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Design strategy for DiCre-mediated inducible gene knockout in the malaria parasite

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We use this protocol and it's working

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Abstract

This protocol describes a detailed strategy for inducibly knocking out a target gene in *Plasmodium* species using the Dimerisable Cre (DiCre) recombinase system, with a primary focus on *Plasmodium falciparum*[1,2]. The approach involves flanking a target genomic region with *loxP* sites ("flox"), priming it for precise DiCre-mediated excision when induced with the drug rapamycin (RAP). We describe a widely used method (in studies like [3,4] for example) for deleting specific functional domains of a gene by incorporating *loxP* sites within synthetic introns (loxPint)[5], combined with simultaneous C-terminal tagging with an epitope tag. This protocol is meant for students and researchers new to designing DiCre strategies, providing step-by-step guidance on selecting the target region for floxing, designing guide RNAs, and creating a repair template for Cas9-enhanced homology-directed repair to introduce *loxP* sites at the desired locus.

Attachments

loxPint(SERA2).gb	LoxP.gb	loxPint(SUB2).gb	3HA.gb
0B	0B	0B	1KB

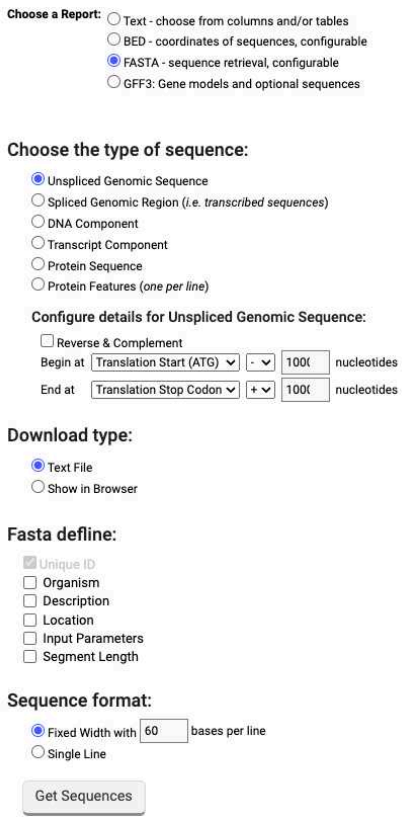
Guidelines

1) This protocol is meant as guidance to get started with designing a DiCre strategy. As with such strategies, there is definitely not "one way" to do it and a lot of variations can exist suited to different needs.
2) If the target gene has introns, exclude any introns within the target region when recodonising. If suitable, replace an existing intron with the loxPint.
3) Sequences of 2 commonly used loxPints, loxP and 3HA tags attached.

Preparing an annotated sequence file for the gene of interest

1 Go to **PlasmoDB**[6] and search for the name or gene ID of the gene of interest.

2 Download the sequence in FASTA format. Select “Download Gene” at the top of the gene page and choose following options



This will generate a FASTA file containing the gene sequence flanked by 1000 nucleotides on either side, for designing homology arms or primers for diagnostic PCR.

3 Open the FASTA file in your favourite sequence editor or molecular biology tool (we use **SnapGene**). Annotate the sequence with gene, intron, exon and the gene’s critical functional or catalytic domain as features. Coordinates of functional domains can be obtained from **PlasmoDB** (Protein features and properties > InterPro Domains).

