

Chapter 3

Nonradioactive Screening of an *M13* Phage Library for A Eucaryotic Microsatellite DNA Sequence

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Introduction

This exercise is part of a series of labs designed to introduce population biology students to basic molecular biology. These labs involve the construction of a random-clone library and the screening of the library.

Table 3.1. Suggested Laboratory Exercises for a Course in Molecular Evolution

<i>Lab</i>	<i>Description</i>
1.	Phenol extraction of genomic DNA from frozen mammalian muscle tissue
2.	DNA restriction digestion and agarose gel electrophoresis of uncut and cut samples
3.	Recovering DNA restriction fragments by electroelution and "Gene-Cleaning"
4.	Ligation of recovered DNA into the cut <i>M13mp18</i> vector and transformation into competent <i>E. coli</i> cells
5.	Plaque lifts and biotinylated-oligonucleotide hybridizations
6. *	Biotin detection and positive plaque isolations (the primary screen)
7.	Secondary (and tertiary) screens for pure clones
8.	PCR amplification of <i>M13</i> clone inserts
9.	Purification of insert DNA and cycle sequencing
10.	Primer design and synthesis
11.	PCR (Polymerase Chain Reaction) of genomic DNA samples from natural populations
12.	Microsatellite analysis of the population data
	* The laboratory exercise described here.

The laboratories are most successful when class size is small, the students have some previous lab experience, and the laboratory assistant is familiar with basic molecular biology methodology. To help contain costs the students work in pairs.

The large number and widespread distribution of microsatellite loci in eucaryotes was first reported in the early eighties (Hamada *et al*, 1982; Tautz and Renz, 1984). These loci are comprised of segments of DNA with very short (one to five base) tandemly repeated sequence motifs such as (TG)_n or (AAT)_n. These short tandem repeats are found in most, if not all, animal and plant species, and can be conveniently typed using the polymerase chain reaction (Litt and Luty, 1989; Weber and May, 1989).

In the human genome the most common microsatellites are those containing the repeats A, AC, AAAN, AAN or AG (where N is C, G or T), in order of decreasing abundance. These five groups comprise about 75% of the microsatellites (Beckman and Weber, 1992). Many other simple

sequence repeats are found at lower frequencies. To date, thousands of human and murine microsatellite loci have been identified and used extensively in genomic mapping (e.g. Human Genome Project). Their medical significance is demonstrated in the recent literature survey summarized in Table 3.2.

The laboratory exercise described here (indicated by * in Table 3.1) represents the detection step of the primary screen of a mammalian DNA library for "positive" (AC)_n microsatellite clones following hybridization of "plaque lifts" to a biotinylated-(GT)₁₂-oligonucleotide probe. This method relies upon the strong affinity of streptavidin for the biotin in a localized probe-target hybrid. The streptavidin is conjugated to alkaline phosphatase (AP), an enzyme that can be assayed readily. A colorimetric assay detects the streptavidin conjugated alkaline phosphatase on the nitrocellulose filter lifts. This exercise, which can be performed by the students in one three hour laboratory session, demonstrates dramatically the power of the screening process.

The library of clones is prepared in bacteriophage *M13* as described by Hudson (1994). If the students have not made their own lifts in a previous lab exercise, hybridized filters can be prepared in advance for them, by the technician, and stored until needed. Once the students perform the detection and identify positive clones, they are shown how to recover viable phage from the corresponding plaques on the Petri plates. In subsequent exercises students learn how these clones are used for microsatellite analysis.

Microsatellites are of particular interest to population geneticists because they often exhibit multiple allelism, in that the number of repeat units at a locus can vary significantly within and between populations (Tautz, 1989; Bruford and Wayne, 1993). Microsatellite allele frequency data are often useful when other natural variation is lacking. Questions of kinship (Queller *et al*, 1993), probability of identity (Paetkau and Strobeck, 1994) and population structure (Paetkau *et al*, 1995) can be addressed.

We use these exercises in the laboratory component of a molecular evolution course where the students develop molecular biology skills. The students generate some allele frequency data from wild populations to analyze.

