CE Based Sequencing Analysis as A Tool for Investigation of Sterility Positives with Fast Turnaround Time

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INTRODUCTION and OVERVIEW

During the development and manufacturing processes of biologics, it is critical to monitor the sterility of pharmaceutical ingredients, water for pharmaceutical use, the manufacturing environment, intermediates and finished products. In addition, Master Cell Bank, each seed lot and cells used in each production run should be tested for adventitious agents including mycoplasma, bacteria, fungi, viruses, and virus-like particles. When microbial growth is observed, an investigation needs to be conducted and FDA guideline requires sterility test isolates be identified to the species level. It has been shown that DNA sequencing is superior in identifying microbes to the species level, when compared against conventional microbiology staining and culture methods. In this poster, we describe a process for sequencing ribosomal RNA gene of microorganism using the GenomeLab GeXP™ Genetic Analysis System that offers fast turnaround time (8 hrs) and high microbial identification accuracy. Upon isolation of nucleic acid from each microbial sample, the gene target is amplified using a polymerase chain reaction (PCR), followed by sequencing reaction utilizing the Dye Terminator Cycle Sequencing (DTCS) on the GenomeLab GeXP Genetic Analysis System. Results are compared against the NCBI microbial library to identify the contaminants.

METHODS

Genomic DNA Isolation: One loopful of bacteria sample from a single colony was re-suspended in 100 ul of sterile water in a microcentrifuge tube. The tube was placed in a boiling water bath for 10 minutes. After one minute spin at 10,000g, the supernatant was used as genomic DNA for PCR. For yeast sample, Genomic DNA was extracted using Beckman Coulter Agencourt Genfind V2 kit with a lysis buffer that contained lysozyme and Lyticase from Sigma.

Target Gene Amplification: For clostridium and yeast contaminants, each PCR reaction (50 ul total volume) contained 5 ul of sample genomic DNA, 1.5 mM MgCl2, 200 nM dNTPs, 200 nM Primers and 0.04 units of AmpliTaq Gold. Cycling conditions were: 95ºC for 5 min followed by 30 cycles of 95ºC for 30sec, 50ºC for 30 sec and 72ºC for 1 min; 72ºC for 10 min and 4ºC forever. For non-pathogenic bacteria contaminant, Takara RR180A kit was used following manufacturer's instructions. Thermal cycling conditions were: 94ºC, 1 min followed by 30 cycles of 94ºC for 30sec, 55ºC for 30 sec and 72ºC for 1 min; 72ºC for 3 min and 4ºC forever.

Post PCR Clean-up: Three alternative methods were used: Agencourt AMPure XP from Beckman Coulter, QIAquick PCR Purification kit from Qiagen, and NucleoSpin Gel and PCR Clean-up kit from Clontech. The process of Agencourt AMPure XP (Figure1 below) takes less than 15 minutes to complete.

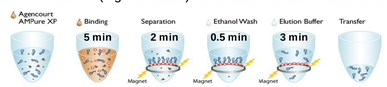


Figure 1. Post PCR clean-up process using Agencourt AMPure XP.

Sequencing and Data Analysis: Sequencing reactions were set up per instructions for the DTCS Quick Start Kit from SCIEX. Sequencing fragments were purified either by ethanol precipitation or using Beckman Coulter Agencourt CleanSeq kit. Separation and data analysis are fully automated on the GenomeLab GeXP Genetic Analysis System. Sequences were submitted to NCBI Database for BLAST search to identify the contaminant.

Figure 2. Basic operator steps for using the GenomeLab GeXP Genetic Analysis System.

