TechNote 302

Molecular Biology

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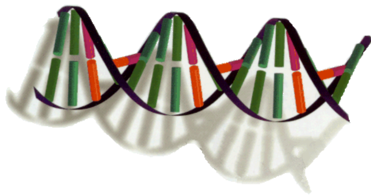

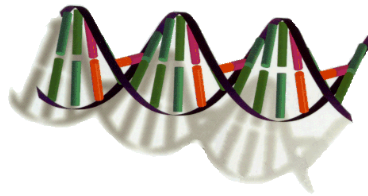

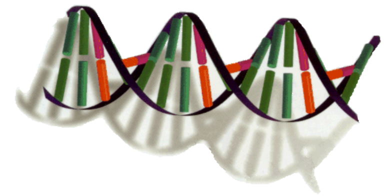

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I. INTRODUCTION

Several types of microspheres are utilized to isolate and purify nucleic acids. Both carboxylate-modified and streptavidin-coated microspheres, together with conjugated probes, can capture target sequences. Examples of this are oligo(dT)-modified microspheres capturing mRNA via their poly A tail and specific oligonucleotide-modified microspheres used in the triple-helical capture of dsDNA. Alternately, carboxylate-modified and silica microspheres have the capacity to nonspecifically bind nucleic acids.[1, 2, 3]

Sections II-A and II-B provide similar protocols using carboxylate-modified superparamagnetic microspheres to facilitate the purification of DNA products without the use of streptavidin-biotin or sequence-specific interactions. Several investigators are making use of this approach in various applications related to the Human Genome Project.

II. DNA PURIFICATION USING SPRI METHODS[14, 15, 16]

The DNA purification method known as Solid-Phase Reversible Immobilization (SPRI) was developed using our carboxylate-modified encapsulated magnetic microspheres. Under conditions of high polyethylene glycol and salt concentration, our microspheres bind both single and double-stranded DNA, including sequencing reaction products, PCR products, M13 phage, lambda phage, plasmids, cosmids, and bacterial artificial chromosomes (BACs). The captured products are then washed with ethanol and eluted in a low ionic strength buffer. This relatively inexpensive method of DNA capture delivers high-quality DNA for sequencing, library construction, and restriction digestion. Proven advantages of this method are low cost, low background, and suitability for automation.

A. Purifying PCR Products

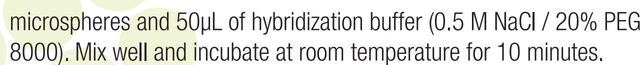
Note: The SPRI PCR method binds DNA based on size, from 200bp to 200 Kbp (BAC DNA isolation). Yields in excess of 80% have been reported.

Table 1: PCR Conditions and Reagents

PCR Primers	
Forward primers are tailed with -21M13 sequences TGTAAAACGACGGCCAGT (18 nt)	
<u>PCR Reagents</u>	
1. 10x PCR Buffer:	100 mM Tris/HCl (pH 9.3) 500 mM KCl 15 mM $MgCl_2$ 0.01% Gelatin
2. 10 mM dNTPs	
3. 10 µM Forward and Reverse Primers	
4. 20 ng/µL Genomic DNA	
<u>Standard PCR Reaction (50µL)</u>	
1. 6.5µL PCR Mix:	10x PCR Buffer (5µL) 10 mM dNTP (0.5µL) Taq Polymerase 1 unit deionized H_2O (Add to make 6.5µL)
2. 41µL Primer Dilution:	10µM F & R Primers (0.5µL) dH_2O (40.5µL)
3. 2.5µL Genomic DNA (50ng)	
<u>PCR Conditions (35 Cycles)</u>	
Initial Predenaturation:	96˚C, 5 minutes
Denaturation:	96˚C, 30 seconds
Annealing:	55-57˚C, 2 minutes
Extension:	72˚C, 5 minutes
Soak:	4˚C, indefinitely

Binding

1. Wash carboxylate-modified encapsulated superparamagnetic microspheres (at 1% solids = 10 mg/mL) 3 times with wash buffer (0.5 M EDTA, pH 8.0). Apply a magnet to the side of the tube for 3 minutes, pulling the microspheres to the side. Aspirate the clear supernatant, and fully resuspend in fresh wash buffer.
2. For each PCR reaction product (50µL), add 10µL of washed



microspheres and 50µL of hybridization buffer (0.5 M NaCl / 20% PEG 8000). Mix well and incubate at room temperature for 10 minutes.

