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White Paper

Benefits of PCR-based mRNA Manufacturing Processes

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mRNA vaccines and therapeutics have become one of the most widely known and growing modalities in recent years.[1,2] While COVID vaccine development occurred in record time, new innovations in manufacturing technologies are critical for successful development of new mRNA applications. Production of these vaccines and therapeutics typically begins with manufacturing of a large quantity of plasmid DNA (pDNA) followed by linearization using restriction enzymes (conventional process).[3] This process is time-consuming and resource intensive as the requirement for a sufficient quantity and quality of pDNA can create a bottleneck in the manufacturing workflow. Moreover, the extensive utilization of genetically modified bacteria and antibiotics is an additional challenge under good manufacturing practice (GMP). To overcome pDNA-associated challenges and bottlenecks, the use of synthetic templates is ideal.[4,5] Several strategies for manufacturing of synthetic DNA have been described and each strategy offers intrinsic advantages as mRNA template. The fastest strategy is based on simple chemical synthesis of oligomeric DNA or longer fragments.[4] Due to the intrinsic size limitation (<3,000 bp), this strategy is only viable for manufacturing of small mRNAs.[6] As robust standard in molecular biology, polymerase chain reaction (PCR)[7] represents a fast alternative for synthesis of diversly sized DNAs. Using PCR to generate the template material for mRNAs is commonly used for small-scale manufacturing. Upscaling the PCR reaction for manufacturing larger amounts of mRNA, e.g., for vaccination, was previously not described. To circumvent the technical challenge in PCR upscaling, isothermal DNA amplification strategies were elaborated.[8] Most of these strategies were developed as analytical alternatives to PCR e.g., if access to thermocyclers was limited or to ensure point-of-care (POC) assessments.[9] However, especially the exponential isothermal strategies are limited by inherent amplification biases like nonspecific amplification or low accuracy.[9,10]

To combine the high quality advantages of synthetic templates with high accuracy and scalability, we developed a proprietary PCR solution. Thereby, we can assign the high accuracy of PCR to mRNA production. This inimitable combination allows mRNA manufacturing with a unique set of advantages.

Advantages of PCR-based mRNA Manufacturing

PCR-based generation of synthetic *in vitro* transcription (IVT) templates enables reliable manufacturing of high-quality mRNA. With this approach, many of the complexities associated with the conventional process are eliminated.

High performance

- through reduced product-related impurities such as trailing and truncated products.
- Increased mRNA homogeneity, stability and prolonged expression.

Increased purity with synthetic template

- ensuring high quality from the start of the process, via cell-free process, mitigating microbial DNA, protein and endotoxins.

Robust scalable process

- Highly reproducible process, leading to reliable mRNA supply. Scaling solution enables mRNA manufacturing from mg to multi-gram scale.

Debottleneck pDNA sourcing

- 10,000x less pDNA required compared to conventional process to manufacture the same amount of mRNA.
- pDNA requirements according to GMP principles only.[11]

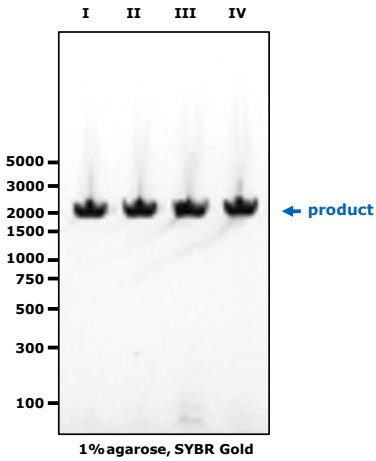
Sequence customization

- including flexible poly(A) tailing during PCR step, circumventing initial pDNA yield issues and truncation when pre-encoded in conventional process workflows.