Figure 3. Sanger Dye Terminator Cycle Sequencing

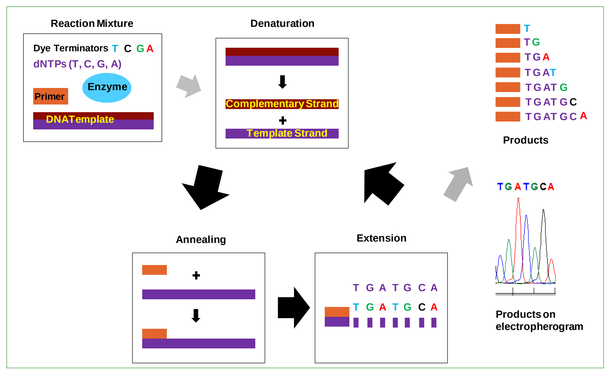
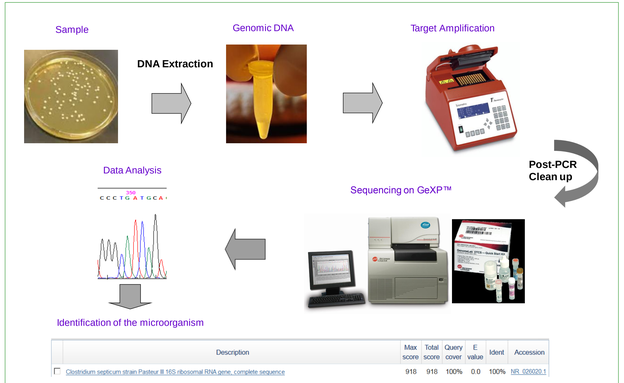


Figure 4. Work Flow for Microbial Identification using GenomeLab GeXP Genetic Analysis System



RESULTS

Identification of *Clostridium septicum* in a sample. The 16s rDNA PCR product was sequenced using DTCS Quick Start Kit. Sequencing fragments were purified by ethanol precipitation and separated on GeXP instrument. Figure 5 shows the sequencing results. After quality based trimming, a 497bp sequence was BLASTed against the 16s rRNA database at NCBI website. Alignment results (Figure 6) indicated that the query sequence was 100% identical to 16s rDNA from *Clostridium septicum.*

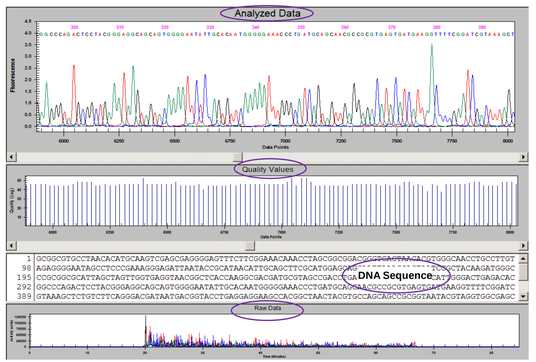


Figure 5. Sequencing results showing raw and analyzed data as well as quality values and DNA sequence. The 16s rDNA PCR product and primer were kindly provided by Dr. Marijke Raymaekers (Virga Jessa Hospital; 3500 Hasselt; Belgium) for a performance test of a new GeXP system in a customer training.

Figure 6. BLAST search results obtained with a 497 bp 16s rDNA sequence in NCBI website.

Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Clostridium septicum strain Pasteur III 16S ribosomal RNA gene, complete sequence	918	918	100%	0.0	100%	NR_026020.1
Clostridium chauvoei strain 2586 16S ribosomal RNA gene, complete sequence	874	874	100%	0.0	98%	NR_026013.1

Identification of *E. coli* in a sample. A non-pathogenic bacteria sample was obtained from Takara, Japan. Genomic DNA was extracted by a quick cell lysis in boiling water bath. Full length 16s rDNA was amplified and sequenced using DTCS Quick Start Kit and GeXP System. Consensus sequence was created in Gene Studio (Figure 7) and submitted to NCBI for BLAST search. Search results (Figure 8) showed query sequence has high homology to pathogenic bacteria and non-pathogenic *E. coli*. Since sample is known to be non-pathogenic, the sample was identified as an *E. coli* species.

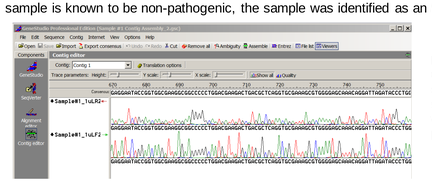


Figure 7. Contig Assembly in Gene Studio to create consensus sequence from sequencing results obtained with different sequencing primers.