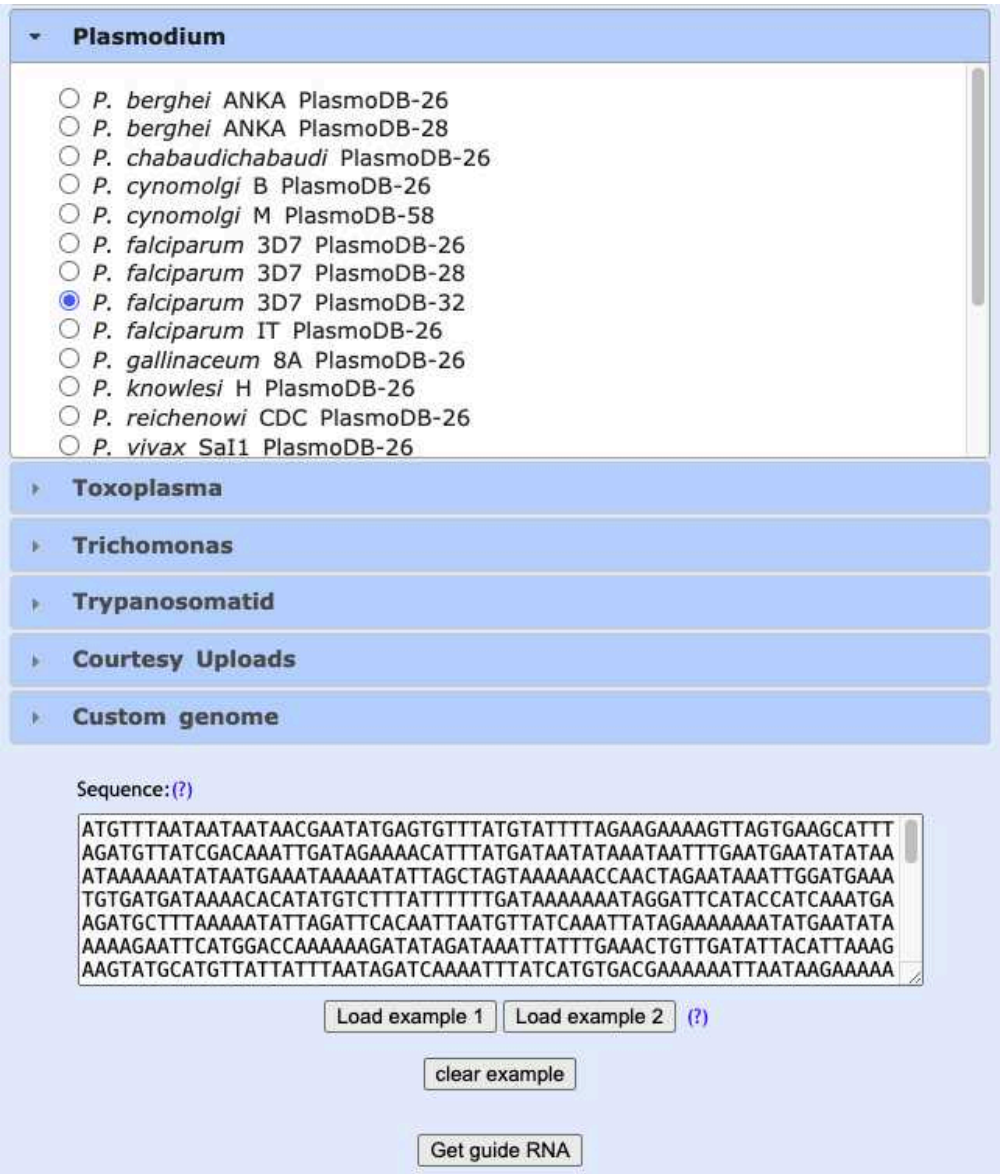
Note: You can skip these steps if you already have access to a fully annotated genome locally.Using a

genome visualisation tool like IGV, you can navigate to the gene locus and export the sequence and annotation as a GenBank file.

wildtype_locus.dna

Selecting the target region to be floxed

4 Predict gRNAs for the entire gene using **Eukaryotic Pathogen CRISPR guide RNA/DNA Design Tool (EuPaGT)**[7].

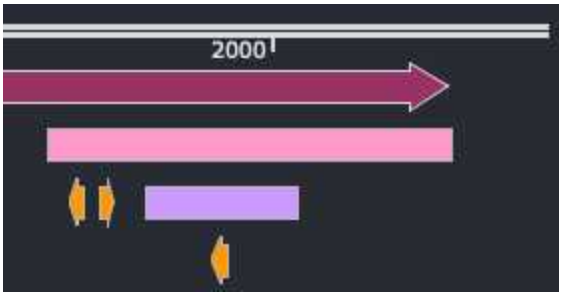


gRNA id	gRNA sequence (PAM "NGG")	Total score	efficiency score based on Doench et al.2014	efficiency score based on CRISPRater	On-target hits in the genome (perfect-match | non-perfect-but-PAM-match)	Off-target hits (perfect-match | nonperfect-match)	In conserved region(?)	GC content	CRISPR-STOP compatible (?)	Flanking microhomolgy pair score	Flanking microhomolgy pair score explanation	Potential problems during transcription
gene_936_revcom	ACACGACAAAGGACTTCTATTGG	0.62	0.55	0.80	1 | 0	0 | 0	no	0.40	no	NA	search is turned off	No problem found
gene_1035	AATATTGGTATCCATAATTGGGG	0.59	0.40	0.77	1 | 0	0 | 0	no	0.25	no	NA	search is turned off	No problem found
gene_497_revcom	ATTTGGCTAAAAATTTGCTTTGG	0.58	0.40	0.74	1 | 0	0 | 0	no	0.25	no	NA	search is turned off	No problem found