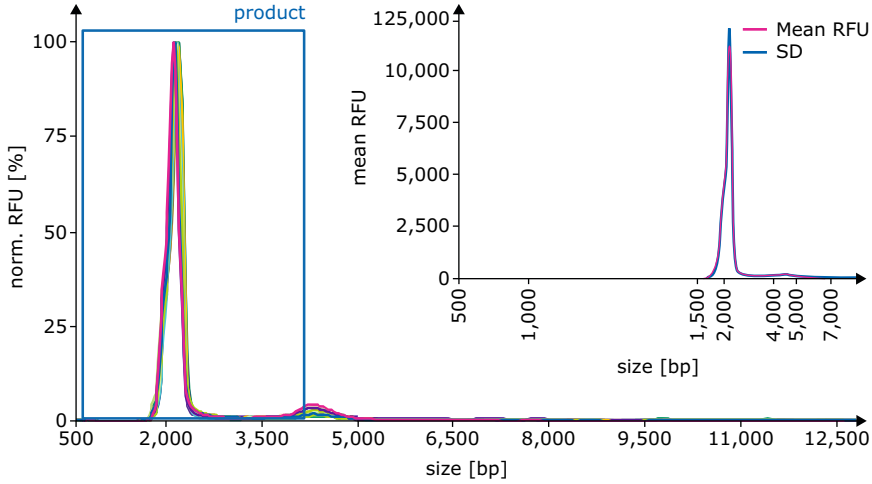
Technical Aspects

PCR-based templates offer a maximum of flexibility, scalability, and quality in mRNA manufacturing. To ensure these benefits, an optimized PCR setup was developed for increased yield and specific amplification with high fidelity.

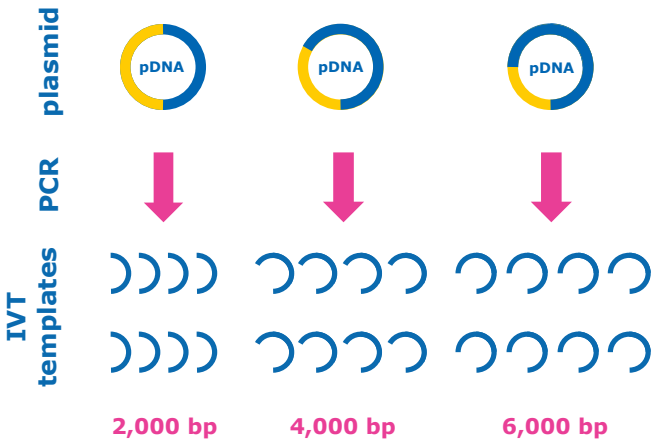
A. Strong amplification



B. High specificity



C. Evaluated template sizes



D. NGS fidelity assessment

PCR-based IVT template	Reads	Bases
2,000 bp – R1	354,610	58,634,357
2,000 bp – R2	354,610	58,873,595
4,000 bp – R1	382,768	65,988,994
4,000 bp – R2	382,768	66,299,497
6,000 bp – R1	384,502	66,862,742
6,000 bp – R2	384,502	67,227,629

E. Amplification performance

mRNA size	PCR protocol	Seq.	Mean error rate*	SD
2,000 nt	AmpTec™ standard process	R1	9.6×10^{-9}	$\pm 1.0 \times 10^{-8}$
2,000 nt		R2		
4,000 nt	AmpTec™ standard process	R1	2.6×10^{-9}	$\pm 1.2 \times 10^{-9}$
4,000 nt		R2		
6,000 nt	AmpTec™ standard process	R1	6.2×10^{-10}	$\pm 3.2 \times 10^{-10}$
6,000 nt		R2		
2,000 nt	AmpTec™ fine-tuned process	R1	3.2×10^{-9}	$\pm 2.7 \times 10^{-9}$
2,000 nt		R2		

F. Fidelity comparison

Biological comparison	Error rate
Taq Polymerase	$\sim 10^{-5}$
E. coli replication	$\sim 10^{-9}$ to 10^{-10}
Likelihood contrast	**Probability to win**
Lottery (6 out of 49)	$\sim 7 \times 10^{-8}$

Figure 1. Specific amplification of PCR-based IVT templates from four separate productions, shown in agarose gel electrophoresis (**A**). High uniformity of crude PCR reactions by CE (**B**). Depicted as normalized (norm.) or mean relative fluorescence units (RFU) with standard deviation (SD): n = 20 productions. PCR-based IVT template reference sequences used for analysis by NGS (**C**). NGS sequence analysis of PCR-based IVT templates based on various reference sequences (**D**). Fidelity assessment of PCR-generated IVT templates. R1 and R2 are individual amplification runs (**E**). Supplementary Table. Contrasts for fidelity assessment of PCR-generated IVT templates by NGS (**F**).[15,16]

Robust PCR Synthesis of Homogenous IVT Templates

Using electrophoretic techniques, characteristic attributes of PCR-based template generation were visualized. With agarose electrophoresis, clear product bands reported efficient amplification by PCR (**Figure 1A**). The complete absence of undesired byproducts further demonstrated the highly specific amplification. Similarly, distinct product signals were observed in capillary electrophoresis (CE); homogeneity of a broad set of individual crude PCR reactions further showed the high reproducibility (**Figure 1B**).

