BioSkryb GENOMICS

## WhitePaper

# Comprehensive, Single-Cell Multiomic Analysis Is Needed for Safer Cell and Gene Therapies Developed with Lentiviral and Adeno-Associated Virus Technologies

## Key Takeaways:

- **Cell and gene therapies based on lentiviral and adeno-associated virus (AAV) can replace dysfunctional genes.**
- **Insertion of lentiviral or AAV-carried genes (transgenes) can result in insertional mutagenesis.**
- **Assessing viral vector dosage, expression levels, and tissue targeting can improve efficacy of cell and gene therapies.**
- **Precise and accurate single-cell whole genome sequencing, enabled by primary template-directed amplification (PTA) found in ResolveDNA® kits, can accurately detect transgene integration, copy number variation, and structural variant changes.**
- **ResolveOME™, a single-cell whole genome and transcriptome workflow, further enables investigators to draw novel links between genomic edits and gene expression, improving understanding of events that may impact safety and efficacy of lentiviral and AAV-based therapies.**

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The public health impact of cell and gene therapies is likely to be measured on the same scale as the great medical advances of the previous three centuries: antibiotics, anesthesia, and vaccines. However, significant challenges remain with today's cell and gene therapy technologies, foremost the potential for inducing unintended and variable genomic edits and gene dysregulation, which can reduce the safety and efficacy of these therapies. With more sensitive single-cell and low input DNA analysis methods, cell and gene therapy developers will have the tools to detect off-target genomic edits and transcriptomic expression changes in single cells and population subsets, increasing confidence in therapeutic safety and efficacy.

## Gene insertion by viral transduction via adeno-associated virus (AAV) or lentivirus can replace missing or dysfunctional genes to treat monogenic diseases

The staple method for gene replacement therapy, gene insertion, typically involves the addition of a wild-type allele to cells in patients lacking a functional copy of that gene. These therapies may utilize transduction via replication-defective lentivirus or adeno-associated virus (AAV) vectors (**Figure 1**). Vectors may be targeted by exploiting tissue-targeting tropism in AAV serotypes.

When the affected tissue is not accessible, gene replacement may also be performed by autologous adoptive cell transfer (ACT). During ACT CD34+ hematopoietic stem cells (HSCs) are harvested, transduced *ex vivo*, and re-introduced to the patient. Some of these cells will ultimately mature into macrophages or dendritic cells residing in the target tissue, where they will secrete the transgene product.[1] These gene therapy approaches require

