B and the corresponding Feed B to try to increase the upstream titres of LVV production from XOFLX™ packaging cell line. The optimal concentrations of Medium B and Feed B managed to increase LVV titres by 60-100% in shake flasks and 2L STR. Further scaling up to 10L STR using the optimal condition resulted in 1.4E8 TU/mL infectious titre in clarified LVV samples. Meanwhile, we tested various pH settings during LVV production in STRs, and found that reducing pH 24 hours post transfection improved LVV infectious titre by ~3 fold.

XOFLX™ producer cell lines, on the other hand, have even greater potential in LVV titre improvements. They not only can be easily scaled up from shake flasks to various scales of STRs without much need of process optimisation, but are also compatible with continuous harvesting process, which can result in much higher LVV yield in each production run. To establish the continuous harvesting process, we firstly tested cell outgrowth and multiple harvests in a scale-down model in shake flasks. Without adding the inducer, by refreshing culture media regularly, we managed to grow the producer cells to high density (>9E6 cells/mL) with good viability (>95%), showing the potential of intensifying the LVV production process. By adding the inducer and keeping the media exchange rate, we managed to do multiple harvests in an extended harvesting window, and the total LVV yield was increased by >5 fold in this intensified process compared to the standard process. Next step, we'll apply the high cell density and media exchange regime to KrosFlo® TFDF® system to establish the proper continuous harvesting process to release further potential of XOFLX™ producer cell lines.

By developing the LVV processes of XOFLX™ packaging and producer cell lines, we managed to significantly improve the LVV titres from both platforms, enhancing the cost-saving benefits and commercial viability of the platforms.

*P0520*

## Towards a directed evolution approach to improve lentiviral vector targeting

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The use of recombinant lentivirus (rLV) holds significant promise for treating a variety of monogenic disorders due to the vector's capacity to transduce a broad spectrum of cell types, including non-dividing cells. Despite this potential, the efficiency and specificity of rLV targeting remain limited, needing strategies to minimize off-target effects and reduce dependency on *ex vivo* gene delivery methods. Here, we develop methods necessary to allow directed evolution approaches to select novel pseudotypes to enhance the cell-type specificity of rLV vectors which incorporate the F and HN glycoproteins from *Sendai virus strain Z.* These F and HN glycoproteins have been used to generate the F/HN pseudotype to target the airway epithelia. Our methodology employs a double-round viral production strategy using a vector genome backbone with intact U3 regions that can conditionally (via provision of Tat) produce viral RNA (vRNA), enabling vector mobilisation. As a proof-of-concept, VSV-G pseudotyped mobilisable rLVs encoding either F, HN or an EGFP transgene under the control of a tetR-sensitive transgene promoter, were produced by transient transfection of HEK293T-tetR (NTR1) cells. These vectors had functional titres of 7.1±2.0e8, 6.5±1.7e8 and 3.0±0.6e8 TU/mL for the F, HN and EGFP variants respectively. In a second round of viral production, these VSV-G pseudotyped particles were used to transduce NTR1 cells. Subsequently, transgene expression (F or HN) was induced with doxycycline and the remaining components necessary for viral production (GagPol, Rev, Tat) were introduced via transient transfection, as well as either HN for the F encoding vector or F for the HN encoding vector. This sequence of events enabled the formation of rLV particles which were self-pseudotyped (i.e. the vector genome in any given vector particle encoded, as appropriate, the F or HN pseudotyping protein found on the particle surface). In the first instance, functional titres achieved were modest - 4.9e4 and 12.4e4 TU/mL for F and HN self-pseudotypes respectively. However, conventional manufacturing scale-up and vector concentration approaches will allow the creation of bar-coded libraries of F and HN sequence variants created by error prone PCR and/or closely related sequence shuffling with sufficient diversity to allow selection of novel pseudotypes via directed evolution. We anticipate this process will allow for the refinement of F and HN glycoprotein variants with enhanced cell-type specificity and improved transduction efficiency.

*P0521*

## Non-integrating lentiviral vectors in the delivery of Cas9 nickase and homologous template for targeted transgene integration

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Genome editing strategies such as base editing and prime editing have been developed to enable safer and more efficient small modifications to the genome. However, in some gene therapy applications the delivery of an entire transgene is still required. In these cases, lentiviral vectors (LVs) are a good option for transgene delivery: they can transduce both dividing and nondividing cells, and have a wide tissue tropism. Furthermore, the packaging capacity of LVs is much larger than that of adeno-associated virus vectors, enabling the delivery of larger transgenes and simpler packaging of targeting constructs. LVs are integrating vectors that preferably integrate within active genes. They thus pose the risk of insertional mutagenesis that could lead to either the inactivation of endogenous genes, or oncogenesis stemming from insertional activation of proto-oncogenes. LVs can be modified to be integration-deficient (IDLVs) with a point mutation in the viral integrase protein, which removes the possibility of insertional oncogenesis. We have tested a strategy combining the benefits of LV delivery and the targeting accuracy of the CRISPR/Cas9 gene editing. To this end, we use a nicking Cas9 (Cas9n) and a single guide RNA (sgRNA) packaged to one IDLV, and a transgene template for homology-directed repair (HDR) and another sgRNA in a second IDLV. This dual IDLV delivery strategy minimizes the risk of off-target editing and ensures that the HDR repair template should be available at all double-strand breaks. In a proof-of-concept study, we delivered an EGFP transgene targeted to the *DAB1* gene in chromosome 1. Flow cytometry was used to quantitate transgene expression, while a junction PCR followed by Sanger sequencing was used to confirm the presence of homologous recombination events. Transduction of 293T cells with the dual transiently expressed IDLVs led to long-lasting EGFP expression resulting from homologous recombination of the transgene to the target site. An analysis carried out by droplet digital PCR showed that a fraction of the transduced cells contained small mutations in the genomic sequence between the sgRNA target sites, which is a known risk of genome editing. Our results show that the simultaneous