



LENTIVIRAL VECTOR CONSTRUCT DESIGN - WHITEPAPER

Improved LVV Manufacturing for In Vivo CAR-T Through Synthetic Biology-Guided Promoter Design

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Improved LVV Manufacturing for In Vivo CAR-T Through Synthetic Biology-Guided Promoter Design

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ABSTRACT

The growing field of in vivo CAR-T leverages the robust anticancer efficacy of ex vivo CAR-T while addressing one of the modality's greatest challenges - manufacturing cost - by moving CAR-T cell manufacturing into the patient. Delivery of lentivirus as a drug product eliminates the need for a multi-step workflow (apheresis, ex vivo modification, lymphodepletion, and reintroduction of modified T cells into the patient) - which is not only costly but also carries risk to the patient in the form of long waits for treatment and side effects of lymphodepletion. However, the administration of lentivirus directly to the patient introduces new safety requirements that were unnecessary when using lentivirus ex vivo to modify T cells - namely, the need for cell type specific targeting to ensure that lentivirus is specifically delivered to the target T cells, and not entirely absorbed by off target tissue sinks. Solutions to this challenge focus on developing T cell specific binders paired with mutant forms of VSV-G that retain fusogenic capability while reducing binding to non-cell type specific receptors. These solutions currently ignore a significant source of off-target lentiviral tropism: targeting mediated by viral surface expression of the therapeutic CAR protein.

Because lentiviral particles acquire their envelope from the producer cell membrane, transcription and translation of the transgene during manufacturing can result in incorporation of the transgene into the viral membrane. The presence of a CAR on the viral surface can redirect vector binding to antigen-positive cells, creating potential safety and efficacy liabilities. We trace the source of this producer-cell "breakthrough expression" to the architecture of HIV-1-derived vectors and describe a construct-level solution that combines a T-cell-specific promoter with cassette inversion. Together, these design elements suppress producer-cell expression while preserving transduction of primary T cells and improving vector performance, demonstrating that product quality and manufacturing efficiency can be enhanced simultaneously rather than traded against one another. The resolution is a property of the construct rather than the process, it is most readily adopted where construct engineering and drug product manufacture sit together — development partners working end to end, from cassette design through vector production to release, can resolve failure modes of this kind before a process inherits them.

1. History of lentiviral vectors in the field

WHERE TODAY'S DESIGN CONVENTIONS ORIGINATE

HIV-1-derived vectors were introduced in the mid-1990s and were distinguished from the gamma-retroviral vectors then in use by a single functional property: the ability to transduce non-dividing cells.¹ Because integration did not require mitosis, targets that had been inaccessible — quiescent lymphocytes, neurons, hepatocytes — came within reach of a stably integrating vector.

The field's shift toward these backbones was accelerated by safety findings rather than by capability alone. Insertional oncogenesis observed in early gamma-retroviral trials, in which vector integration near a proto-oncogene drove clonal proliferation,² established that vector architecture is a determinant of clinical outcome and not merely of transduction efficiency. Design attention moved to the vector's own transcriptional elements.

Two developments followed: Third-generation packaging systems removed the accessory genes, rendered the system Tat-independent, and distributed the remaining functions across separate plasmids, so that reconstitution of a replication-competent genome required multiple improbable recombination events.³ In parallel, self-inactivating (SIN) design deleted the U3 enhancer–promoter from the 3' long terminal repeat, limiting mobilization of the integrated provirus and reducing the risk of insertional activation of neighboring genes.⁴

WHAT THE SIN DELETION DOES AND DOES NOT DO

The SIN modification deletes U3 from the 3' LTR of the transfer construct, so that the integrated provirus in the target cell lacks a functional LTR promoter. It does not remove promoter activity from the 5' end during production: there, the Tat-dependent viral U3 promoter is replaced outright by a constitutive heterologous promoter — typically CMV or RSV — which drives Tat-independent transcription of the packageable genomic RNA. Producer-cell transcription across the construct is therefore a designed feature of the system, not a defect in it.

These changes had a consequence for expression that is easy to overlook. With the enhancer–promoter removed from the integrated 3' LTR, transgene expression in the target cell became the responsibility of an internal promoter placed within the cassette. Selection of that promoter was governed by the requirements of *ex vivo* manufacture, where the vector is a reagent applied to cells and subsequently washed away: strong, well-characterized, broadly active promoters were the rational choice, and EF-1 α , CMV and MND became convention. Approved *ex vivo* products established this manufacturing template, and it remains in use. What it did not have to account for is a setting in which the vector particle is itself the administered product.

