

April 2026

**Keywords or phrases:**Synthetic DNA, Polymer, Lipid-Based Nanoparticles, Preformed Vesicles, Transfection, *In vivo* Delivery

# Improved *in vivo* Protein Expression and Durability Using dbDNA™ Delivered With Sartorius Non Viral Delivery Solutions

Pauline Urquia<sup>1</sup>; Claire Guéguen, PhD<sup>1</sup>; Lisa Caproni, PhD<sup>2</sup>; Mitchell Brun, PhD<sup>2</sup>; Milica Vukovic, PhD<sup>2</sup>; Verna McErlane, PhD<sup>2</sup>; Patrick Erbacher, PhD<sup>1</sup><sup>1</sup> Sartorius Polyplus, 75 rue Marguerite Perey, Illkirch, France<sup>2</sup> Touchlight Genetics Ltd, Morelands & Riverdale Buildings, Hampton, UK

Correspondence

Email: [claire.gueguen@sartorius.com](mailto:claire.gueguen@sartorius.com)

## Introduction

Gene Therapy approaches have historically relied on viral delivery systems, predominantly Adeno-Associated Virus, but drawbacks include limited payload capacity, complex manufacturing processes, and anti-vector immune responses that can limit *in vivo* efficacy. Non-viral DNA and RNA approaches offer compelling alternatives, yet their broader application has been hindered by lower delivery efficiency compared with viruses, which have naturally evolved highly effective routes into cells.

Effective non-viral delivery systems depend not only on the route of administration and mechanisms of cellular uptake, but critically, on the selection of an appropriate DNA vector. Whether polymer-based, liposomal, or lipid-nanoparticle formats, these delivery platforms are continuously advanced and optimized at Sartorius to enhance efficacy, specificity and targeting. Enzymatically produced DNA has emerged as an alternative to plasmid-based vectors, offering advantages in manufacturing and biological performance. Touchlight has developed a portfolio of DNA molecules optimized for any size payload and compatible with all gene therapy and gene editing platforms.



Touchlight's pioneer molecule, doggybone DNA (dbDNA™) is a linear double-stranded construct with covalently closed ends. Megabulb DNA (mbDNA™) is a circular single-stranded DNA molecule with a short double-stranded stem loop that is compatible with homology directed repair (HDR) and transient expression platforms. Touchlight's suite of custom circles contains single-stranded circles (sscDNA), hybrid single-stranded circles (hsscDNA), and double-stranded circles (dscDNA). These circles are compatible with homology-independent targeted integration (HITI), recombination, transposition, and transient expression platforms. All Touchlight molecules offer the benefits of reduced immunogenicity, flexible sequence architecture that lacks bacterial backbone elements commonly present in bacteria- or phage-derived DNA (such as antibiotic resistance genes and origins of replication) with an inbuilt copy number advantage per mass of DNA and allow for high sequence complexity and secondary structures.

Efficient formulation plays a central role in enabling reliable *in vivo* delivery of nucleic acids to target tissues. However, many established technologies are associated with manufacturing and operational challenges, including dependence on specialised microfluidic platforms and constraints imposed by complex Intellectual Property landscapes.

Access to straightforward, user-friendly formulation approaches has the potential to accelerate research and development by lowering technical barriers, thereby enabling early-stage investigators to more readily explore and optimise non-viral gene delivery strategies as they advance their therapeutic concepts.

The two reagents employed in this study were designed for use without specialized instrumentation apart from a micropipette. No formulation expertise is required, and the reagents are ready for use within 15 minutes.

*In vivo*-jetPEI® is a cationic polymer reagent renowned for its versatility in delivering nucleic acids such as DNA, siRNA, miRNA, shRNA, and other oligonucleotides across a wide range of animal models, including mice, rats, rabbits, llamas, and monkeys. Its ability to target multiple organs through systemic and local administration has been validated by over 650 peer-reviewed publications, establishing it as a cornerstone in the fields of *in vivo* nucleic acid delivery for fundamental research, oncology, gene therapy development, and vaccination. Furthermore, *in vivo*-jetPEI® is available in both research and GMP grades, facilitating a smooth transition from laboratory research to clinical applications.

