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Identification of Nuclear Hormone Receptor Homologs by Screening Libraries with Highly Degenerate Oligonucleotide Probes

Bruce Blumberg

1. Introduction

Orphan nuclear receptors have been identified, using a variety of methods over the years. The first were identified by low-stringency hybridization, using known receptors as probes. This strategy has been successful, because members of the steroid receptor superfamily contain a conserved DNA-binding domain, and share regions of similarity in the ligand-binding domain. These conserved regions may also be used to design polymerase chain reaction primers that have been used to identify new receptors, primarily members of known families. The recent explosive increase in DNA sequences from EST and genomic sequencing projects has also allowed the identification of new family members. The *Caenorhabditis elegans* genome has recently been sequenced and shown to contain a large variety of putative nuclear receptor genes, some of which may be represented in mammalian genomes. The question remains of how to identify potentially highly divergent mammalian homologs. One possibility is to wait until such sequences appear in the rapidly growing sequence databases from rodent and human genome projects. This method has been used to identify a novel member of the steroid receptor superfamily ***(1–3)*** and may ultimately result in the identification of others. For those who do not wish to wait, or who work on model organisms whose genome projects are not well advanced (e.g., *Xenopus*), there is no substitute for directly isolating the relevant cDNAs.

Polymerase chain reaction-based methods require two oligonucleotides. Designing two appropriate sequences may not always be possible, because either sequence information is lacking or there is insufficient sequence conser-

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vation. This can be overcome by screening with a single oligonucleotide whose sequence is derived from amino acids in a region conserved in sequence among members of a gene family. Various oligonucleotide-screening strategies have been employed for this purpose in the past, including "guessmers," in which the wobble position of each codon is derived from a codon frequency table for the species in question, and the substitution of inosine in the wobble position, which may allow multiple types of sequences to be detected. Each of these methods introduces a bias that may result in an unsuccessful screen. The author prefers the use of oligonucleotides that represent all possible codons for the amino acids to be matched. These fully degenerate oligonucleotides are guaranteed to hybridize precisely to the target sequence, and will always result in successful screens, if a few precautions are taken.

The most serious problem with using highly degenerate oligonucleotides to probe blots results from the inability to predict which sequence, out of a family of sequences, is actually hybridizing to the target. Since the G:C content of each differs, one cannot easily pick a hybridization and washing temperature that minimizes the number of false hybridization signals. This problem can be overcome by the use of 3.0 *M* tetramethylammonium chloride (TMAC) in the washing buffer ***(4–6)***. In this method, one hybridizes at low stringency, and washes at high stringency, reducing the number of false positives. 3.0 *M* TMAC stabilizes A:T base pairs such that they melt at the same temperature as G:C base pairs. This has the effect of making the melting temperature of any hybrid strictly a function of the length of the hybridized region. A collateral benefit is that TMAC sharpens the melting profile of DNA duplexes, so that hybrids that melt over a 5–10°C range in the presence of Na+ melt within 1–1.5°C in TMAC. Using the TMAC method, it is possible to distinguish between hybrids differing in length by as few as 1 bp ***(6)***. The author et al. have used the TMAC method to identify novel nuclear hormone receptors ***(7–9),*** homeobox genes ***(10–13)***, Transforming growth factor β family members ***(14)***, and P450 family members ***(15)***.

Previously, a degenerate oligonucleotide (TGY GAR GGN TGY AAR GGN TTC TT) was used to identify novel members of the steroid receptor superfamily ***(7)***. This DNA sequence corresponds to the highly conserved amino acid sequence, CEGCKGFF, found in the P-box ***(16)*** of the DNA-binding domain of many nuclear receptors. It is straightforward to use a similar approach to identify vertebrate homologs of the many recently identified *C. elegans* ORFs. **Figure 1** shows a representative selection of the different P-box sequences from *C. elegans* thought to encode nuclear hormone receptors. Although there are only 25 P-box sequences, these represent more than 200 receptor DNA-binding domains. Because *C. elegans* does contain receptors that harbor the

Fig 1

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CAACAAFF
CDGCKGFF        CDACKMFF
CEGCKGFF        CESCKAFF        CESCKGFF
CFGCKGFF
CGACAAFF
CHGCKAFF
CKACAAFF        CKGCKTFF
CLACAAFF        CLGCKTFF
CNGCKTFF        CNGCKGFF        CNACKMFF
CRACTAFF        CRACAAFF        CRGCNAFF
CSACGSFL        CSACSSFF
CTACASFF
CVGCKTFF
CYACKMFF        CYGCKGFF


