An Effective Way to Study for Mismatch Repair(MMR) Activity in Bacteria

Parth H. Desai and Eric A. Josephs
The Joint School Of Nanoscience and Nanoengineering (JSNN)
University of North Carolina at Greensboro (UNCG)

Abstract

Mismatch Repair (MMR) is a guardian that ensures the recognition and removal of mis-incorporated nucleotides during replication in a cell. Loss of MMR results in the increased rates of spontaneous mutation in a eukaryotic or bacterial cell. Study of MMR can give insight into the molecular details of diseases like Cancer and antibiotic resistance of bacteria. Earlier studies of *E.coli* MMR system was understood outside of the cell in a predefined system which prevents us to get the direct knowledge of the *in vivo* scenario. There is a need for an efficient way to study MMR system inside the cell. Our focus is to use oligonucleotide recombination, to improve the isolation of genomic DNA containing specific mutations. Here, we have developed a technique to selectively enrich the DNA with specific mutations, which can provide new insight into the MMR as it occurs in living cells.

Introduction

- For my master's thesis, I have been developing new biotechnologies to study how cells avoid genetic mutations during replication. This mutation avoidance is a Mismatch Repair (MMR) process which is a key guardian of genetic information. Proteins associated with MMR recognize mismatched nucleotides on the DNA, then remove and restore the original genetic information before the incorrect nucleotide can be passed on to the next generation.
- This technique makes use of a 'genotypic' screen, so we can isolate the rare mutations from the DNA itself, rather than 'phenotypic' screens that require those mutations to change the phenotype of the organism. In our technique (Genotypic selection method), we continuously degrade unmutated or wild-type DNA using a thermostable restriction enzyme while using a polymerase chain reaction (PCR) to enrich the DNA with the mutation.
- First, we validated this approach to enrich the rare mutations on the plasmid DNA containing mutations of the recognition site of the thermostable restriction enzyme and found it to be highly selective during this assay.

Figure 1. Our approach where we use ApeKI restriction enzyme that will eliminate the unmutated DNA with every round of PCR cycle to enrich the mutated DNA with flanking mutations

- We use oligonucleotide recombination technique to see the compatibility of our method to enrich the rare mutations introduced in the cell.
- Oligonucleotide Recombination is a genome editing technique which uses 50-90nt oligonucleotides that are complementary to a plasmid or genomic DNA and contains mismatched nt within it and when the beta recombinase from the λ phage is also expressed during transfection, oligonucleotide recombination efficiency increases during replication on the lagging strand[1].

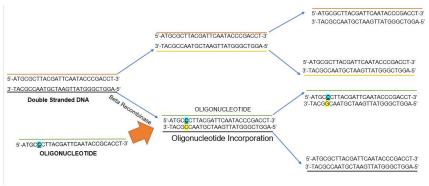

Figure 2. Schematic of lambda beta mediated oligonucleotide recombination and its incorporation during replication in the E.coli genome

- A challenge of using oligonucleotide recombination directly to study MMR is that incorporation rate is low to quantify the MMR activity and depends on the specific oligonucleotide sequence, where it is being integrated, and other factors.
- A SPORE assay uses oligonucleotides containing phosphorothioate bonds as chemical protection. During a SPORE assay[2,3], a synthetic oligonucleotide is designed to contain chemically protected, MMR-inactive 'control' mismatch and an unprotected, MMR-active 'probe' mismatch.
- This allows researchers to normalize 'MMR efficiency' by 'oligonucleotide incorporation efficiency' and provides a quantitative characterization of mismatch repair in a single experiment for different mutational strains and different types of mismatches.

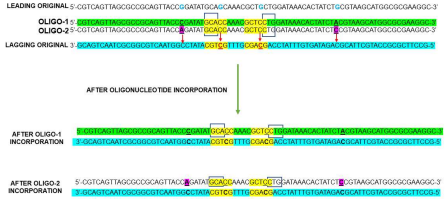

Figure 3. SPORE oligonucleotide recombination and incorporation with induced mismatches (Blue box represents the phosphorothioate bonds, yellow boxes show the ApeKI sites (control mutations) , underline and bold nucleotides are flanking mutations (probe mutations)

- Then, we combined our approach with SPORE assay to study MMR in living *Escherichia coli*, a model organism to study MMR. That technique typically uses a phenotypic screen, but for the first time, we could use genotypic selection.
- The results suggest that this approach could be a powerful new tool to address several questions about how MMR is coordinated in living cells that would otherwise be nearly impossible to study.
- Here, we have developed a molecular technique to isolate rare mutations from a population in a way that allows us to directly quantify MMR activity as it occurs in