Washing

1. Magnetically separate the microspheres from solution, aspirate the supernatant, and resuspend in 150µL 70% ethanol.
2. Repeat the wash.
3. Magnetically separate the microspheres for 2 minutes, then aspirate the supernatant.

Elution

1. Air dry for 2 minutes. Resuspend the microspheres in 20µL of elution buffer (10 mM Tris-Acetate, pH 7.8) and incubate at room temperature for 5 minutes.
2. Magnetically separate the microspheres and remove the DNA-containing supernatant for testing and sequencing.

B. Purifying ssDNA from M13 Bacteriophage

Grow single-stranded M13 bacteriophage in a deep-well plate, centrifuge to pellet the host cells or filter through carboxyl-derivatised 0.45µm filter plates. Transfer the supernatant, or filtrate, into a new microtiter plate, containing the SDS to lyse the phage. Add the magnetic microspheres to the binding buffer (PEG/salt) and incubate at room temperature for 10 minutes. The microsphere/DNA complex is washed and the DNA eluted into the appropriate volume for DNA sequencing. This procedure is similar to that used for isolation of PCR products.

Binding

1. Wash carboxylate-modified encapsulated superparamagnetic microspheres (10 mg/mL) 3 times with wash buffer (0.5 M EDTA, pH 8.0).
2. For each M13 phage supernatant (100µL), add 10µL of washed microspheres and 120µL of hybridization buffer (0.20 M $MgCl_2$ / 26% PEG 8000). Mix well and incubate at room temperature for 10 minutes.

Washing

1. Place the microtiter plate on a magnet for 2 minutes.
2. Wash the microspheres 2 times with 150µL 70% ethanol. *Note*: High ionic strength wash solutions retain *slightly* more DNA, but they also introduce residual salts that may interfere with downstream sequencing.

Elution

1. Air dry for 2 minutes. Resuspend the microspheres in 50µL of elution buffer (10 mM Tris-Acetate, pH 7.8) and incubate at room temperature for 3 minutes. *Note*: Other low ionic strength solutions will also elute the DNA from the microspheres, including sucrose, formamide, etc.
2. Magnetically separate the microspheres and remove the supernatant for testing and sequencing.

III. NUCLEIC ACID ADSORPTION TO SILICA MICROSPHERES[4,5,6]

DNA can be isolated by using a standard alkaline lysis preparation to release DNA from bacterial cells, together with the nucleic acid binding properties of silica microspheres. The precipitated lysate is cleared of cellular debris and precipitated proteins by filtration through a coarse prefilter. This filtered DNA lysate is then adsorbed to silica microspheres in the presence of a binding solution. While the DNA is bound, impurities and salts are removed by washing, and the DNA is subsequently released by elution in a low salt buffer.

DNA Purification

1. Centrifuge bacterial cells (1.5mL in 2.0mL 96-well plates) for 10 minutes at 1300 G.
2. Decant and resuspend in 50µL GTE solution (33 mM glucose, 17 mM Tris-HCl, 7 mM EDTA•Na_2, 100µg RNase A/mL, 2mg lysozyme/mL) by vortexing.
3. Lyse cells by adding 50µL freshly prepared NaOH-SDS solution (266 mM NaOH, 1.3% w/v SDS), mix gently, and incubate at room temperature for 5 minutes.
4. Neutralize lysed cell solution by adding 50µL of potassium acetate solution (3 M KoAc, 5 M HOAc).
5. Mix cells gently by rocking, then incubate on ice for 10 minutes.
6. Transfer lysates to a coarse prefilter (NucleicA from Millipore), filter using a vacuum manifold (Millipore MAVM 096 01), and collect into 0.65µm Durapore filter plate (Millipore MADV N65) containing 2mg of washed silica beads/well.

Particle Washing

1. Combine 2mL of 0.9µm (10% solids) silica microspheres with 10mL 0.5 M EDTA•Na2 in a 50mL conical centrifuge tube.
2. Mix and spin at 2000 rpm in a tabletop centrifuge for 5 minutes.
3. Discard supernatant, and resuspend silica microspheres in 10mL of 0.5 EDTA•Na_2 by vortexing.
4. Repeat this process 3 times.
5. Resuspend microspheres in 10mL binding buffer (2.5 M NaCl, 20% PEG 8000) by vortexing.
6. Add 100µL of the bead / binding buffer solution to each well of the filter plate, and store at 4˚C until ready for use.