C   E   G   C   K   G   F   F
TGY GAR GGN TGY AAR GGN TTC TT
```

Fig. 1. P-box sequences for known and putative C. elegans nuclear receptors. The oligonucleotide we have successfully used and the corresponding P-box sequence is shown at the bottom.

CEGCKGFF P-box, it is not unreasonable to suppose that vertebrate homologs will exist for at least a subset different identifiable *C. elegans* P-box sequences uncovered by the genome project. The use of P-box oligonucleotides to screen allows one to identify the particular receptor cloned in a single sequencing reaction.

2. Materials

1. 5.0 *M* TMAC stock solution: TMAC is hygroscopic; therefore, one must prepare a stock solution at a nominal concentration of 5 *M*, then precisely quantitate it with a refractometer (*see* **Note 1**). Calculate the molarity (*M*) from the following formula:

   M = (refractive index – 1.331)/0.018 (*see* **Note 2**)

2. TMAC washing buffer: 3.0 *M* TMAC, 0.05 *M* Tris-HCl, pH 8.0, 0.2 m*M* ethylenediamine tetraacetic acid (EDTA).
3. Standard oligonucleotide hybridization buffer (*see* **Note 3**): 6.6 × NET (final 1 *M* NaCl, 0.1 *M* Tris-HCl, 6 m*M* EDTA), 5 × Denhardt's solution, 0.05% sodium pyrophosphate (NaPPi), 0.1% sodium dodecyl sulfate, 0.1 mg/mL, yeast RNA, 125 U/mL heparin.
4. 20X NET: 3 *M* NaCl, 0.3 *M* Tris-HCl, pH 8.3, 18 m*M* EDTA.
5. 6X standard sodium citrate (SSC), 0.05% NaPPi.

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3. Methods

3.1. Designing an Oligonucleotide

3.1.1. Considerations

1. It is useful to also use the screening oligonucleotide for identifying the recombinant clones by sequencing; therefore, try to design the oligo so that a sequence diagnostic for the family is read when it is used as a sequencing primer. This makes the classification of positive cDNAs and the identification of false positives rapid.
2. Since chemically synthesized oligonucleotides are built from the 3' to 5' ends, the 3' end of the oligonucleotide must be unique, or else you will have to make several different oligonucleotides.
3. The degeneracy in the 3'-most 11 nucleotides (nt) makes a difference when using the screening oligonucleotide as a sequencing primer. For best results, try to keep this below 16-fold. To sequence 1 pmol of template (optimal amount), use a molar excess of primer approximately equal to the degeneracy in the 3'-most 11 nt.

3.1.2. Rules of Thumb

1. Use oligonucleotides 20–30 nt in length. The author prefers 23-mers for most screenings.
2. Avoid sequences containing Ser. If this is not possible, make two separate oligonucleotide pools, one with the AGY codons, and the other with the TCN codons.
3. Avoid codon usage tables. Make the oligonucleotides a completely degenerate version of the amino acid sequence. Do not make combination guessmers and degenerate probes (*see* **Note 4**).
4. Try not to make the oligo self-complementary, if that can be avoided.
5. Do not purify degenerate oligonucleotides by ion-exchange chromatography. Pharmacia and others recommend purification on ion exchange resins under alkaline conditions, but, in the author's experience, this fractionates oligonucleotides by sequence, and biases the composition of individual fractions.
6. Synthesize degenerate oligonucleotides at the 1 μ*M* scale. Most instruments use the largest amount of excess reagents at this scale, and this translates to decreased bias in the resulting oligonucleotide pool.
7. Have the supplier purify the oligo by polyacrylamide gel electrophoresis (PAGE) or reverse phase cartridge. PAGE is superior, because RP cartridge purification does not remove any trityl-on failure sequences that may be present. This could result in a mixed size population (*see* **Note 5**).
8. Alternatively, purify the (detritylated) oligonucleotide on a 15% polyacrylamide-8M urea gel. Second choice (for a trityl-on) oligonucleotide would be to purify it on a RP cartridge, such as NENsorb-Prep.

3.1.3. Calculating the Number of Expected False Positives

1. Under the best circumstances, one will still expect to find some false positives that result from random matches between the oligonucleotide and the target DNA sequences.

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2. The *a priori* statistical probability of finding a matching sequence in any random DNA sequence is a function of the total number of nt to be screened, size of the oligonucleotide, number of contiguous matches required, and size of the oligonucleotide pool.

$$N = C(2)(n - h + 1)p/4^{h}$$