## Viral Vector–Based Cell & Gene Therapies

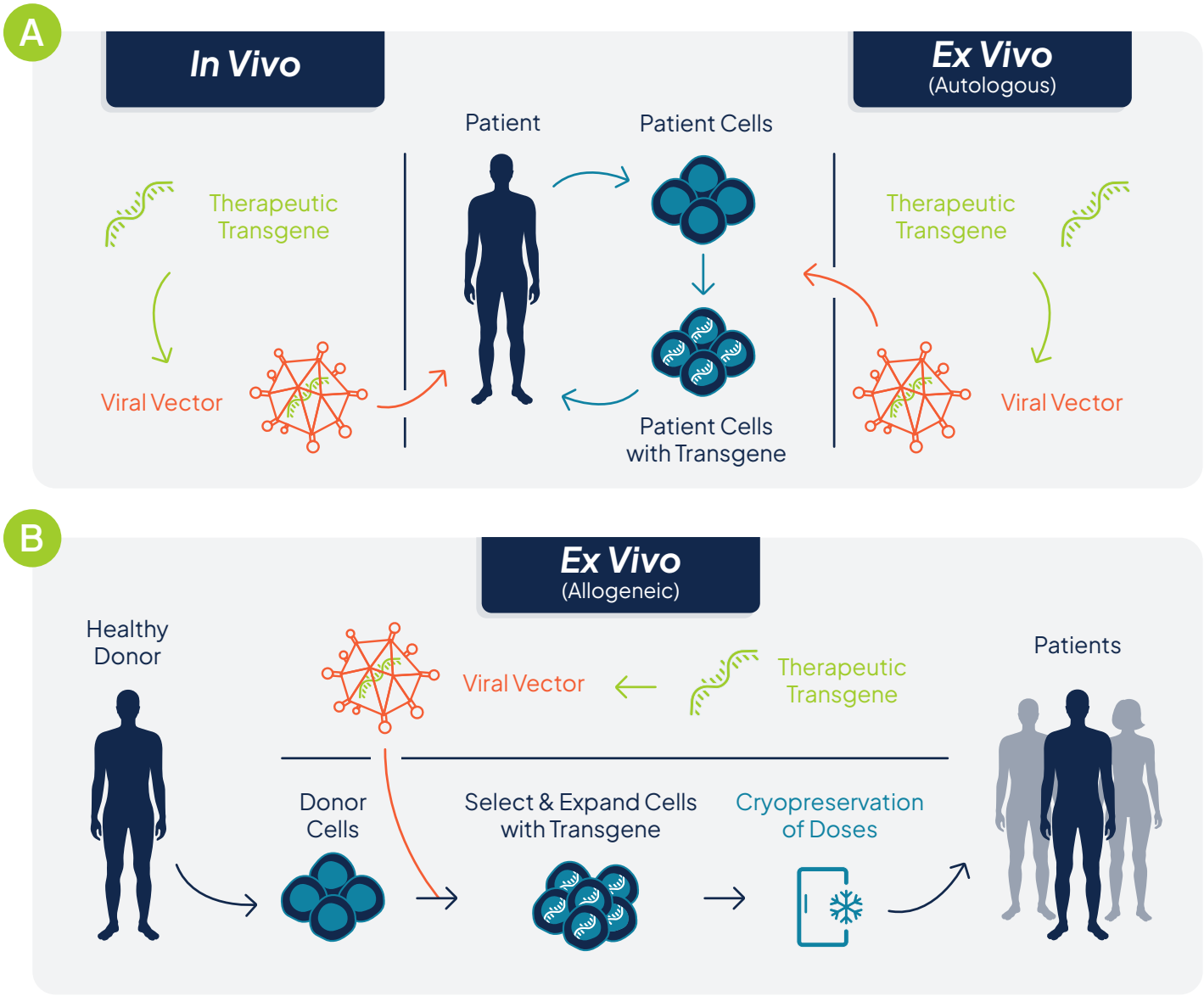


**Figure 1. Viral gene therapies can replace disease-associated genes. A)** For a personalized approach, the viral vector can either be introduced directly to the patient (*in vivo* transduction) or can be used to transduce cultured cells isolated from the patient for later autologous transplant (*ex vivo* transduction). **B)** For an "off-the-shelf" approach, healthy donor cells can be cultured and transduced, then transplanted to allogeneic patients. All methods involve delivery of the therapeutic transgene via a replication-defective viral vector.

successful introduction of the transgene into the host cells and subsequent expression of the gene product (protein).

Multiple cell and gene therapies utilizing these vectors have earned approval in the United States in recent years. The first AAV therapy to earn FDA approval, Luxturna® (Voretigene neparvovec-rzyl), treats recessive retinal dystrophy by adding a functional *RPE65* allele to light-sensing rod cells *in vivo*. Two years into a five-year post-marketing trial, patients treated with Luxturna have shown a dramatic and durable improvement in both visual acuity and light sensitivity, with few adverse events recorded.[2]

Therapies based on *ex vivo* transduction of autologous cells using lentiviral vectors have also recently received approval. Zynteglo® (betibeglogene autotemcel), for example, treats transfusion-dependent ß-thalassemia, a hemoglobin deficiency in which patients cannot survive without regular blood transfusions. Treatment involves harvest of hematopoietic stem cells, insertion of a modified ß-globin gene, and reintroduction. In 89% of patients treated during the clinical trial, transduced cells successfully took residence in patient bone marrow and produced sufficient healthy blood cells to remove the need for further transfusions.[3]

These therapies are transformative. However, cell and gene therapies based on lentiviral and AAV-mediated gene delivery are not without risks, most notably malignancies arising from insertional mutagenesis.

