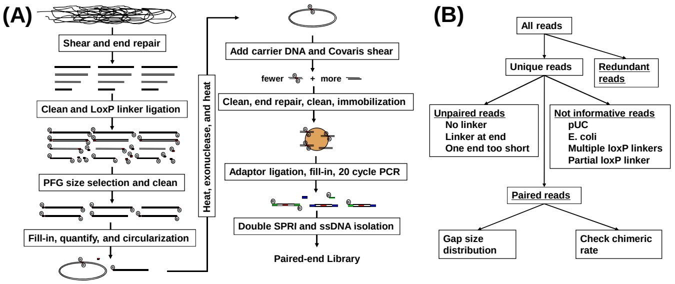
# Large Gap Size Paired-end Library Construction for Second Generation Sequencing

Ze Peng[1], Matthew Hamilton[1], Jeff Froula[1], Aren Ewing[1], Brian Foster[1], and Jan-Fang Cheng[1]
1Lawrence Berkeley National Laboratory

## Abstract

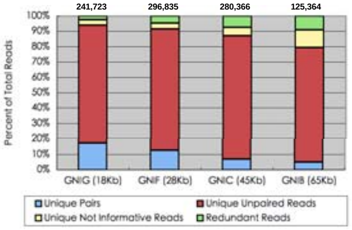
**Fosmid or BAC end sequencing plays an important role in de novo assembly of large genomes like fungi and plants. However construction and Sanger sequencing of fosmid or BAC libraries are laborious and costly. The current 454 Paired-End (PE) Library and Illumina Jumping Library construction protocols are limited with the gap sizes of approximately 20 kb and 8 kb, respectively. In the attempt to understand the limitations of constructing PE libraries with greater than 30Kb gaps, we have purified 18, 28, 45, and 65Kb sheared DNA fragments from yeast and circularized the ends using the Cre-loxP approach described in the 454 PE Library protocol. With the increasing fragment sizes, we found a general trend of decreasing library quality in several areas. First, redundant reads and reads containing multiple loxP linkers increase when the average fragment size increases. Second, the contamination of short distance pairs (<10Kb) increases as the fragment size increases. Third, chimeric rate increases with the increasing fragment sizes. We have modified several steps to improve the quality of the long span PE libraries. The modification includes (1) the use of special PFGE program to reduce small fragment contamination; (2) the increase of DNA samples in the circularization step and prior to the PCR to reduce redundant reads; and (3) the decrease of fragment size in the double SPRI size selection to get a higher frequency of LoxP linker containing reads. With these modifications we have generated large gap size PE libraries with a much better quality.**

## Paired-end Library Construction and Data Analysis Processes



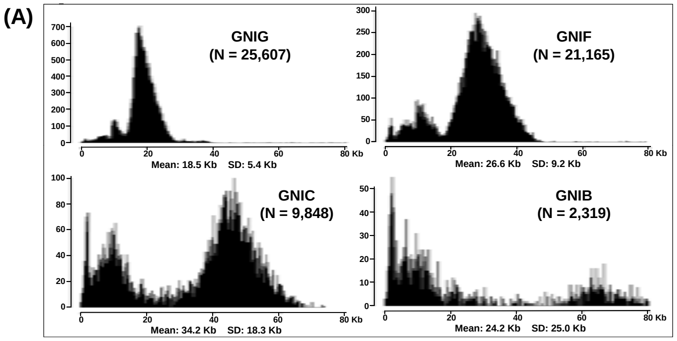
(A) This is a modified version of the 454 Recombi Paired-end Library Construction Protocol. We use pulsed-field gels to size select fragments greater than 20Kb in size. We increase the amount of DNA in the circularization step to 600ng. The pUC carrier DNA is treated with UV to reduce the chance of amplification. We also use Covaris sonicator to shear circularized DNA. The flow of sequence data analysis is shown in (B). All reads are cross-matched against each other to identify redundant reads (greater than 95% nucleotide matches). The remaining reads are grouped into 3 major categories including "not informative", "unpaired", and "paired" reads. The paired reads must have more than 15 bases of sequences on both sides of the loxP linker. Only unique paired reads are used to check for chimera and gap size distribution.