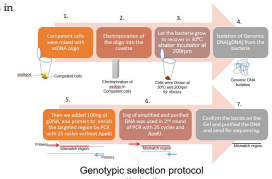

Figure 4. Workflow of "Genotypic selection protocol"

Results and Discussion

1. Testing the efficiency and sensitivity of ApeKI using plasmid DNA.

- We used two plasmid Target and Non-Target and sequence alignment is shown in the Figure 1. We tried to enrich the highlighted Non-Target ApeKI sequence (GGAGC) [in the box and (T)] with our method.
- Results are shown in the Figure 2 (A), (B), (C).
- Here, we can see quantitatively that the Non-Target (NT) sequence is significantly enriched over Target (T) plasmid sequence after PCR with ApeKI in all the ratios. After ApeKI enrichment, Sanger sequencing revealed that the signals initially mixed as 1e2:1 Target: Non-Target plasmids were on average 85.47% Non-Target sequence with a standard deviation of 8.27% across all sites that differ in sequence.
- 1e4:1 dilutions were, after enrichment, 79.32% non-target with a standard deviation 10.29% across all differing sites, and 1e6:1 dilutions were, after enrichment 70.48% Non-Target sequence with a standard deviation of 15.28% across all sites.
- In summary, for 1e2:1, 1e4:1, and 1e6:1-fold dilutions, that is an enrichment of ~85-fold, 7900-fold, and 705000-fold enrichment, relatively.

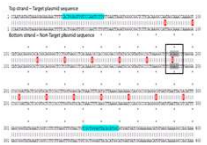

Figure 5. The Compared sequence of Two Distinct Plasmids.

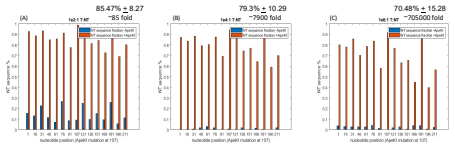

Figure 6. Quantitative Enrichment of Non-Target (NT) Sequence Fraction from the Mixed Sequences

Conclusion : We have confirmed that our approach with ApeKI can enrich the targeted site as well as the flanking nucleotides across the plasmid DNA sequence efficiently.

2. Testing the efficiency of ApeKI to enrich successful Oligonucleotide Recombination mutations in the *E. coli* genome.

- We used oligonucleotide sequence to induce dG to dC single mutation at lagging strand of *galK* gene which will convert GCTGC site to GCTCC. This will create only one ApeKI site mutation.
- We used another gene location on the *E.coli* genome, *araD* gene in which two ApeKI restriction sites are separated by four nucleotides. We converted GCAGC to GCACC and GCTGC to GCTCC by inducing dG to dC mutation.

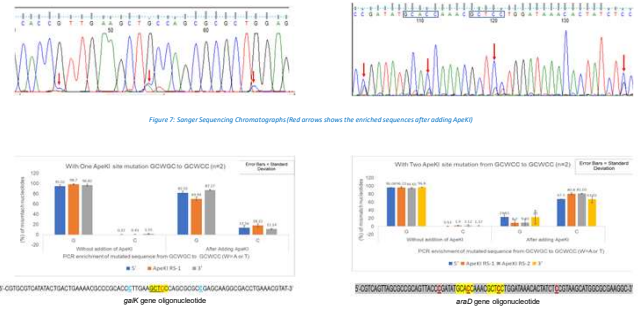

Figure 7: Sanger Sequencing Chromatographs (Red arrows shows the enriched sequences after adding ApeKI)

Figure 8: Quantitative analysis From Chromatograph (Comparison of enriched DNA sequence before and after adding ApeKI)

Without adding ApeKI- The mean (%) of mutated sequence on the galK gene after oligonucleotide recombination			After adding ApeKI- The mean (%) of mutated sequence on galK gene after oligonucleotide recombination	
	G -Wild Type (n=2)	C- Mutated (n=2)	G – Wild Type (n=2)	C- Mutated (n=2)
5'	95.02 ± 2.54	0.37 ± 0.19	82.02 ± 3.10	13.56 ± 4.13
ApeKI RS (Restricton site)-1	98.7 ± 1.14	0.43 ± 0.61	69.94 ± 3.75	18.22 ± 3.18
3'	96.82 ± 1.85	1.55 ± 0.97	87.27 ± 0.96	11.14 ± 1.17

