Metodologias para edição de genomas (CRISPR-Cas9)

Profa. Dra. Chirlei Glienke

BIOGEMM/UFPR

Origem do mecanismo de CRISPR

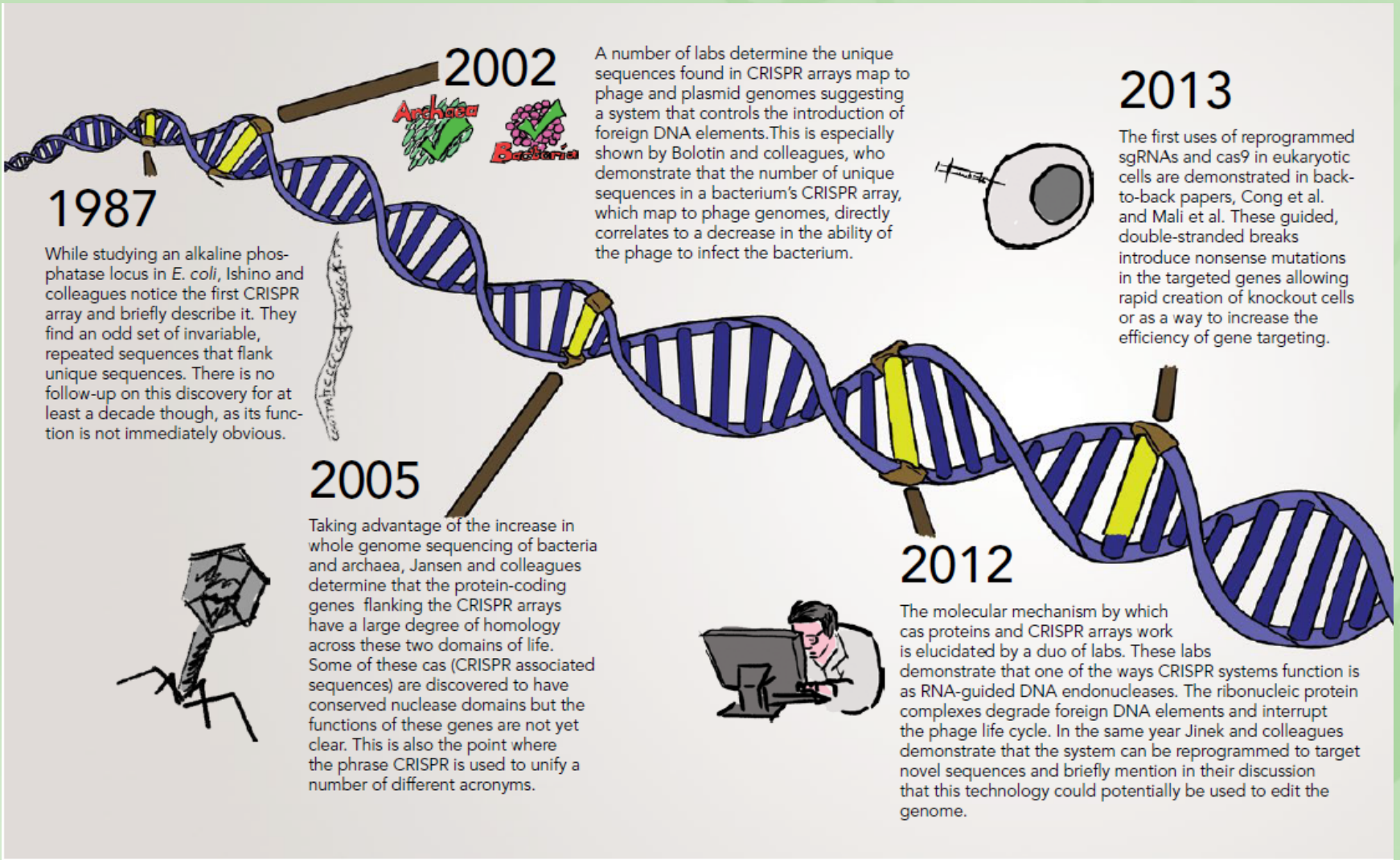


Figure 1 | The Discovery and Development of CRISPR and Cas9

DOI: 10.23861/EJBM201631754

Premio Nobel em química em 2020

Jennifer Doudna (University of California Berkeley) e Emmanuelle Charpentier (Max Planck Unit for the Science of Pathogens, Berlin)

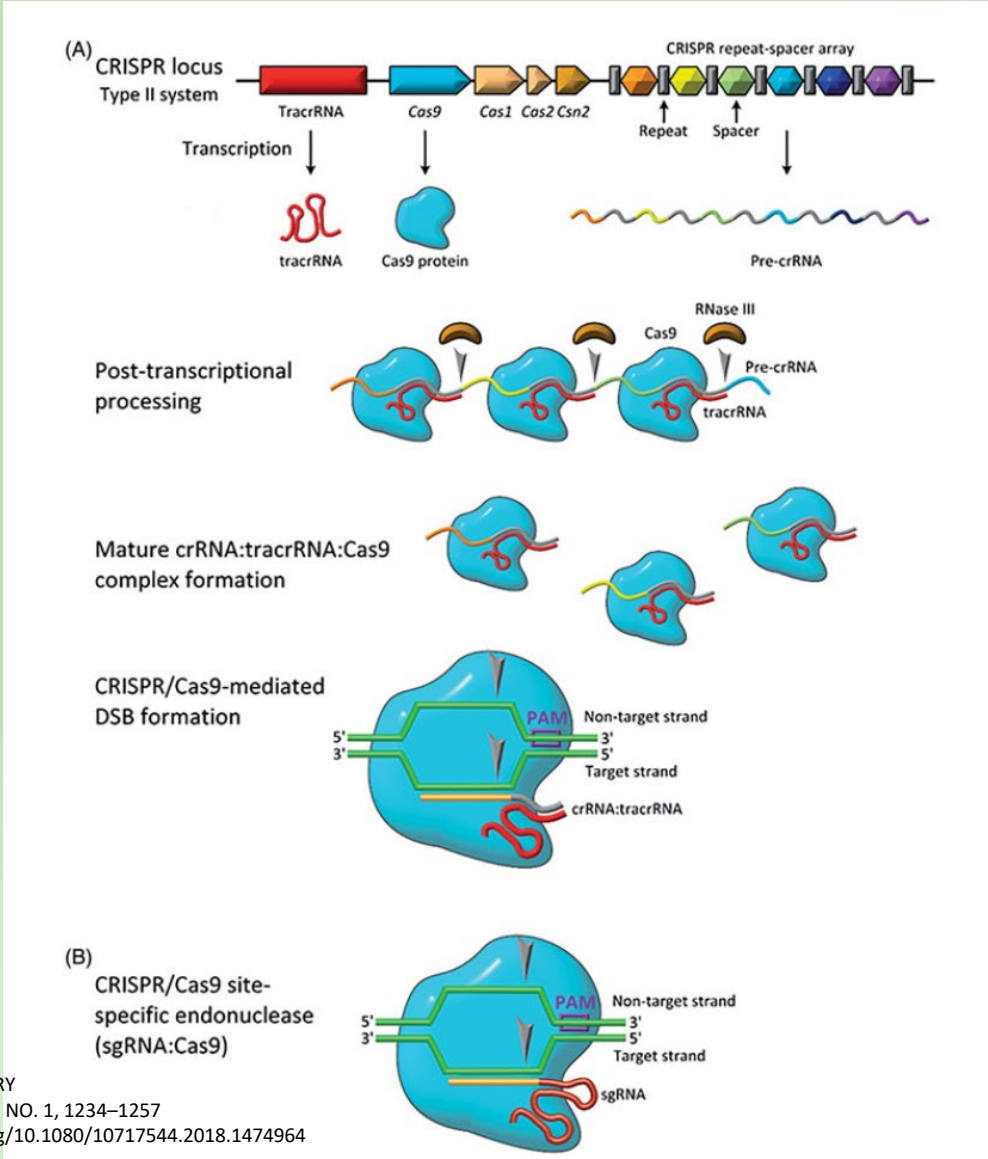
Sistema CRISPR – Cas

1. Bactérias e archaea desenvolveram sistemas de defesa adaptativos mediados por RNA, chamados de repetições palindrômicas curtas regularmente intercaladas (CRISPR) que protegem os organismos de vírus e plasmídeos invasores

2. Esses sistemas de defesa dependem de pequenos RNAs para detecção específica de sequência e silenciamento de ácidos nucléicos exógenos

3. Os sistemas CRISPR / Cas são compostos por:
   a) genes cas organizados em operon (s)
   b) CRISPR que consiste em sequências de direcionamento de genoma (chamadas espaçadores) intercaladas com repetições idênticas

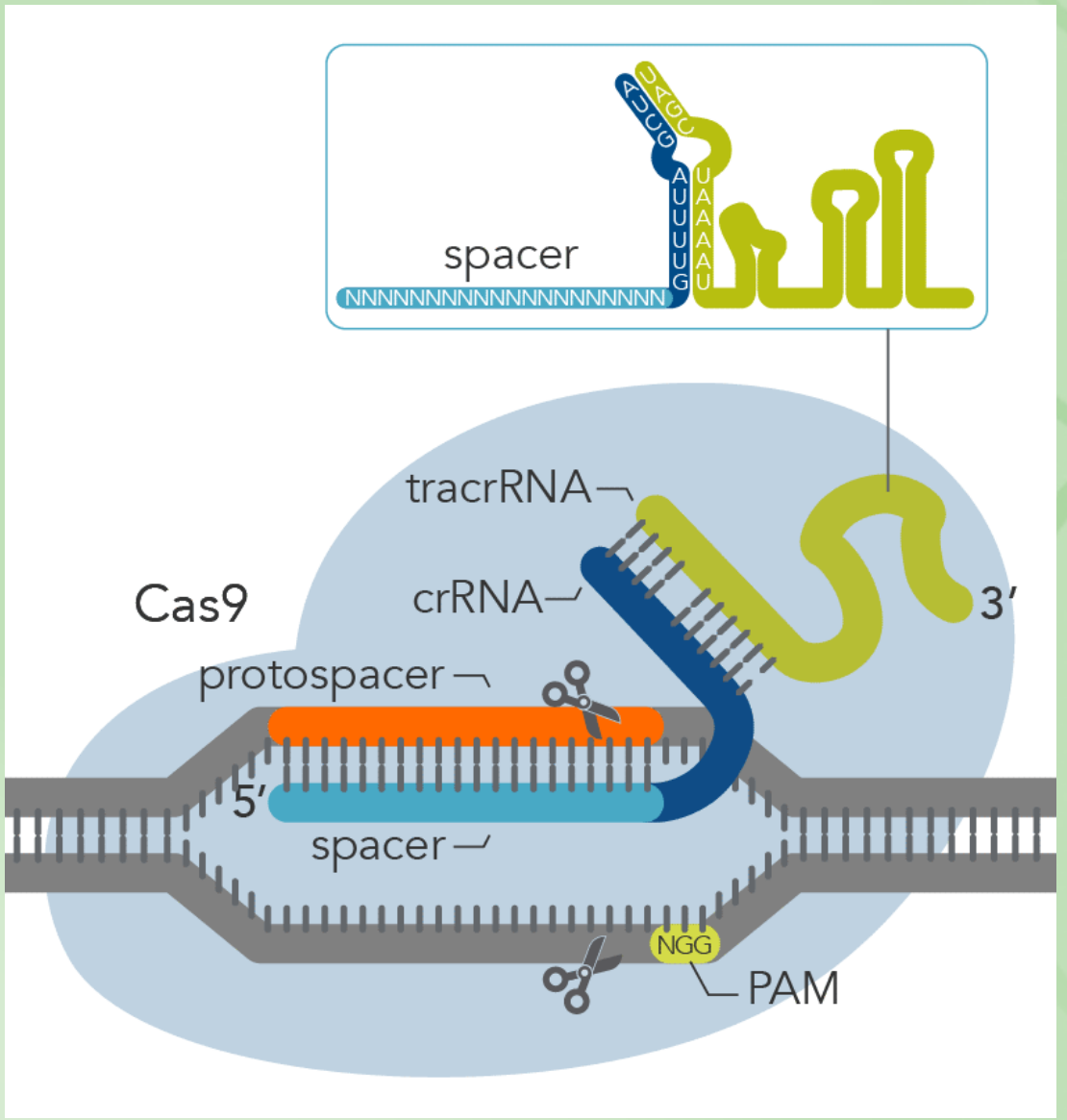
Biologia do sistema CRISPR / Cas tipo II



- (A) Representação genômica de CRISPR / Cas9 juntamente com produtos de transcrição / tradução relevantes.
- (B)CRISPR / Cas9 projetado para edição de genes específicos do local (sgRNA: Cas9).
- Setas cinza indicam locais de quebras de nucleotídeo de fita simples.