2. HIV virology and the origin of breakthrough expression

WHY THE PRODUCER CELL TRANSCRIBES THE TRANSGENE

The long terminal repeat comprises three regions — U3, R and U5 — of which U3 carries the enhancer and promoter elements, including a TATA box and binding sites for host transcription factors.

Transcription of the viral genome initiates at the U3/R boundary of the 5' LTR and terminates within the 3' LTR, so a single promoter generates the full-length transcript that serves both as the packaged genome and as the template from which subgenomic messages are spliced.

In the virus, this promoter is weak until the viral protein Tat binds the TAR element in the nascent transcript and recruits host elongation machinery. Third-generation systems dispense with Tat, driving the transfer construct from a heterologous constitutive promoter instead.³ The point for manufacture is that the transcript encoding the vector genome is produced abundantly in the producer cell by design — it must be, since it is the material to be packaged.

Export of that transcript is itself regulated. The unspliced genomic RNA carries the Rev response element (RRE), and its transit to the cytoplasm depends on Rev binding there — the mechanism by which the virus exports an intron-containing transcript the host would otherwise retain or degrade. One template thus serves two fates: packaging as genome, and translation of whatever open reading frames it carries.

Against this background, two routes to transgene protein in the producer cell follow directly, and they are additive. The first is **internal promoter activity**: a ubiquitous promoter does precisely what it was selected to do — drive strong expression in essentially any cell — and the HEK293 production line is such a cell. The transgene is transcribed throughout the production window, when it is required only in the target cell. The second follows from the paragraph above: where the expression cassette lies in the **same sense** as the vector genome, the abundant genomic transcript is itself a transgene mRNA, carrying the coding sequence into the cytoplasm on a transcript the process cannot do without. The second route is the more consequential of the two, because it is indifferent to the promoter placed in front of the transgene: it operates whenever the cassette is sense-oriented, and it is driven by the one transcript vector production cannot dispense with. Together these two routes give the phenomenon this paper is concerned with.

Breakthrough expression is transcription and translation of the therapeutic transgene in the producer cell during vector manufacture, arising from either route and irrespective of the internal promoter's intended cell specificity — the transgene breaks through the transcriptional controls placed around it, and is expressed in the cell that makes the vector rather than the cell the vector treats. Because the second route needs no internal promoter of its own, both sense-oriented configurations are affected whatever promoter they carry, as Section 4.2 demonstrates directly (Figures 2 and 3).

Two consequences organize the remainder of this paper. Because particles bud from the producer cell membrane, transgene product synthesized during manufacture is carried into the purified product rather than left in the bioreactor — the subject of Section 3. And because the two routes respond to different interventions, closing one does not close the other: a selective promoter addresses internal promoter activity while leaving the genomic transcript untouched. That proposition is examined against data in Section 4.

3. Why breakthrough expression is a drug product risk

FROM MANUFACTURING ARTIFACT TO CMC AND SAFETY CONCERN

Titer, product quality, and cost per batch

Transcription and translation of a protein for which the producer cell has no use diverts biosynthetic resources during the production window. The effect is amplified where the transgene product is cytotoxic, membrane-active, or a constitutively signaling receptor, and it is variable in a way that is difficult to control by process means. Because most of a batch's cost — plasmid, media, bioreactor occupancy, downstream processing and release testing — is incurred irrespective of yield, a reduction in titer raises cost per dose almost proportionately.

Loss of targeting control

A transgene product displayed on the vector envelope confers a second, undesigned tropism. In a chimeric antigen receptor (CAR) construct, surface-displayed CAR directs the particle toward antigen-positive cells, competing with the engineered envelope (a binder that selects target cells, a fusogen that drives entry). The particle acquires a targeting determinant that no one specified and no release assay measures.

Antigen sink and dose escalation

Surface-displayed receptor binds shed or soluble antigen from any source, and mistargeted particles are consumed by antigen-positive cells that were never the intended recipients. Each such event removes a particle from the population available to transduce the target, so the dose required to achieve a given effect rises — and with it both the innate immune burden the patient must absorb and the number of doses that must be manufactured.