Complementing this, *in vivo*-jetRNA®+ is a non-viral delivery solution specifically designed for *in vivo* mRNA delivery. *in vivo*-jetRNA®+ is composed of loadable lipid-based nanoparticles that we will refer to as preformed vesicles (PFV). These PFVs ensure high delivery efficiency and safety which make them a simplified and innovative alternative to traditional lipid nanoparticles (LNPs). Its user-friendly two-step protocol enhances its appeal for researchers seeking rapid, simple and reliable mRNA delivery methods.

In this study, we evaluated the complexation of plasmid DNA (pDNA) and dbDNA™ using *in vivo*-jetPEI® and *in vivo*-jetRNA®+ transfection reagents. In addition, we investigated the biodistribution and stability of luciferase expression following retro-orbital (RO) and intramuscular (IM) administration, providing insights into the efficacy and potential applications of these delivery systems in various experimental contexts.

## 2. Materials and Methods

### 2.1. Design of Synthetic DNA

A dbDNA™ vector containing a CMV promoter, a Firefly luciferase (Luc2) gene and the SV40 late polyadenylation signal was manufactured using Touchlight's enzymatic process. The dbDNA™, 2555bp in length, was provided in 10mM Tris HCl, pH 7.9. Plasmid DNA matching the dbDNA™ expression cassette (extracted from *E. coli* using Qiagen EndoFree Gigaprep kits) was also supplied. Both dbDNA™ and pDNA underwent standard QC testing including endotoxin assays to ensure suitability for *in vivo* delivery.

### 2.2. Particle Characterization

Mixes of DNA with polymers and PFVs were characterised for size and polydispersity index (PDI) using dynamic light scattering (DLS, Malvern Zetasizer, Worcestershire, UK). Prior to ZetaSizer analysis, the transfection mixes were diluted twenty-fold in water to a concentration of 2.5 µg/mL DNA.

### 2.3. Animal Experiments

All animal studies were conducted in accordance with the French Animal Care guidelines and protocols were approved by the Direction des Services Vétérinaires. Six-week-old female OF1 mice 22–24 g were obtained from Charles River Laboratories (Lyon, France) and subjected to a week of quarantine and acclimation period before use.

### 2.4. *In vivo* Delivery via Intravenous Injection

On the day of administration, *in vivo*-jetPEI<sup>®</sup>/Luc2 DNA and *in vivo*-jetRNA<sup>®</sup>+/Luc2 DNA complexes were prepared according to the manufacturers' recommendations. Briefly, 10 µg of Luc2-encoding DNA were first diluted in the provided glucose 5% followed by the mixing-in of *in vivo*-jetPEI<sup>®</sup> with a N/P ratio of 8 or were first diluted in the provided mRNA buffer followed by the mixing-in of *in vivo*-jetRNA<sup>®</sup> with a ratio of 1 µg mRNA:2 µL of *in vivo*-jetRNA<sup>®</sup>+. Following an incubation of 15 minutes at room temperature for both transfection reagent, complexes were injected by RO route (10 µg DNA per mouse). One or seven days after injection, mice were anesthetized by CO<sub>2</sub> inhalation. The organs of interest were dissected, rinsed in PBS (x 1) and mixed with Precellys<sup>®</sup> homogenizer in 1 mL for spleen and in 2 mL for lungs and liver of lysis buffer x1 (Promega).

Each organ mix was frozen at -80 °C, thawed and an aliquot of 0.5 mL was taken for luciferase analysis. The aliquot was centrifuged for 5 min at 12,000 rpm at 4 °C. Luciferase enzyme activity was assessed on 5 µL of organ lysate supernatant using 100 µL of luciferin solution (Promega). The luminescence (expressed as RLU) was measured by using a luminometer (Centro LB 960, Berthold) and normalized per mg of organ protein with Pierce BCA Assay Protein Kit (Thermo-Fisher).

