

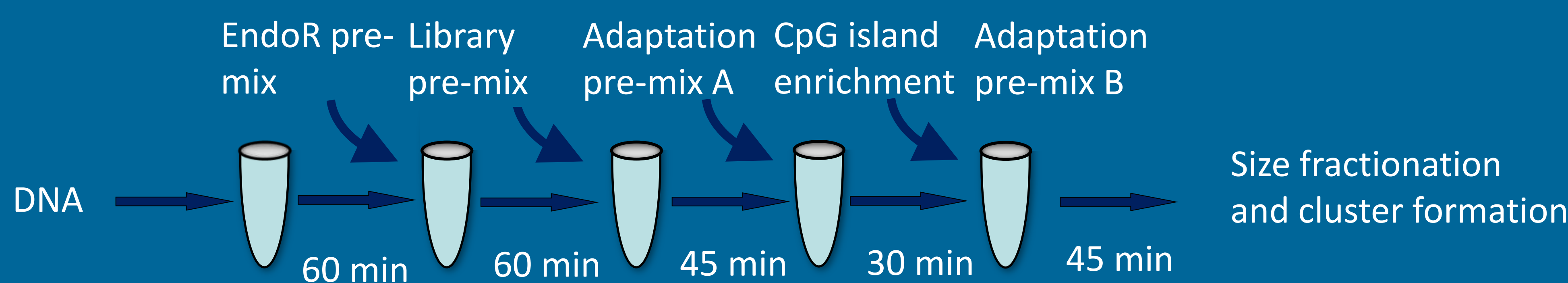
Simple, Automatable NGS Library Preparation Method for Global DNA Methylation Profiling in Cancer Research and Diagnostics



E. Kamberov, T. Kurihara, J. Langmore, V. Makarov, J. M'Mwirichia, Rubicon Genomics, Ann Arbor, MI
kamberov@rubicongenomics.com; + 1 734 677 2894 ; www.rubicongenomics.com

