision and cell integrity.

---

3. Laboratory Activities

The hands-on component was a central feature of the Training School, designed to bridge theoretical understanding with practical experimentation.

3.1 Objectives

The main goal was to compare **different delivery materials** (plasmid DNA vs. mRNA) for the expression of a reporter gene (GFP) and to demonstrate the **delivery of CRISPR/Cas9 components** for targeted gene knockout in **primary T cells**. Participants were guided through both the editing preparation and editing assessment stages, allowing them to understand each step of the workflow. To ensure reproducibility and reduce variability with a previous COST-sponsored training activity in **Granada**, a set of reagents were prepared and distributed from Granada to Freiburg. In particular, we received **T cell activation reagents** and single guide RNA (**sgRNA**) targeting specific human genes, such as *TRAC*, *PDCD1* and *B2M*. In addition, we received **mRNA encoding for different editing enzymes**. The use of these shared reagents provided a consistent experimental foundation between the two training schools, enhancing the comparability of results and ensuring that participants across both

locations operated within a unified methodological framework. This collaborative approach not only strengthened the scientific rigor of the program but also reinforced the spirit of cooperation and knowledge exchange that underpins the COST initiative.

3.2 Experimental Design

Participants were divided into two parallel groups to ensure personalized supervision and effective use of resources:

- **Group 1** and **Group 2** alternated between "Editing Preparation" and "Editing Assessment" sessions on different days.

Editing Preparation (led by Maria Silvia roman Azcona)

- Verification of T cell activation via flow cytometry using the surface marker CD25.
- Preparation of cells (counting, washing, and recovery media setup) and reagents (plasmid, mRNA, CRISPR/Cas9 components).
- Execution of the electroporation procedure and recovery of transfected cells.

Editing Assessment (led by Sibtain Haider)

- Evaluation of delivery efficiency based on GFP expression and post-electroporation recovery using flow cytometry.
- Assessment of gene editing efficiency at the TRAC locus via surface staining and flow cytometric analysis of T cell receptor (TCR) loss.
- Genetic validation through sequencing: DNA extraction, PCR amplification, and deconvolution of sequencing data to quantify editing outcomes.

3.3 Learning Outcomes

Through these practical sessions, participants gained:

- Hands-on experience with **electroporation-based delivery** of genome editing materials.
- A comparative understanding of **plasmid vs. mRNA delivery** efficiency.
- Familiarity with **flow cytometry techniques** for phenotypic and functional readouts.
- Insight into **molecular validation** workflows for confirming editing events.

These activities effectively linked lecture content to laboratory practice, reinforcing the theoretical lessons with concrete experimental data.

---

4. Conclusions

The *Training School on the Impact of GE Delivery Tools on Target Cells* successfully provided a comprehensive educational experience spanning the full workflow of genome editing, from the design of editing reagents and delivery vectors to data analysis and safety evaluation.

Participants acquired theoretical knowledge of state-of-the-art delivery systems and genome editing tools while gaining practical expertise through guided laboratory sessions in T-cell editing and flow-cytometric and genotypic assessments.

The combination of expert lectures, active discussion, and collaborative experiments fostered a vibrant learning environment that encouraged knowledge exchange and future collaboration across European research groups. The Training School thus contributed meaningfully to strengthening scientific capacity in genome engineering and translational gene therapy.