where N = the number of expected random matches/haploid genome, C = the genome size (or the complexity of the library * the average size), n = the length of the probe, h = the number of matches required, p = the total number of different oligonucleotides in the pool.

a. For 10^6 cDNA clones, of average length 2 kb, screened with a mixture of 512 different 23-mers under two mismatch conditions, we get the following: $N = (2000)(10^6)(2)(23 - 21 + 1)(512)/4^{21}$, which is 1.4 expected random matches.
b. An important factor here is the degeneracy of the probe (*see* **Note 6**).
c. In the above calculation, the value for C is the total number of independent base pairs screened.
d. If the cDNA library used were amplified, then one would use the number of independent clones or the number screened, whichever is smallest, for C. For a genomic Southern, use the size of the genome. For a genomic library, divide the genome size by the size of the average insert.

3. Unfortunately, DNA sequences are not random; therefore, one must also search the oligonucleotide sequence against the DNA database, to ensure that it does not accidentally hybridize to repetitive sequences or other sequences that may interfere with the screening.
4. Sometimes this initial screening can help to identify unknown members of the gene family under investigation ***(17)***.
a. The FINDPATTERNS program of UWGCG works well for this purpose.
b. Since DNA has two strands, one must also search with the complement of the screening oligonucleotide.

Au: findpatterns OK?

3.2. Labeling the Probe

1. It is important to use enough probe, and to make it hot enough.
2. A standard, high-density library screen (e.g., duplicate filters from 10 150-mm plates, with 100,000 plaques each) would require ~200 pmol oligonucleotide. For a 23-mer, this is 760 ng (7.6 ng/pmol).
3. In subsequent purifications, 200 pmol oligonucleotide is adequate for only 24 filters, or so (100 mm), because, depending on the degeneracy of the probe, one might well not be in probe excess with the higher numbers of phage present during plaque purification. To convince yourself that this is true, do the following calculations.
a. Assume 10^7 phage/plaque (conservative for λZAP or gt10), 50 positive plaques/plate average, 24 filters (duplicates from 12 plates), and 200 pmol 1000-fold degenerate oligo.
b. Calculating molecules of target: $(10^7)*(50)*(24) = 1.2 \times 10^{10}$ targets.

Au: 100,000 correct?

Au: spell out ZAP & gt10

c. Calculating molecules of probe: 200 pmol of oligo = (6.02×10^{23} molecules/ mol * 200×10^{-12} mol) = 1.2×10^{14} molecules of probe, divided by the degeneracy (1024 fold) = 1.1×10^{11} of each probe species, assuming 100% labeling. In practice, 100% labeling is not possible, moreover, there will be sequences to which the probe hybridizes only moderately, thus diluting out the available probe for true positives.

4. Be sure to make the probe hot enough.
   a. Use severalfold molar excess of γ^{32}P-adenosine triphosphate (6000 Ci/m*M*, or greater) whenever possible.
   b. Pure γ^{32}P-adenosine triphosphate (e.g., NEG-002Z, New England Nuclear) works best but is expensive when labeling large amounts of probe. One can substitute a crude preparation (e.g., NEG-035C) for this purpose; however, the probes are not as completely labeled. This is a reasonable trade-off considering that it is 5–10-fold less expensive (*see* **Note 7**).
   c. After the labeling reaction is completed, remove the unincorporated label by two consecutive spun columns using Sephadex G-25 or equivalent (*see* **Note 8**).

3.3. Plaque Lifts

1. Plating the library
   a. For first-round screens, plate the library to obtain 50–100 K plaques/150-mm round plate, or 250,000–500,000 per 22×22-cm bioassay dish.
   b. Lift duplicate filters (3 min first lift and 6 min second lift), and do not even consider purifying signals that do not duplicate (*see* **Note 9**).
   c. Process a convenient number of plates at a time, e.g., 6–12, depending on how facile you are. Spread the filters out on large sheets of filter paper until all lifts have been finished, and allow them to dry at room temperature.

Au: hours meant? If so, use "h". Minutes meant, use "min". Please check thruout

2. For first-round screens, place the filters, plaque-side-up, on blotter paper saturated with 0.5 *M* NaOH-1.5 *M* NaCl for 3 min, then on 0.5 *M* Tris-HCl, pH 7.5-1.5 *M* NaCl for 3 ,om, then on 2X SSC for 3 min.
