DyeTerminator Removal Kit on the Microlab® STARlet: enabling automation technologies for Sanger (Cycle) sequencing

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.

Eliminate manual procedures for removal of DyeTterminator from cycle sequencing reactions.
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.

Figure 1:
Microlab® STARlet: Automated and robust removal of salts and unincorporated dyes from DyeTerminator sequencing reactions, with Nimagen's magnetic bead technology.

NIMAGEN

HAMILTON

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 transfered 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.

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.

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.

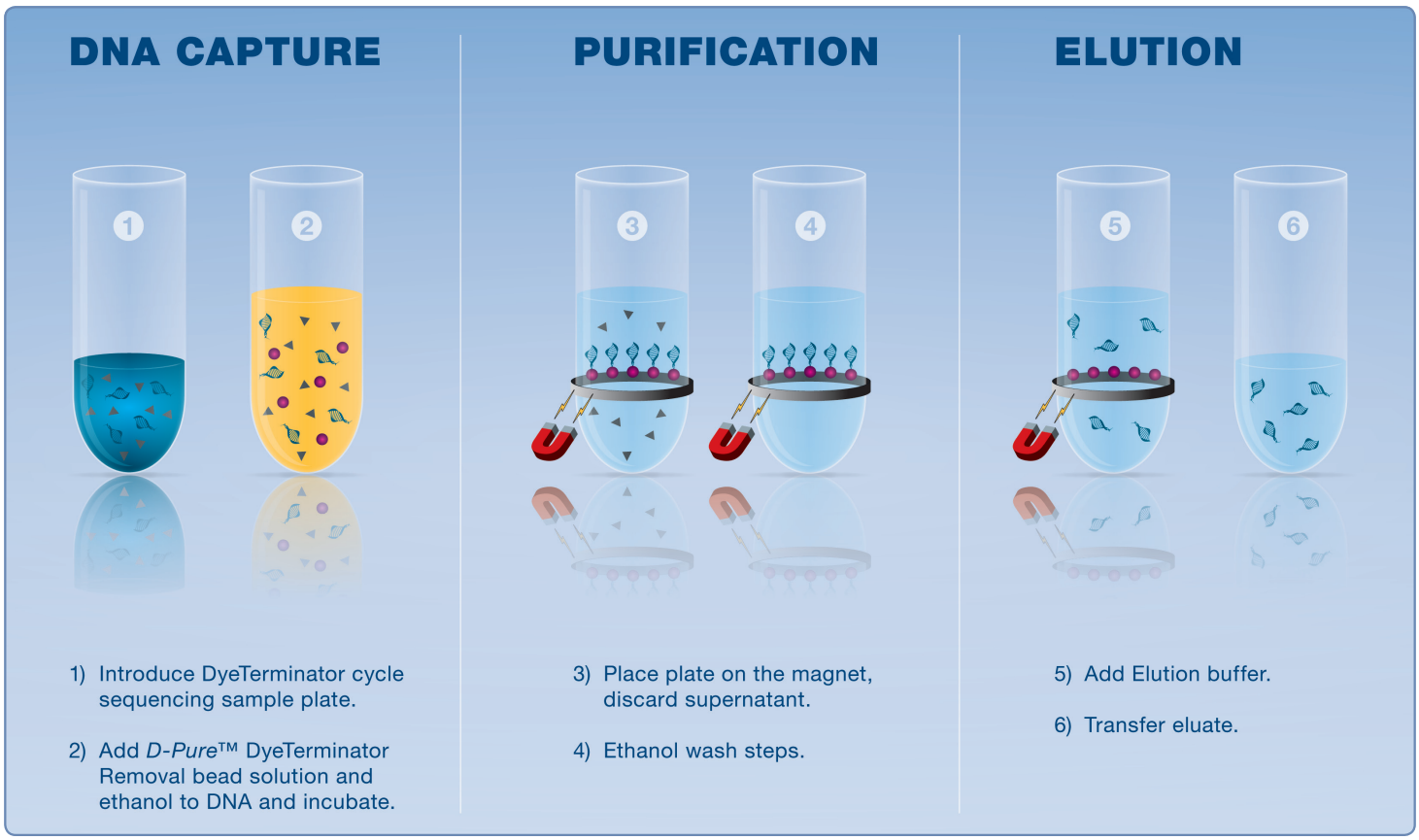
Application software

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.