Characterization and CMC risk

A vector whose envelope composition varies with transgene expression is harder to specify, harder to release consistently between batches, and harder to defend in a regulatory filing. An uncontrolled attribute becomes a comparability liability at every subsequent process change.

THE TWO EFFECTS COMPOUND

Reduced titer and escalating dose act on cost of goods from opposite directions, and multiply rather than add: producer-cell expression lowers the number of doses recovered from a batch, while antigen sequestration and mistargeting raise the number of particles each dose must contain. Batches per patient is the ratio of the two, so reductions in titer and escalations in dose compound — before allowing for the release testing and comparability work the additional batches themselves require.

Lentiviral particles bud from the producer cell membrane and incorporate the transmembrane proteins resident there at the time of budding. This is a property of the platform — the same property that makes envelope pseudotyping and surface engineering possible — and it is the mechanism that carries producer-cell transgene expression out of the bioreactor and into the product. Four consequences follow.

The clinical stakes of surface-displayed transgene product are established in the literature. In a reported case, a patient treated with CD19-directed CAR-T cells relapsed at nine months with CD19-negative leukemia in which the malignant cells aberrantly expressed the anti-CD19 CAR; the CAR gene had been introduced into a leukemic B cell, and the CAR product bound the CD19 epitope *in cis* on that cell's own surface, masking it from recognition and conferring resistance to the administered product.⁵ Subsequent work identified a mechanism by which such transduction may occur: anti-CD19 CAR displayed at the surface of lentiviral vector particles promotes transduction of antigen-expressing cells.⁶ That relapse arose during *ex vivo* manufacture, in which malignant cells present in the apheresis material were transduced, rather than from producer-cell expression; taken together, however, the two reports establish that surface-displayed transgene product carries demonstrated clinical consequence and that particle-surface display is a route by which it acts.

The distinction between the two settings also indicates why the concern grows rather than diminishes with direct administration. In *ex vivo* use the vector is a reagent: it is applied to cells, then removed, and the product released is a characterized cell population. The transduction of a malignant cell required the coincidence of contaminating disease in the starting material — a hazard addressable by selection and sorting. Where the vector particle is itself the drug product, no downstream step removes what the producer cell placed on the envelope; it is present in the vial, in the specification, and in the administered dose, and it circulates in a patient whose antigen-positive compartment may be the disease. The mitigation available *ex vivo* is not available, and the exposure is systemic.

4. How synthetic biology resolves an inherited misdesign

PROMOTER ENGINEERING, ORIENTATION, AND INTERDEPENDENCE

4.1 Promoter engineering

A promoter is useful in this application only if it satisfies three constraints simultaneously. It must be sufficiently selective to be functionally inactive in the production line and in non-target tissue; sufficiently strong in the target cell to deliver therapeutic expression; and sufficiently compact to leave transgene capacity intact. The design specification for the GeneFab T-cell promoter series states these explicitly: **on** in primary T cells at a strength comparable to EF-1 α ; **off** in hepatocytes, the principal site of off-target vector accumulation after systemic administration; **off** in HEK293T, to minimize incorporation of transgene product into the viral envelope; within <750 bp.