3. When processing of each filter or set of filters is completed, transfer to sheets of dry filter paper, and allow to air-dry. Bake nitrocellulose or nylon filters at 80°C for 30 h ***(18)***. Nylon filters may be UV crosslinked, but this does not increase signal strength.
4. Wash the dried filters for 15 min in 0.05 *M* NaOH, with shaking. This step removes debris from the filters, enhances the signal, and reduces the background considerably.
5. Rinse the filters in five, 3-min changes of dH_2O, to ensure removal of NaOH. Check the final wash with pH paper to ensure that NaOH has been removed.

3.4. Hybridization

1. Prehybridize overnight in hybridization buffer at 42–46°C (*see* **Note 11**).
2. Calculating the hybridization temperature.

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a. Optimally, one should hybridize at T_m – 5°C for perfectly matched probes. This is impossible for mixed-probe populations; hence, calculate the maximum number of possible A and T residues possible for the probe, then estimate the minimum T_m assuming T_m = 4°C *(G + C) + 2°C *(A + T).
b. Hybridize at ~10–15°C below this T_m (*see* **Note 12**) 46°C works well, in practice.
3. Overnight hybridization is sufficient. Longer times give higher background.
4. The volume is not critical, since the probe cannot self-anneal. Use about 10–15 mL/bag.
5. Incubation with agitation is not necessary, but not harmful, if a shaker or hybridization oven is available.

3.5. Washing

1. This is the most critical step. The most important factor in a successful screen is careful and skillful washing. Do not take shortcuts or deviate from the protocol.
2. Calculate the correct washing temperature:
   a. Obtain the T_m for a specific-length oligonucleotide from the figure in Wood et al. paper ***(6)***.
   b. Assume that T_m is reduced by 1°C for each % mismatch.
   c. Wash at T_m-5°C – (mismatch reduction)
   d. For a 23-mer at 1 mismatch, this is 65°C – 5 – 4 = 56°C.
3. After hybridization, remove the filters to a 500 mL–1 L container of 6X SSC, 0.05% NaPPi . Rinse for 2 min at room temperature to remove unhybridized probe.
4. Remove the SSC, add a fresh aliquot, and incubate at room temperature, with shaking for 15 min. Take care that the filters move around freely and do not stick to each other or to the container.

Au: 15 min? Pls check all

5. Add a sufficient amount of 3 *M* TMAC wash buffer, so that the filters can move freely when agitated. Incubate 15 min at room temperature (*see* **Note 14**).
6. Place the filters in a seal-a-meal bag, and leave sufficient area for the filters to move around freely (*see* **Note 15**).
7. Add 200 mL preheated TMAC wash buffer, and place the bag with filters into a preheated water bath. Anchor the corners so that the bag does not move, but allows the filters to move freely.
8. Incubate with shaking for 15 min. Cut the corner of the bag, remove the TMAC, add another preheated aliquot, and repeat the washing (*see* **Note 6**).
9. Place the filters back into the larger container and wash with 6X SSC, 0.05% NaPPi to remove TMAC, which smells bad and leaves a sticky residue on the filters.

3.6. Detecting the Positive Signals

1. Place the wet filters between sheets of plastic wrap, and fold the edges over to prevent drying.
2. Tape the filters securely to a sheet of used X-ray film or other suitable transparent or translucent support.

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3. Place tiny dots of radioactive ink on the tape, for orientation purposes
4. Expose to film for 1–3 days with intensifying screens at –80°C (*see* **Note 17**).
5. Orient the cassette (bottom to top) as sample, screen, film, screen. When using BioMax MS screens and film, one screen is typically sufficient. In this case, the orientation should be sample, screen, film.

3.7. Purifying Positive Signals

1. After developing the film, orient the films to the filters by aligning the spots of radioactive ink with the signals they produce.
2. Transfer the filter labels and the registration marks in the filter to the films.
3. Align the registration marks on the first and second lift, and circle signals that duplicate.
4. After finishing all filters, place the film with the signals circled on a light box.
5. Align the holes in the plate with the registration marks on the film.
6. Pick a region surrounding the signal, with the blunt end of a Pasteur pipet (or a yellow tip cut to about one-third its original length.
7. Transfer the plug to a 1.5-mL microcentrifuge tube containing 1 mL SM buffer. Allow the phage to diffuse out of the plug for at least 4 h, and preferably overnight.