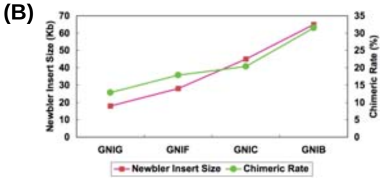
## Quality of Long Gap Size PE Libraries



We constructed 4 libraries with DNA isolated from Saccharomyces cerevisiae S288C. We used this completed genome of 12,156,676 bases to evaluate the limitations of the current approach for constructing long gap size paired-end libraries. The fragments isolated from the PFG range from 18 to 65Kb. The names and fragment sizes of these libraries are shown in the left bar graph. The number of reads generated from these libraries are shown on the top of the graph. The large amount of unpaired reads seen in all 4 libraries were caused by the short sequence read length (avg. 300bp) and the long library inserts (avg. 600bp).

## Gap Size Distribution and Chimeric Rate

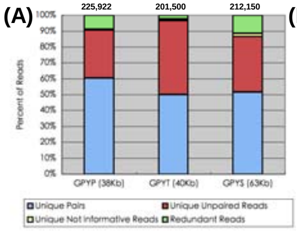


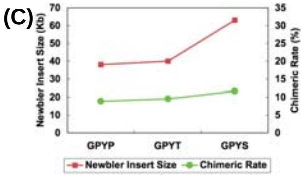


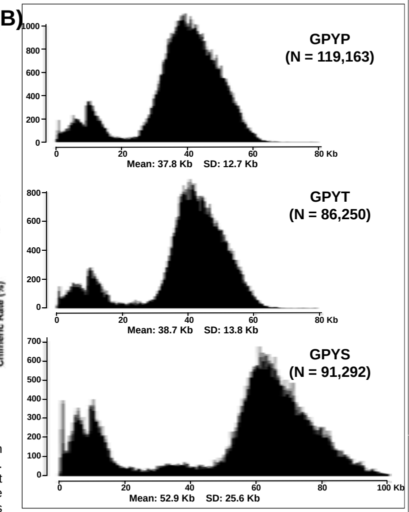
We mapped all the unique pairs of these libraries to the yeast genome and plotted the gap size distribution as 0.5Kb bins in (A). We've seen an increasing amount of small fragment contamination as the targeted fragment size increases. Chimeric pairs include two reads with wrong orientation or separated greater than 80Kb. We've seen an increase of chimeric rate as the targeted fragment size increases.

## Improvement of the Long Gap Size PE Library Construction

| Steps to improve | Old process | New Process | Effect |
|---|---|---|---|
| Pulsed-field gel size selection | Once | Twice or two discontinuous pulse cycles | Reduce small fragments |
| DNA concentration in circularization | 6 ng/ul | 3 ng/ul | Reduce chimeric rate |
| Sonication shearing | 500-700 bp | 200-400 bp | Increase reads with loxP linkers |

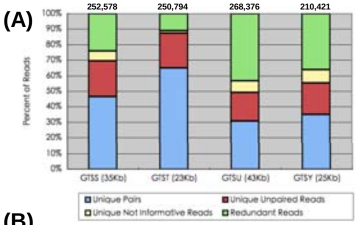




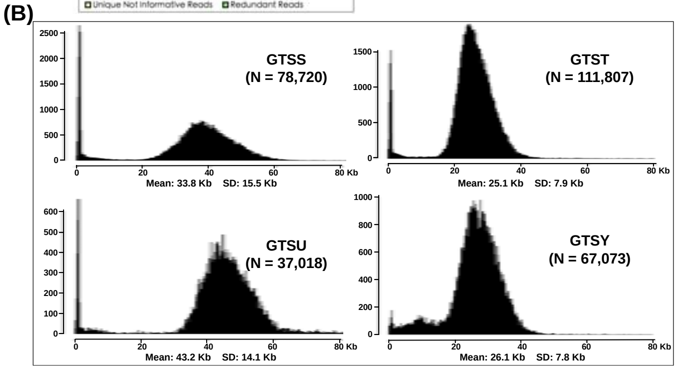


We constructed 3 long gap size libraries from the same yeast DNA using the improved steps. The sequence analysis shows significant improvements in the representation of unique pairs (A), the contamination of small fragments (B), and the reduction of chimeric pairs (C).

