Protein-binding matrices for phenol-free extraction of DNA and RNA modifying enzymes: applications for genetic engineering and high throughput

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Even though biotechnology methods have advanced, some purification techniques remain the same as they were decades ago, making it difficult to develop efficient strategies. Removal of unwanted enzymes from post-reaction mixture to purify modified nucleic acids suitable for downstream applications is an essential step in many genetic engineering protocols which until now relies mainly upon liquid organic extraction. Organic extraction is associated with several intrinsic drawbacks: toxicity of both phenol and chloroform, inhibition of subsequent enzyme activity with remaining traces of phenol, loss of purified nucleic acids in the organic phase, time- and labour-consuming procedures and waste disposal care. Therefore, it is of high interest for researchers to be able to modify DNA and RNA in multi-step protocols without interrupting the procedure for phenol-chloroform extractions and subsequent alcohol precipitation.

In a course of phenol-free genetic engineering protocols development, Clonogene has formulated and comprehensively tested four protein-binding matrices (PBM) as an efficient alternative to conventional liquid organic extraction (Fig. 1). PBM selectively binds proteins from aqueous mixture with nucleic acids and therefore deproteinate DNA and RNA without sample dilution îâ reaction buffer change. Purified nucleic acids are immediately ready for downstream applications.

MATRIX	FEATURES
BlueSorb™	Removes the bulk of enzymes and proteins from nucleic acid and oligo samples before electrophoresis or chromatography
QuickClean™	Quantitative, removes enzymes from samples with high protein load; applicable for most genetic engineerin protocols; cleans-up radio- and fluorescein-labelled DNA. cDNA. and aRNA probes
deENZYME™	advanced for-all-purposes matrix with highest protein binding capacity; efficiently removes aggressive enzymes; suited to 96-well plates in à slurry format
EnzyLock™	resistant to drying-rehydration; intended for pre-loading of disposable devices and 96-well filtered plates

Figure 1. Protein-Binding matrixes chart

Protocol

The following is the standard batch extraction procedure: for a given volume of reaction mixture to be deproteinated, one-tenth volume of thoroughly mixing PBM suspension was added. The tube was vortex or pipette mixed for 10-20 seconds and centrifuged at 14 000 rpm for 1 minute to pellet a PBM-enzyme complex.In all employed assay procedures, enzyme removal was defined as the complete absence of its detectable activity in PBM-extracted supernatant îâ iiltrate.

PBM features

PBM has successfully extracted all tested enzymes and proteins, over 100 species in total. The matrices allow the cleanup of virtually any type of nucleic: single- îâ double-stranded DNA and RNA, RNA-DNA heteroduplexes, covalently closed or linear plasmid DNA, and oligonucleotides. PBM work well in all common reaction buffers in the presence of proteinaceous enzyme activity enhancers and other additives. The matrices generate nucleic acids which are ready for immediate use and do not interfere with downstream enzyme activity. Since PBM is non-toxic, it does not required special precautions and waste disposal care.

In pilot experiments, it has been demonstrated that PBM quantitatively removes a variety of DNA and RNA modifying enzymes (Fig. 2). Later, the matrices were successfully applied for key genetic engineering techniques. Among these are manipulation with genomic and plasmid DNA

DNA Modifying	Enzymes
Polymeras & end Polishing	DNA Polymerase I, Klenow, T4 DNA polymerase, Pfu
Thermostable polymerases	over 15 enzymes from top Vendors
Restriction Endonucleases	over 60 enzymes
Nucleases	DNase I, Exonuciease III, Mung Bean Nuclease, S1-Nuclease, Uracil-DNA Glycosylase
Ligases	T4 and E.coli DNA ligase

RNA Modifying	Enzymes
Polymerases	M-MuLV and AMV reverse transscriptases, SP6/T3/T7 RNA polymerases
Ribo nucleases	RNase A, RNase H
Other	Polynucleotide kinase, alkaline phosphatase

Figure 2. List of extracted enzymes

[1], cDNA synthesis and cloning [2], PCR clean-up, nested inserts generation [3], and in vitro transcription [4]. Many other advanced protocols are now under development and optimisation. Figure 3 illustrates efficacy of ten thermostable DNA polymerases removal with single deENZYME extraction.

High-throughput purification

As biotechnology has turned to high-throughput techniques and automation, further employing of liquid organic extraction of nucleic acids modifying enzymes from post reaction mixture makes it difficult to employ robotics multi-step genetic engineering protocols. Using multi-well filtered plates, deENZYMF was first successfully employed for high throughput applications in à sluny format: assay mixtures were arrayed in à conventional 96-well plate, followed by the addition of the matrix slurry. The mixtures were then transferred to a 96-well filtered plate, followed by filtration or centrifugation. Purified filtrates, containing deproteinated DNA or RNA were simultaneously collected in a fresh plate, followed by subsequent analysis or further modification.