DRUG DELIVERY
2018, VOL. 25, NO. 1, 1234–1257
https://doi.org/10.1080/10717544.2018.1474964

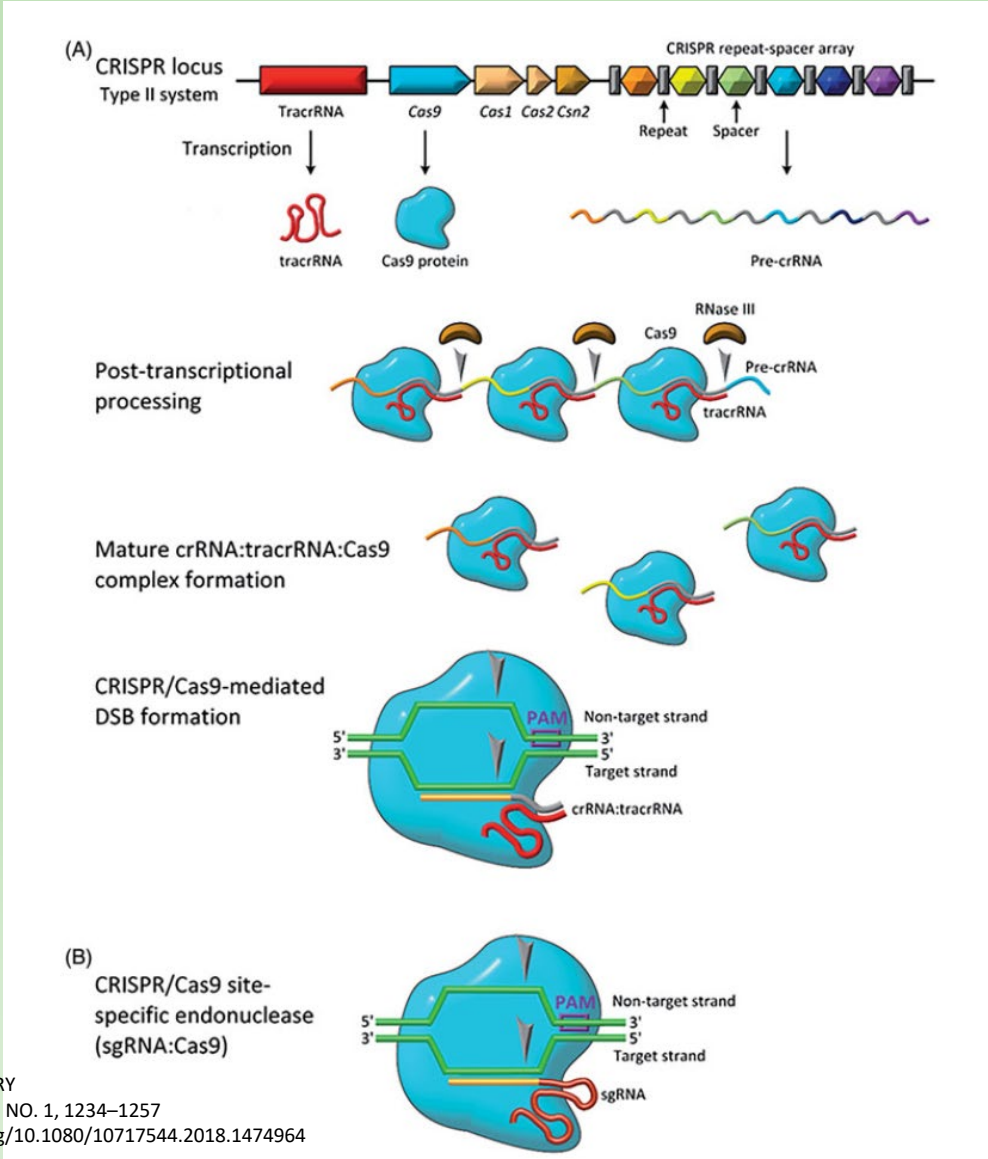
Componentes do Sistema CRISPR-Cas9



- RNA guia:
  - Spacer (região que tem complementariedade com o DNA)
  - crRNA (Complementariedade com o tracrRNA - trans-activating crRNA)

- PAM (protospacer adjacent motif):
  - NGG para a Cas9 nativa
  - Sequencia de reconhecimento da Cas9 (fita complementar)
  - Abertura da molécula

Biologia do sistema CRISPR / Cas tipo II



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Edição genética via CRISPR / Cas9

- Dois métodos tradicionais
  - transformação mediada por protoplasto (PMT)
  - **transformação mediada por Agrobacterium (AMT)**
    - Maior eficiência
  - CRISPR / Cas9: repetição palindrômica curta regularmente interespaçada agrupada (em tandem) / proteína associada (CRISPR / Cas9)
  - clustered regularly interspaced short palindromic repeat/associated protein system (CRISPR/Cas9)

CRISPR

- Ferramenta de edição de genomas descrita para:
  - Nocaute
  - inserção
  - substituição de genes

- CRISPR tipo II mais populares têm dois componentes:
  - (i) uma endonuclease Cas9 associada a CRISPR de Streptococcus pyrogenes e
  - (ii) um RNA guia único (sgRNA), que é a fusão de um RNA CRISPR precursor (precrRNA) e um RNA CRISPR transativador (tracrRNA) 19

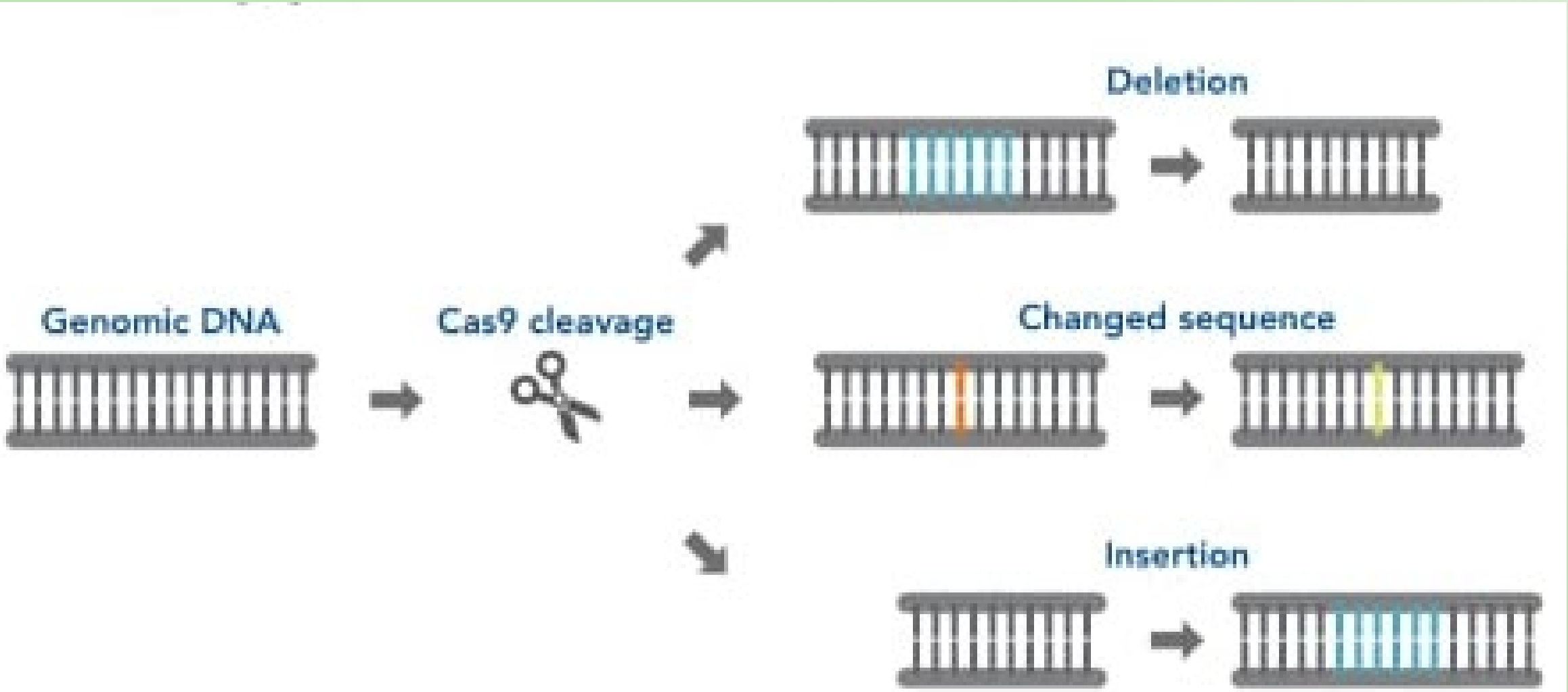
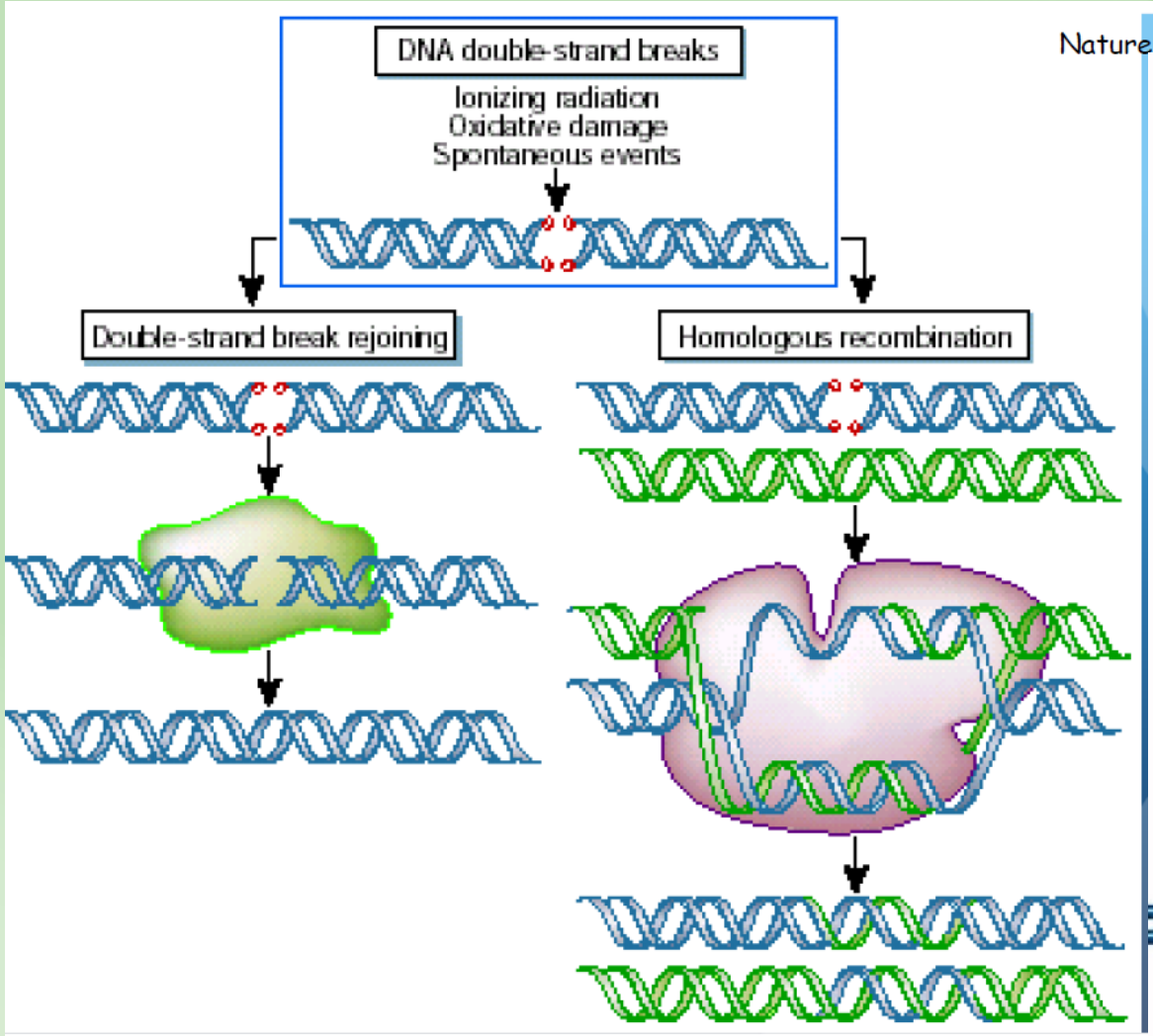