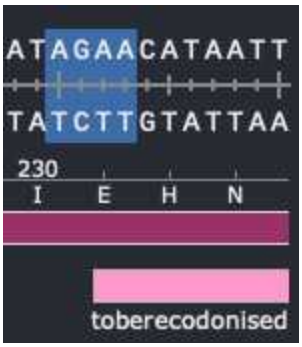
5 Choose 2 or 3 gRNA target sites with good scores (efficiency scores > 0.5, aiming for the highest) close to either the functional domain or near the C terminal end of the gene. Make sure that the sequences are unique and not present in any other region of gene. Add them as features in wildtype_locus.dna.

guide target sites in orange.

6 Choose a target region that-
i) begins upstream of the gene's critical functional or catalytic domain and extends to the end of the gene,
ii) starts immediately after an AG in an AG-AT/AA/GA/GT sequence to mimic the exon-intron boundary in *Plasmodium*[8](this will be where loxPint would be inserted later). If such a junction is not available within a reasonable distance upstream of the functional domain, an AA-AT/AA/GA/GT sequence could serve as an alternative.
iii) includes at least two of the selected gRNAs within 100 bp of its boundaries. While this proximity is ideal for efficient Cas9-mediated HDR, distances up to 500 bp have been successfully used in our experience.



target region in pink.