Materials

Buffers:

- #1. 0.1 M Tris-Cl (pH 7.5), 0.15 M NaCl
- #2. 3% (w/v) BSA (bovine serum albumin, Fraction V, Sigma #A-9647) in buffer #1
- #3. 0.1 M Tris-Cl (pH 9.5), 0.1 M NaCl, 50 mM MgCl₂

stop buffer: 20 mM Tris-Cl (pH 7.5), 0.5 mM EDTA (ethyline-diamine-tetraacetate)

sterile saline: 0.15 M NaCl Dissolve 8.7 g in one liter and autoclave.

streptavidin-alkaline phosphatase conjugate (streptavidin-AP)*

dye: NBT * (nitroblue tetrazolium, 75mg/mL in 70% dimethylformamide)

chromogenic substrate: BCIP* (5-bromo-4-chloro-3-indooylphosphate, 50 mg/mL in dimethylformamide; also known as X-phosphate)

* available together in a kit (one BluGene System[®], GIBCO/BRL-Canadian Life Technologies, Inc., #18279-018, cost ~\$100 US per kit)

large (150 x 15 mm) plastic Petri dishes (Fisher Scientific # 8-757-14), one case
 curved forceps (one per group)
 500 mL plastic wash bottles (two per group)

2 liter beakers, plastic (one per group)
 sterile wide-bore Pasteur pipettes (one container per bench)
 sterile 1.5 mL microcentrifuge tubes (one container per bench)

Table 3.2. Some Inherited Diseases Caused by Expanded Microsatellites.

Disease or Condition	Mode of Inheritance	Microsatellite	Normal Number of Repeats	Expanded Number of Repeats	Repeat DNA Localization
<i>fragile ch. syndromes:</i>					
FRAX A ¹	X-Linked	CGG	~20	200-1000	5' untranslated
FRAX E ²	X-Linked	GCC	6-25	200-1000	??
FRAX F ³	X-Linked	GCC	6-29	300-500	ORF
FRA 16A ⁴	autosomal	CCG	16-49	1000-2000	??
<i>ataxias:</i>					
SCA 1 ⁵	autosomal dominant	CAG	~20	36-121	ORF
SCA 3 ⁶ (Machado-Joseph's)	autosomal dominant	CAG	13-36	68-79	ORF
SCA 6 ⁷	autosomal dominant	CAG	4-16	21-27	ORF
Friedreich's ⁸	autosomal recessive	GAA	10-21	200-900	intronic
DRPLA ⁹ (Haw River Syndrome)	autosomal dominant	CAG	3-25	54-68	ORF
<i>others:</i>					
myotonic dystrophy ¹⁰	autosomal dominant	CTG	5-30	»30	3' untranslated region
Kennedy's ¹¹	autosomal dominant	CAG	~20	38-66	ORF
Huntington's ¹²	autosomal dominant	CAG	11-34	42-100	ORF
Numbers refer to references p. 49					

Biotin Detection and Positive Plaque Isolation Primary Screen

Genomic DNA can be fragmented by endonuclease restriction and cloned in a bacteriophage vector such as *M13* for growth in *E. coli*. The resulting viral plaques which form on the lawn of bacterial cells can be replica-transferred onto nitrocellulose filters. These "plaque lifts" represent thousands of clones that can be screened for any specific DNA sequence easily. The localized single-stranded *M13* DNA's, each with their own individual genomic DNA insert, are bound to the filters and presented for hybridization to a labeled, sequence-specific DNA probe.

In our case lifts have been exposed to a labeled probe specific for one class of microsatellites. Microsatellites come in a number of types but all are very short tandemly repeated DNA sequences. They are quite common and tend to be dispersed throughout eucaryotic genomes. We have used a nonradioactive, biotin-end-labeled (GT)₁₂ oligonucleotide DNA probe. It can hybridize to microsatellites of the type (AC)_n, which are dispersed throughout the mammalian genome and consequently will be found in our library of cloned DNA's.

The determination of which clones contain (AC)-repeat microsatellites is accomplished by detection of the hybridized probe. We shall do this here by taking advantage of the extremely high affinity of the protein avidin for the vitamin biotin ($K_d \sim 10^{-15}$ M).

In this lab we use streptavidin-conjugated alkaline phosphatase to detect the localized biotin. Streptavidin is a protein isolated from *Streptomyces avidinii* which has better binding properties than avidin itself. We allow the conjugated streptavidin to bind the immobilized biotin on the filters, wash away unbound conjugate, and then stain for any remaining alkaline phosphatase enzyme activity. This is detected by the development of visible purplish-blue spots of precipitated dye over the positions of microsatellite-bearing clones on the filter. Then we go back to the plates from which the lifts were made to recover viable phage particles from the corresponding plaques. This is done by aligning the corresponding filter and plate and "picking" the appropriate plaques into individual microcentrifuge tubes. At this point we will have completed a primary screen and should have some putative positives for further analysis.