### 2.5. *In vivo* Delivery via IM Injection

On the day of administration, *in vivo*-jetRNA<sup>®</sup>+/Luc2 DNA complexes were prepared according to the manufacturers' recommendations. Briefly, 15 µg of Luc2-encoding DNA were first diluted in the provided mRNA buffer followed by the mixing-in of *in vivo*-jetRNA<sup>®</sup> with a ratio of 1 µg DNA:2 µL of *in vivo*-jetRNA<sup>®</sup>+. Following an incubation of 15 minutes at room temperature for both transfection reagent, complexes were injected by IM route in quadriceps (15 µg DNA per mouse). One or seven days after injection, mice were anesthetized by CO<sub>2</sub> inhalation. Quadriceps were dissected, rinsed in PBS (x 1) and mixed with Precellys<sup>®</sup> homogenizer in 1 mL of lysis buffer x1 (Promega). Each organ mix was frozen at -80 °C, thawed and an aliquot of 0.5 mL was taken for luciferase analysis. The aliquot was centrifuged for 5 min at 12,000 rpm at 4 °C. Luciferase enzyme activity was assessed on 5 µL of organ lysate supernatant using 100 µL of luciferin solution (Promega). The luminescence (expressed as RLU) was measured by using a luminometer (Centro LB 960, Berthold) and normalized per mg of organ protein with Pierce BCA Assay Protein Kit (Thermo-Fisher).

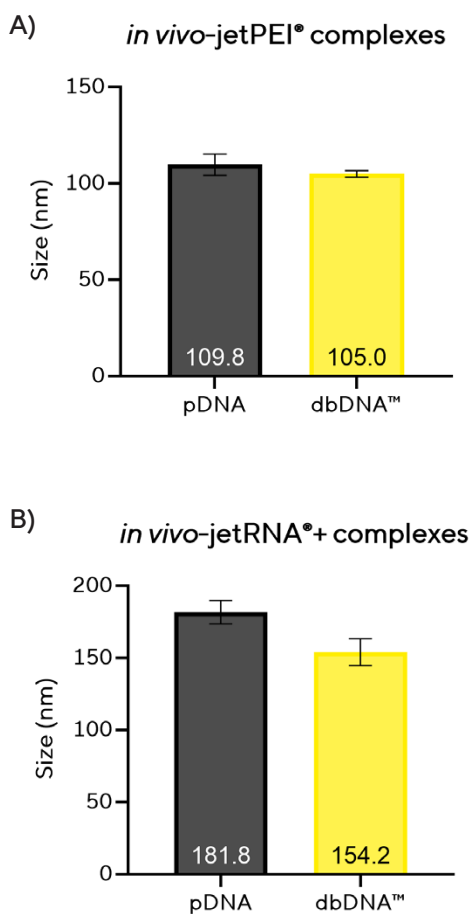
### 2.6. Statistical Analyses

Statistical analyses were conducted with GraphPad Prism 10. Data were reported as mean. The statistical analyses were described in the figure legends. Two-way ANOVA analysis were performed when comparing multiple groups. \*\*P-values < 0.005, \*\*\*\*P < 0.0001 were considered statistically significant.

### 3. Results

#### 3.1. Smaller Complexes With dbDNA™ With *in vivo*-jetPEI® and *in vivo*-jetRNA®+

It is well established that the size and nature of the nucleic acid influence the size of complexes formed with *in vivo*-jetPEI® or *in vivo*-jetRNA®+. Prior to injection, particle size was measured by DLS, and the intensity mean hydrodynamic diameters are reported in Figure 1 for both reagents complexed with either pDNA or dbDNA™. For *in vivo*-jetPEI® complexed with 10 µg of DNA, dbDNA™ resulted in a slight reduction in complex size. In contrast, for *in vivo*-jetRNA®+ complexed with 15 µg of DNA, dbDNA™ led to a pronounced decrease in particle size.



**Figure 1: Impact of DNA on complexes sizes with *in vivo*-jetPEI® (A) or *in vivo*-jetRNA®+ (B).**

A. *in vivo*-jetPEI® complexation with 10 µg DNA,  
B. *in vivo*-jetRNA®+ complexation with 15 µg DNA.  
Mean with SD of intensity (nm), size measured by DLS.