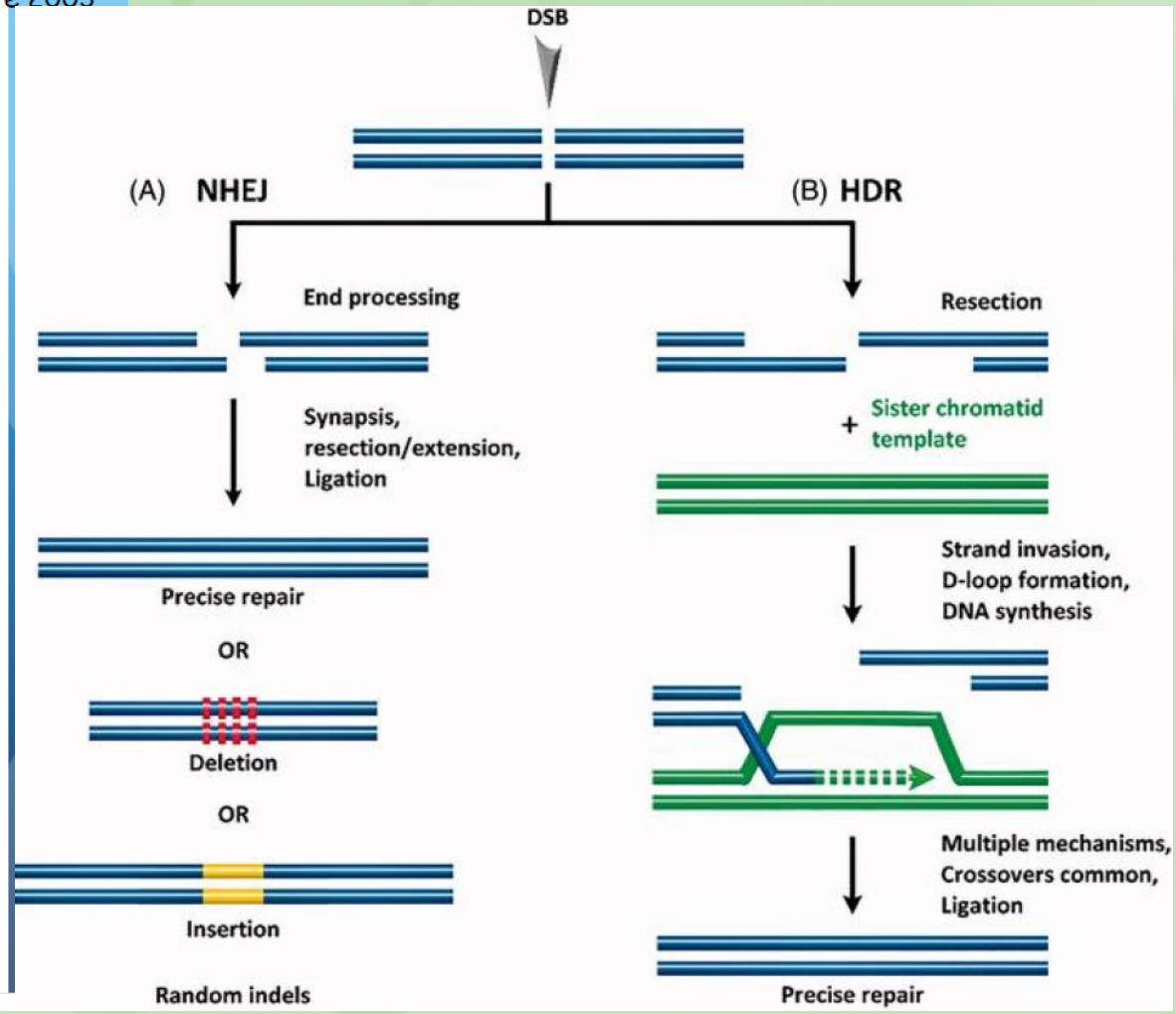
Figure 3. Possible outcomes after genomic DNA cleavage. Cellular DNA repair pathways such as NHEJ can lead to deletions, changed sequences, and small insertions.

https://go.idtdna.com/rs/400-UEU-432/images/IDT_The%20CRISPR%20basics%20handbook.pdf

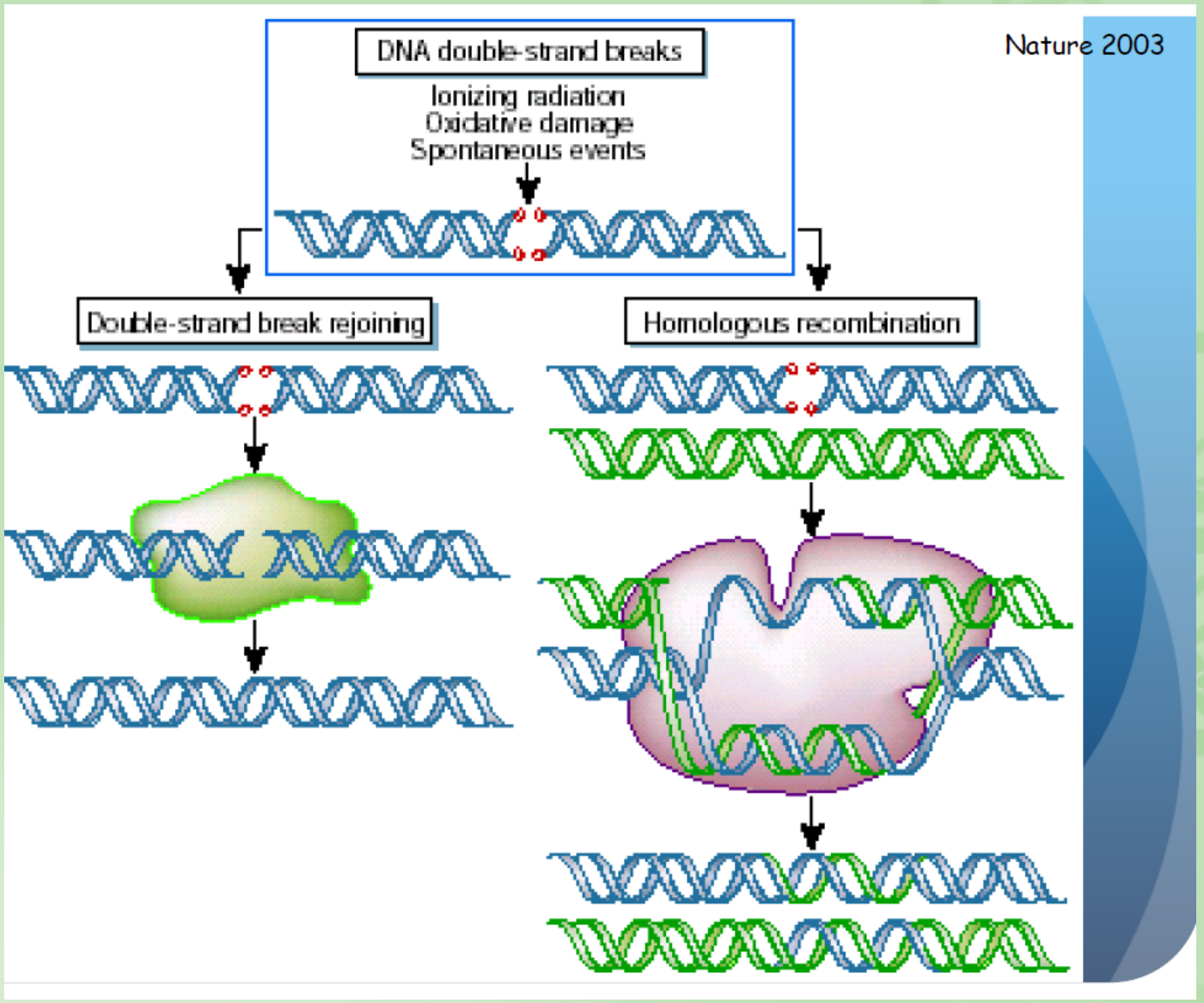
Mecanismos de reparo de dupla quebra de DNA

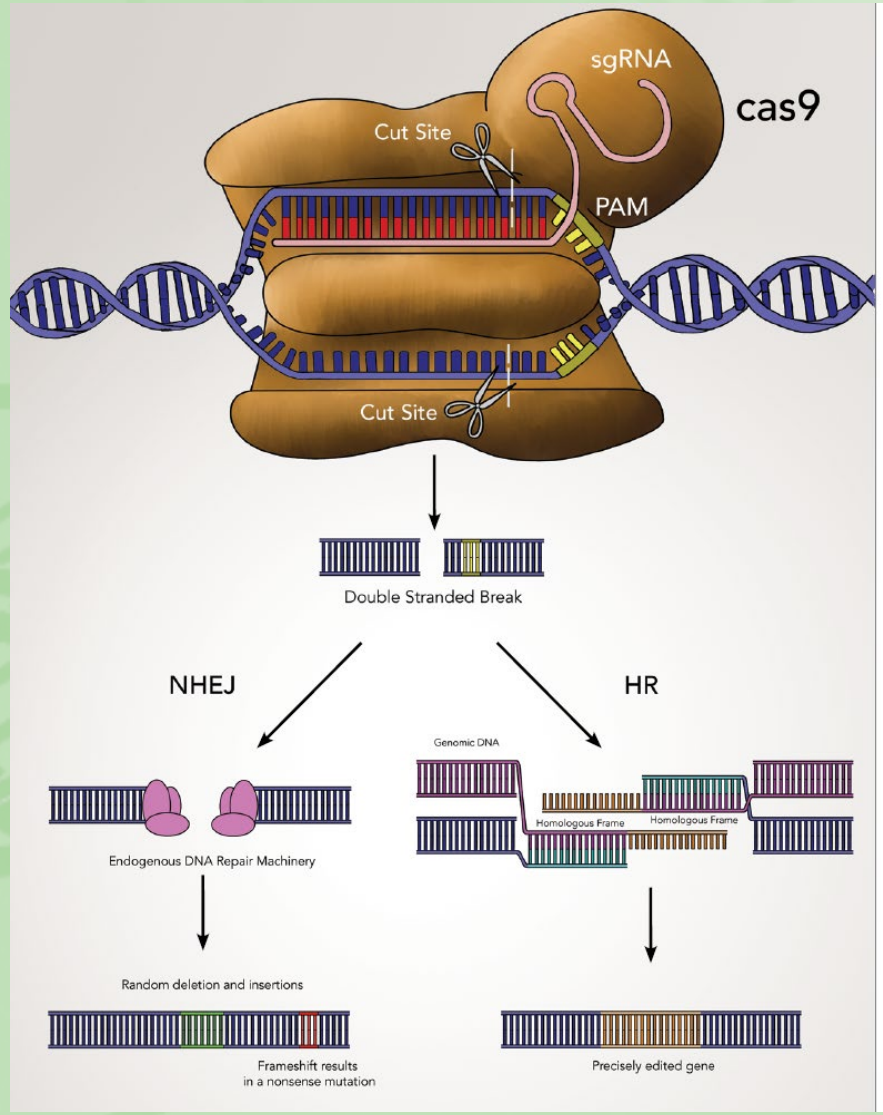


Nature 2003



Reparo de dupla quebra de DNA e CRISPR Cas9



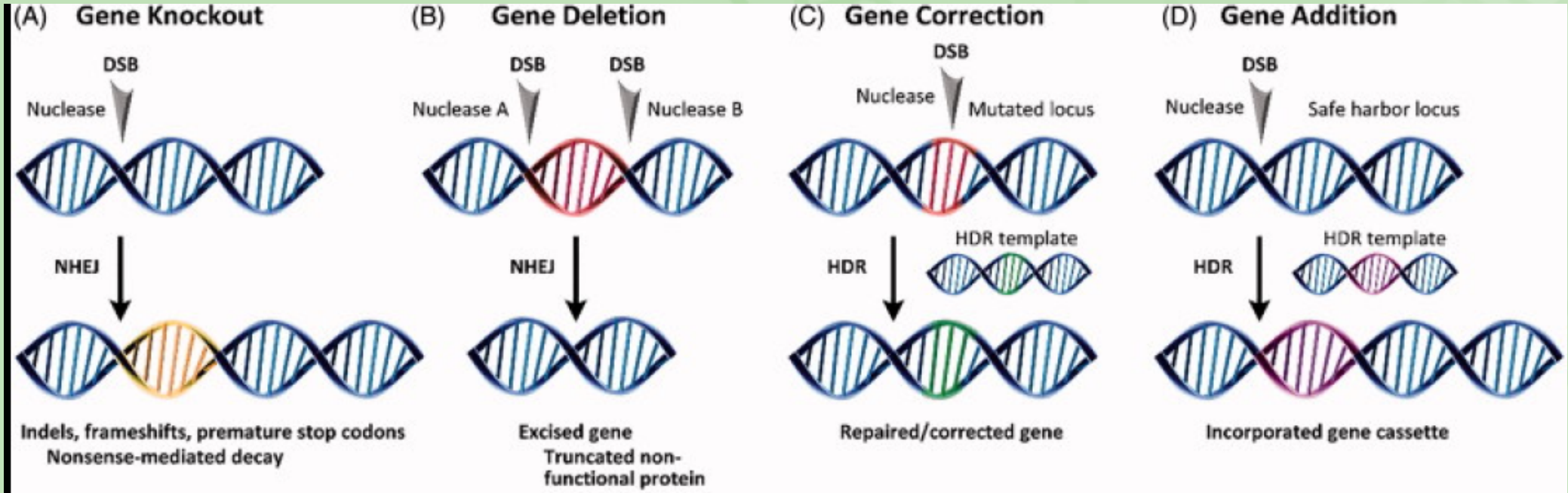


DOI: 10.23861/EJBM201631754

Figure 4. HDR can incorporate new sequences into genomic DNA. Small sequences can be changed for purposes of gene correction or modification (left), and large sequences such as whole genes can be added coincidentally (right).