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



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 CycleSequencing 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.

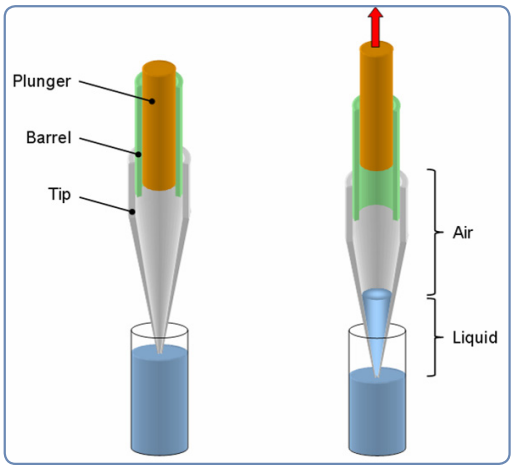


Figure 3: Air displacement pipetting principle: The liquid is aspirated into and dispemsed from the disposable tip by the movement of a plunger. No system liquid is involved.

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*™ DyeTerminator 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*™ DyeTerminator 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.

Kit	Mean quality value	S/N
NimaGen´s D-Pure™ DyeTerminator kit	41	± 300
DNA purification kit from vendor 2	42	± 250
DNA purification kit from vendor 3	41	± 100
DNA purification kit from vendor 4	42	± 20

Figure 4: Validation results for the four sequencing sample purification kits. The table shows mean values from the average basecall quality and the signal noise ratio of the sequencing reactions.

The cost per purification reaction is very different for the four kits. Therefore, the Genetics Department decided to use NimaGen´s *D-Pure*™ DyeTerminator kit on the Microlab® STARlet in the future for the sequencing projects since its cost-perfomance 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.

DNA capture

- Place 384-well plate on magnet position
- Read barcode of all sample plates
- Resuspend *D-Pure*™ Cleaning Beads by aspiration/dispensing and add 5µl into each sample in the 96-well sample plates
- Add ethanol into the wells of the sample plate to achieve a concentration of 85%
- Aspirate entire samples from sample plate, dispense into 384-well plates on the magnet and wait 30sec. for the beads to settle
- Combine samples from four 96-well plates in one 384-well plate
- Incubate 180sec. per plate

Purification

- Discard supernatant
- Wash 2 times with 30µl 85% ethanol, incubate beads in ethanol for 30sec.
- Remove supernatant
- Air dry for 450sec.

Elution

- Add 30µl of *D-Pure*™ elution buffer to each well
- Mix while on the magnet and incubate for 600sec.
- Discard empty sample plates and load elution plates
- Transfer 25µl of eluate into elution plate

Figure 5: Visual workflow: Sequence purification in 384-well plates

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.

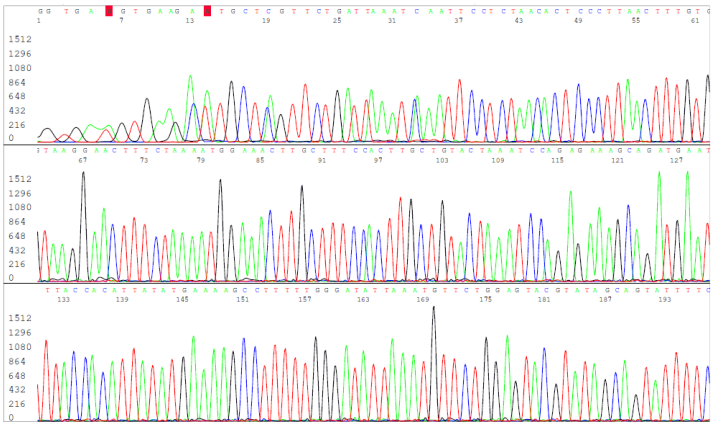


Figure 6: Chromatogram of a sequencing reaction purified with D-Pure™ DyeTerminator Removal kit. Signals are homogeneous

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
Labware requirements	**Part number**
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™ DyeTerminator 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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