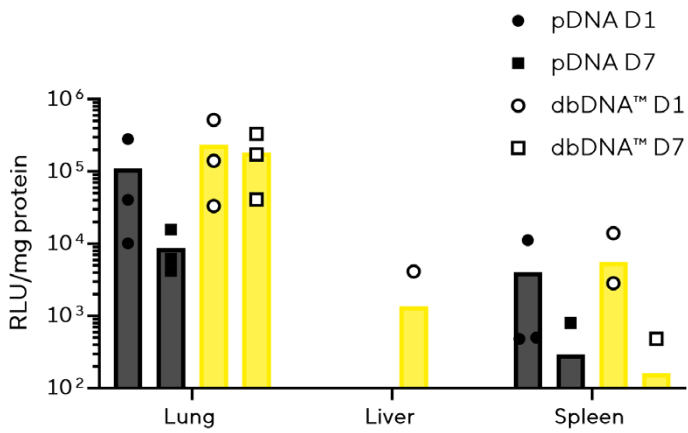
#### 3.2. dbDNA™ Complexes With *in vivo*-jetPEI® Demonstrate Higher and Longer Luciferase Expression *in Lungs*

Firstly, luciferase expression was measured at one and seven days after RO injection of 10 µg pDNA and dbDNA™ encoding Luc2 using the *in vivo*-jetPEI® delivery system (Figure 2). The expression levels are quantified in relative light units per milligram of protein (RLU/mg protein) across three major organs: lungs, liver, and spleen.

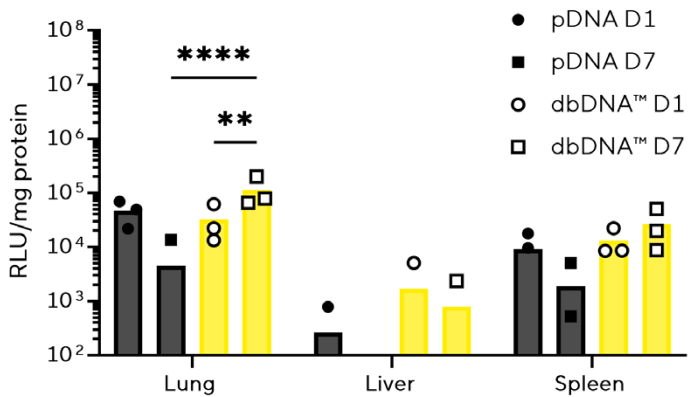
As expected and described in the literature<sup>12</sup> with *in vivo*-jetPEI® delivery system, the lungs and spleen exhibit measurable luciferase activity ( $4 \times 10^3$ - $2 \times 10^5$  RLU/mg protein) one day after injection of both DNAs, reflecting the distribution and uptake of the DNA across these different tissues.

Notably, higher luciferase expression is achieved in the lungs ( $1$ - $2 \times 10^5$  RLU/mg protein) compared to the spleen ( $4$ - $6 \times 10^3$  RLU/mg protein), indicating effective transfection and gene expression in this organ. In the lungs, one day post-injection, dbDNA™ shows slightly higher luciferase expression ( $2 \times 10^5$  RLU/mg protein) compared to pDNA ( $1 \times 10^5$  RLU/mg protein). Interestingly, seven days after dbDNA™ injection, luciferase expression remains similar to the levels observed one day post-injection. Additionally, seven days after DNA injection, dbDNA™ maintains higher luciferase expression ( $2 \times 10^5$  RLU/mg protein) compared to pDNA ( $9 \times 10^3$  RLU/mg protein) suggesting that dbDNA™ expression is more stable than pDNA. In the spleen, slightly higher luciferase expression is achieved with dbDNA™ ( $6 \times 10^3$  RLU/mg protein) compared to pDNA ( $4 \times 10^3$  RLU/mg protein) one day post-injection. However, seven days after the injection, luciferase expression decreases to near-background levels for both DNAs ( $2$ - $3 \times 10^2$  RLU/mg protein). Finally, in the liver, only dbDNA™ demonstrates luciferase expression ( $1 \times 10^3$  RLU/mg protein) one day post-transfection.