IVT Template Fidelity Assessment

To confirm high-yield amplification with high fidelity, PCR-based IVT templates were analyzed using next generation sequencing (NGS). Various reference sequences with different lengths were used (**Figure 1C**). High coverage was ensured by sequencing the same position several thousand times (**Figure 1D**).

The assessment was further restricted to high quality sequencing data. A Phred quality score of at least 30 was used to guarantee a base call accuracy of at least 99.9%.

Based on the data and established procedures, error rates in the range of 10^{-9} to 10^{-10} were determined. These error rates were in the same range as *Escherichia coli* (*E. coli*) replication, which represents the origin of plasmid-based IVT templates. A second optimized PCR protocol was developed, and the PCR product fidelity was analyzed using NGS with the same high-quality standards. While significantly increasing the yield, high fidelity was maintained (**Figure 1E**).

High Purity of PCR-based IVT Templates

Purity of PCR-based IVT templates was assessed with a focus on residual *E. coli* proteins. Here, residual *E. coli* proteins were not detected (**Figure 2**). In contrast, significant levels of residual *E. coli* protein were detected in batches of purified plasmid-based templates, which can carry over into the IVT reaction.

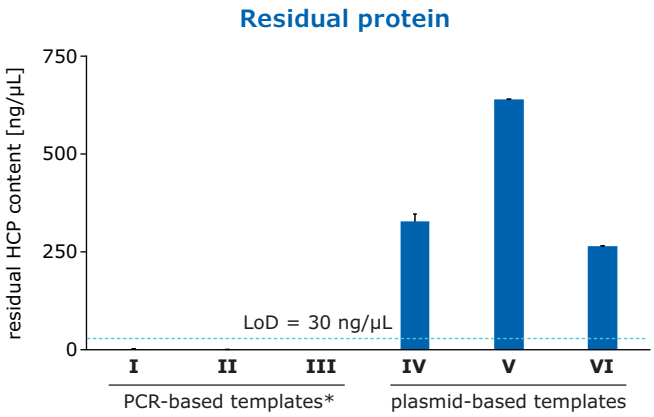


Figure 2. PCR-based generation of IVT templates enabled improved purity profiles. For plasmid-based IVT templates, as used in conventional processes, high levels of proteins were detected by an *E. coli* host cell protein (HCP) specific ELISA (enzyme linked immunosorbent assay). Plasmid-based IVT templates were commercially obtained. Mean value, error bars indicate standard deviation. *non-diluted samples

High Reproducibility of mRNA Synthesis

The high reproducibility of PCR-based template generation further enables mRNA synthesis with definite sizes. In CE, the definite signal of crude PCR products is reflected in the final mRNA products (**Figure 3**).