Procedure - Biotin Detection

1. In a large plastic Petri dish rehydrate your dried hybridized filter, together with a filter test strip (dot blot of spots containing 0, 2, 5, 10 and 20 pg biotinylated DNA, provided for you), in 25 mL of buffer #1 (Appendix A) for 5 min., followed by 25 mL buffer #2 for 10 min. Using your forceps to retain the filters in the dish, decant and discard used buffers down the sink drain.
2. Just before use, dilute 21 μ l streptavidin-AP conjugate into 21 mL buffer #1. Replace the buffer #2 from step 1 with this solution. Incubate 10 min. at room temperature.
3. Transfer the filters to a 2 liter beaker containing 600 mL buffer #1. Swirl gently and occasionally over 10 min.
4. Decant the buffer and repeat this wash step with a fresh 600 mL of buffer #1.
5. Decant the wash and replace with 200 mL buffer #3. Incubate for 10 min. at room temperature. This step adjusts the pH to allow enzyme (AP) activity.
6. Next is the colorimetric detection of the probe-target hybrid with the streptavidin-alkaline phosphatase-conjugate, a chromogenic substrate (BCIP or X-phosphate) and dye (NBT).

Transfer the two filters to a new Petri dish containing freshly-prepared dye mix (Appendix B) and incubate under low light conditions (say, in a cupboard or drawer) until dark bluish spots the size of plaques appear (usually within 30 min. to an hour). A check of the test strip will indicate whether the detection system is working and how sensitive it is.

7. When the spots have developed, decant the dye mix and replace with stop buffer. Five min. later the filters can be air-dried on paper towels and stored until needed. Fading of the color will occur if the filters are stored in the light for long periods.
8. Using the positions of the "positives" on the filter, go back to the corresponding plate of plaques, and orient the two with respect to one another. With sterile wide-bore Pasteur pipettes pick individual *M13* phage plaques into 200 μ l aliquots of sterile saline (Appendix A) in 1.5 mL microcentrifuge tubes. These will be used for subsequent replating and secondary (and tertiary, if need be) rescreening, until pure, well-isolated positive clones are obtained for further use and analysis.

Notes for the Instructor

This laboratory exercise, and the others in the series from which it is drawn, should not be attempted without the assistance of a laboratory assistant who is adept in the basic techniques of molecular biology. Although simple in execution, the exercise requires considerable expertise and time in the preparations. Samples of hybridized filter lifts prepared in advance should be tested with the detection system to ensure success in the student laboratory.

Safety considerations: The instructor should make the students aware of the hazards associated with the teratogen dimethylformamide, the solvent in which both the BCIP and the NBT are dissolved. Although they handle only very small volumes we supply disposable gloves and review the MSDS with them. All other reagents are non-toxic.

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Appendix A *Buffer Preparation*

Stock Solution	mL for Buffer #1	mL for Buffer #3	mL for stop buffer
1.0 M Tris, pH 7.5	1,000	0	20
1.0 M Tris, pH 9.5	0	100	0
5.0 M NaCl	300	20	0
1.0 M MgCl ₂	0	20	0
0.5 M EDTA, pH 8	0	0	1
distilled water	8,700	860	979
final volume	10,000	1,000	1,000

Buffer #2 is prepared by dissolving 30 g BSA (bovine serum albumin, Fraction V, Sigma #A-9647) in 1,000 mL of Buffer #1.

Sterile saline : 0.15 M NaCl Dissolve 8.7 g in one liter and autoclave.

Appendix B *Dye Mix*

Dye mix is prepared fresh in a Petri dish under subdued lighting. NBT and BCIP are part of the BluGene kit (See Materials).

Caution: wear gloves when handling this. Dimethylformamide is harmful.

22.5 mL buffer #3

99 µl NBT dye solution (75 mg/mL nitroblue tetrazolium in 70% dimethylformamide)

75 µl BCIP chromogenic substrate solution, also known as X-phosphate (50 mg/mL 5-bromo-4-chloro-3-indoylphosphate in dimethylformamide)

Swirl gently to mix.