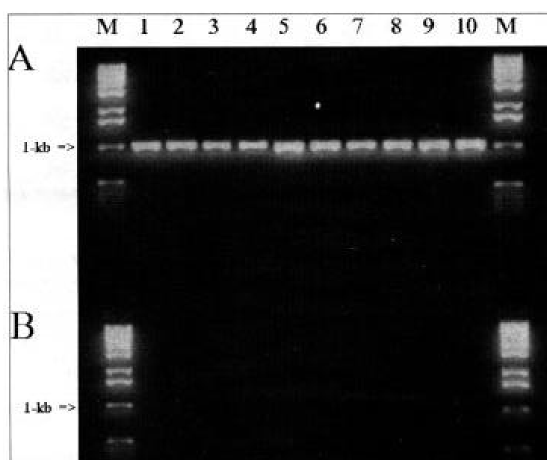

Figure 3. Reaction mixtures without enzymes (A) were extracted with deENZYME, followed by addition of thermopolymerases and PCR cycling. Complete reaction mixtures containing thermopolymerases (A) were one time extracted with deENZYME, followed by PCR cycling. Ì : 1-Kb DNA Ladder. Lines: 1 - AmpliTaq (PE Applied Biosystems), 2 - Stoffel Fragment (PE), 3 - Taq (Boehringer), 4 - Expand High Fidelity (Boehringer) 5 - Taq2000 (Stratagene), 6 - TaqPlus (Stratagene), 7- Pfu (Stratagene), 8 - PLATINUM Taq (Life Technologies), 9 - Taq (Promega), 10 - Tet-Z (Dialat). Reaction buffers according to the suppliers.

Òî meet the requirements of high throughput single-step enzyme removal, Clonogene has developed EnzyLock that resists drying-rehydration cycling without decreasing its protein binding capacity. Disposable devices, pre-loaded with dried EnzyLock, can be stored at room temperature for at least one year, being readily available for sample loading and processing. The matrix has been successfully employed in conjunction with vacuum-driven 96-well filtered plates for simultaneous removal of restriction endonucleases (Fig. 4) as well as many other enzymes and proteins. The advantage of EnzyLock-based deproteination is the single-pass format, without the need of matrix ñîïditioning îä performing of nucleic acids binding, washing and elution steps.

Oncoming applications

Preliminary data demonstrates that absorbed enzymes and proteins can be subsequently eluted from the matrices with high concentration of urea. Therefore, PBM might be further employed to concentrate proteinaceous compounds from diluted aqueous solutions.

Conclusions

Protein Binding Matrices are non-toxic environmental friendly adsorbents that remove proteins from aqueous mixture with nucleic acids high efficiency. The matrices are universal, e.g. they bind all tested types of enzymes

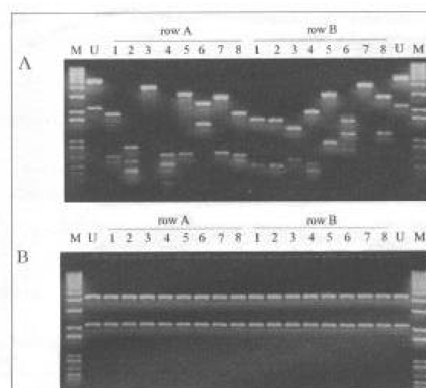

Figure 4. Reaction mixtures without enzymes (A) were filtered through 96-well plates preloaded with dried EnzyLock, followed by template plasmid DNA and restriction endonucleases addition and incubation at +37°C Complete reaction mixtures containing enzymes (B) were filtered through 96-well plates preloaded with dried EnzyLock, followed by template plasmid DNA addition and incubation at +37°C for 18 hrs. M: 1-Kb DNA Ladder. U: untreated plasmid DNA. Enzymes: Rsa I, Pal I, Sca I, Alu I, Dra I, Pvu II, Hae III, Ssp I, Msp I, Sau 3AI, Hpa II, Hinf I, Pvu I, Xho II, Nhe I, Bgl I. Reaction buffers from suppliers.

and proteins, both of natural and recombinant origin, and deproteinate all types of nucleic acids. Since PBM offers àvery flexible and convenient way for faster and reliable enzyme extraction, it has been successfully applied to àvariety genetic engineering protocols. Òï addition, EnzyLock and deENZYMF allow to perform high-throughput sample processing in convenient 96-well plate format and thus might be suitable for robotic workstations. Hence, employing PBM to deproteinate modified DNA and RNA that are immediately suited for downstream applications is a highly developed alternative to traditional liquid organic extraction and to the use of DNA isolation kits.

References:

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