DNA Binding

1. Filter DNA lysate into the Durapore filter plate containing the bead / binding buffer solution. Cover and vortex for 10 seconds.
2. Incubate at room temperature for 10 minutes.
3. Remove liquid from the plate by vacuum filtration, leaving the DNA / silica particle complex behind.
4. Wash the solid phase to remove salt and impurities by adding 200µL 70% EtOH to the filter plate, with subsequent filtering until the excess ethanol is removed.

DNA Elution

1. Add 20-60µL of either 10 mM Tris (pH 7.5), H2O, or TE (pH 7.5) (10 mM Tris base, 1 mM EDTA•Na_2) to each well of the filter plate containing the silica particle-DNA complex.
2. Incubate the filter plate at ambient temperature for 5 minutes, to release DNA from the particles.
3. Pull the sequence ready eluate through the filter and collect in a 96-well tray.

TIPS

- If nucleic acids are being purified for PCR purposes, GuSCN-containing buffers may be purified from contaminating nucleic acids by filtration over glass columns.
- All glassware should be autoclaved.
- During washing, ensure that silica particles are completely resuspended (no clumps). If necessary (during the pre-binding wash step), tough clumps may be broken using bath or probe sonication, without damage to the particles.

IV. PREPARATION OF MAGNETIC OLIGO(DT) AND OTHER OLIGONUCLEOTIDE MICROSPHERES[6]

In-house attachment of oligonucleotides to magnetic beads can be an economical alternative to buying pre-made reagents from commercial sources. The following is an efficient procedure for covalent coupling of oligo(dT) or other oligonucleotides in a one-step reaction procedure. This procedure may be used to modify 80mg of beads with 1.6mg 5'-amino-modified oligonucleotides in 50mL of coupling buffer in a 50mL tube. This article specifies magnetic microspheres, however, carboxylate-modified non-magnetic microspheres could be used as well. If that is the case, simply use centrifugation rather than magnetic separation.

Required Materials

1. 5'-amino-modified oligo$(dT)_{30}$ or 5'-amino-modified oligonucleotide
2. 1-methylimidazole
3. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)
4. Carboxylate-modified magnetic microspheres
5. Imidazole Buffer, pH 7.0
6. SSC Buffer: (20x Recipe) 175.3g (3 M) NaCl, 88.2g (0.3 M) Na_3Citrate• 2 H_2O. Fill to 800mL with H_2O, adjust to pH 7.0 with 1 M HCl, and fill to 1L with H_2O.
7. PBS Buffer: (10x Recipe) 80g NaCl, 2g KCl, 14.4g Na_2HPO_4•7H_2O, 2.4g KH_2PO_4. Fill to 800mL with H_2O, adjust to pH 7.4 with 1 M HCl, and fill to 1L with H_2O.

Washing

1. Wash microspheres 2 times in 100 mM imidazole buffer, pH 7.0.
2. Separate the beads each time by pulling them to the side of the tube using a strong magnet.
3. Aspirate the supernatant and resuspend in fresh 100 mM imidazole buffer, pH 7.0.

Binding

1. Mix 1mg of microspheres with 20µg of 5'-amino-modified oligonucleotide in 0.6mL freshly prepared coupling buffer (100 mM EDC in 100 mM imidazole buffer, pH 7.0). *Note*: Store EDC desiccated at 4˚C, and use only freshly prepared buffer.
2. Incubate for 3 hours at 50˚C. During the procedure, the beads should be rotated or inverted continuously, so that they do not settle out of solution.

Second Washing

1. After incubation, collect the microspheres with a magnet and aspirate the supernatant.
2. Wash the microspheres 3 times with 2X SSC buffer containing 0.5% SDS, at room temperature.
3. Wash 2 times with RNase-free water at 65˚C.
4. Resuspend the microspheres in PBS containing 0.2% NaN_3, and store in aliquots at a concentration of 5 mg/mL at 4˚C.

The binding capacity of the modified microspheres may be determined by extracting target sequences [mRNA if using oligo(dT)] with decreasing amounts of beads in parallel tubes, and measuring the amount of eluted RNA by wavelength scanning from 220-300nm or by blotting (Southern or Northern).

