Application note

Top 3 reasons for automation of this assay
Targeted re-sequencing involves large numbers of PCR products as templates for cycle sequencing reactions. Clean up of sequencing reactions is critical for success of the projects.

Increase throughput.
Reduce sample-to-sample variation.

Introduction

Genome-wide targeted gene re-sequencing is used to elucidate genetic mechanisms of common diseases by identification of nucleotide alterations. It will enable the high-resolution analysis of genetic variation between individuals within populations. Gold standard for validating and completing such sequencing projects is fluorescent dye-terminator (Sanger) cycle sequencing followed by automated capillary electrophoresis. It created increasing demand for fast, robust and automated workflows and high standardization without compromises in data quality which resulted in the widespread adoption of robotic liquid handlers in DNA sequencing laboratories. The process involves linear amplification (cyclecycling) using PCR products as a template, cleanup and re-suspension in a buffer solution for sequencing. One of the major bottlenecks in the sequencing workflow is removal of unincorporated dye terminators and salt ions from the sequencing reactions before loading onto the Genetic Analyzer.

Methods such as ethanol precipitation, sephadex filtration, and other, non-magnetic bead based systems, require manual steps such as centrifugation and/or vortexing and are therefore not well suited for full automation.

In this application note we describe how removal of salts and unincorporated dyes using D-Pure™ DyeTerminator removal kit from NimaGen, www.nimagen.com, in a fully automated procedure with the Microlab® STARlet greatly increases throughput and reproducibility in sequencing sample purification.
Reliability and quality for automated sequencing sample purification

The new D-Pure™ DyeTerminator Removal kit from NimaGen was fully automated on Hamilton’s Microlab® STARlet instrument.

Method description

Magnetic beads of the D-Pure™ DyeTerminator removal kit are transferred into the sequencing samples and ethanol is added to a final concentration of 85%. The samples are transferred to a new 384- (or 96-) well plate and beads are captured on a magnet. Only two wash steps without bead resuspension are required before clean DNA is recovered by adding the elution buffer of the kit. The sequencing samples are transferred into 96-well plates which can be introduced into the Genetic Analyzer. The purified sequencing products are analysed on Applied Biosystems 3730xl Genetic Analyzers, using POP-7™ and 50cm capillary arrays.

System description

The deck is manually loaded with micro-plates, tips and reagents. Up to eight 96-well sample plates can be run at a time and DNA is captured in two 384-well plates. Elution plates are provided in two stacks. Two tip box modules hold 50µl tips for the CO-RE 96 Probe Head which transfers the samples and beads, ethanol and buffer. Plate movements during the process are performed by the CO-RE Gripper.

Application software

The validated method was developed using Microlab® VENUS software. It includes the method itself, definitions for labware and liquids and controls the entire multistep pipetting, incubation, and recovery process.

Kit description

The D-Pure™ DyeTerminator Removal kit consists of magnetic beads for sequencing product capture and elution buffer. Each component has been optimized for removing salts and unincorporated dye terminators from DNA sequencing reaction mixtures.

Evaluation

D-Pure™ DyeTerminator Removal kit from NimaGen was tested at the Genetics Department, Radboud University

Figure 2: NimaGen’s D-Pure™ DyeTerminator kit principle

DNA CAPTURE
1) Introduce Dye Terminator cycle sequencing sample plate.
2) Add D-Pure™ Dye Terminator Removal bead solution and ethanol to DNA and incubate.

PURIFICATION
3) Place plate on the magnet, discard supernatant.
4) Ethanol wash steps.

ELUTION
5) Add Elution buffer.
6) Transfer eluate.
Medical Center in comparison with three other kits from three different vendors. All four kits were magnetic bead-based for DNA capture and elution. BigDye® Terminator Cycle Sequencing samples, ranging from 200bp to 1000bp, were purified on the Microlab® STARlet using the kits and the method described above. Sequencing signal quality, reproducibility, dye-blob removal and signal-to-noise (S/N) were analyzed in the test phase. The kits were ranked according to their performance, their automation potential, and their cost efficiency.