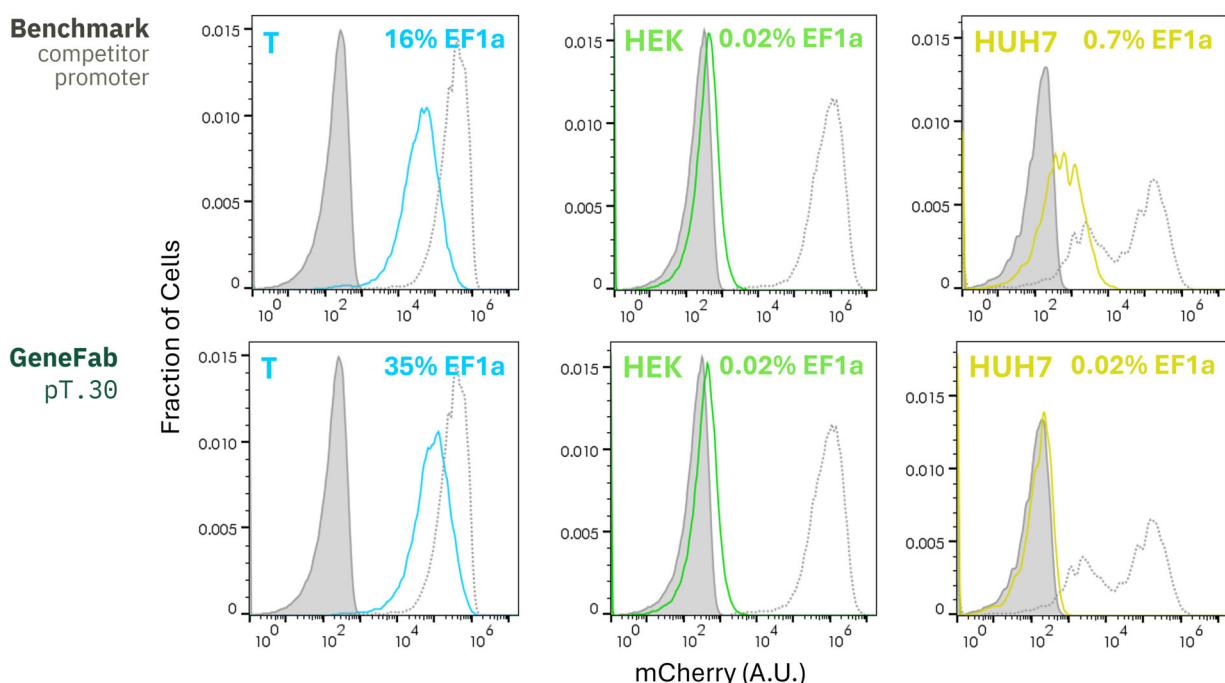


Figure 1. Reporter expression by cell type for a benchmark T-cell promoter and GeneFab pT.30. Cells were transduced with reporter lentiviral vector and analyzed by flow cytometry; abscissa, mCherry fluorescence intensity (log scale); ordinate, fraction of cells. Filled grey indicates untransduced controls and dotted traces an EF-1 α -driven control in the same cell type. Values are expressed as a percentage of the EF-1 α control: pT.30 reached 35% in primary T cells against 16% for the benchmark, and 0.02% in both HUH7 and HEK293T against 0.7% for the benchmark in HUH7.

Two features of this profile bear on a program decision. The separation between on- and off-target expression is approximately three orders of magnitude, rather than a partial reduction — which is what allows the promoter to function as a containment element and not merely as an expression tuner. And the 750 bp footprint leaves headroom for larger transgenes, multicistronic designs, and the regulatory elements a modern construct requires.

4.2 Cassette orientation

Promoter selectivity addresses transcription initiating within the cassette, but not the transcript that carries the vector genome. Inverting the expression cassette relative to that genome removes the coding sequence from the sense of the genomic transcript, so that transcript can no longer serve as a transgene mRNA. Four configurations — constitutive or T-cell-specific promoter, in forward or reverse orientation (Figure 2) — were compared to breakthrough expression. Neither modification sufficed alone (Figure 3): constitutive promoter inverted gave 25% expression relative to control; as a forward specific promoter still gave 7.4%, because a sense-oriented cassette leaves the readthrough open. Only in combination — a specific promoter in an inverted cassette — did signal fall to minimal detection.