## Long Gap Size PE Library Construction of Fungal Genomes



We constructed 4 long gap size paired-end libraries from 3 fungal DNA - Mucor circinelloides (GTSS and GTST), Neurospora tetrasperma (GTSU), and Spathaspora passalidarum (GTSY). The number of reads generated from these libraries are shown on the top of the bar graph (A). The increase of redundant reads in GTSS, GTSU, and GTSY were resulted from a higher loss of DNA during the library construction process. For each of these 3 genomes, we only have a draft assembly to locate the resulted pairs. The gap size distribution plots are shown in (B). We could not accurately determine chimeric pairs without a finished genome.



## Test Assembly of Two Fungal Genomes with Long Gap Size Libraries

A

| Spathaspora passalidarum (GC 37%) | | | | |
|---|---|---|---|---|
| | Test assembly 1 | Test assembly 2 | Test assembly 3 | Current assembly |
| 454 std | 438.60 Mb | 438.60 Mb | 438.60 Mb | 438.60 Mb |
| New 26Kb 454 PE | | 67.41 Mb | | |
| Fosmid ends | | | 23.34 Mb | 23.34 Mb |
| Old 23Kb 454 PE | | | | 172.65 Mb |
| | | | | |
| Scaffold Count | N/A | 35 | 32 | 47 |
| Scaffold Length | N/A | 13.23 Mb | 13.31 Mb | 13.27 Mb |
| N50 Scaffold Number | N/A | 3 | 3 | 4 |
| N50 Scaffold Length | N/A | 2.03 Mb | 2.06 Mb | 1.75 Mb |
| ≥1Kb Contigs Number | 155 | 152 | 135 | 153 |
| ≥1Kb Contigs Length | 13.00 Mb | 13.03 Mb | 13.03 Mb | 12.98 Mb |
| N50 Contigs Number | 24 | 20 | 18 | 22 |
| N50 Contigs Length | 153.94 Kb | 211.37 Kb | 205.22 Kb | 196.77 Kb |

B

| Mucor circinelloides CBS277.49 | | | | | | | |
|---|---|---|---|---|---|---|---|
| | Test assembly 1 | Test assembly 2 | Test assembly 3 | Test assembly 4 | Test assembly 5 | Test assembly 6 | Test assembly 7 |
| 454 std | 2,255 Mb | 2,255 Mb | 2,255 Mb | 2,255 Mb | | | 2,255 Mb |
| New 23 Kb 454 PE | | 81 Mb | | | | 81 Mb | 81 Mb |
| New 35 Kb 454 PE | | | 80 Mb | | | | |
| Fosmid ends | | | | 39 Mb | 39 Mb | | |
| pMCL ends | | | | | 229 Mb | 229 Mb | |
| pUC ends | | | | | 156 Mb | 156 Mb | |
| Old 5kb 454 PE | | | | | | | 677 Mb |
| Old 8kb 454 PE | | | | | | | 868 Mb |
| | | | | | | | |
| Scaffold Count | N/A | 766 | 1075 | 850 | 501 | 602 | 565 |
| Scaffold Length | N/A | 38.01 Mb | 36.06 Mb | 42.34 Mb | 38.99 Mb | 38.16 Mb | 37.62 Mb |
| N50 Scaffold Number | N/A | 6 | 111 | 8 | 5 | 5 | 5 |
| N50 Scaffold Length | N/A | 2.50 Mb | 0.97 Mb | 1.41 Mb | 2.78 Mb | 3.15 Mb | 3.35 Mb |
| ≥1kb Contigs Number | 2,507 | 2,451 | 2,506 | 2,447 | 2 | 2,007 | 2,296 |
| ≥1kb Contigs Length | 34.92 Mb | 34.88 Mb | 34.87 Mb | 34.98 Mb | 35.75 Mb | 35.63 Mb | 35.02 Mb |
| N50 Contigs Number | 464 | 440 | 457 | 455 | 329 | 340 | 415 |
| N50 Contigs Length | 27 Kb | 28 Kb | 27 Kb | 27 Kb | 37 Kb | 34 Kb | 30 Kb |

We ran two sets of test assemblies of the Spathaspora passalidarum (Table A) and Mucor circinelloides (Table B ) sequences using Newbler. The top halves of the tables show the type of libraries and amount of sequences used in the assemblies. The bottom halves of the tables show the assembly stats. The results show that 454 large insert paired-ends and the Sanger fosmid ends generate similar number of scaffolds and scaffold sizes in the whole genome assemblies (test assemblies 2 and 3 of Table A, and test assemblies 2 and 4, and test assemblies 5 and 6 of Table B).

## Acknowledgements

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