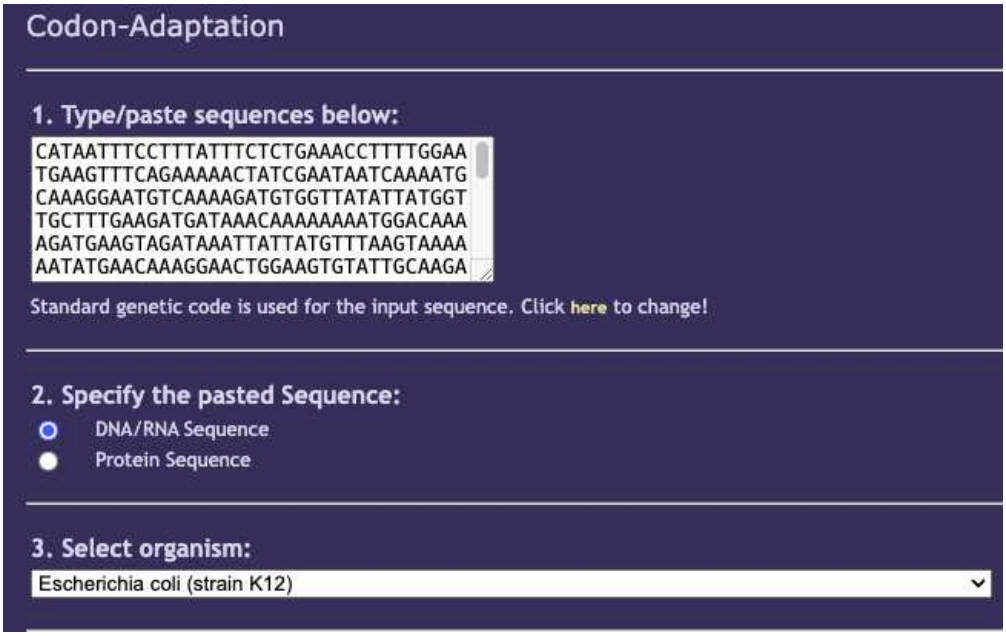


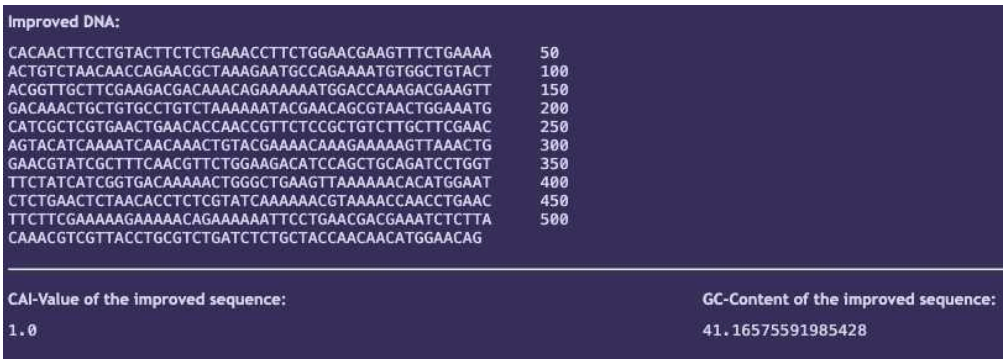
Exon-intron junction

Designing the repair template

7 Recodonise the selected target region (excluding any introns and the stop codon) using the **JAVA Codon Adaptation Tool**[9].

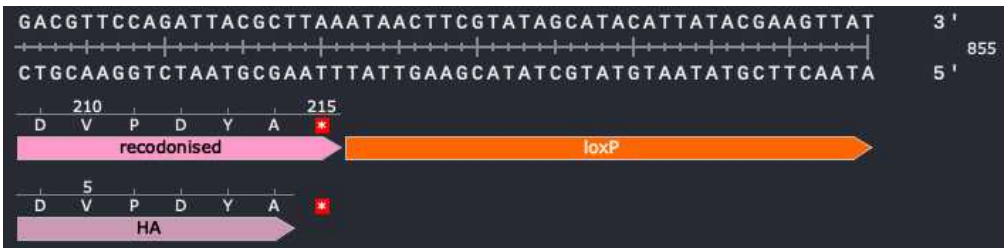
Note: This tool is preferred as it achieves higher base substitution rates compared to codon optimisation tools typically provided by gene synthesis companies. Another good alternative is **VectorBuilder**.





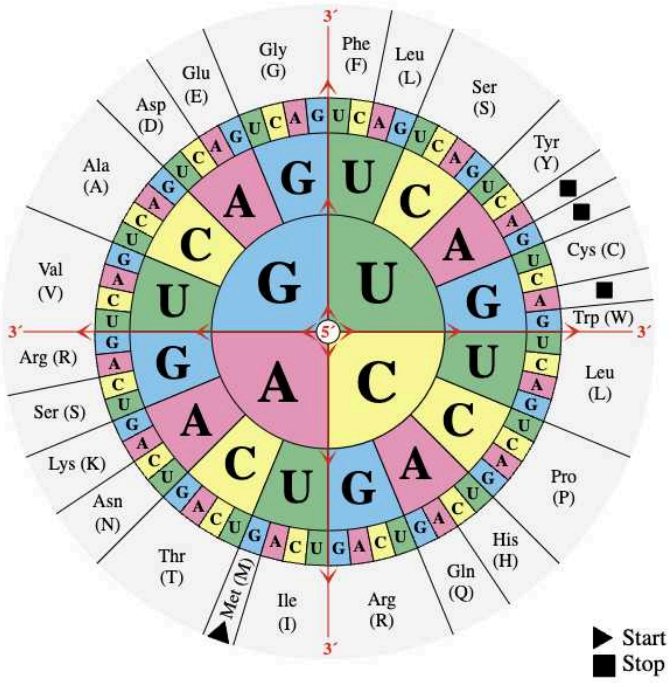
8 Create a new DNA sequence file with the following sequences arranged in this order-
i) a *loxPint* sequence,
ii) the recodonised sequence from Step 7.
ii) a triple HA tag sequence (or any peptide tag of your choice).
iv) a stop codon (TAA), and finally
v) a *loxP* sequence

syn_gene.dna

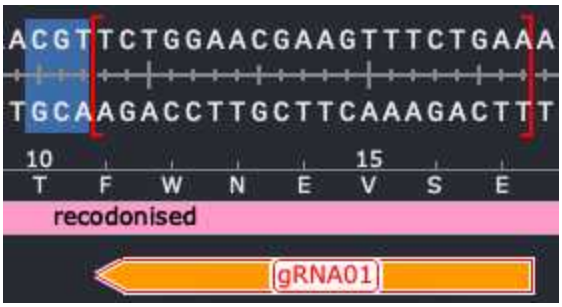


Take care to add the stop codon *after* the tag.

