Engineering circular ribonucleic acids (circRNAs) for improved protein production

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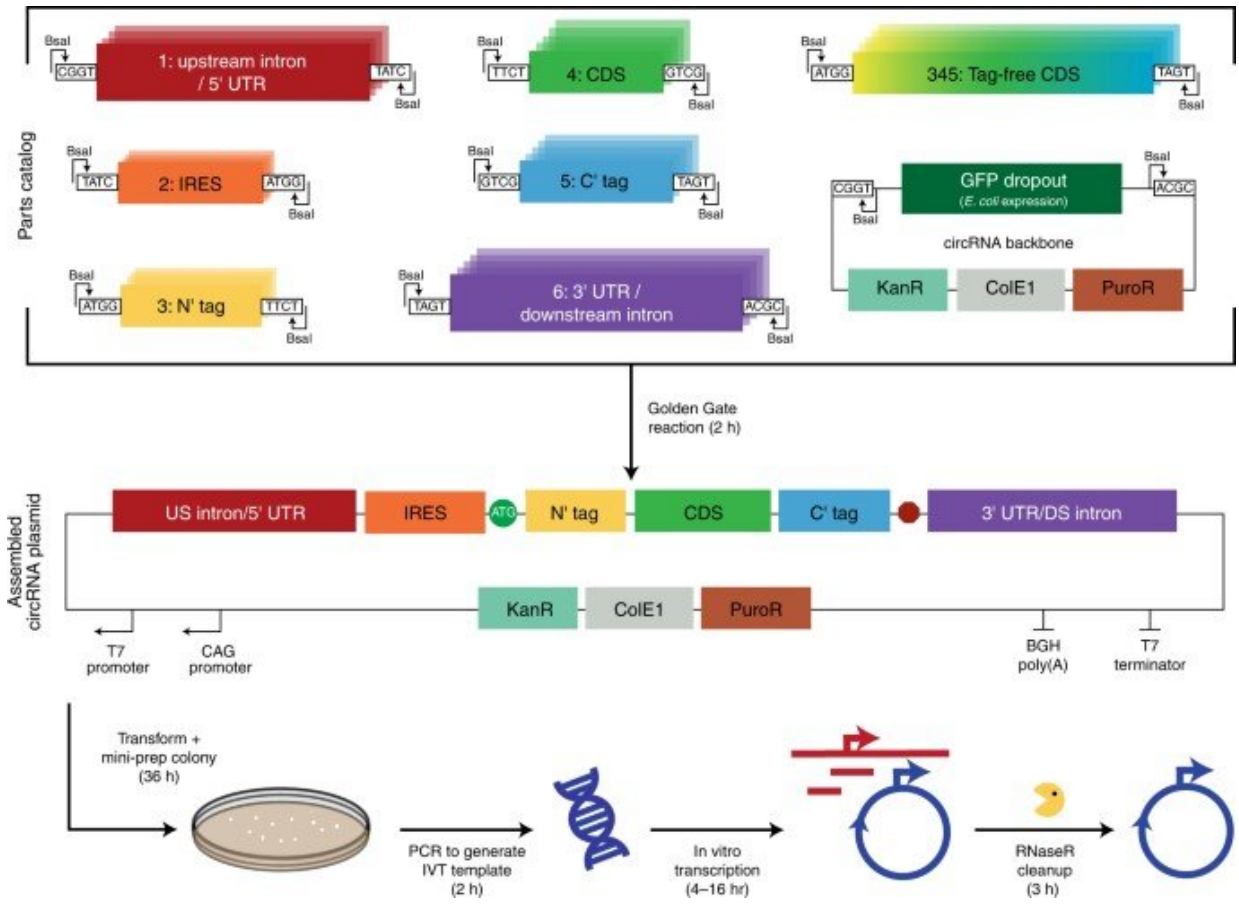