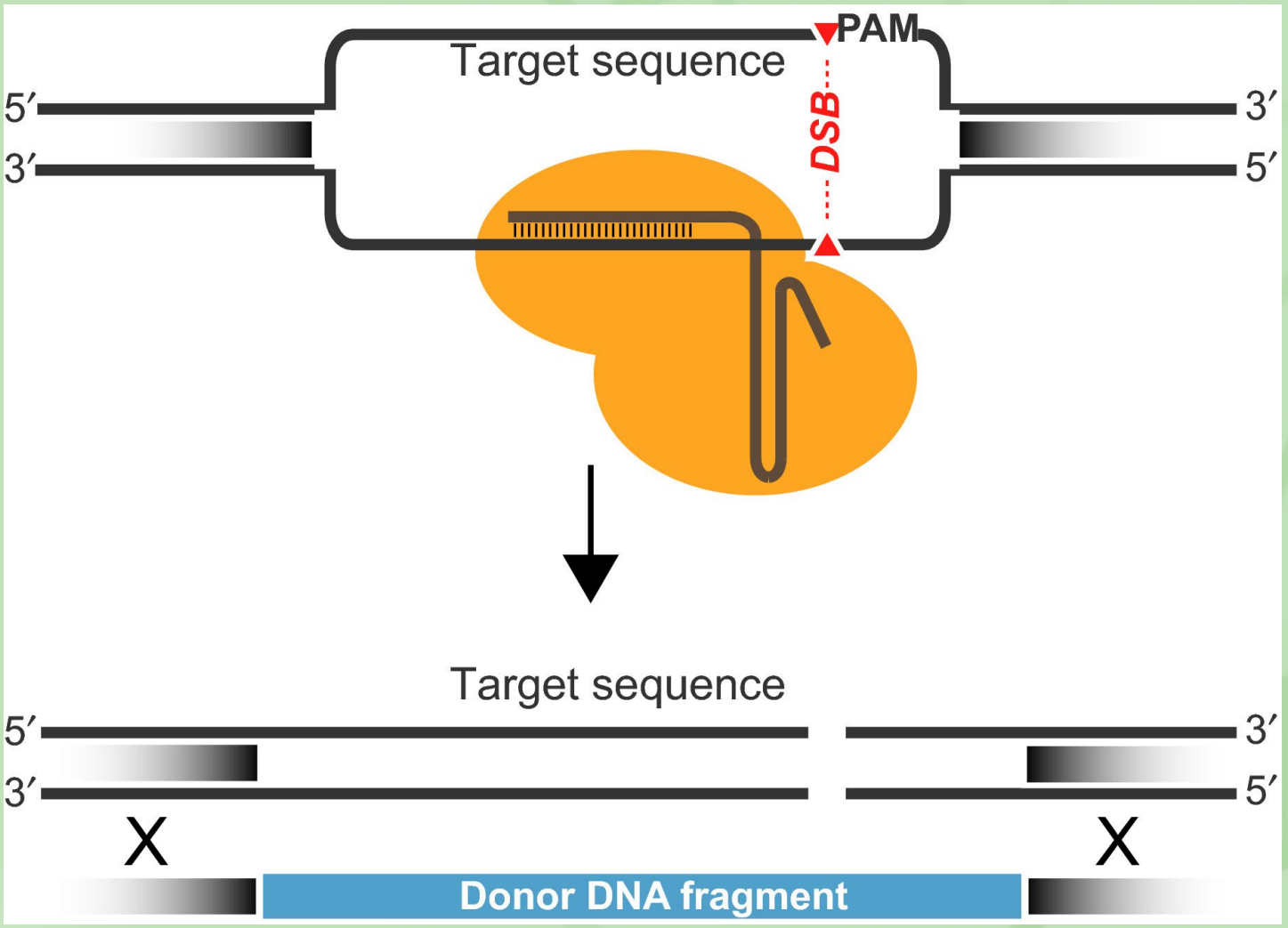
https://go.idtdna.com/rs/400-UEU-432/images/IDT_The%20CRISPR%20basics%20handbook.pdf

Edição de DNA - CRISPR Cas9



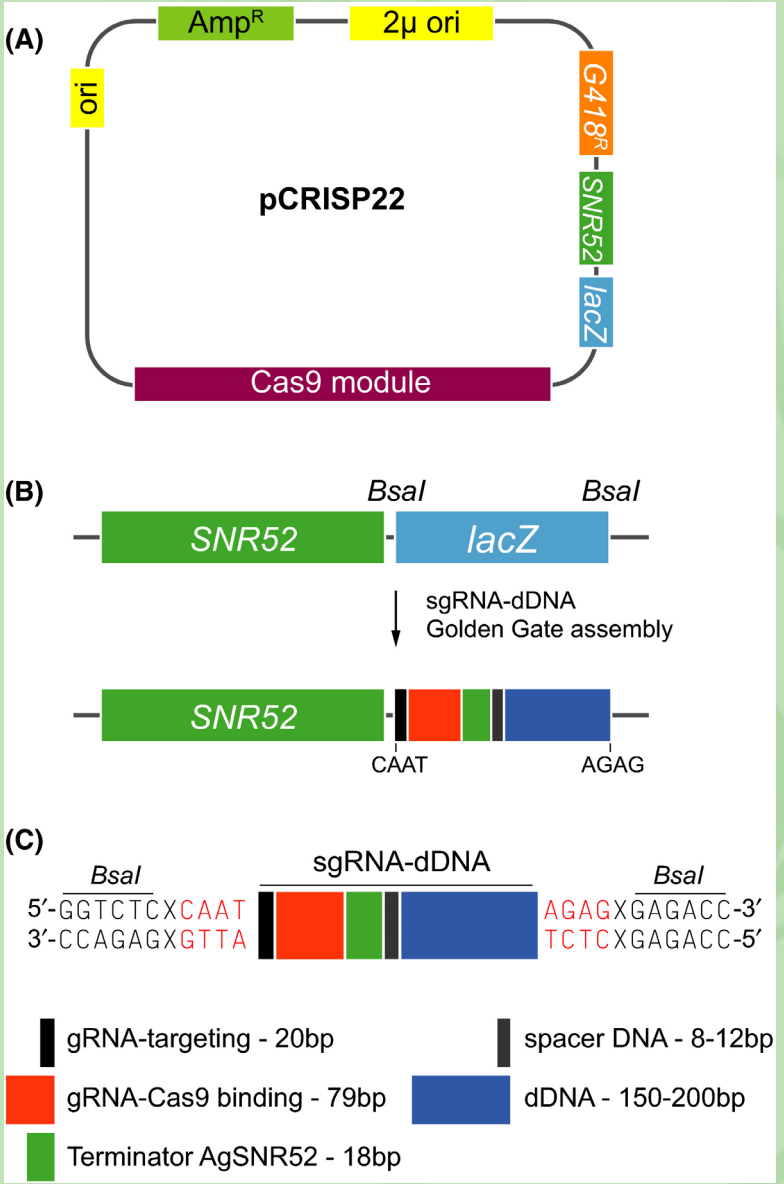
Drug Deliv. 2018; 25(1): 1234-1257.
Published online 2018 May 25. doi: 10.1080/10717544.2018.1474964
PMCID: PMC6058482
PMID: 29801422
Delivering CRISPR: a review of the challenges and approaches
Christopher A. Lino, Jason C. Harper, James P. Carney, and Jerilyn A. Timlin

One-vector CRISPR/Cas9 - edição genética do fungo *Ashbya gossypii*



Microbial Biotechnology, Volume: 12, Issue: 6, Pages: 1293-1301, First published: 05 May 2019, DOI: (10.1111/1751-7915.13425)

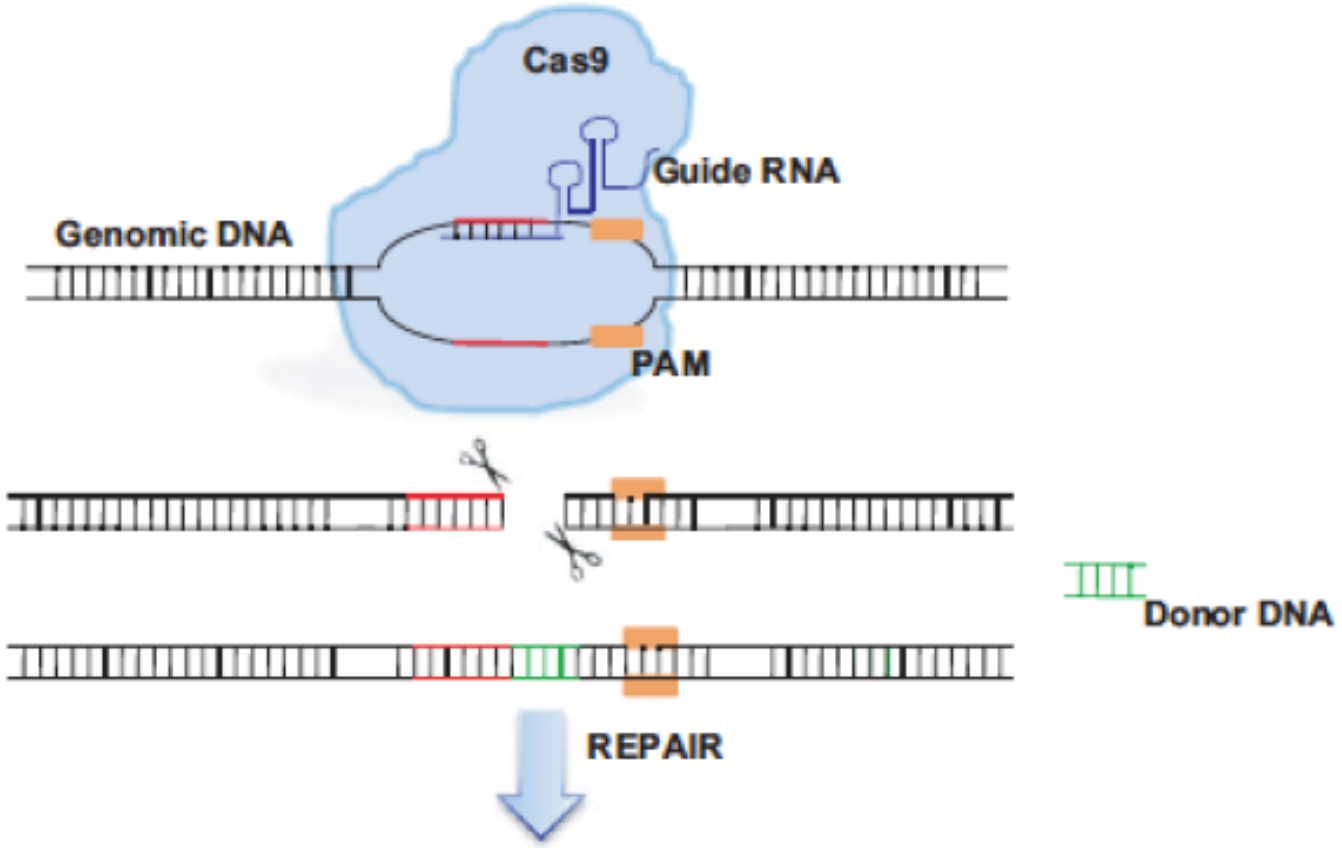
One-vector CRISPR/Cas9 - edição genética do fungo *Ashbya gossypii*



Microbial Biotechnology, Volume: 12, Issue: 6, Pages: 1293-1301, First published: 05 May 2019, DOI: (10.1111/1751-7915.13425)