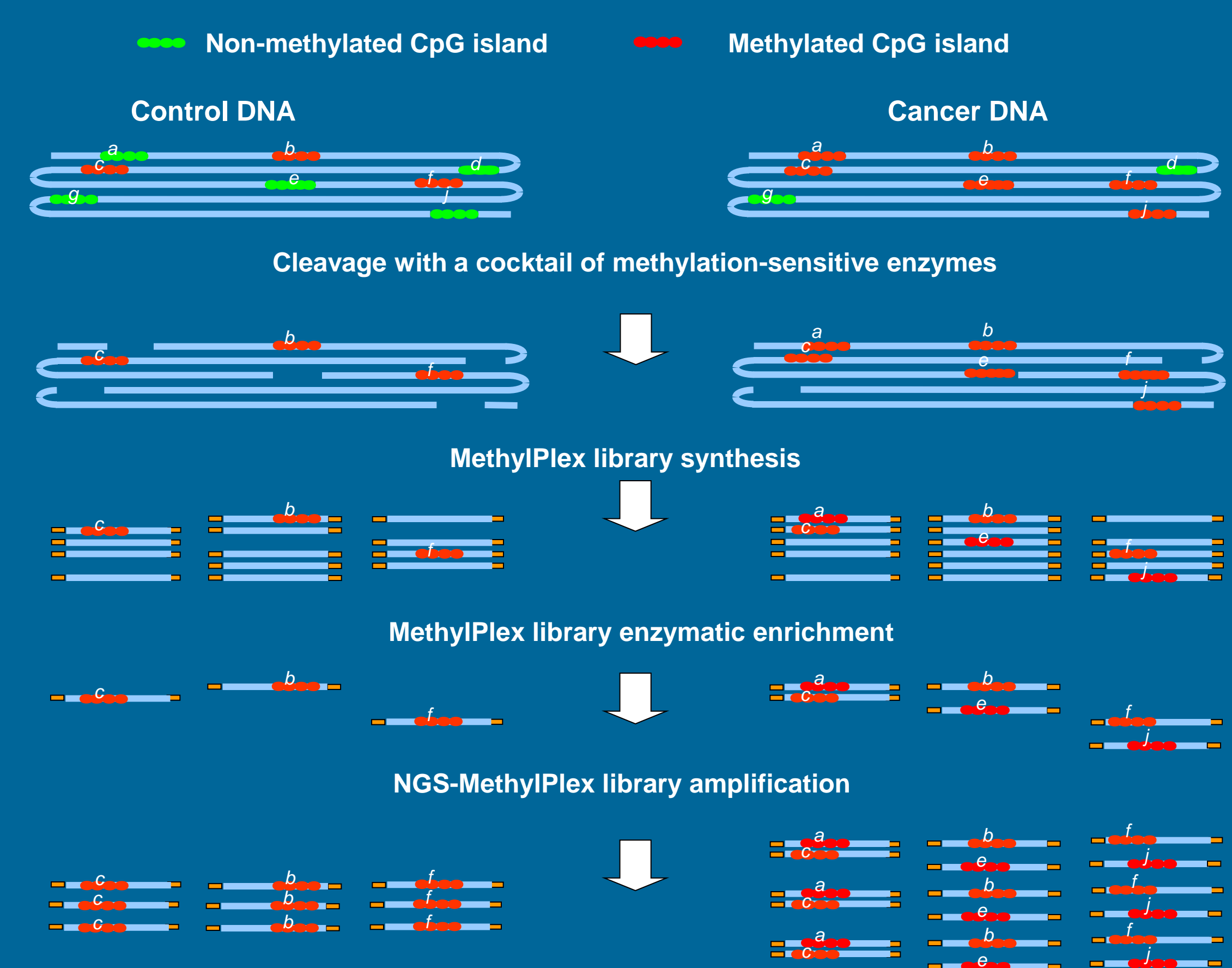
Next-generation DNA sequencing has revolutionized epigenetic research by enabling genome-wide sequencing and profiling of differentially methylated DNA regions associated with cancer and other diseases. Current protocols for preparing libraries for NGS DNA methylation analysis usually require microgram quantities of DNA, mechanical fragmentation, preparation of DNA ends, ligation of adaptors, gel size fractionation, library amplification, numerous purification steps, and isolation of methylated DNA by immunoprecipitation (MeDIP) or magnetic beads binding (MBD), which together require days of preparation time. Here we present a streamlined, automatable process (MethylPlex-NGS) to prepare libraries for methylation analysis using the Illumina Genome Analyzer™ (GA) and results of genome-wide methylation profiling of human prostate cancer and benign cell lines. Compared to other sample preparation methods MethylPlex-NGS is substantially simpler, more sensitive, faster, requires only 20 - 50 ng of input DNA, and 4 hrs total preparation time. The MethylPlex-NGS process is initiated by digesting input DNA with methylation-sensitive restriction enzymes, followed by attachment of universal sequences and subsequent PCR amplification. A second round of enzymatic treatment depletes non-GC rich sequences, and an additional amplification ensures enrichment of highly methylated DNA fragments. MethylPlex-NGS libraries were synthesized from genomic DNA isolated from LNCaP prostate cancer cells, PrEC benign prostate epithelial cells, and 12 tissue samples from localized tumors, metastatic tumors, and control samples. Libraries were sequenced using the Illumina Genome Analyzer™ (GA). LNCaP cells exhibited a seven-fold enrichment for promoter CpG island methylation compared to PrEC cells. Eight hundred and thirty unique gene promoters methylated only in LNCaP, but not in PrEC cells have been identified, of which 789 mapped to the Oncomine database. The data was corroborated using "Methyl-Profiler" qPCR, bisulfite sequencing, and a functional strategy using gene expression arrays following treatment with the demethylating agent 5-Aza. In addition, a cancer-specific methylation pattern was identified that encompasses 1,045 genes in metastatic samples and 1,436 in localized plus metastatic samples combined, which may play a pivotal role in tumor progression.