Sequences producing significant alignments:

Select All None Selected:0

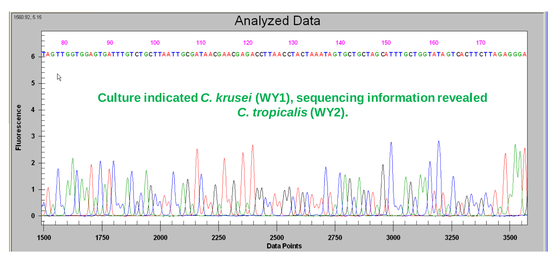
Alignments | Download | GenBank | Graphics | Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Escherichia fergusonii strain ATCC 35469 16S ribosomal RNA gene, complete sequence	2649	2649	100%	0.0	99%	NR_074900.1
Shigella flexneri strain ATCC 29903 16S ribosomal RNA gene, partial sequence	2649	2649	100%	0.0	99%	NR_026331.1
Escherichia coli strain NBRC 102203 16S ribosomal RNA gene, partial sequence	2643	2643	100%	0.0	99%	NR_114042.1
Escherichia fergusonii strain ATCC 35469 16S ribosomal RNA gene, partial sequence	2639	2639	100%	0.0	99%	NR_027549.1
Escherichia fergusonii strain NBRC 102419 16S ribosomal RNA gene, partial sequence	2638	2638	100%	0.0	99%	NR_114079.1
Shigella sonnei strain CECT 4887 16S ribosomal RNA gene, partial sequence	2638	2638	100%	0.0	99%	NR_104026.1
Shigella flexneri 2a str. 301 strain 301 16S ribosomal RNA, complete sequence	2625	2625	100%	0.0	99%	NR_074892.1
Escherichia coli O157:H7 str. Sakai strain Sakai 16S ribosomal RNA, complete sequence	2615	2615	100%	0.0	99%	NR_074891.1
Shigella boydii strain 3594 16S ribosomal RNA gene, partial sequence	2615	2615	100%	0.0	99%	NR_104901.1
Escherichia coli str. K-12 substr. MG1655 strain K-12 16S ribosomal RNA, complete sequence	2615	2615	100%	0.0	99%	NR_102804.1
Shigella boydii SB227 strain SB227 16S ribosomal RNA, complete sequence	2610	2610	100%	0.0	99%	NR_074893.1
Shigella sonnei Ss046 strain Ss046 16S ribosomal RNA, complete sequence	2604	2604	100%	0.0	99%	NR_074894.1
Shigella dysenteriae Sd197 strain Sd197 16S ribosomal RNA, complete sequence	2588	2588	100%	0.0	99%	NR_074895.1
Escherichia albertii strain Albert 19960 16S ribosomal RNA gene, partial sequence	2588	2588	100%	0.0	99%	NR_025569.1
Shigella dysenteriae strain ATCC 13313 16S ribosomal RNA gene, partial sequence	2580	2580	100%	0.0	99%	NR_026332.1
Escherichia coli strain U 5/41 16S ribosomal RNA gene, partial sequence	2567	2567	98%	0.0	99%	NR_024570.1

Figure 8. BLAST search results obtained in NCBI website with a 1443bp 16s rDNA sequence from Sample#1. Sequences produced high homology to query sequence are 16s rDNA from pathogenic bacteria (*Escherichia Fergusonii; Escherichia Albertii; Escherichia coli O157* and *Shigella* species) and non-pathogenic bacteria (*Escherichia coli NBRC 102203, Escherichia coli* strain K12 and *Escherichia coli* Strain U5/41). Since the sample is non-pathogenic, the sample is identified as an *E. coli* species.

Identification of a Wild Yeast 2 contaminant in a beverage sample. The microbial contaminant was initially typed as *Candida krusei (Wild Yeast 1)* by culture method by a commercial vendor. The 18s rDNA was sequenced using DTCS Quick Start Kit and GeXP System. After quality based trimming, the 18s rDNA sequence (Figure 9) was submitted to NCBI for BLAST search. The contaminant was identified as *Candida tropicalis (Wild Yeast 2)*. This identification was further confirmed by experiments using XP-PCR technology on GeXP System.

Figure 9. The 18s rDNA sequence of the beverage contaminant and its alignment results in NCBI.



CONCLUSIONS

- Sanger Sequencing is more specific and reliable than traditional microbial identification methods such as culture typing.
- Capillary Electrophoresis analysis of ribosomal RNA gene sequence on GenomeLab GeXP Genetic Analysis System is simple, fast and accurate.