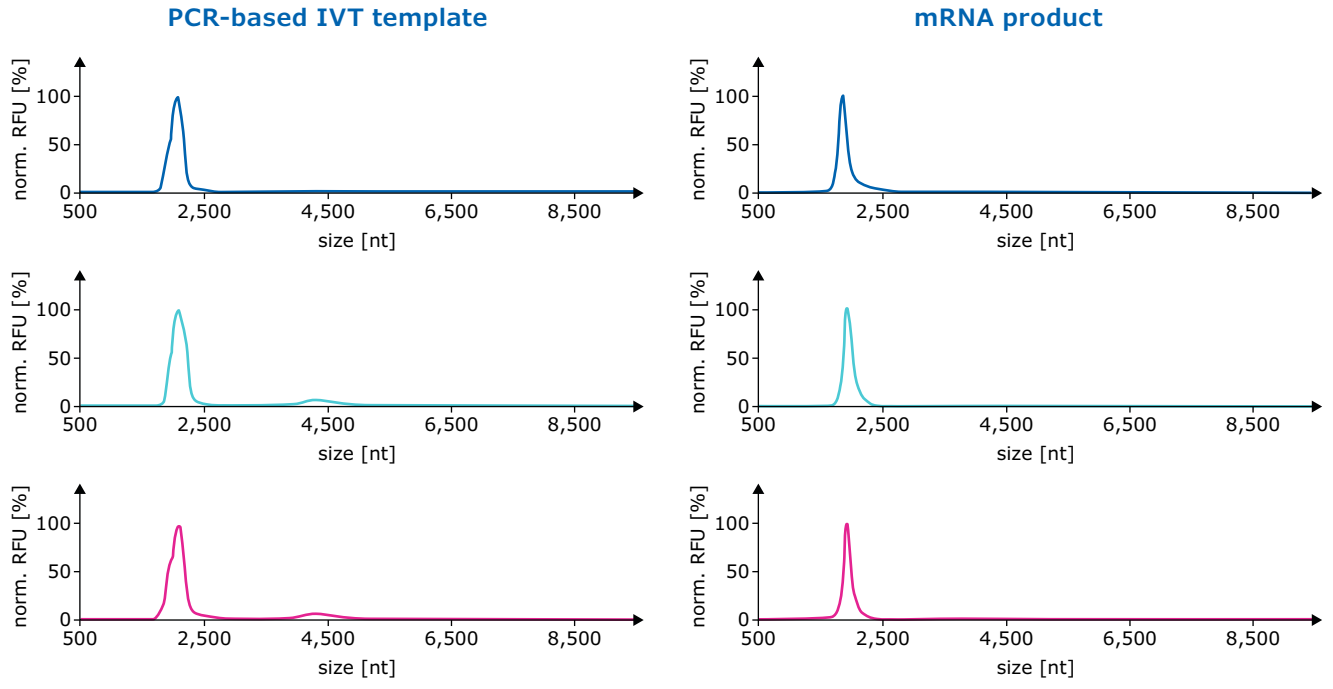


Figure 3. Robust and reproducible mRNA synthesis using PCR-based IVT templates. Data shown from 3 individual productions.

Improved mRNA Integrity

PCR-based mRNAs were contrasted with competitor mRNAs manufactured via conventional processes using linearized plasmids. For an appropriate integrity comparison in CE, mRNAs purified by a simple silica membrane workflow were used. The PCR-based upstream process resulted in a clearly defined product at the expected product size. In contrast, several undefined signals were detected in the mRNA generated using linearized plasmids (**Figure 4**).

Exact mRNA size conformity was further analyzed by CE. With the PCR-based mRNA, nearly 100% of the expected sizes were detected. This contrasted with mRNAs generated by conventional processes based on linearized plasmids. Here, mRNAs exhibited a heterogeneous distribution and a size deviation of approximately 16% (**Figure 4**). A heterogenous size distribution may affect the performance of mRNA in clinical applications.