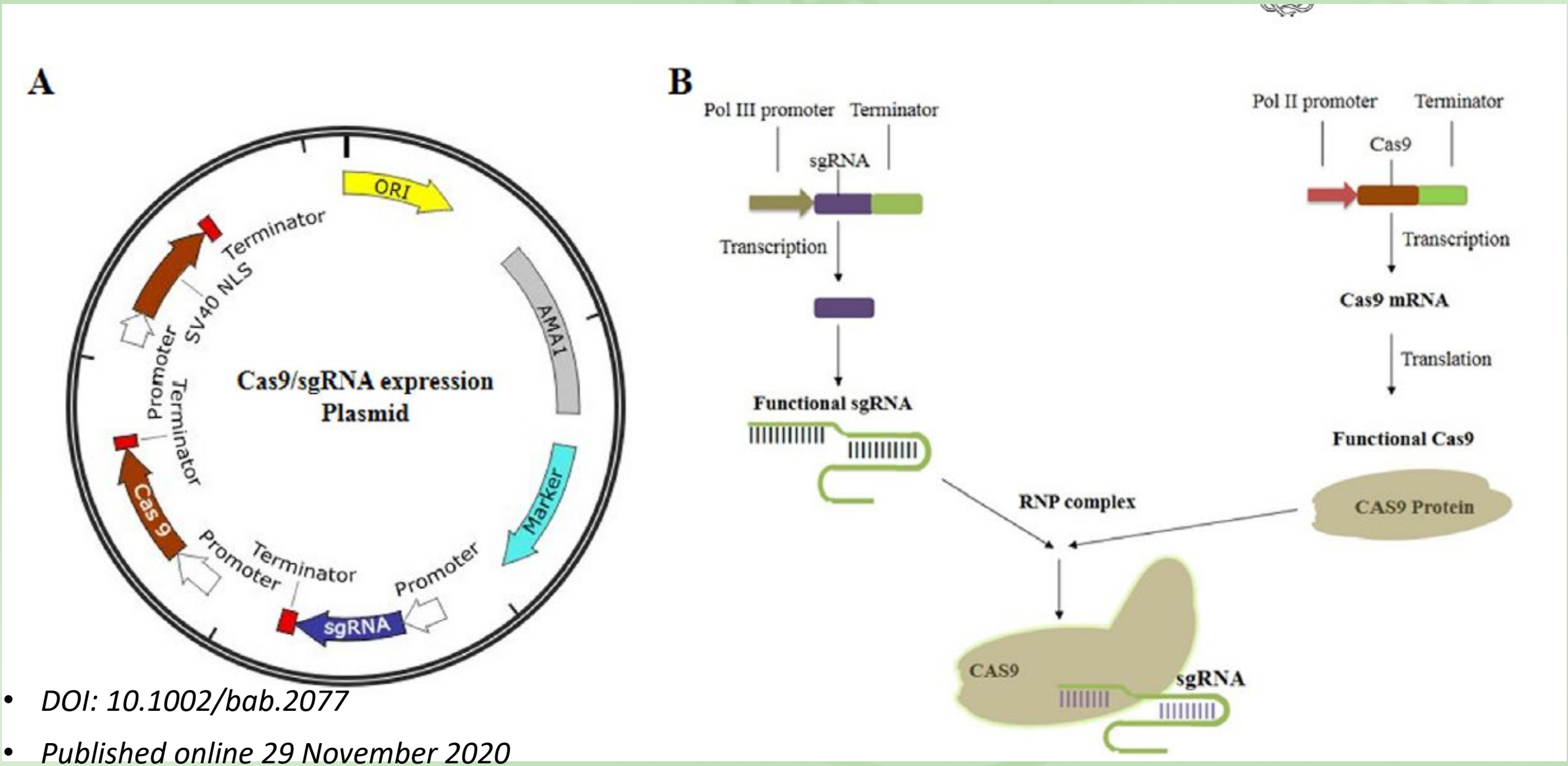
Genetic Modification	Application	Nuclease Activity	gRNA
Knock-out	Permanently remove gene function	Cas or Cas9n	gRNA targeting 5′ exon or essential protein domains
Knock-in	Generate a specific sequence change	Cas or Cas9n	gRNA targeting region of interest
Interference	Reduce gene expression	dCas-repressor	gRNA targeting gene promoter elements
Activation	Increase gene expression	dCas-activator	gRNA targeting gene promoter elements

CRISPR-Cas9

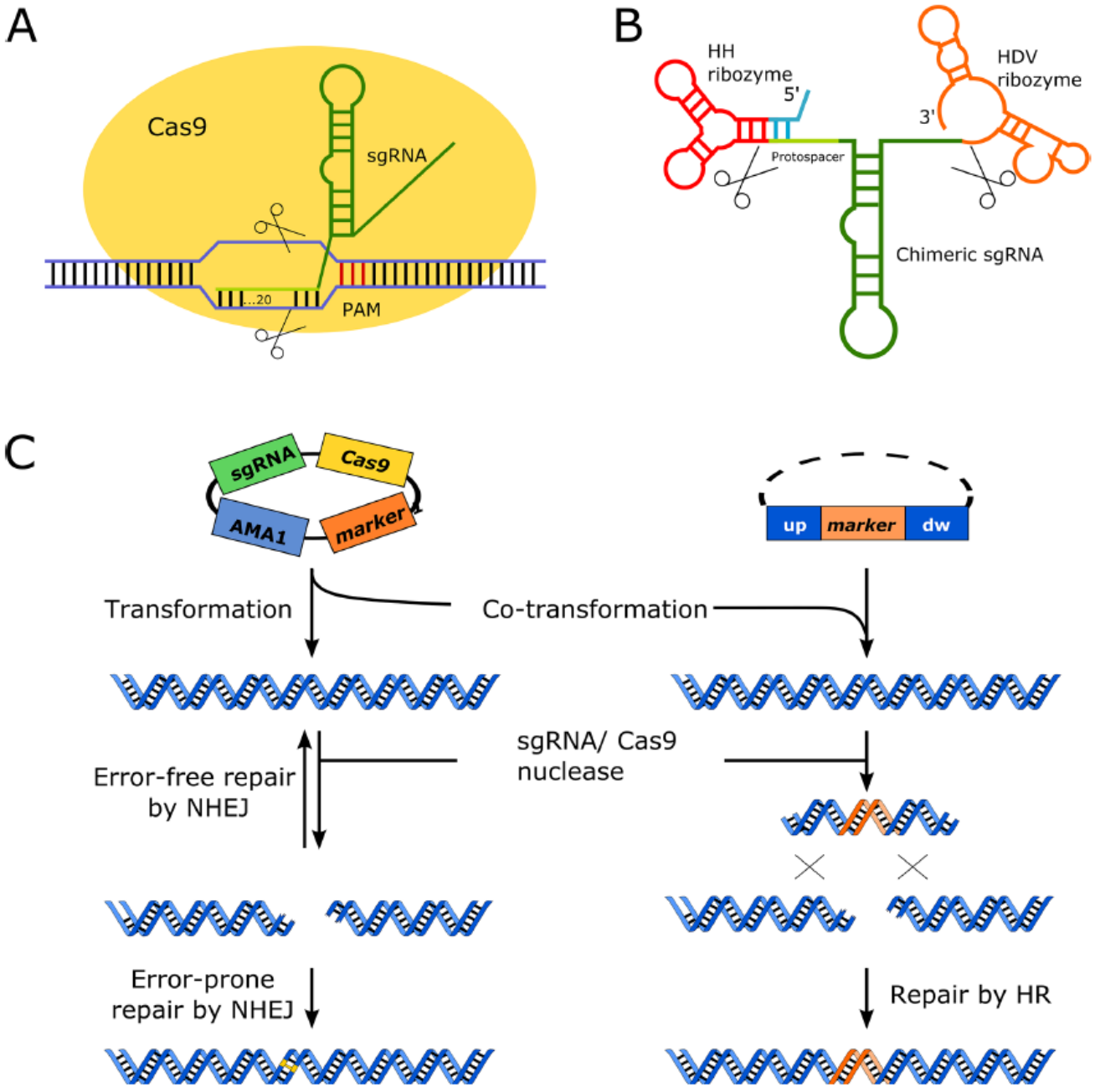


1. O sgRNA (RNA guia) reconhece a sequência de DNA alvo e forma um duplex de DNA / RNA no local de reconhecimento.
2. A endonuclease Cas9 então cliva esse duplex de DNA / RNA.
3. As quebras de DNA de fita dupla resultantes são reparadas por união de extremidade não homóloga ou por reparo dirigido por homologia (HDR), se um molde de DNA para o reparo for cotransformado nas células.
4. O sistema de reparo de HDR é particularmente eficaz para knock-outs completos de genes quando acoplado a ribonucleoproteínas Cas9 (RNPs) duplas montadas in vitro que têm como alvo as extremidades 5 'e 3' da região de codificação do gene.