Overall, these results underscore the efficacy of *in vivo*-jetPEI® as a delivery system for pDNA and dbDNA™, achieving notable luciferase expression in lungs and demonstrating its potential for targeted gene expression *in vivo*. Furthermore, these data highlight the differential luciferase expression between dbDNA™ and pDNA across various organs. dbDNA™ exhibits slightly higher luciferase expression in all tested organs one day after injection and shows particularly more stable expression in the lungs seven days post-injection compared to pDNA.



**Figure 2: Luciferase expression in lungs, liver and spleen after one and seven days after RO injection of pDNA or dbDNA™ complexed with *in vivo*-jetPEI®.** D1: Day 1, D7: Day 7. Mean (n = 3 biologically independent animals).



**Figure 3: Luciferase expression in lungs, liver and spleen, one and seven days after RO injection of pDNA or dbDNA™ complexed with *in vivo*-jetRNA®+.** 10 µg of DNA have been injected in mice by RO after complexation with *in vivo*-jetRNA®+ (Ratio 1:2). Lungs, spleen and liver were analysed one (D1) and seven (D7) days after injection. Mean (n = 3 biologically independent animals). Statistical significance was calculated with a two-way ANOVA with multiple comparison test: \*\*P < 0.005, \*\*\*\*P < 0.0001.

### 3.3. Longer Expression in the Lungs and Spleen With dbDNA™ Complexes With *in vivo*-jetRNA®+

*In vivo*-jetRNA®+ is a ready to use solution composed of PFVs formulated for *in vivo* mRNA delivery and can also be used to complex other nucleic acids, including DNA. In Figure 3, pDNA and dbDNA™ encoding Luc2 were complexed with *in vivo*-jetRNA®+ and administered to mice by RO injection at 10 µg DNA per mouse. After one and seven days post-injection, organs were harvested and luciferase activity was quantified (RLU/mg protein).

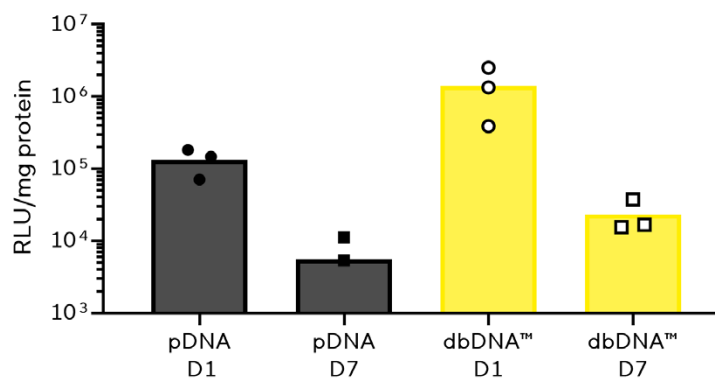
Consistent with its mRNA delivery profile<sup>3,4</sup>, *in vivo*-jetRNA®+ drives high protein expression in the lungs and spleen with reduced off-target expression in the liver. A similar organ distribution was observed with DNA-containing particles (pDNA or dbDNA™), with approximately 5×10<sup>4</sup> RLU/mg protein in lungs and 1×10<sup>4</sup> RLU/mg protein in spleen one day after injection. One day post-injection, lung and spleen signals were comparable between the pDNA and dbDNA™ constructs. Seven days post-injection, however, pDNA-driven expression had declined across organs, whereas dbDNA™ maintained splenic expression and produced an approximately one-log higher signal in the lungs.

These findings are concordant with results obtained using *in vivo*-jetPEI® (Figure 2) and indicate that dbDNA™ supports more durable (and in the lungs, enhanced) expression across multiple organs following intravenous administration. Given the increase observed seven days after the injection, expression appears to be sustained for at least one week post-administration.

### 3.4. dbDNA™ Complexes With *in vivo*-jetRNA®+ Demonstrate Higher Luciferase Expression in Muscle

Injection by IM route is commonly used for vaccine delivery. An increase in the duration of expression extends exposure to antigens and can be beneficial in increasing the effectiveness of vaccination. Both types of DNA were therefore complexed with *in vivo*-jetRNA®+ and were injected into mice into the quadriceps of the hind leg at a dose of 15 µg DNA per mouse. One and seven days after injection, the muscles were harvested and luciferase expression was analysed (Figure 4).