## Unintended consequences of lentiviral and AAV-based therapies include genomic integration that can result in insertional mutagenesis and give rise to neoplastic changes in patients

Insertional mutagenesis is a potential consequence of lentiviral and AAV-based therapies, as both strategies share a similar mechanism of action, whether edits occur *in vivo* or *ex vivo*. Lentiviral vectors, carrying packaged RNA, enter cells via membrane fusion and utilize reverse transcriptase and integrase to add transgene sequences to the host cell genome.[1] AAV vectors, carrying packaged single-strand DNA (ssDNA), enter cells via receptor-mediated uptake and utilize the host cell's DNA repair mechanisms to synthesize double-stranded DNA (dsDNA), primarily in the form of extrachromosomal episomes.[4] Episomes are not replicated during mitosis, so they tend to be shed in actively dividing cells, but AAV sequences are occasionally integrated into the host cell genome, where they will remain in clonal descendants of that cell.[5]

While wild-type AAV preferentially integrates within specific sites, replication-defective AAV vector integration events can occur anywhere in the genome, often within transcription units.[6] Lentivirus integration location shows a similar preference for transcription units and gene-rich regions.[7] Both of these vectors have the potential for insertional mutagenesis. The implications of transgene insertion into a coding sequence vary based on the insertion location and the function of the disrupted gene. The consequences of integration into noncoding regions are uncertain, but such events do pose a safety concern since many noncoding regions are involved in regulation of tumor suppressor genes and/or oncogenes.[6]

Unfortunately, these are not hypothetical dangers. In a historic example, a subset of γc-deficiency (SCID X1) patients who received autologous CD34 bone marrow cells with retrovirus-mediated γc gene transfer developed T cell acute lymphoblastic leukemia 2-14 years after treatment.[8] Analysis of these patients revealed that in the leukemic cells, the γ retroviral vector inserted into the promoter of the LMO2 protooncogene. Similarly, Skysona®

(elivaldogene autotemcel), an approved human therapy for cerebral adrenoleukodystrophy utilizing CD34+ HSCs transduced with lentivirus, carries a warning for the potential of hematologic malignancy due to Lenti-D lentiviral vector integration into proto-oncogenes.[9] In more recent times with newer targeting approaches, researchers have observed an unusually high rate of hepatocellular carcinoma (HCC) in mice receiving neonatal AAV treatment. This apparent tumorigenic effect of AAV transduction is associated with transgene insertion into the *Rian* locus, a long noncoding RNA (lncRNA) region with roles in gene regulation and epithelial to mesenchymal transition.[6] These cautionary tales highlight the importance of analyzing where viral vectors integrate into the host genome both in pre-clinical models and clinical trial participants.

## Assessing viral vector dosage, expression levels, and tissue targeting can improve efficacy of cell and gene therapies

Beyond evaluating where transgenes integrate into the host genome, additional concerns exist around transgene expression level and tissue location. Modern lentiviral and AAV vectors for cell therapy have been chosen for their limited immunogenicity, and their cassettes refined to minimize the potential for expression of oncogenic genes near a transgene integration site.[10] Concerns remain, however, due to uncertainties surrounding the immunogenic potential of transgene-expressed proteins, their therapeutic expression range, their clearance rate, and the stability of their expression levels. Understanding these factors is vital to ensuring the long-term safety of any gene therapy, predicting its efficacy, and optimizing its dosage prior to and during human trials.[11]

Characterizing transduction efficiency and gene expression are particularly critical for gene therapies administered systemically, which cause transduction events throughout the body—not limited to diseased organs and tissues. Some AAV variants do exhibit tropism, preferentially targeting muscle, liver, central nervous system, or peripheral nervous system tissue, and therapies may employ them to direct transgenes primarily to disease-affected tissues.[6] However, off-tissue targeting occurs even with tropic vectors, potentially resulting in undesirable transgene expression and side effects. Quantification of off-tissue transduction events is necessary to assess clinical risks and adjust methods or dosage to optimize outcomes.