MethylPlex-NGS workflow for methylation profiling on Illumina GA. Total time: 4 hrs

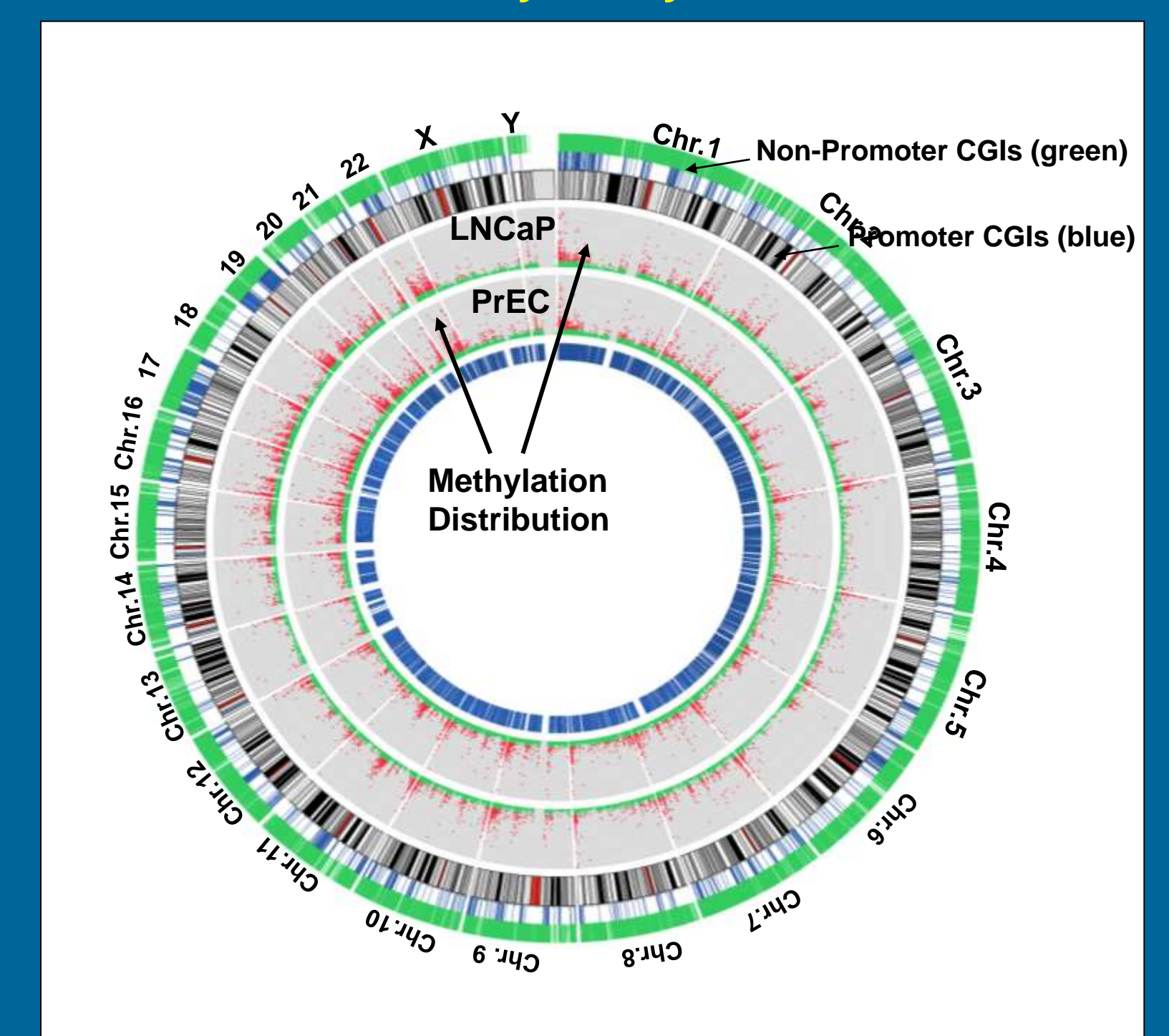


The MethylPlex-NGS process is initiated by digesting input DNA with methylation-sensitive restriction enzymes, followed by attachment of universal sequences and subsequent PCR amplification. A second round of enzymatic treatment depletes non-GC rich sequences, and an additional amplification ensures enrichment of highly methylated DNA fragments. MethylPlex-NGS libraries were synthesized from 50 nanograms of genomic DNA isolated from LNCaP prostate cancer cells, PrEC benign prostate epithelial cells, and 12 tissue samples representing localized tumors, metastatic tumors, and control samples. Libraries were sequenced using the Illumina Genome Analyzer™ (GA). A Hidden Markov Model algorithm detected enriched regions from mapped reads obtained in each sequencing run, and while both cell lines had a total of ~55,000 methylated regions each, LNCaP cells exhibited a seven-fold enrichment of promoter CpG island methylation compared to PrEC cells. Eight hundred and thirty unique gene promoters have been found to be methylated only in LNCaP, but not in PrEC cells, of which 789 mapped to the Oncomine database. The data was corroborated using "Methyl-Profiler" qPCR, bisulfite sequencing, and a functional strategy using gene expression arrays following treatment with the demethylating agent 5-Aza. Several methylated regions were validated by multiple independent approaches including bisulfite sequencing. Detailed promoter analysis revealed diverse methylation patterns around transcription start sites, including direct methylation of CpG islands, methylation of regions flanking CpG islands, and methylation of sites devoid of CpG islands. Methylated promoters correlated with gene repression, and there was an enrichment of novel regions methylated in LNCaP cells that were also methylated in prostate cancer tissues. In addition, a cancer-specific methylation pattern was identified that encompasses 1,045 genes in metastatic samples and 1,436 in localized plus metastatic samples combined, which may play a pivotal role in tumor progression (our acknowledgment to Dr. Arul Chinnaiyan and his team from University of Michigan).