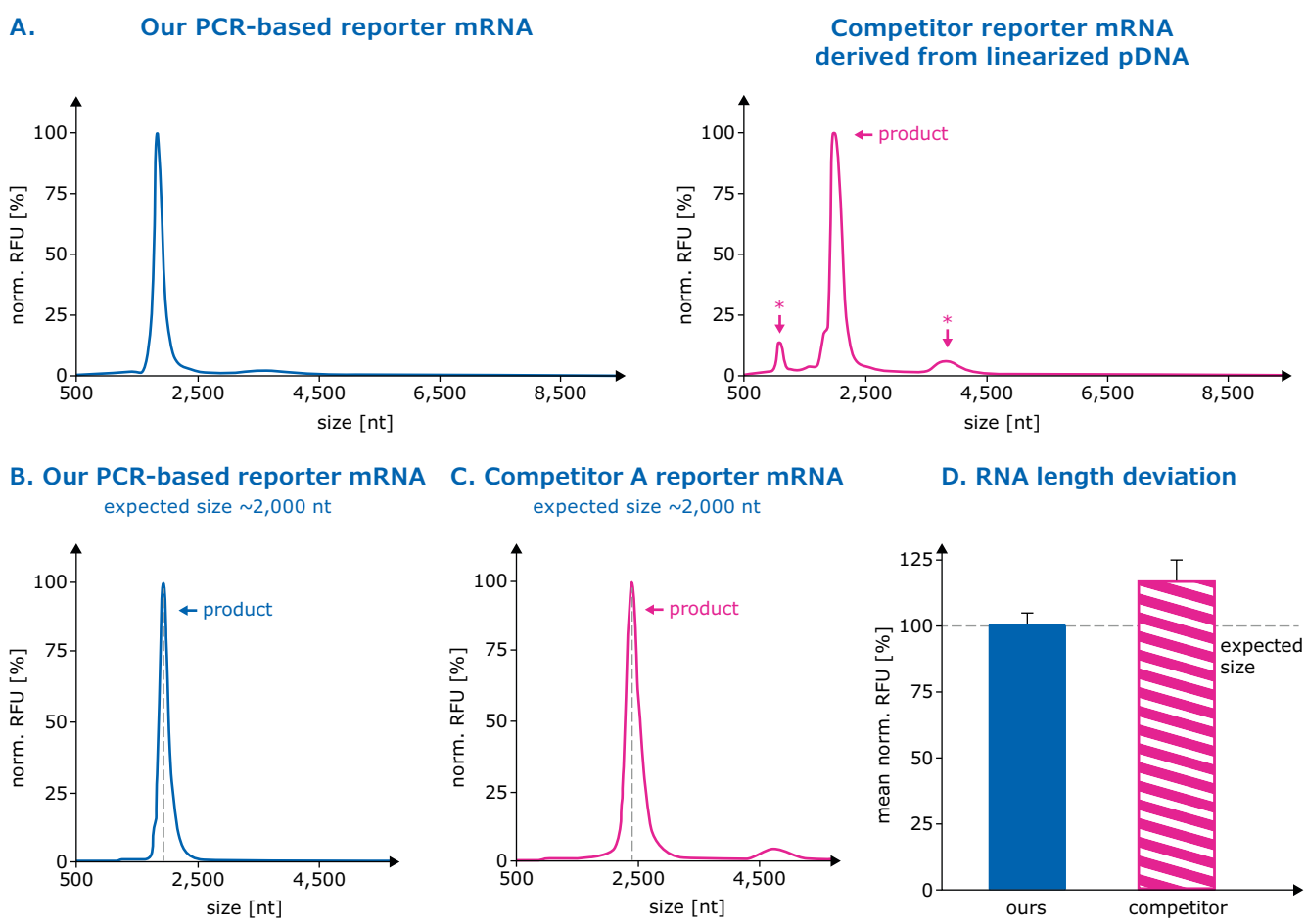


Figure 4. Comparison of mRNA integrity from the PCR-based method comparison to conventional plasmid-based synthesis. *undefined signal (**A**). Robust and reproducible mRNA integrity in CE using PCR-based manufacturing (**B**). In contrast to conventional processes based on linearized plasmids (**C**). Mean mRNA length deviation (n = 5) with error bars indicating SD (**D**), vertical dashed line indicates highest signal.

Finally, mRNAs generated by the PCR-based manufacturing process were comprehensively analyzed with focus on critical quality attributes (**Table 1**). The assessment conclusively confirmed that the proprietary PCR-based workflow produces mRNA with high quality and especially with improved purity, and integrity.

Critical quality attribute	Analytical procedure	Observed values
Residual total protein	NanoOrange™ Fluorogenic assay	<0.71 ng/µg mRNA
mRNA purity	A260/A280	2.33
mRNA length deviation	Capillary electrophoresis	2%
mRNA integrity	Capillary electrophoresis	90%
Residual template	qPCR	2.33 × 10^{-2} ppm
dsRNA	Dot blot	0.7%
5′ capping	LC-MS	>99%
Poly(A) length	LC-MS	123 A

Table 1. Summary of quality attributes of mRNA produced using PCR-based IVT templates.

Conclusion

PCR-based manufacturing of mRNA is a robust alternative to linearized plasmid-based synthesis with multiple advantages.

From an operational perspective, PCR-based IVT templates overcome bottlenecks in plasmid supply. Additionally, the need for enzymatic linearization with potential byproducts is eliminated. NGS analysis demonstrated that an elaborated PCR setup enabled the synthesis of IVT templates with highest fidelities. The introduction of the poly(A)-tail, directly during PCR, provides high flexibility towards optimized translation efficiencies.[17,18] The definite size of the primer-encoded poly(A)-tails is further transferred to the final mRNAs. Hence, undesired recombination events as observed in *E. coli* for plasmid-encoded poly(A)-tails are also excluded.[12,13,14]

Since the PCR-based production process is cell-free, introduction of microbial DNA, microbial proteins, and endotoxins is minimized. The improved purity profile is directly reflected in the final mRNA products (**Table 1**).

In summary, the presented PCR-based process robustly generates high quality mRNA with reproducible performance. The process design allows manufacturing of mRNAs to meet clinical as well as commercial needs.