V. COVALENT ATTACHMENT OF DNA TO MICROSPHERES

There are several methods for the covalent attachment of nucleic acids to microspheres. Most of the methods involve placing a functional group at the 5' end of the DNA strand and employing a suitable reagent to link the tagged DNA to the surface of the microsphere. For example, to attach an amine-tagged DNA fragment to carboxyl-modified microspheres, carbodiimide is used as the coupling reagent. If the DNA fragment is labeled with a thiol group and the bead's surface is amine-modified, a heterobifunctional coupling reagent may be employed. Also, if the fragment has an amine group and the beads are surface-functionalized with either amine groups or hydroxyl groups, cyanuric chloride can be employed.[11]

Methods and papers using silica beads and functionalized paramagnetic beads, such as epoxy-modified, hydrazide-modified, and others have been published and are available upon request.12 Our TechNote 205 provides protocols for covalently attaching ligands using a variety of standard chemistries. This may be downloaded directly from our website, **www.bangslabs.com**.

VI. TRIPLE-HELIX-MEDIATED AFFINITY CAPTURE (TAC)[7,8,9]

Triple-helix formation has proven to be a powerful and fairly general approach to DNA targeting. It is based upon the specific binding of pyrimidine oligonucleotides to a polypurine strand in duplex DNA, forming a local triple-helical structure. Studies have demonstrated the potential of local triple-helix-mediated capture for the enrichment and screening of recombinant DNA libraries, as well as for the purification of polymerase chain reaction (PCR) products. This application makes use of streptavidin-coated microspheres and biotinylated oligonucleotides.

Buffers

1. GTE Solution: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0
2. Binding Buffer: cold 3 M KOAc, pH 5.0
3. Elution Buffer: 50mM Tris-HCl, pH 9.0
4. Washing Buffer: 10mM NaOAc, pH 5.8, 100 mM $MgCl_2$

Making Triplex Microspheres

1. Wash 1mL streptavidin-coated microspheres (at 1% solids) 2 times with PBS (10 mM Na_2HPO_4, pH 7.5, 0.15 M NaCl). Resuspend in 1mL PBS.
2. Add 200 pmol biotinylated capture oligonucleotide. Incubate at room temperature for 30 minutes.
3. Wash the beads 2 times with PBS, 2 times with elution buffer, and 2 times with binding buffer. Store in PBS at 4˚C until used.

Capture

1. Spin 1.5mL of overnight culture in a microcentrifuge (at approximately 12,000 G) for 20 seconds to form a pellet. Discard supernatant.
2. Resuspend cell pellet in 100µL of GTE solution. Vortex to dissolve completely.
3. Add 200µL 0.2 M NaOH / 1% SDS solution. Invert to mix. Incubate on ice for 5 minutes.
4. Add 150µL binding buffer solution. Invert to mix. Incubate on ice for 5 minutes.
5. Spin in microcentrifuge for 5 minutes to form a pellet. Transfer supernatant to fresh tube.
6. To bind, add 100µL of Triplex Beads. Leave at room temperature for 15 minutes.

7. To wash, immobilize the beads against the side of the tube with a magnet and wash 1 time with 100µL washing buffer. Carefully pipette out all of the liquid.
8. To elute, add 50µL elution buffer. Leave at room temperature for 10 minutes.
9. Immobilize the beads against the side of the tube and transfer the supernatant to a fresh tube.

VII. PURIFICATION OF DNA SEQUENCING REACTIONS

A fundamental aspect of modern molecular biology is DNA sequence analysis. In order for the DNA to be accurately sequenced, and the background noise reduced, it is necessary to first remove impurities that are remnants of the sequencing reaction (enzymes, salts, unreacted dye terminators, etc.). The following procedure outlines how streptavidin-coated magnetic microspheres can simplify this process.

Standard protocols exist for DNA sequencing.3,4,5 Automated sequencing instrumentation is offered by PE Biosciences (ABI PRISM®) and Amersham Life Science (Thermo Sequenase dye terminator cycle sequencing core kit), among others. Therefore, this protocol is specific to using streptavidin-coated superparamagnetic microspheres to purify and isolate DNA for gel sequencing. The reaction is as follows.