IM injection of complexes containing either dbDNA™ or pDNA elicited measurable luciferase activity in muscle one day post-injection. Figure 4 shows that dbDNA™ produced a higher signal than pDNA at 24 h (dbDNA™,  $1 \times 10^6$  RLU/mg protein; pDNA,  $1 \times 10^5$  RLU/mg protein), corresponding to approximately a tenfold increase. Overall expression declined after seven days post administration however, expression from dbDNA ( $2 \times 10^4$  RLU/mg protein) remained higher than pDNA ( $5 \times 10^3$  RLU/mg protein), suggesting that dbDNA™ may be advantageous for vaccination applications, where robust and sustained transgene expression is expected to enhance immunogenicity and, consequently, vaccine efficacy.



**Figure 4: Luciferase expression in muscle, one and seven days after IM injection of pDNA or dbDNA™ complexed with *in vivo*-jetRNA®+.** 15 µg of DNA have been injected in mice by IM injection after complexation with *in vivo*-jetRNA®+ (Ratio 1:2). Quadriceps were analysed one day (D1) and seven days (D7) after injection. Mean (n = 3 biologically independent animals).

## 4. Discussion

Touchlight's dbDNA™ platform provides inherent manufacturing flexibility, supporting both scale-up (targeting large indications) and scale-out (required for neoantigen vaccines) production while offering the advantages of a functionally smaller DNA vector. When effectively delivered, dbDNA™ enables efficient and durable transgene expression, supporting success in both non-viral gene therapy and DNA vaccines.

The efficacy of DNA (of any format) benefits from the use of either a delivery device or a formulation to facilitate cell transfection; delivery of naked pDNA via needle and syringe alone has a long history of limited clinical success. While a wide range of easy-to-assemble, off-the-shelf formulations with diverse chemistries have long been available for *in vitro* transfection, access to formulations suitable for *in vivo* applications has remained more challenging.

The availability of polymer- and lipid-based delivery reagents from Sartorius provides researchers with a practical opportunity to evaluate the impact of formulation strategies in *in vivo* models, thereby accelerating the optimization of DNA therapeutics and vaccines.

This study shows that both *in vivo*-jetPEI® and *in vivo*-jetRNA®+ can effectively deliver pDNA and dbDNA™, with the choice of delivery reagent and injection route influencing the biodistribution of the complexes and the resulting organ-specific luciferase expression. Across all tested organs, Touchlight's dbDNA™ consistently provides increased and more sustained luciferase expression than pDNA, offering key benefits for vaccination and non-viral gene therapy applications. Notably, dbDNA™ exhibited sustained expression in the lung and muscle tissues, suggesting enhanced stability and potential for long-term gene expression. This characteristic could be particularly advantageous in applications such as vaccination, where prolonged transgene expression is crucial for improved immunogenicity. Additionally, the use of *in vivo*-jetRNA®+ resulted in reduced off-target expression, offering a more targeted approach to gene delivery. Superior expression offers the possibility of dose sparing, with reductions in DNA dose consequently also reducing the amount of formulation, further reducing cost. Additionally, some formulations (especially when delivered systemically) may exhibit tolerability challenges in the animal, limiting the dose that can be delivered without toxicity. Dose sparing while maintaining protein expression levels is a key advantage of dbDNA™. Overall, these findings underscore the efficacy of these delivery systems, with dbDNA™ emerging as a promising candidate for achieving robust and sustained gene expression *in vivo*.

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## Germany

Sartorius Stedim Biotech GmbH  
August-Spindler-Strasse 11  
37079 Goettingen  
Phone +49 551 308 0

## USA

Sartorius Stedim North America Inc.  
565 Johnson Avenue  
Bohemia, NY 11716  
Toll-Free +1 800 368 7178

 **For further information, visit**  
[sartorius.com](https://www.sartorius.com)