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Pilot Project Proposal

Prepared for GP-write

Name of Project:

Recombinase-Mediated Assembly (RMA)

Proposer and Contact Information:

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Background:

Currently, the oligonucleotide fragments generated by traditional phosphoramidite chemistry must be assembled in a time-consuming and error-prone manner, limiting the size of DNA that can be created. Gibson Assembly is a decent choice, but relies on correct sticky-end pairing to align fragments. While acceptable when only a small number of fragments are being joined, it quickly becomes overwhelmed, leading to erroneous ligations.

One alternative relies on the action of the RecA-like recombinase UvsX, an enzyme involved in T4-like phage replication. This protein (in concert with its accessory proteins UvsY and gp32) spontaneously forms a nucleofilament on ssDNA and then “scans” nearby dsDNA for a homologous sequence, whereupon it will insert the ssDNA, disrupting the double helix and allowing other enzymes (polymerases, ligases) to act.

UvsX's chief use today is as an alternative to heat-based PCR amplification, operating at a constant temperature and generating amplicon for downstream detection, operating similarly to traditional PCR. It should be possible to utilize this enzyme for 'one-pot' assembly of an arbitrary number of fragments, generating dsDNA many kilobases in length, with minimal errors.

It is recommended to do a cursory reading on the UvsX protein's function in homology searching and strand invasion, as well as RecA's role in dsDNA break repair, as this will elucidate their mechanism of action. Unlike other “recombinase” proteins with which most are familiar, these enzymes will attach to any ssDNA – and perform a highly processive reaction that potentially renders traditional sticky-end ligation obsolete.

Technical Idea:

(See Figure 1 for diagram)

dsDNA fragments with a single 5' overhang ~20bp in length are generated by a suitable technique. For the sake of clarity, we will refer to the 5' overhang as male, and the other end of the fragment (blunt-ended dsDNA) as female. Each fragment's male end is homologous to the female end of one other fragment in the mixture, ensuring that the assembly will occur in an orderly fashion.

The recombinase coats the male end, and the nucleofilament begins to seek the matching female end of another fragment. Once a match is found, strand invasion occurs, displacing the 3' strand of the female end. Ligation occurs between the 5' strand of the female end and the 3' underhang of the male end. The displaced 3' female end is degraded by Exonuclease T, which is specific to ssDNA and proceeds exclusively 3'→5'.

Once the displaced end has been degraded down to its dsDNA 'base', ligation occurs between this base and the 5' overhang from the male strand. A polymerase may be required to ensure that the ends are flush, depending on the proclivity of Exonuclease T in this situation. This results in a new, double-length fragment that is able to continue the reaction.

While this reaction has a decent number of moving parts (the actions of the accessory proteins UvsY and gp32 are excluded for clarity), it has the potential to incorporate a high-fidelity error-checking system into the assembly of fragments.

Utility:

Bridging the gap between short synthetic fragments and entire chromosomes is an essential step in the the GP-write project. Synthesizing a genome appears a nearly impossible effort without some improvement to the assembly process, and any promising solution merits investigation.

In combination with an Isothermal Amplification Array (*see other proposal*), this technique provides a pipeline from very short oligos all the way up to chromosome-sized DNA, with a significant reduction in the labor involved versus traditional methods.

Figure 1

Recombinase-Mediated Assembly