A modular cloning platform for circRNA enables rapid design–build–test cycles. Schematic describing the modular cloning platform used to create template plasmids for circRNA synthesis. Parts 1–6 corresponding to the upstream intron and 5′ UTR, IRES, N-terminal (N′) tag, coding sequence (CDS), C-terminal (C′) tag and 3′ UTR and the downstream intron were individually cloned into part plasmids via Golden Gate reactions (Supplementary Fig. 1). Part plasmids and the circRNA backbone were then combined in a second Golden Gate reaction to

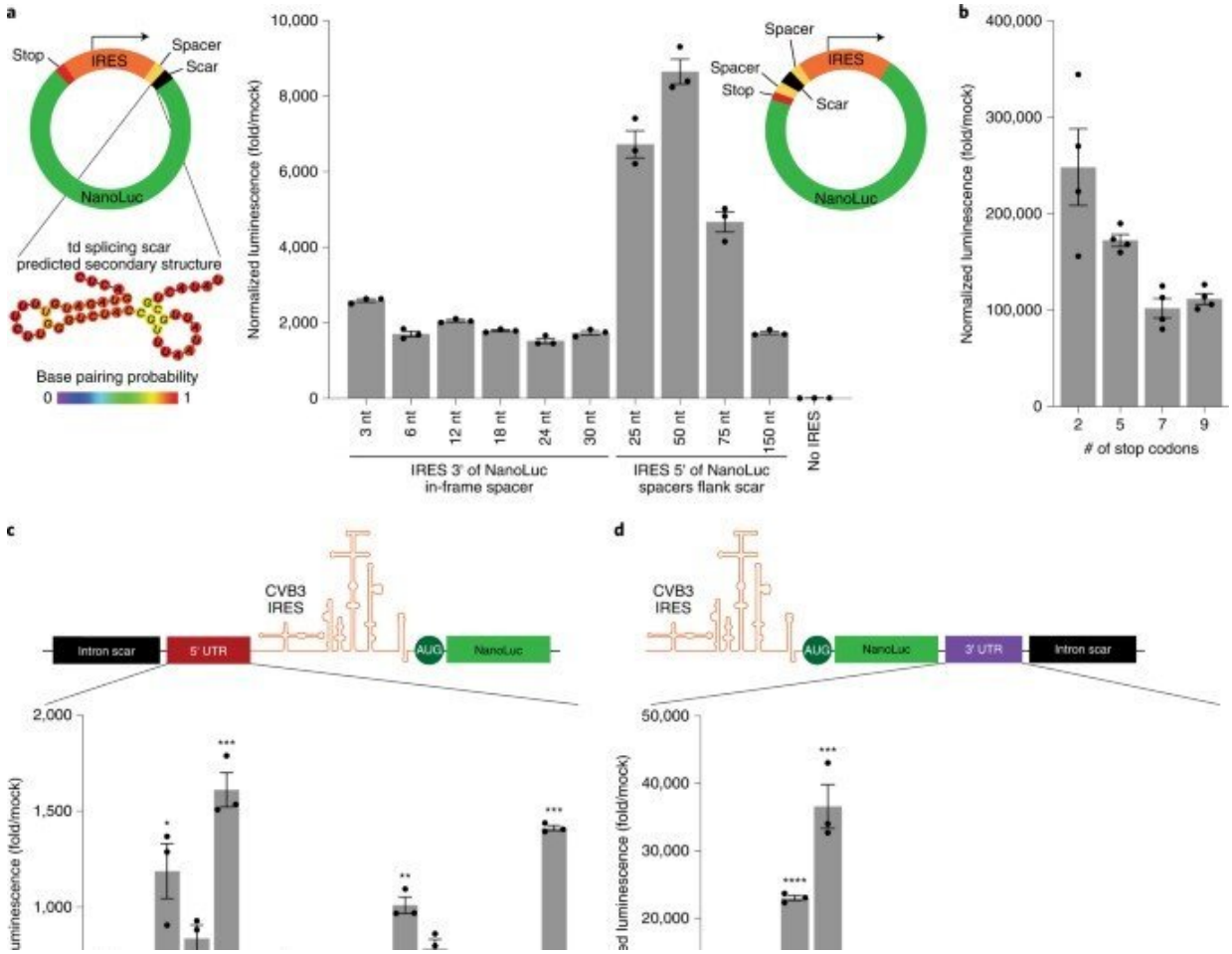
create a circRNA plasmid. The circRNA backbone contains a CAG promoter enabling circRNA transcription after transient transfection in cellulo, a T7 promoter enabling IVT, homology sequences that assist with RNA circularization, low-structure regions that facilitate RNaseR processivity and a bacterially expressed GFP dropout sequence to negatively select for incorrect assemblies. If a CDS without N′ or C′ tags was used, parts 3–5 were replaced with a single part. PCR products from circRNA plasmids were subsequently used as templates for IVT to synthesize RNA. Lastly, RNaseR cleanup was performed to digest linear RNAs and isolate circRNA. DS, downstream. Credit: *Nature Biotechnology* (2022). DOI: 10.1038/s41587-022-01393-0