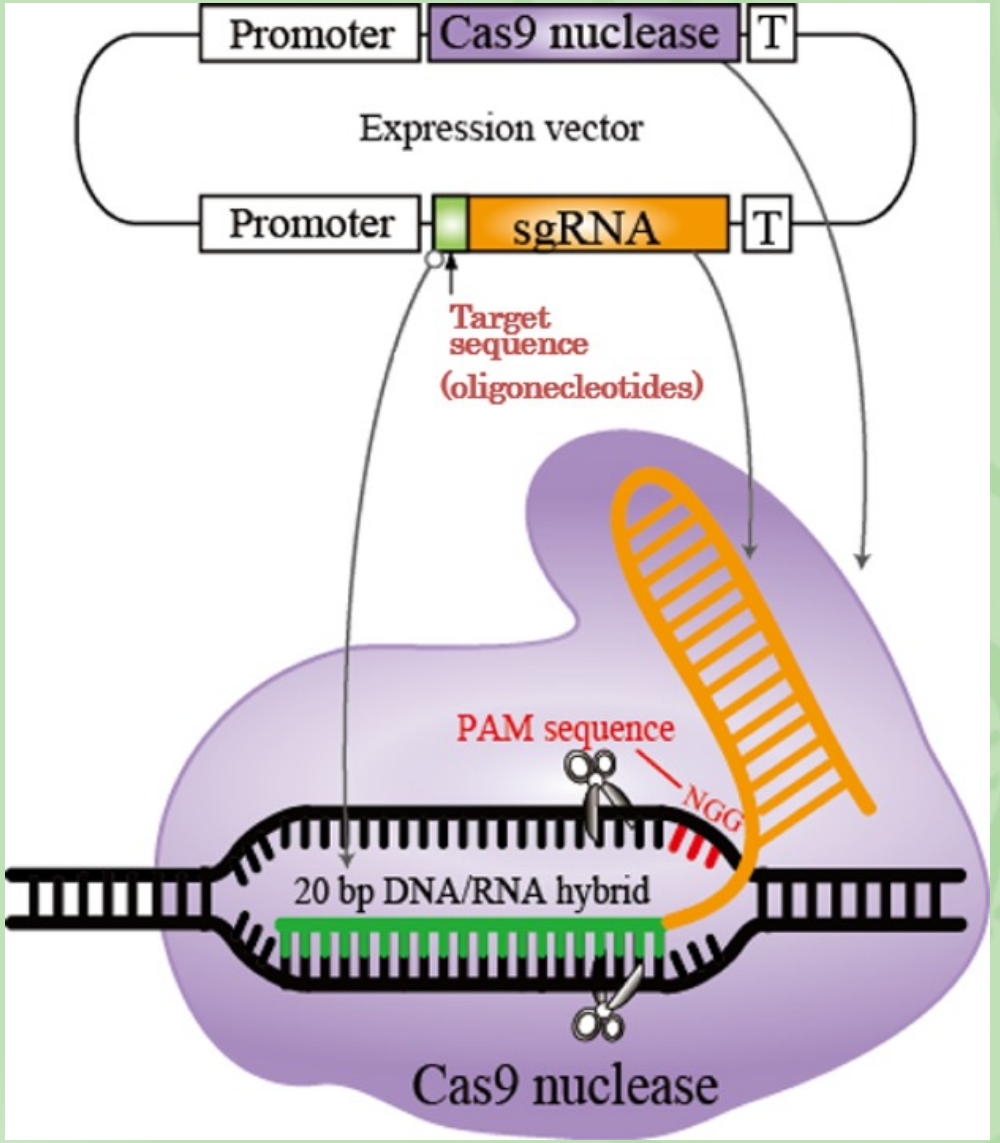
Edição de DNA - CRISPR Cas9



- *DOI: 10.1002/bab.2077*
- *Published online 29 November 2020*

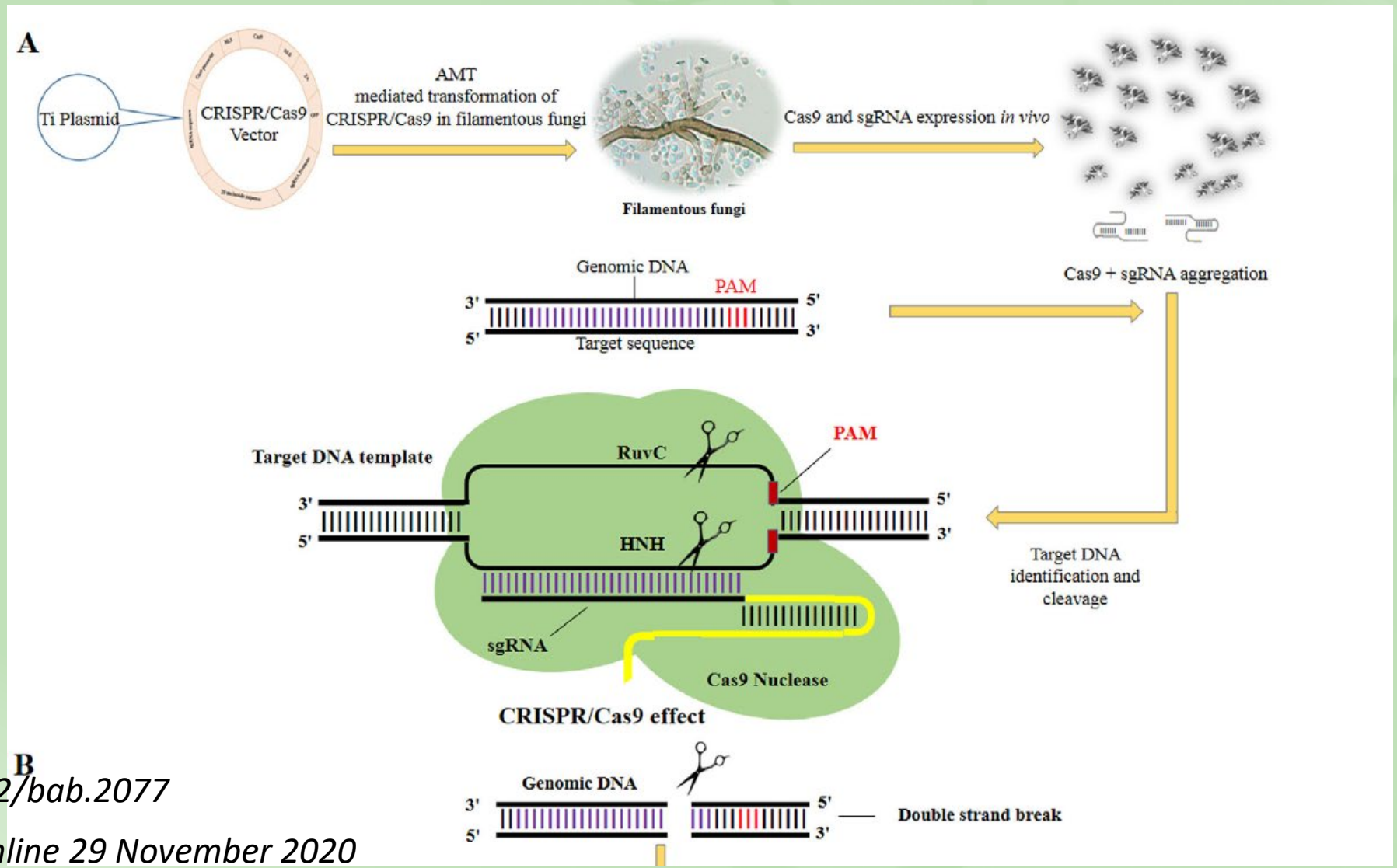

A
Cas9
sgRNA
...20
PAM
B
HH ribozyme
5'
HDV ribozyme
3'
Protospacer
Chimeric sgRNA
C
sgRNA
Cas9
AMA1
marker
up
marker
dw
Transformation
Co-transformation
Error-free repair by NHEJ
sgRNA/ Cas9 nuclease
Error-prone repair by NHEJ
Repair by HR

Sistema CRISPR / Cas feito sob medida para substituição dirigida de genes altamente eficiente no fungo brusone do arroz



Biotechnology and Bioengineering, Volume: 112, Issue: 12, Pages: 2543-2549, First published: 03 June 2015, DOI: (10.1002/bit.25662)

Edição de DNA - CRISPR Cas9



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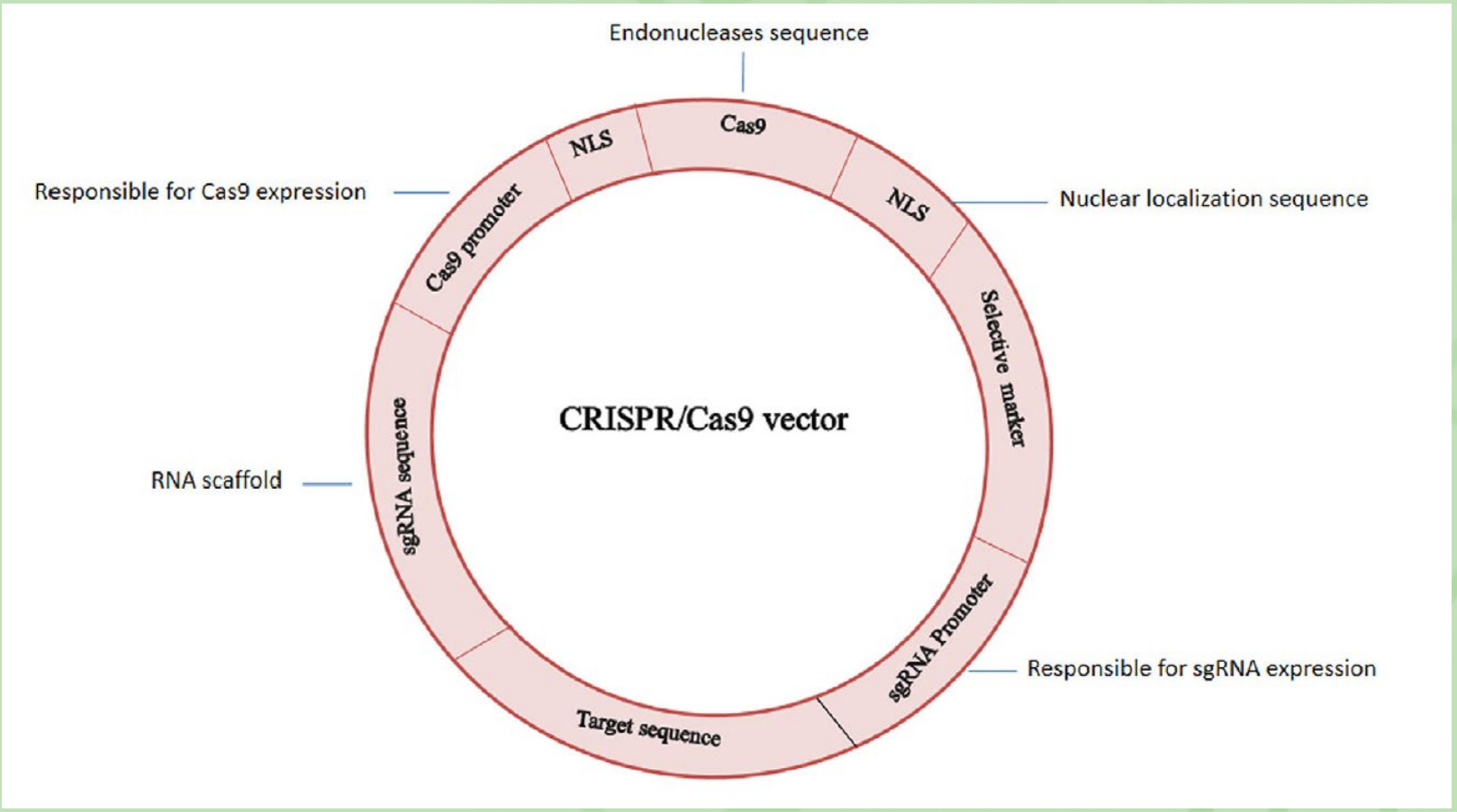

- *DOI: 10.1002/bab.2077*
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Edição genética via CRISPR Cas9 e Cas12a (Cpf1) (nucleases)

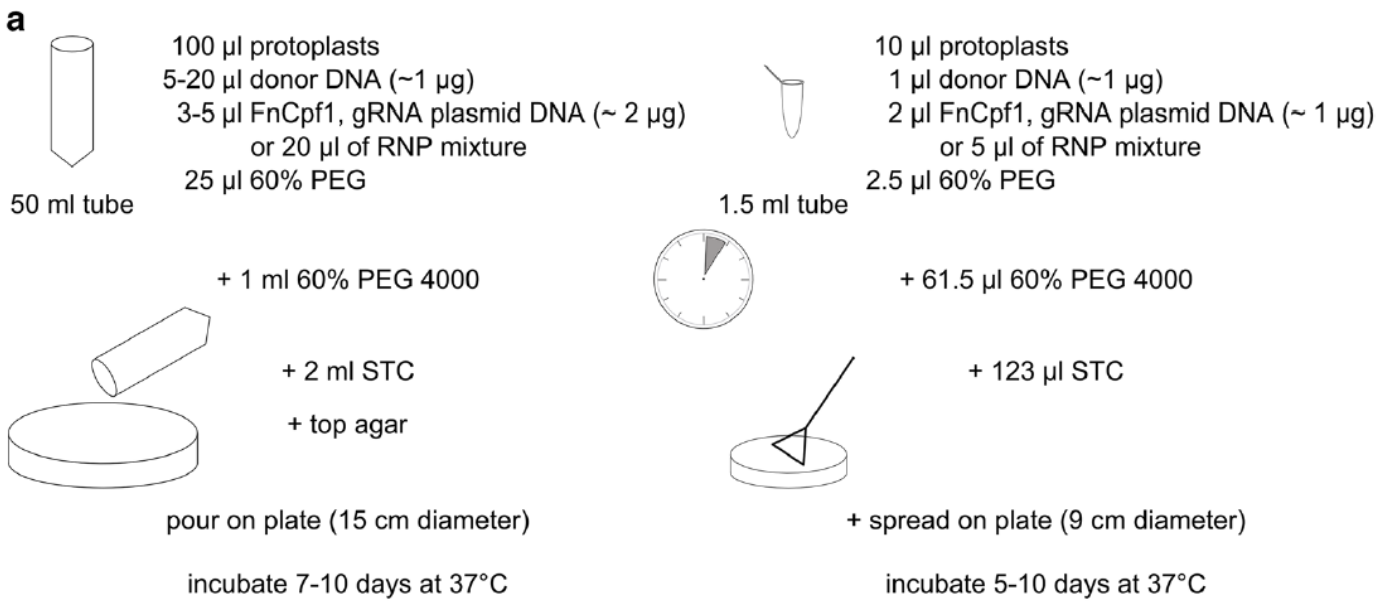
- Nuclease Cas9 de *Streptococcus piogenes* (SpCas9)
- Nucleases Cpf1 de *Francisella novicida* (FnCpf1) e *Acidaminococcus* sp. (AsCpf1)
- Em comparação com Cas9, Cpf1 reconhece sequências PAM ricas em T e não precisa de um crRNA de ação trans (tracrRNA) devido à atividade única de nuclease dupla que cliva não apenas o DNA alvo, mas também seu próprio CRISPR-RNA (crRNA)
- A sequência de reconhecimento:
  - SpCas9 é 5' - NGG - 3 '
  - FnCpf1 é 5' - TTN - 3 '
  - AsCpf1 é 5' - TTTN - 3 '

Linhagens modelo de fungos filamentosos para as quais as ferramentas de edição de genes CRISPR já foram estabelecidas

Species	CRISPR protein	Nuclease delivery	References
Aspergillus fumigatus	Cas9	Plasmid-based and RNP-based	[36, 37]
A. nidulans	Cas9, Cpf1	Plasmid-based	[9]
A. niger	Cas9, Cpf1	Plasmid-based and RNP-based	[5, 9]
A. oryzae	Cas9	Plasmid-based	[38]
Neurospora crassa	Cas9	Plasmid-based	[39]
Penicillium chrysogenum	Cas9	Plasmid- based and RNP-based	[33]
Thermothelomyces thermophilus	Cas9	Plasmid-based	[12]
Trichoderma reesei	Cas9	Plasmid-based	[40]