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References

1. Qin S, Tang X, Chen Y, Chen K, Fan N, Xiao W, Zheng Q, Li G, Teng Y, Wu M, Song X. mRNA-based therapeutics: powerful and versatile tools to combat diseases. Signal Transduct Target Ther. 2022 May 21;7(1):166.
2. Liu C, Shi Q, Huang X, Koo S, Kong N, Tao W. mRNA-based cancer therapeutics. Nat Rev Cancer. 2023 Aug;23(8):526-543.
3. Whitley J, Zwolinski C, Denis C, Maughan M, Hayles L, Clarke D, Snare M, Liao H, Chiou S, Marmura T, Zoeller H, Hudson B, Peart J, Johnson M, Karlsson A, Wang Y, Nagle C, Harris C, Tonkin D, Fraser S, Capiz L, Zeno CL, Meli Y, Martik D, Ozaki DA, Caparoni A, Dickens JE, Weissman D, Saunders KO, Haynes BF, Sempowski GD, Denny TN, Johnson MR. Development of mRNA manufacturing for vaccines and therapeutics: mRNA platform requirements and development of a scalable production process to support early phase clinical trials. Transl Res. 2022 Apr;242:38-55.
4. Gaur RK, Krupp G. Preparation of templates for production of ribozymes and substrates. Methods Mol Biol. 1997;74:69-78.
5. de Mey W, De Schrijver P, Autaers D, Pfitzer L, Fant B, Locy H, Esprit A, Lybaert L, Bogaert C, Verdonck M, Thielemans K, Breckpot K, Franceschini L. A synthetic DNA template for fast manufacturing of versatile single epitope mRNA. Mol Ther Nucleic Acids. 2022 Aug 17;29:943-954.
6. Tossberg JT, Esmond TM, Aune TM. A simplified method to produce mRNAs and functional proteins from synthetic double-stranded DNA templates. Biotechniques. 2020 Oct;69(4):281-288.
7. Mullis K, Faloona F, Scharf S, Saiki R, Horn G, Erlich H. Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. Cold Spring Harb Symp Quant Biol. 1986;51 Pt 1:263-73.
8. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 2000 Jun 15;28(12):E63.
9. Zhao Y, Chen F, Li Q, Wang L, Fan C. Isothermal Amplification of Nucleic Acids. Chem Rev. 2015 Nov 25;115(22):12491-545.
10. Oliveira BB, Veigas B, Baptista PV. Isothermal Amplification of Nucleic Acids: The Race for the Next "Gold Standard". Front. Sens. 2021 Sept 2; 752600.
11. https://www.ema.europa.eu/en/documents/other/questions-and-answers-principles-gmp-manufacturing-starting-materials-biological-origin-used-transfer-genetic-material-manufacturing-atmps_en.pdf
12. Grier AE, Burleigh S, Sahni J, Clough CA, Cardot V, Choe DC, Krutein MC, Rawlings DJ, Jensen MC, Scharenberg AM, Jacoby K. pEVL: A Linear Plasmid for Generating mRNA IVT Templates with Extended Encoded Poly(A) Sequences. Mol Ther Nucleic Acids 5, 2016; E306.
13. Trepotec Z, Geiger J, Plank C, Aneja MK, Rudolph C. Segmented poly(A) tails significantly reduce recombination of plasmid DNA without affecting mRNA translation efficiency or half-life. RNA 25, 2019: 507-518
14. Preiss T, Muckenthaler M, Hentze MW. Poly(A)-tail-promoted translation in yeast: implications for translational control. RNA 4, 1998; 1321-31.
15. McInerney P, Adams P, Hadi MZ. Error Rate Comparison during Polymerase Chain Reaction by DNA Polymerase. Mol Biol Int, 2014; 287430.
16. Fijalkowska IJ, Schaaper RM, Jonczyk P. DNA replication fidelity in Escherichia coli: a multi-DNA polymerase affair. FEMS Microbiol Rev 36, 2012; 1105-1121.
17. Li CY, Liang Z, Hu Y, Zhang H, Setiasabda KD, Li J, Ma S, Xia X, Kuang Y. Cytidine-containing tails robustly enhance and prolong protein production of synthetic mRNA in cell and in vivo. Mol Ther Nucleic Acids 30, 2022; 300-310.
18. Xiang K, Bartel DP. The molecular basis of coupling between poly(A)-tail length and translational efficiency. eLife 10, 2021; e66493.

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