## Characterizing lentiviral and AAV-based cell and gene therapy products with single-cell genomics and transcriptomics

Lentiviral or AAV-based therapies hold immense promise, but, as discussed in the previous sections, thorough characterization should be made of these products during therapy development, pre-clinical, and clinical testing. Today there is greater scrutiny around characterizing unintended consequences of gene editing. For example, in the United States, the Food and Drug Administration (FDA) is creating guidelines for safety assessments when creating gene products that include human genome editing.[12]

While best practices are being developed by regulatory agencies, what features should investigators require in their assays and analysis plans?

**Single-cell resolution.** Transgene insertion mediated by lentiviruses or AAVs may not occur in all cells or in the same location in the genome. Only through single-cell analysis can investigators successfully identify the number of insertional events in individual cells.

**Even allelic balance.** Transgene insertions may disrupt one or more copies of an endogenous gene. Only through assays demonstrating good allelic balance can investigators be confident that both copies of a gene are without insertional mutations.

**High sensitivity and precision in mutation calling.** Lentiviral and AAV-mediated transgene insertions can result in copy number and structural variant changes. Leveraging an assay with high precision and sensitivity enables researchers to confidently determine whether unintended mutations have occurred.

**Uniform whole genome coverage.** Transgene insertions can occur throughout the genome and insertions in non-coding regions can result in insertional mutagenesis. An assay with uniform genome coverage enables better detection of insertion sites.

**Bioinformatics analysis support.** Single-cell whole genome sequencing has unique bioinformatics considerations. Identifying a collaborator or service provider with expertise in single-cell genomics is a critical aspect in streamlining data analysis.

With these considerations in mind, investigators require a comprehensive solution that enables them to analyze the entire genomes of single cells.

ResolveDNA from Bioskryb Genomics leverages primary template-directed amplification (PTA) to enable precise and accurate single-cell whole genome sequencing. PTA chemistry, available only through BioSkryb Genomics in ResolveDNA and ResolveOME kits, overcomes biases, allelic dropout, low and variable genome coverage, poor reproducibility, and artifacts associated with existing whole genome amplification approaches.[13, 14] This patented chemistry has been used to characterize the insertion sites of transgenes and catalog the number of copy number variations and structural variations that occur in individual cells following gene editing.[14]

For investigators who want to move beyond inference of gene expression and define the mechanisms of gene expression on a single-cell level, BioSkryb Genomics offers ResolveOME.[15] ResolveOME provides single-cell genome and transcriptome amplification in a unified workflow. This approach combines PTA-mediated whole genome amplifications with full-transcript reverse transcription, allowing novel links to be drawn between genomic and transcriptomic changes. For example, in acute myeloid leukemia cells with acquired resistance that exhibited increased *CEBPA* expression without genomic copy number increases, ResolveOME enabled the identification of a candidate distal promoter/enhancer SNP approximately 20kb 5' of the *CEBPA* transcriptional start site.[15] Deploying ResolveOME in lentiviral or AAV-mediated gene edited cells could lead to similar discoveries, notably determining insertion sites for transgenes, their expression level, and whether their expression has altered the cell type.

These complex analyses require bioinformatics support. Supporting the analysis and interpretation of data generated with ResolveDNA and ResolveOME is BaseJumper®. BaseJumper is a bioinformatics platform built for biologists that enables multiomic data analysis. With this suite of products, along with custom service offerings through ResolveServices℠, BioSkryb Genomics provides solutions for more thorough characterization of lentiviral and AAV-based therapies.