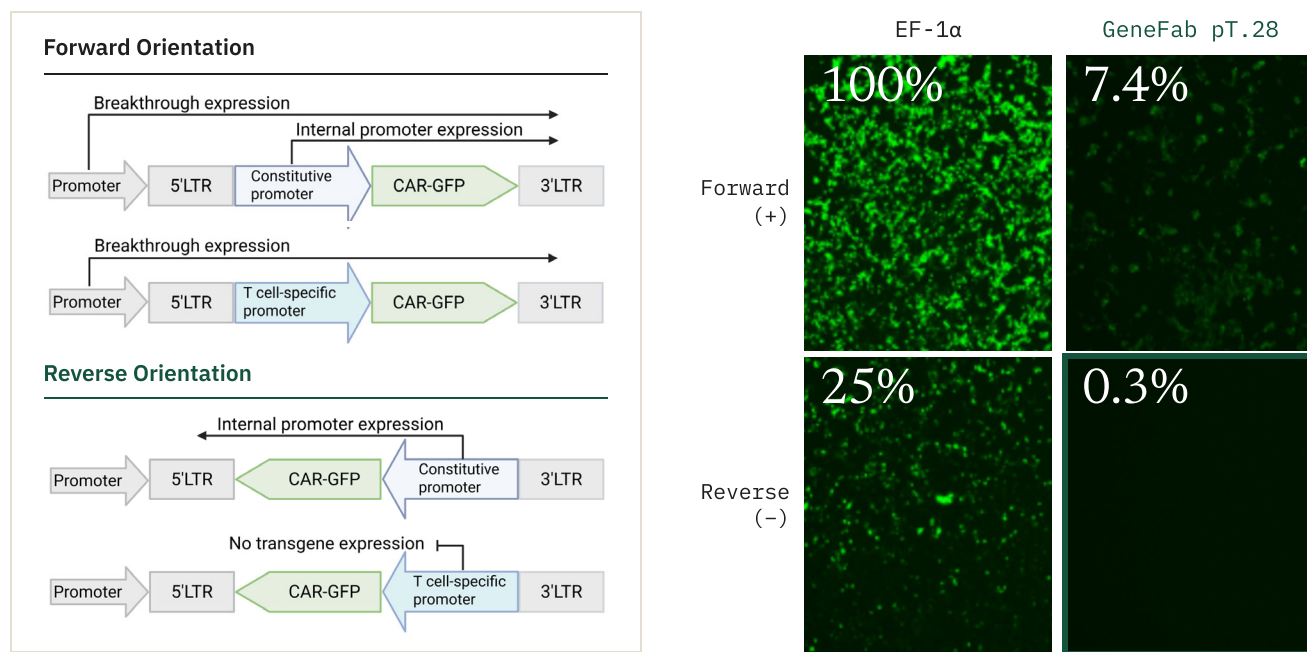


Figure 2. The four cassette configurations. Forward (a, b): breakthrough proceeds from the 5' promoter with *either* promoter. Reverse (c, d): the transgene is antisense to the genomic transcript, so breakthrough cannot occur; (c) retains convergent internal expression, (d) none.

Figure 3. CAR-GFP expression in HEK293 producer cells across the configurations of Figure 2. Transient transfection at production ratios, imaged for GFP; signal normalized to EF-1α forward. Promoter alone gave 7.4% of control, inversion alone 25%, combined 0.3%.

4.3 Why the two modifications are interdependent

The two modifications depend on each other in both directions. Section 4.2 established the first: inversion closes the readthrough route a selective promoter cannot reach. The dependency also runs the other way: inversion is safe only where the promoter is silent in the producer cell. Inverting a cassette that retains an *active* promoter places two convergent transcription units in the same producer cell — the promoter on one strand, the genomic transcript on the other — and the resulting double-stranded RNA triggers host pathways that suppress titer. The effect has been shown: in interferon-competent producers it acts through protein kinase R, and in HEK293T, where no interferon response exists, through an RNA-interference pathway; in both, disabling the sensor restores the lost titer.^{7,8} The mechanism differs by cell, but the dependency does not — a titer penalty appears wherever an active promoter generates the antisense strand (demonstrated in Section 4.4).

A promoter that is functionally inactive in the producer line resolves this by construction: with no initiation inside the cassette during production, no antisense strand is generated and the genomic RNA has nothing to anneal with. Selectivity is therefore not a refinement of the orientation change but the precondition that makes inversion usable, while inversion is what allows a selective promoter to close the route selectivity cannot reach. The same silence resolves a second liability: with no transgene product made during production, none is available for incorporation into the particle envelope, so neither the mistargeting nor the antigen sequestration described in Section 3 has a source.

4.4 Functional titer across the four configurations

Functional titer was subsequently determined for all four configurations, and the result is more informative than the prediction that motivated it. Inversion moves titer in opposite directions depending on the promoter it carries: with EF-1 α it **reduced** titer, as the convergent-transcription mechanism predicts; with pT.28 it **increased** titer, and inverted pT.28 gave the highest yield of the four.

This result provides direct observational support for the interdependence argued in Section 4.3: the titer penalty arises only where an active promoter generates the antisense strand, and removing that activity reverses the penalty rather than merely avoiding it. A plausible account is that transgene product synthesized in the producer cell burdens vector assembly, so yield and containment improve together.