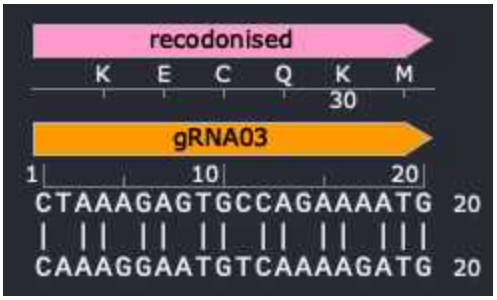
9 If not already achieved during recodonization, further modify the gRNA target sites by introducing silent base substitutions to disrupt the PAM site (NGG). If disrupting the PAM site is not feasible, introduce as many mismatches as possible within the target sequence to minimize off-target effects.



By Mouagip - Codons aminoacids table.png, Public Domain, https://commons.wikimedia.org/w/index.php?curid=5986132

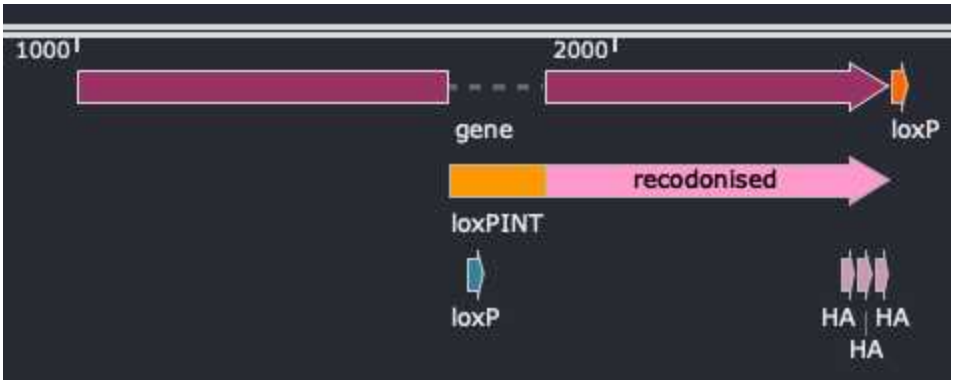


PAM site AGG changed to ACG (highlighted)

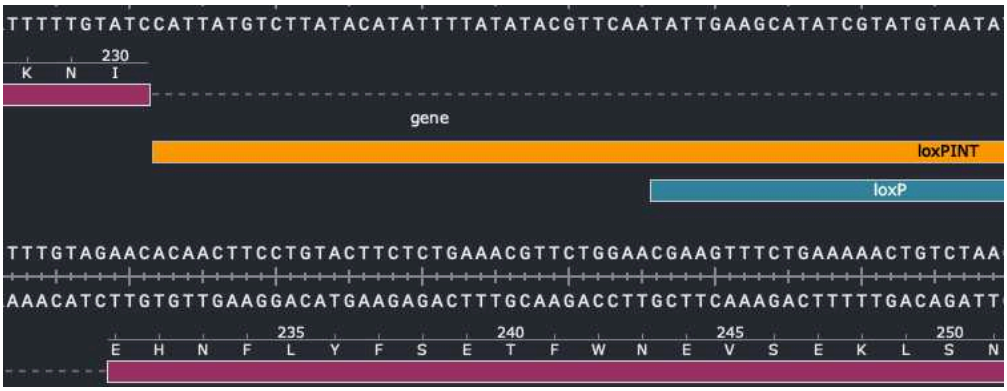


or silent mismatches (top) introduced in gRNA sequence (bottom)

10 Verify the accuracy of the strategy by replacing the target region in the gene with this designed repair sequence and ensure that the translated protein sequence (across the inserted loxPint) remains unchanged.