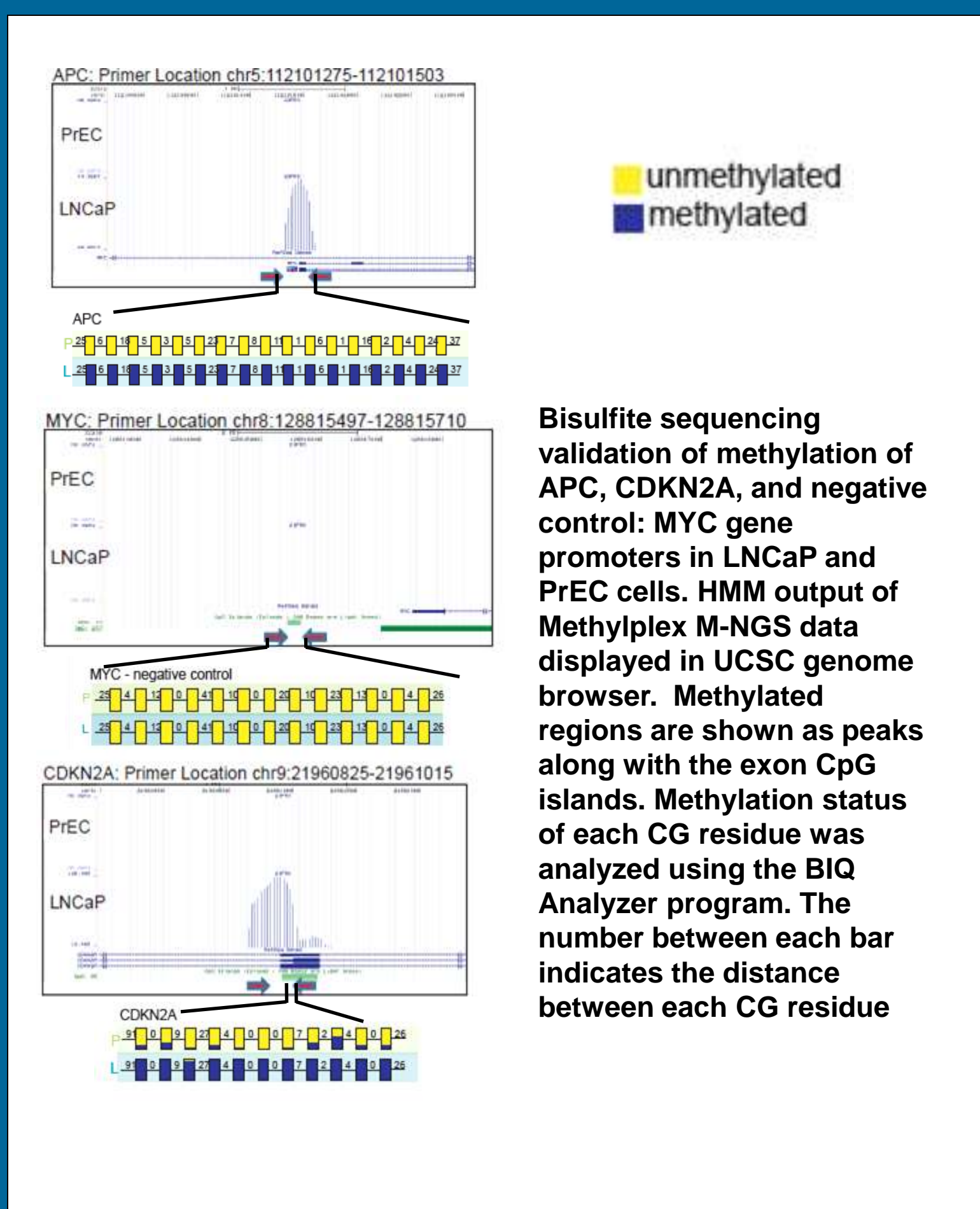
MethylPlex Selectively Amplifies Methylated Promoter Sequences