Without adding ApeKI-The mean (%) of mutated sequence on the araD gene after oligonucleotide recombination			After adding ApeKI- The mean (%) of mutated sequence on araD gene after oligonucleotide recombination	
	G -Wild Type (n=2)	C- Mutated (n=2)	G – Wild Type (n=2)	C- Mutated (n=2)
5'	96.08 ± 0.34	0.52 ± 0.73	25.15 ± 9.76	67.5 ± 0.43
ApeKI RS (Restriction site)-1	96.21 ± 1.92	1.9 ± 0.76	8.7 ± 7.6	80.8 ± 2.98
ApeKI RS (Restriction site)-2	94.65 ± 1.56	2.12 ± 2.36	9.33 ± 9.02	81.03 ± 0.71
3'	96.8 ± 0.22	1.17 ± 0.94	23 ± 16.62	67.02 ± 8.31

Figure 9: Tabular representation of Quantitative analysis from Chromatographs (Numerical Values of G and C nucleotides before and after adding ApeKI)

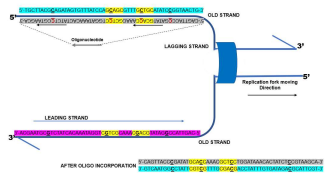

Figure 10: Schematic representation of oligonucleotide incorporation on araD gene lagging strand (Blue), Oligonucleotide (Grey), lagging strand (purple), Two ApeKI sites intend to mutate (yellow), Bold and Underlined nucleotides (Flanking nucleotides)

Conclusion : Above results indicate that the strategy to screen simultaneously for two mutated ApeKI recognition site mutations has worked significantly better than the screen using only one, and we got more efficient signals from the sequencing.

- We can see significantly stronger enrichment of all four mutations (two flanking the ApeKI sites) introduced by this oligonucleotide.
- This suggests that using two ApeKI separate recognition sites for genotypic screening helps to enrich the targeted mutations.

3. SPORE assay using genotypic selection:

- We use two different oligonucleotides (SPORE-oligo 1 & oligo 2) on the araD gene where control mismatches are protected using phosphorothionate bonds and dA-dC as probemismatches with dC-dC silent mismatches (Figure 3).
- We perform the "Genotypic selection protocol (Figure 4)" with the above-mentioned oligonucleotides.

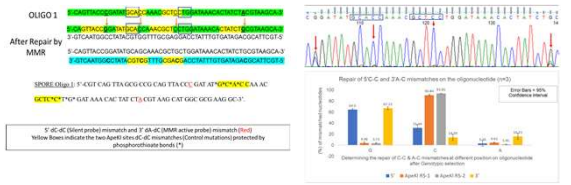

Figure 11. SPORE oligo 1 (Oligonucleotide Sequence, Sanger sequencing Chromatograph, Quantitative graphs)

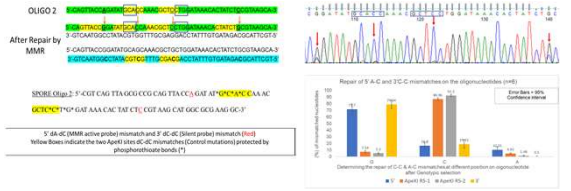

Figure 12: SPORE oligo 2 (Oligonucleotide Sequence, Sanger sequencing Chromatograph, Quantitative analysis)

Summary

- We have developed a new genotypic selection method to screen for the selected mutant DNA.
- We saw 705000-fold enrichment of rare mutations present across the DNA plasmid.
- We saw that this method works best with two ApeKI restriction sites mutations in the genome.
- This method when combined with SPORE assay helped us to study the MMR process on *araD* gene lagging strand.
- It suggests the difference in the repair of dA-dC mismatch on the lagging strand.

Future Directions

- Further optimization and validation is required to estimate the sensitivity of this technique to study MMR-like processes.
- MMR process should be studied with different mutants of MMR proteins in E.coli to understand whether MMR is replication dependent or independent process.
- Time dependent studies of MMR process should be studies as it occurs in the living cells.
- This method should be applied to the organisms where phenotypic selection can not be performed or oligonucleotide recombination efficiency is poor.

References

1. *Costantino and Court, 2003, PNAS, doi: 10.1073/pnas.2434959100 .*
2. *Eric A. Josephs and Piotr E. Marszalek, 2017, Nucleic Acids Research, doi: 10.1093/nar/gkw1339.*
3. *Eric A. Josephs and Piotr E. Marszalek, 2018, DNA Repair, doi: 10.1016/j.dnarep.2018.06.002.*
4. *Gu et al. Genome Biology, 2016, doi: 10.1186/s13059-016-0904-5.*

Acknowledgements

- Dr Eric A. Josephs.
- Josephs lab members and Undergraduate Summer interns.
- The study was supported by,
- UNCG Startup funds and JSNN Startup funds
- NIH grants-1R21AI146876 & 1R35GM133483

Contacts

Parth H. Desai
phdesai@uncg.edu

Eric A. Josephs
eajosephs@uncg.edu