Note that the nuclease can be delivered to the cell either plasmid encoded or as a purified protein, which has to be preassembled with in vitro transcribed gRNA to form ribonucleoproteins (RNPs)

Reduzindo a escala do procedimento de transformação



b

approach/target(s)	RNP			plasmid		
	mean [%]	analysed transformants	n	mean [%]	analysed transformants	n
pks4.2	100	10	2	100	10	4
pks4.2 & snc1	-	-	-	100 & 40	15	2
				100 & 56	16	

c

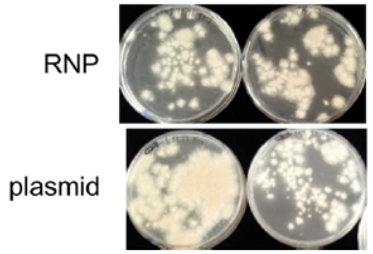


Fig. 4 Downscaling the transformation procedure. **a** Comparison of medium- and small-scale transformation, details on the methodology can be found in "Methods" section. **b** Table comparing the genome editing efficiency of small-scale transformation between the RNP- and plasmid-based approach, n = number of transformations performed. **c** Backside of four transformation plates from small-scale transformations are shown, which demonstrate that a reduced number of transformants will be gained with the small-scale protocol when compared to the medium-scale protocol (see Fig. 3b)

Kwon *et al. Fungal Biol Biotechnol (2019) 6:15* https://doi.org/10.1186/s40694-019-0079-4

Sistema CRISPR-Cas9 para edição genética do fungo *Fusarium proliferatum*

ID	Protospacer sequence 5′→3′	PAM site
sgRNA1175	TCACCCCCGAGTACCGCTGT	AGG
sgRNA9269	TGATGCGTATCTGGAAATGA	AGG

Table 1. sgRNAs used in this study

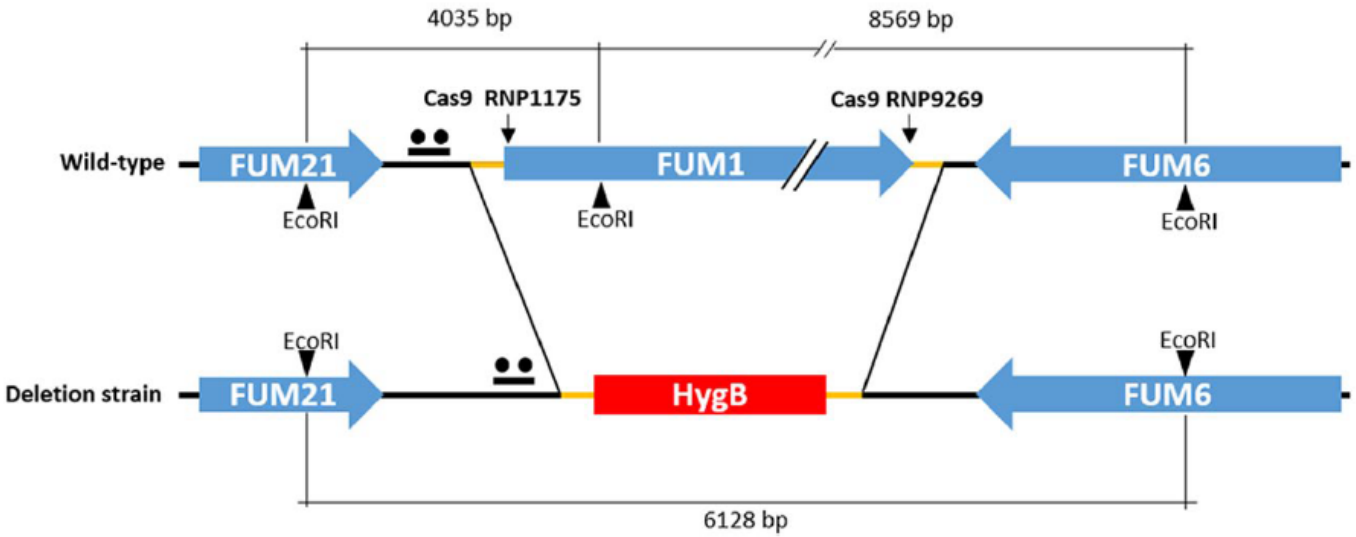


Figure 1. Schematic representation of the *FUM1* gene deletion by *in vitro*-assembled dual Cas9 ribonucleoproteins coupled with homology directed repair (HDR). The cleavage sites of the *in vitro*-assembled Cas9 RNP1175 and RNP9269 (↓), the 50 bp microhomology regions for HDR (orange segment), the *Eco*RI cut sites, and the pks*FUM1*-specific probe (●●) are represented for the genomic locus of the wild-type ITEM 7595 and Δ*FUM*1 deletion strain.

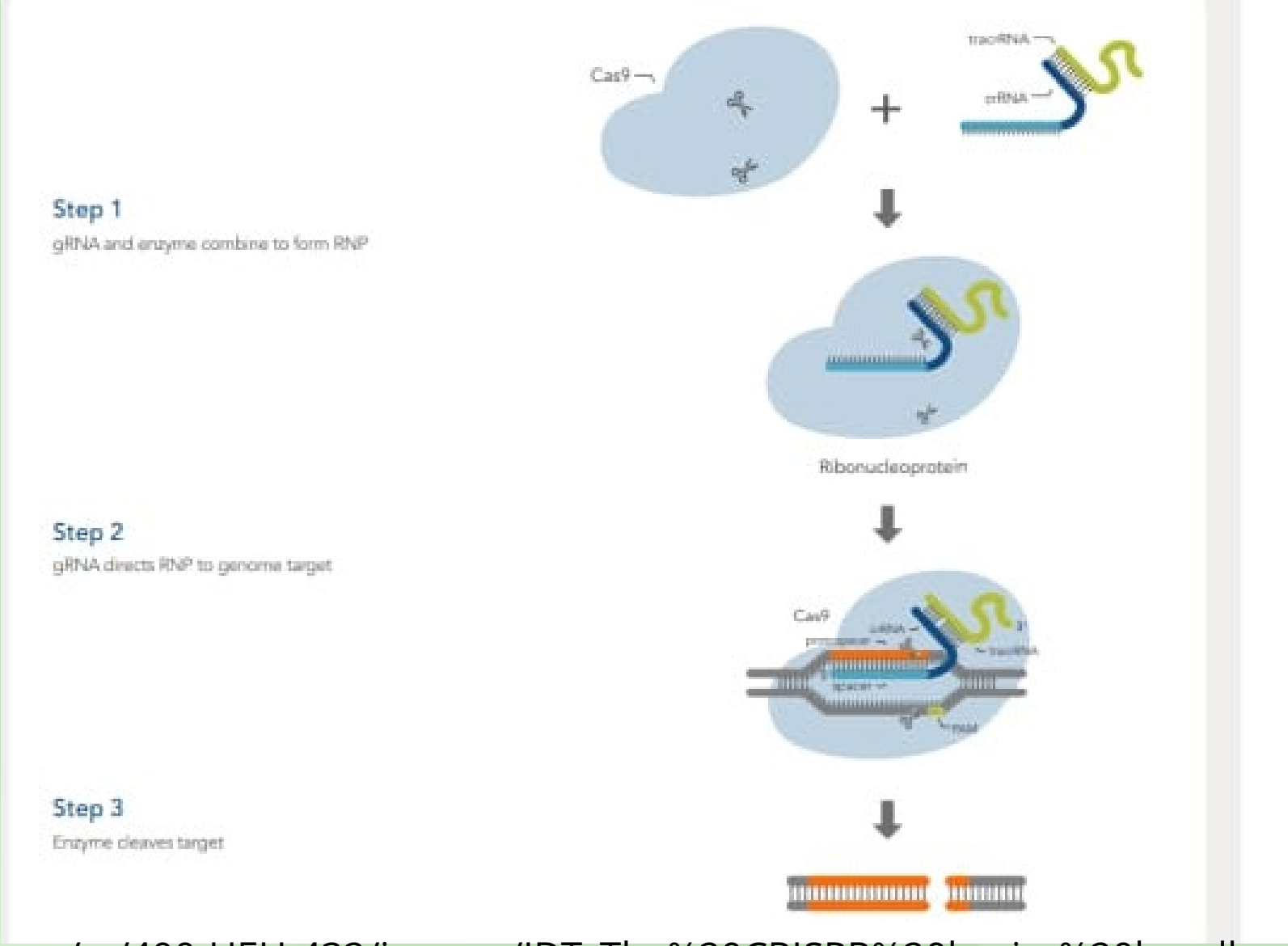
1. Deleção de gene cluslter (8000bp) em um único evento de transformação

2. Sistema CRISPR-Cas9 acoplando um sitio específico de quebra de DNA de fita dupla mediada por duas ribonucleoproteínas Cas9 (recombinação em microhomologia requerendo apenas regiões de 50 pb que flanqueiam o gene alvo)

3. Este sistema reduz o risco de mutações fora do alvo e minimiza o risco de alterar qualquer gene adjacente à região alvo