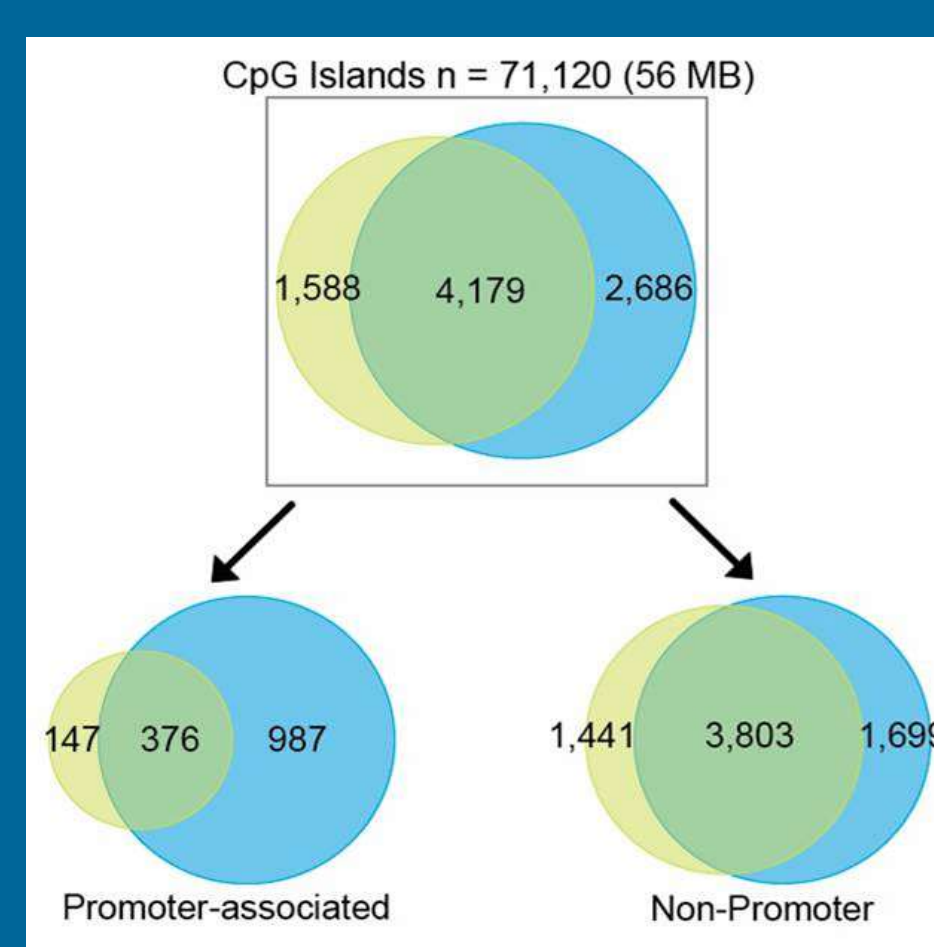
Circos Diagram Summarizing Methylation Detected by MethylPlex-NGS