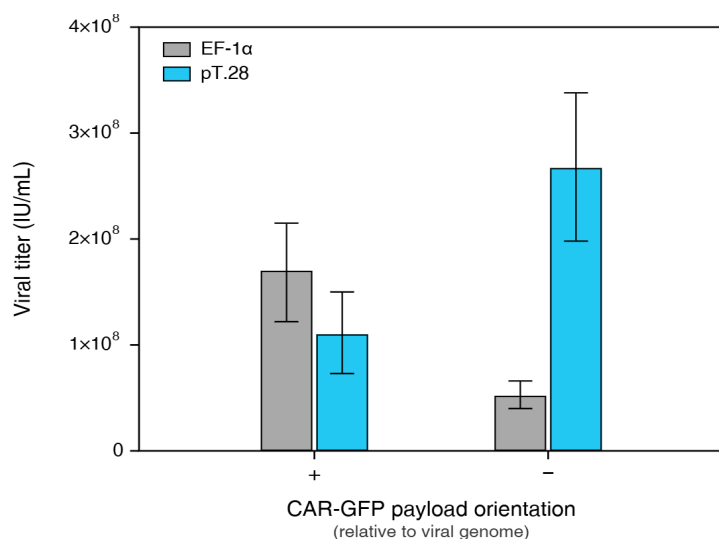


Figure 4. Functional titer (IU/mL) of vector produced with EF-1 α or GeneFab pT.28 driving the CAR-GFP payload, in sense (+) and antisense (-) orientation relative to the vector genome. Bars show mean $\pm$ SD.

Target-cell potency is unaffected by the orientation change: in primary T cells, pT.28 delivered 29% of the EF-1 α control in the sense orientation and 34% in the antisense orientation (data not shown). Orientation is a producer-cell variable, not a determinant of expression in the transduced cell — as Section 2 predicts, since the genomic transcript that makes orientation consequential is abundant only during production.

4.5 Concurrent improvement in containment and yield

Producer-cell containment and vector yield are frequently treated as competing objectives, on the assumption that transcriptional attenuation during manufacture carries a process-performance cost. The data presented above do not support that assumption. A promoter matched to the target cell, in a cassette inverted relative to the vector genome, removes that risk: attenuating transcription in the producer cell raised functional titer rather than costing it.

**Titer and product quality**

Inactive in HEK293, so inversion generates no antisense transcript and no dsRNA. Yield does not merely hold — it is the highest of the four configurations (Figure 4).

Mistargeting control

No transgene product on the particle surface, so the fusogen–binder pair alone determines tropism. No epitope masking, and no undesigned second specificity.

Antigen sink and dosing

Nothing on the envelope binds shed antigen, so vector is not consumed before it arrives — and expression in the transduced T cell is unchanged at 34% of EF-1 α .

Taken with Figure 1, these results describe a construct in which transgene product is not available for envelope incorporation during manufacture, and in which the promoter remains inactive in hepatocytes and the production line after administration. The undesigned tropism described in Section 3 therefore has no source in this configuration, since a particle cannot display a protein the producer cell did not synthesize. Containment and efficiency are delivered by the same two design decisions.

5. Why Manufacture with GeneFab?

Seamless tech transfer

No re-qualification of the promoter in a different host cell, and no re-derivation of its expression behavior at production scale. The behavior recorded in Figure 1 is that of the system in which the material will be manufactured.

Existing analytical assays

Identity, potency and residuals assays are already established against these constructs, so analytical development is not on the critical path for a program that adopts them.

No external process development

The construct arrives matched to a running platform, rather than requiring a platform to be built around it. Drug product manufacture sits under one roof: payload optimization, process and analytical development, vector production, purification, formulation and fill, and release.

The scope of what transfers without further work, and what requires bridging activity for a given payload and target indication, is program-specific and best established in a project review.

From promoter to release, one party accountable

Breakthrough expression is a construct problem that no process parameter reaches — and a construct fix is only worth having if the process it enters was built to receive it. GeneFab engineers the promoter, designs the cassette around it, and manufactures the drug product: three decisions that determine each other, taken together rather than handed between vendors.

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About GeneFab

GeneFab is a contract development and manufacturing organization (CDMO) founded in 2023 to advance cGMP-compliant cell and gene therapies. Guided by a vision to make innovative genetic medicines available to all, the company partners with innovators to design, develop, and manufacture transformative therapies through an integrated technology platform spanning synthetic biology, in vivo vectors, and autologous and allogeneic ex vivo cell therapy production. From its Bay Area, California facilities, GeneFab pairs rigorous science with operational excellence to enable safe, scalable, end-to-end delivery of next-generation therapies.

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