**Technology**

Hamilton’s air displacement pipetting technology provides reliable, consistent walk-away liquid handling automation with only minimal maintenance requirements (Fig. 3). The absence of a system fluid is very advantageous and eliminates extended, time consuming rinsing cycles and risks of leakage.

**Results**

Validation reports for the four different kit brands showed that three of the four kits – including D-Pure™ Dye Terminator Removal kit - had the same performance with mean quality values of 42 (Fig. 4) and very good signal homogeneity and dye-blob removal (Fig. 6). Signal-to-noise (S/N) ratios were in the same range also. However, the samples purified using the D-Pure™ Dye Terminator Removal kit had an overall better S/N value (±300). All four tested kits were suited for an automated workflow because they were all using magnetic beads for DNA purification. No manual step was involved and they all could be run on the Microlab® STARlet using essentially the same protocol with the same ease of use.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Mean quality value</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NimaGen’s D-Pure™ Dye Terminator kit</td>
<td>41</td>
<td>± 300</td>
</tr>
<tr>
<td>DNA purification kit from vendor 2</td>
<td>42</td>
<td>± 250</td>
</tr>
<tr>
<td>DNA purification kit from vendor 3</td>
<td>41</td>
<td>± 100</td>
</tr>
<tr>
<td>DNA purification kit from vendor 4</td>
<td>42</td>
<td>± 20</td>
</tr>
</tbody>
</table>

The cost per purification reaction is very different for the four kits. Therefore, the Genetics Department decided to use NimaGen’s D-Pure™ Dye Terminator kit on the Microlab® STARlet in the future for the sequencing projects since its cost-performance relation is significantly better than that of the other vendors, with no compromise in data quality and recovery.

Purification of 4 x 96 sequencing samples using the D-Pure™ kit is completed in 50 minutes. Up to eight 96-well plates can be processed in 1 hour and 40 minutes without user intervention. Eight 96-well plates are processed per run and 4 runs per day are performed. Deck capacity - and therefore walk-away time - may be increased by integrating additional plate stackers.
Discussion

Hamilton, Radboud University Medical Center and NimaGen have developed a method for fully automated sequence purification with maximum throughput, quality and reliability. There is no need for manual intervention such as centrifugation or vortexing. Samples processed with the Microlab® STARlet are clean and ready for analysis on an automated sequencer. The purified products showed high stability and low peak degradation with very low variation in sample-to-sample signal strength resulting in reduction of overloaded samples, less need for re-injection and less effort for signal normalization, compared to manual purification.

### System requirements

| Part number | Microlab® STARlet, CO-RE 96 Probe Head 1000µl, 2 x 1000µl pipetting channels, CO-RE Gripper, 1x NTR tip carrier, 3x plate carriers | 173000-804 / HAMILTON |
| 96 Wash Station Dual (Wash station for 96 disposable tips, two wash chambers) | 190247 / HAMILTON |
| 4x Multiflex carrier base | 188039 / HAMILTON |
| Multiflex liquid dispenser trough 96 | 188115APE / HAMILTON |
| 4x Multiflex NTR1 module | 191420 / HAMILTON |
| 8x Multiflex PCR plate module 96 | 188049 / HAMILTON |
| 2x MultiflexMTPFixationFrameAgentcourt | 188295APE / HAMILTON |
| 3x Multiflex DWP/ 384 tip box module | 188042 / HAMILTON |
| 2x Multiflex plate stack module (landscape) | 188044 / HAMILTON |
| System dimensions: width: 1124mm, height: 903mm, depth: 795mm |
| Microlab® STAR 50µl Vol. CO-RE Tips, without filter | 235947 / HAMILTON |
| 3x Seahorse Bioscience Reservoirs | 201244-100 |
| FrameStar 384, blue frame, 50 plates | 4ti-0384/B / Bioke |
| Superplate PCR Detection plate | BC-2100 / Thermo Scientific |
| Reagents |
| D-Pure™ Dye Terminator Removal kit including: bead solution and elution buffer | DP500 / NimaGen, NL-Nijmegen |
| BigDye® Terminator v1.1 Cycle Sequencing Kit | 4337452 (5000 rxn) / Applied Biosystems |
| Ethanol |

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