(2019) 9:19836 | https://doi.org/10.1038/s41598-019-56270-9

Passo a passo da Edição via CRISPR Cas9



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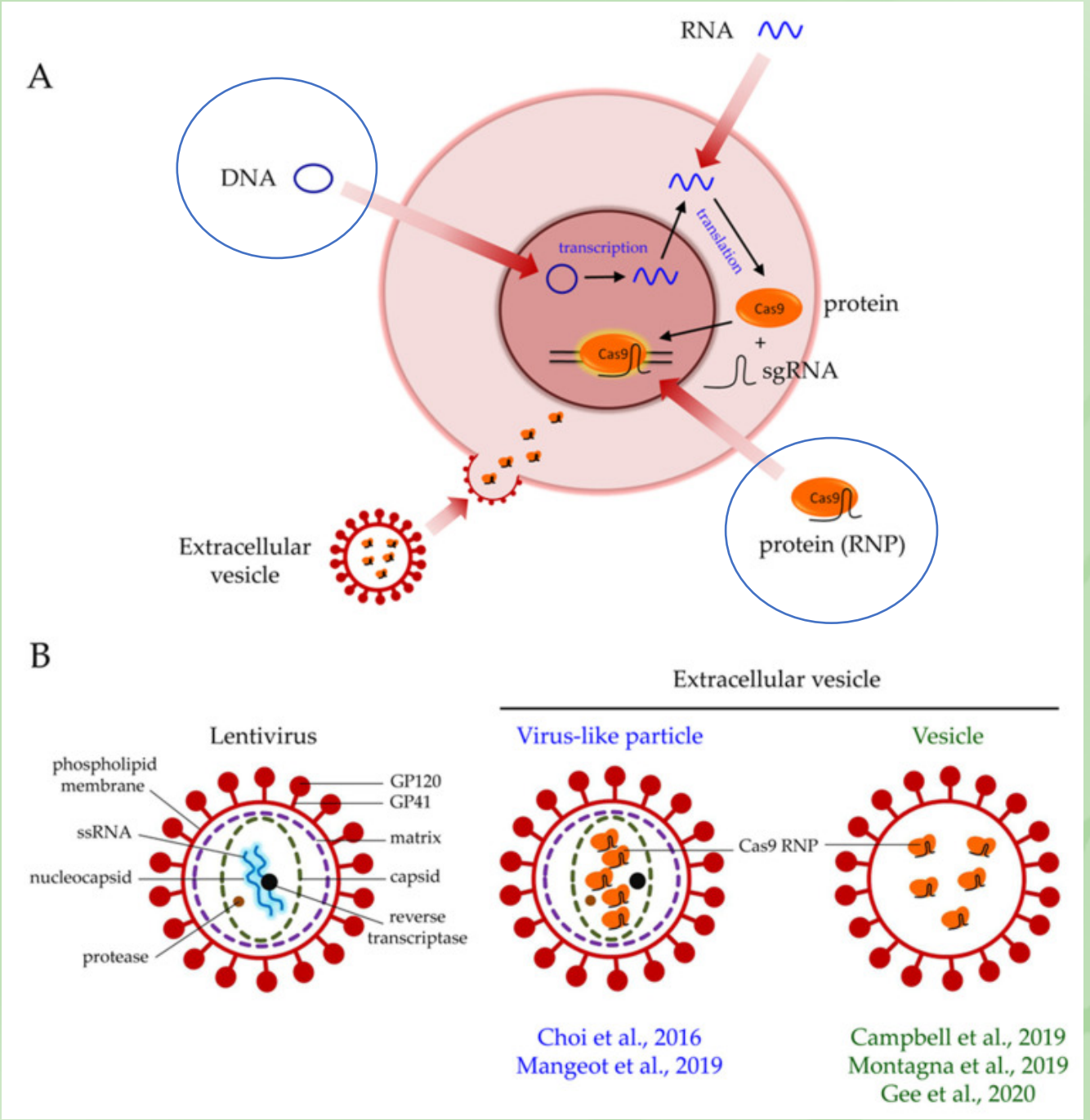
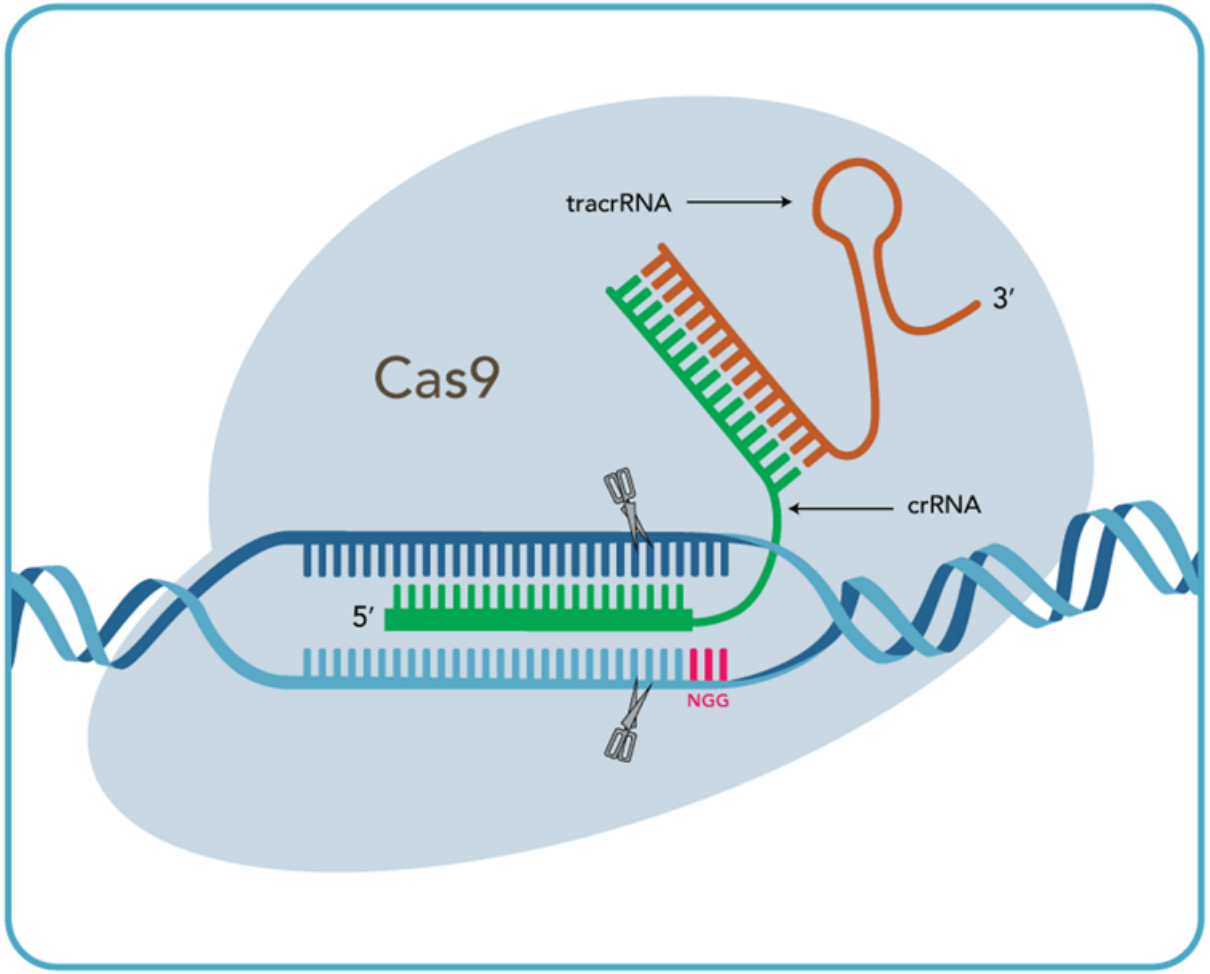


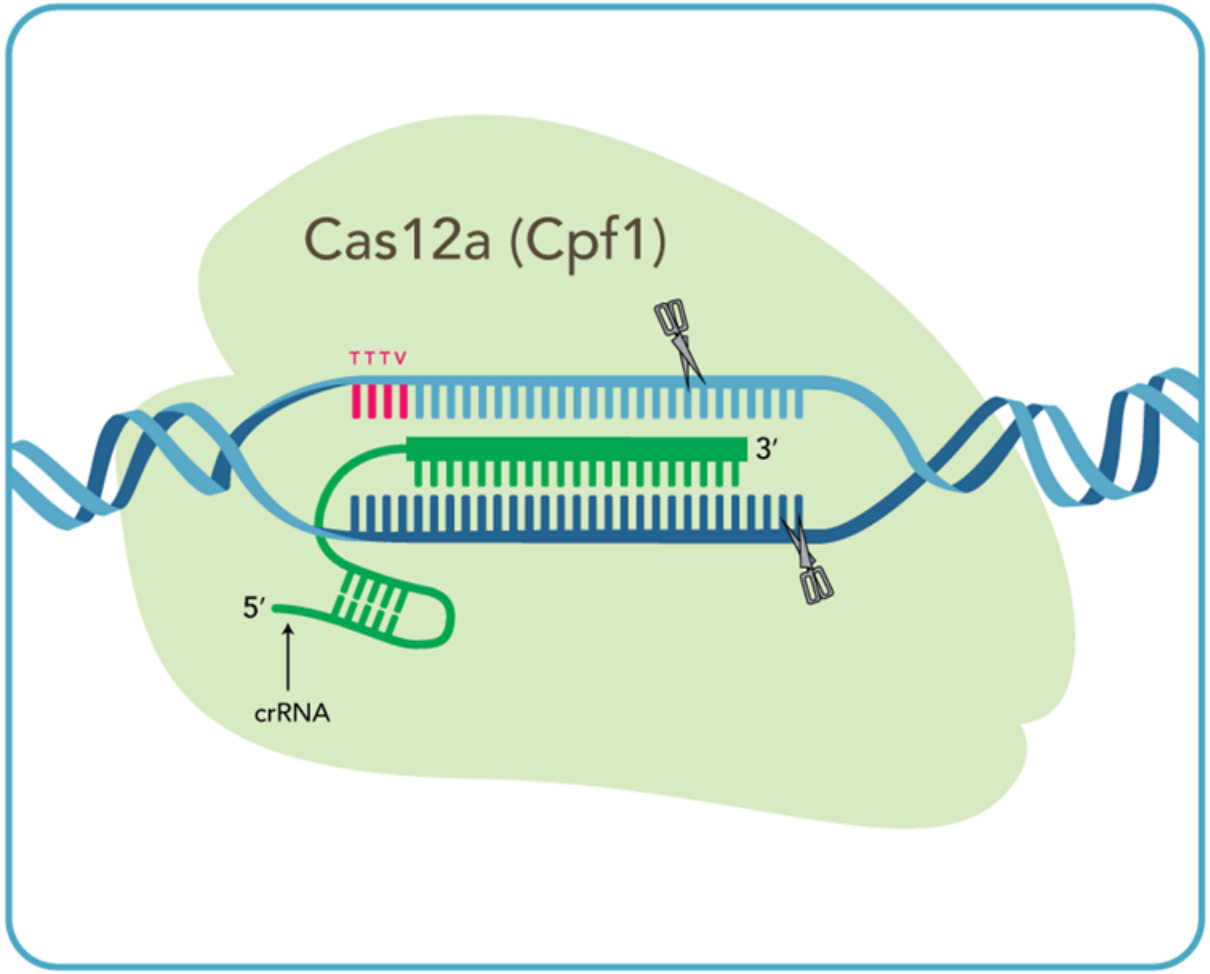
- Biomolecules. 2020 Jun; 10(6): 839. doi: 10.3390/biom10060839

Orientação prática para a implementação da tecnologia CRISPR em fungos filamentosos com base nos dados obtidos para *Thermothelomyces thermophilus*

	Plasmid-based approach	RNP-based approach
Preparation of nuclease	Cloning of the nuclease into a plasmid prior transformation is mandatory. When constitutively expressed, risk of off-targets should be considered. When present on AMA-plasmid, the risk should be lower but still present	Cloning of the nuclease into a plasmid allowing heterologous expression, e.g. in *E. coli*, is a prerequisite. Once established and purified, the nuclease can be aliquoted and stored prior to use. As the protein does not become expressed in the targeted fungus, the risk of off-targets should be very small
Preparation of guide RNA	Plasmid-based, thus more stable during handling and storage	Involves in vitro transcription, hence potentially sensitive to handling errors
Transformation procedure	Easy	Easy but requires preassembly of RNPs
Transformation rate	Very high also with four targets	Very high for single and double targets Low for three and four targets
Single-targeting efficiency of FnCpf1, AsCpf1, SpCas9	Locus-dependent	Locus-dependent
Multiplex-targeting efficiency of FnCpf1	High (34 % ± 6 % in this study)	Low (13 % ± 2 % in this study)
MTP-based down-scaling for FnCpf1	Possible with no loss in efficiency with respect to single and double targeting	Possible with no loss in efficiency with respect to single targeting*

Endonucleases Cas9 e Cas12a (Cpf1)





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Endonucleases Cas9 e Cas12a (Cpf1)

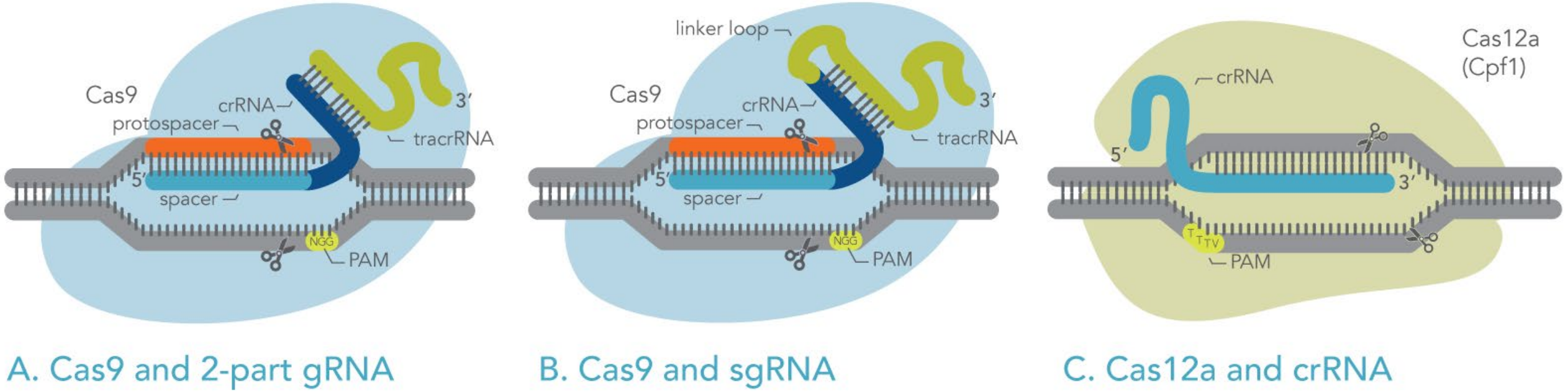
	Cas9 system	**Cas12a system**
Applications	General genome editing	•For species with AT-rich genomes •For regions with limiting design space for use of the CRISPR-Cas9 system

Ribonucleoprotein components	•gRNA options:
	  • crRNA and tracrRNA
	  • sgRNA
	•Cas9 endonuclease
		•crRNA
		•Cas12a endonuclease

PAM sequence†	NGG	•TTTV for Cas12a V3 •TTTN for Cas12a *Ultra*

https://www.idtdna.com/pages/products/crispr-genome-editing?utm_source=google&utm_medium=cpc&utm_campaign=ga_crispr_cas&utm_content=ad_group_cas9&gclid=CjwKCAjwtJ2FBhAuEiwAIKu19m1SCZSmhNJ8ykolMbomT3zKjsJOzEYHKb3yhk4ePHNbtBciTPXpzxoCZpkQAvD_BwE

Esquema de ribonucleoproteínas ativas contendo enzimas Cas ligadas a RNAs guia



A. Cas9 and 2-part gRNA B. Cas9 and sgRNA C. Cas12a and crRNA

(**A**) Cas9 with 2-part guide RNA, comprising crRNA and tracrRNA. (**B**) Cas9 with single-guide RNA. (**C**) Cas12a with crRNA (gRNA).

https://www.idtdna.com/pages/technology/crispr/crispr-genome-editing