Circular ribonucleic acids (circRNAs) are a promising platform for gene expression studies as a stable and prevalent ribonucleic acid in eukaryotic cells, which arise from back-splicing. In a new report now published in *Nature Biotechnology*, Robert Chen and a team of interdisciplinary researchers at Stanford University, California, U.S., developed a systematic approach to rapidly assemble and test features affecting protein production based on synthetic circular RNAs. The team maximized translation of the circRNA by optimizing fine elements to implement design principles to improve circular RNA yield by several hundred-fold. The outcomes facilitated an increased translation of the RNA of interest, when compared to messenger RNA (mRNA) levels, to provide durable translation in vivo.

Developing circular RNA (circRNA) in the lab

Therapeutics based on ribonucleic acids span across messenger RNA (mRNA), small interfering RNAs (siRNA) and microRNAs (miRNA) with expansion into modern medicine including small molecules, biologics and cell therapeutics. For example, the lately popular mRNA vaccines can be designed in the lab and developed at a rapid pace to respond to evolving and urgent medical crises. Coding RNAs can be

circularized into circRNAs to extend the duration of protein translation, based on RNA molecules that covalently join head-to-tail. Bioengineers have also advanced the synthesis of circular long transcripts into circRNAs. However, the fundamental mechanisms of initiating translation to form circular RNA or messenger RNA differ due to the lack of a 7-methylguanylate (M^7G) cap on the circular RNAs. As a result of this, researchers need to thoroughly examine the principles of circular RNA translation to build better therapies and potentially surpass the translational capacities of mRNA. To examine this aspect, the team developed a modular high-throughput platform to build and test synthetic circular RNAs for optimized translation and improved protein yields.



Optimization of RNA non-coding elements enable stronger circRNA translation. (a) NanoLuc activity after transfection of HeLa cells with circRNAs containing either a 3′ or a 5′ IRES and spacer sequences of varying lengths. When the IRES is 3′ to the NanoLuc reporter, translation through the td splicing scar is unavoidable. The predicted secondary structure of this scar is shown. NanoLuc activity was normalized to constitutive firefly luciferase activity from the same sample and then divided by values from mock transfection. Data are mean ± s.e.m. for n = 3 biological replicates. (b) NanoLuc activity at 24 hours after transfection of HeLa cells with circRNAs containing the indicated number of stop codons. NanoLuc activity was normalized to constitutive firefly luciferase activity from the same sample and then divided by values from mock transfection. Data are mean ± s.e.m. for n = 4 biological replicates. (c) NanoLuc activity after transfection of HeLa cells with circRNAs containing different 5′ spacer sequences. NanoLuc activity was normalized to constitutive firefly luciferase activity from the same sample and then divided by values from mock transfection. Data are mean ± s.e.m. for n = 3 biological replicates. *P = 0.0213, **P = 0.0051 and ***P