Validation of differentially methylated regions predicted by MethylPlex-NGS in LNCaP and PrEC cells

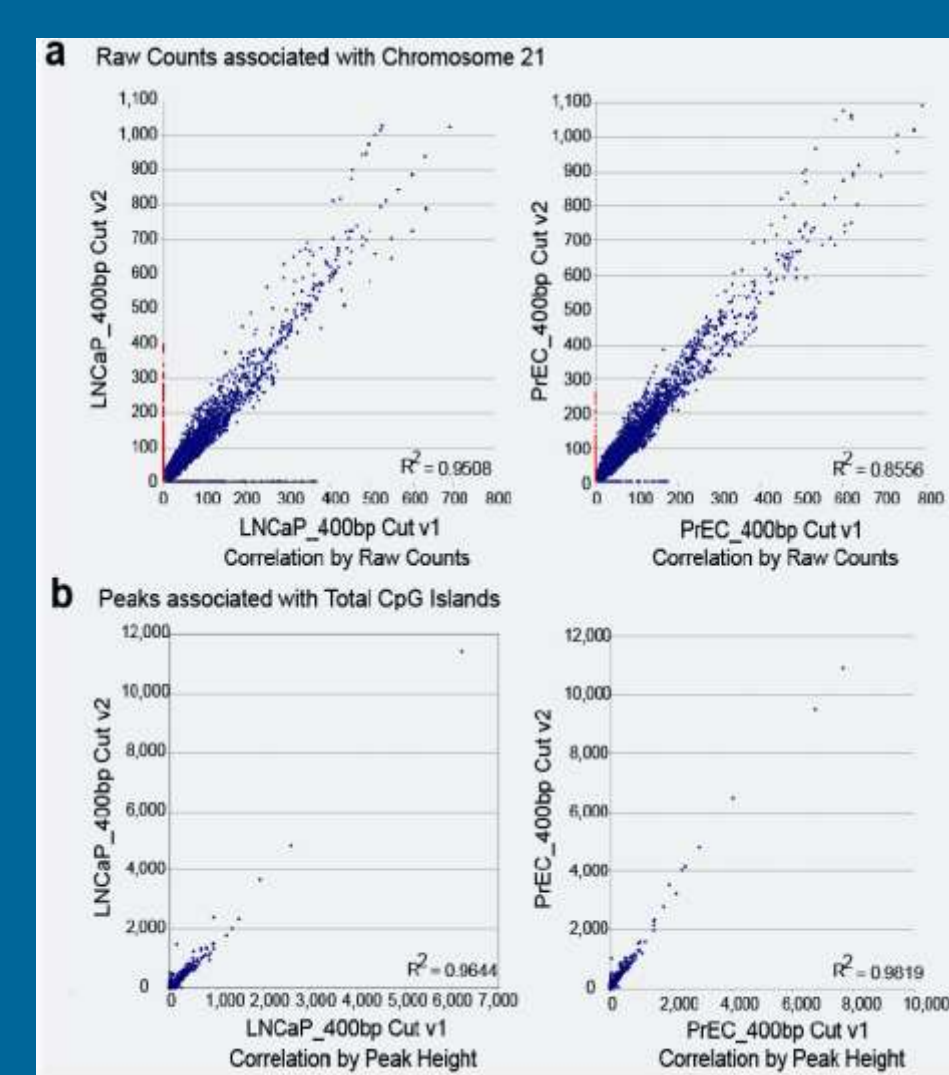


Venn diagram representing overlap between the regions methylated in LNCaP and PrEC cells



Overlap between methylated regions in LNCaP and PrEC cells is ~ 70% (green). While there is a 7 fold increase in the methylation of promoter associated CpG islands in LNCaP (blue) compared to PrEC cells (yellow). This difference is not seen in non-promoter associated CpG islands.

Regression analysis of MethylPlex-NGS mapped reads and HMM output

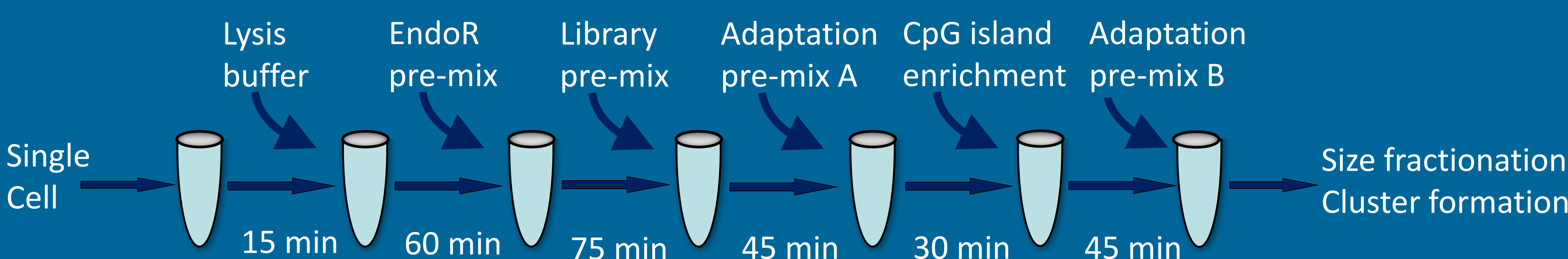


There is high correlation between sequencing runs (a) Reads that mapped to chromosome 21 in LNCaP and PrEC runs are compared using window size of 25bp. In LNCaP samples, a total of 33,627 reads were present at 25 bp windows with R² value of 0.9508, and in PrEC, 37,406 reads with R² value of 0.8556 were observed. (b) Linear regression analysis of all DNA methylation that occurred on CGIs showed high correlation (R² value = 0.9398 and 0.9819, n=5,734 and 4,966, respectively).

Conclusions:

- MethylPlex-NGS can be used for accurate profiling of CpG methylation in as little as 20 - 50 ng DNA.
- MethylPlex-NGS makes Methyl-seq as simple and useful as RNA-seq.
- MethylPlex-NGS will soon be available as beta test kit for profiling methylation in DNA samples.
- Rubicon is developing PicoPlex-NGS WMA for single cell methylation profiling. A beta test kit is scheduled for release in 2011.
- Rubicon is seeking collaborators interested in early access to MethylPlex-NGS and PicoPlex-NGS WMA for profiling methylation in DNA specimens and in single cancer, embryo, and stem cells.

Introducing PicoPlex-NGS WMA for Single Cell Methylation Profiling. Total time : 4.5 hrs



PicoPlex-NGS WMA is under development. Rubicon is seeking collaborators interested in early access to PicoPlex-NGS WMA for profiling methylation in single cancer, embryo, and stem cells.