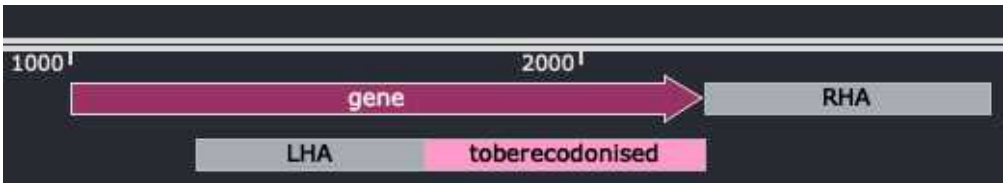
integrated_locus.dna



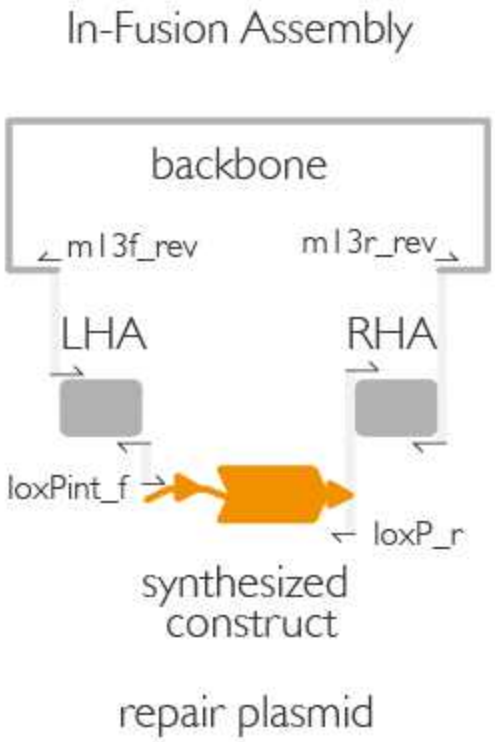
verify amino acid sequence is unchanged after inserting loxPint

Designing the repair construct for assembly using Gibson/In-Fusion cloning

11 Select 400–500 bp regions flanking either end of the target region as homology arms, ensuring that amplification primers 20-25 bps long with a melting temperature (Tm) of 55–60 °C can be designed (*P. falciparum* has a highly AT-rich genome).



12 Design primers to amplify homology arms from genomic DNA with 20 bp overhang sequences homologous to the ends of the synthesised construct and the vehicle plasmid backbone.



Common primers to amplify backbone-
1) m13f_rev : ACTGGCCGTCGTTTTAC
2) m13r_rev : GTCATAGCTGTTTCCTG

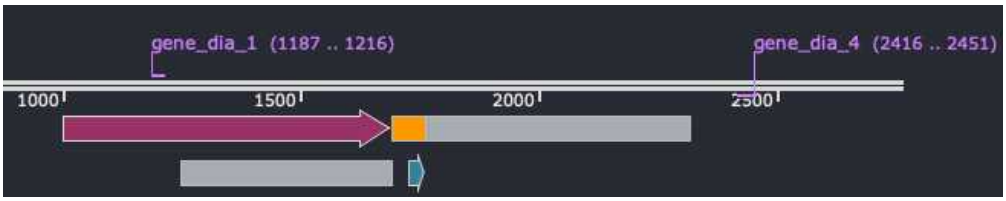
Common primers to amplify synthesized construct-
3) loxPint_f : GTAATACAGAATATGTATAAAATATATGCAAG (for SUB2:loxPint)
4) loxP_r : ATAACTTCGTATAATGTATGCTATACGAAGTTATTTAAGCG

Primers to amplify LHA and RHA (20 bp overhangs in bold)-
5) LHA_F : **GTTGTAAAACGACGGCCAGT**-specific sequence
6) LHA_R : **TTATACATATTCTGTATTAC**-specific sequence
7) RHA_F : **CATACATTATACGAAGTTAT**-specific sequence
8) RHA_R : **ACACAGGAAACAGCTATGAC**-specific sequence

Designing diagnostic PCRs

13 Design two primers on outside RHA and LHA regions (dia_1 and dia_2) to check for integration and excision. You can additionally create an excised locus file (by deleting region between two loxP sites and retaining one loxP site) to easily check for expected amplicon size or map sequenced amplicons.

gene_dia_1 - loxP_R for 5' integration and loxPint_F - gene_dia_4 for 3' integration



excised_locus.dna
gene_dia_1 - gene_dia_2 to check for excised locus upon RAP treatment.

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