Reagents

1. Streptavidin-coated microspheres (supplied at 1% solids)
2. Binding Buffer: 1X TES (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, pH 8.2) and 0.2% Tween® 20
3. Wash Buffer: 1X TES buffer
4. Elution Buffer: 10 mM EDTA, 95% formamide, 0.05% bromophenol blue (or other DNA stain)

Procedure

1. Add 20µL microsphere suspension and 20µL binding buffer to each reaction vessel (per 50µL volume). *Note*: These volume ratios are a baseline that may require optimization for your particular sequencing reaction.
2. Incubate for 15 minutes with gentle mixing.
3. Wash 2 times (by magnetic separation) with 10µL wash buffer and 1 time with 10µL H_2O.
4. Elute DNA by resuspending in 6.5µL elution buffer at 90˚C for 5 minutes, with mixing.
5. Separate microspheres magnetically, retain supernatant.
6. Load supernatant onto gel for sequencing. *Note*: While the elution step is required, the final wash step is optional, in that the microspheres can be added along with the supernatant to the gel walls without interfering with the sequencing.

VIII. COUPLING / RELEASE OF NUCLEIC ACIDS USING STREPTAVIDIN-COATED MICROSPHERES[13]

Biotinylated oligonucleotides are a useful tool in many fields of biological research, particularly as probes for hybridization studies. The major problem encountered in the application of this system lies in the difficult reversal of the interaction between immobilized streptavidin and the biotinylated material, necessitating rather drastic elution conditions. An example of an application where disrupting the biotin/streptavidin interaction is particularly desirable is when biotin is used as an affinity tag for the purification of biological molecules. Another example is in the screening of randomized RNA libraries for novel catalytic RNAs. This application involves the covalent modification of the active ribozymes with a biotin-tag and the subsequent purification by streptavidin-coated beads.

Current elution procedures either involve highly denaturing conditions using chaotropic reagents, such as 50% guanidine isothiocyanate/formamide (the disadvantage being that these reagents also release significant amounts of streptavidin) or competition of streptavidin-bound biotinylated nucleotides with a high concentration of free biotin. The following procedure takes advantage of the lability of the streptavidin / biotin bond in the presence of 2-mercaptoethanol (2-ME). The advantage of this procedure is that it is a simple and inexpensive method to release streptavidin-bound biotinylated oligonucleotides under relatively mild and controllable conditions that avoid the presence of free biotin or chaotropic agents in the eluted sample.

Reagents

1. Streptavidin-coated microspheres (supplied at 1% solids)
2. Biotinylated oligonucleotide probe
3. 0.15 N NaOH
4. TTL Buffer: 100 mM Tris-HCl (pH 8.0), 0.1% Tween® 20, 1 M LiCl
5. TT Buffer: 250 mM Tris-HCl (pH 8.0), 0.1% Tween® 20
6. TTE Buffer: 250 mM Tris-HCl (pH 8.0), 0.1% Tween® 20, 20 mM Na_2 EDTA (pH 8.0)
7. Hybridization Mixture: 2 M $NaClO_4$, 0.4% SDS, 20 mM $MgSO_4$, 10% 8000 MW PEG
8. Elution Buffer: 1.0 M NaCl, 50 mM MOPS (pH 7.4), 5.0 mM EDTA, 2.0 M 2-ME

Binding Procedure

1. Transfer 1mg (0.1mL) streptavidin-coated microspheres to 0.5mL centrifuge tube.
2. Separate, decant and then rinse in 200µL TTL buffer. *Note*: Separate polymeric and silica microspheres via centrifugation; use a magnet for superparamagnetic microspheres.
3. Separate, decant, and resuspend in 20µL TTL buffer.
4. Add biotinylated probe in the amount corresponding to the manufacturer's reported binding capacity, bringing microspheres to a final volume of 25µL.
5. Incubate for 15 minutes at room temperature (18-25˚C) in TTL buffer with gentle mixing.
6. Separate probe/microsphere conjugates, decant, and wash pellet in 0.15 N NaOH (to remove any nonspecifically bound probe).
7. Rinse 2 times in TT buffer, with appropriate separation steps.
8. Resuspend in TTE buffer, incubate at 80˚C for 10 minutes, and decant to remove any unstable biotin/streptavidin couplings.
9. Resuspend in 100µL hybridization mixture for storage.

Elution Procedure

1. Resuspend in 100µL elution buffer for 2-3 minutes at room temperature (or heated to 95˚C).
2. Wash and precipitate supernatant with ethanol to recover eluted nucleic acid.
3. Resuspend streptavidin-coated microspheres in TTL buffer for reuse, or storage buffer (hybridization mixture).

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