To talk to a BioSkryb Genomics scientist about how ResolveDNA, ResolveOME, ResolveServices, and BaseJumper can empower research programs using viral vectors, email a member of our team at:

## References


1. Xu, K. (Ed.). (2011). Viral Gene Therapy. InTech.
2. Fischer, M., Maier, R., Suhner, A., et al. (2022, May). PERCEIVE study report: Real-world safety and effectiveness of voretigeneneparvovec. Association for Research in Vision and Ophthalmology (ARVO) 2022 Annual Meeting.
3. Koh, A., Jamuar, S. (2023). Therapeutics in paediatric genetic diseases: Current and future landscape. Singapore Medical Journal, 64(1), 7. https://doi.org/10.4103/singaporemedj.SMJ-2021-376
4. Hauck B, Zhao W, High K, Xiao W. Intracellular viral processing, not single-stranded DNA accumulation, is crucial for recombinant adeno-associated virus transduction. J Virol. 2004 Dec;78(24):13678-86. doi: 10.1128/JVI.78.24.13678-13686.2004.
5. Berns KI, Muzyczka N. AAV: An Overview of Unanswered Questions. Hum Gene Ther. 2017 Apr;28(4):308- 313. doi: 10.1089/hum.2017.048.
6. Sabatino DE, Bushman FD, Chandler RJ, Crystal RG, Davidson BL, Dolmetsch R, Eggan KC, Gao G, Gil- Farina I, Kay MA, McCarty DM, Montini E, Ndu A, and Yuan J. Evaluating the state of the science for adenoassociated virus integration: An integrated perspective. Molecular Therapy Vol. 30 No 8 August 2022. DOI:https://doi.org/10.1016/j.ymthe.2022.06.004
7. Ronen K, Negre O, Roth S, Colomb C, Malani N, Denaro M, Brady T, Fusil F, Gillet-Legrand B, Hehir K, Beuzard Y, Leboulch P, Down JD, Payen E, Bushman FD. Distribution of lentiviral vector integration sites in mice following therapeutic gene transfer to treat β-thalassemia. Mol Ther. 2011 Jul;19(7):1273-86. doi: 10.1038/mt.2011.20.
8. Fischer A, Hacein-Bey-Abina S. Gene therapy for severe combined immunodeficiencies and beyond. J Exp Med. 2020 Jan 6;217(2):e20190607. doi: 10.1084/jem.20190607. PMID: 31826240; PMCID: PMC7041706.
9. SKYSONA (elivaldogene autotemcel) suspension for intravenous infusion package insert. fda.gov. 9/2022.
10. Baker AH, Herzog RW."Did Dendritic Cell Activation, Induced by Adenovirus-Antibody Complexes, Play a Role in the Death of Jesse Gelsinger?. Molecular Therapy. Vol. 28 No 3 March 2020. DOI:https://doi.org/10.1016/j.ymthe.2020.02.010
11. Belov A, Schultz K, Forshee R, Tegenge MA. Opportunities and challenges for applying model-informed drug development approaches to gene therapies. CPT Pharmacometrics Syst Pharmacol. 2021 Apr;10(4):286-290. doi: 10.1002/psp4.12597.
12. United States Department of Health and Human Services Food and Drug Administration. Therapy Products Incorporating Human Genome Editing. March 2022. https://www.fda.gov/media/156894/download
13. Gonzalez-Pena, V., Natarajan, S., Xia, Y., Klein, D., Carter, R., Pang, Y., Shaner, B., Annu, K., Putnam, D., Chen, W., Connelly, J., Pruett-Miller, S., Chen, X., Easton, J., Gawad, C. (2021). Accurate genomic variant detection in single cells with primary template-directed amplification. Proceedings of the National Academy of Sciences, 118(24). https://doi.org/10.1073/pnas.2024176118
14. Data on file
15. Marks, J. R., Zawistowski, J. S., Salas-González, I., Tate, T. A., Morozova, T. v, Blackinton, J. G., Arvapalli, D. M., Velivela, S. D., Harton, G. L., Gawad, C., Weigman, V. J., Hwang, E. S., West, J. A. A. (2023). Unifying comprehensive genomics and transcriptomics in individual cells to illuminate oncogenic and drug resistance mechanisms. BioRxiv, 2022.04.29.489440. https://doi.org/10.1101/2022.04.29.489440

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