8. For subsequent rounds of screening, dilute the primary plaque 1000-fold, and plate three dilutions (e.g., 1, 5, and 25 µL). Select the plate with an appropriate number of plaques for lifting filters.
   a. For a second-round screen, pick a plate that has about 100–500 plaques and one higher density for each positive.
   b. Pick only one positive signal per second round plate and try to pick one that is separated from surrounding plaques by the widest margin. If this margin is >5 mm all around, the plaque is probably pure. If not, perform another round of screening.
   c. For third round screens, 25–50 plaques/plate is good but do not use a plate with less.

Au: spell out SM

3.8. Sequencing Positive Clones

1. Another advantage of using degenerate oligonucleotides to screen cDNA libraries is that the cDNAs may be directly identified by sequencing them with the screening oligo.
2. In general, best results are obtained when sequencing 1 pmole DNA with a 1–2-fold molar excess of primer.
3. For degenerate primers, a larger molar excess is required. A rule of thumb is to calculate the degeneracy in the 3'-most 11 positions of the primer, then use this molar excess. For optimal results, the amount must be calculated and titrated for each different primer.

4. Notes

1. If you do not have a refractometer, find someone who does. If a refractometer cannot be found, use a brixmeter (e.g., 28–62% sugar, Fisher no. 13-946-60B).

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Read the TMAC as % sucrose, and convert this to refractive index, using the table in the CRC handbook entitled "Index of refraction of aqueous solutions of sucrose." 5 *M* TMAC has a refractive index approximately equal to that of 50% sucrose.

2. The author usually buys a whole case of TMAC from Fisher: Dissolve it all at ~5 *M*, pool into a large flask, mix well, filter to remove debris, then measure a small aliquot. Dispense the stock into conveniently sized aliquots. Store in tightly sealed bottles, to avoid absorption of H_2O and subsequent changes in concentration.
3. Filter the hybridization buffer through a 0.45-µm filter, preferably composed of the same type of membrane that will be used for hybridization and store at 4°C. This filtration step results in reduced background.
4. The author has never failed to clone the desired sequence using completely degenerate oligonucleotides, but has had bad experiences with probes containing inosine instead of mixed nucleotides. Consequently, these are not recommended.
5. The authors has had excellent success with degenerate oligonucleotides produced by Genosys (www.genosys.com).
6. One can use pool sizes of up to 3000 or so, but the best results are obtained with 1024-fold degenerate (or less) 23-mers. The oligonucleotide the author used for identifying novel orphan receptors was 512-fold degenerate.
7. Use the purified isotope (NEG-002Z) for labeling the probe for high-density, first round screening, then use the crude isotope (NEG-035C) to label the probe for subsequent rounds of purification.
8. One spun column typically gives ~95% removal of the unincorporated label, however, considering the amounts of isotope in use here (1–10 mCi), this leaves too much free label (50–500 µCi) remaining. This can result in high background. The second column reduces this to 2.5–25 µCi, which is acceptable.
9. Ensure proper alignment by poking asymmetric, vertical holes through the filter and into the plate during the first adsorption. After all of the first lifts are complete, place the second filter on each plate, then hold each plate up to the light, and precisely duplicate the hole pattern. Performing duplicate lifts for first- and second-round screens is important to ensure that only true positives are picked; it is optional (but safer) for third-round screens.
10. It is important for subsequent signal strength that high-density filters from first round screens be processed by capillary action. For second- or third-round screens, it is acceptable to process the filters by immersion in containers of the solutions. Incubate at 3' with enough shaking to keep the filters from sticking to each other.
11. This extended prehybridization reduces background considerably. Prehybridization temperature is not critical. For convenience, one typically uses the same temperature for both hybridization and prehybridization.
12. The author typically hybridizes up to 25 filters/bag, and uses 200 pmol of labeled probe/hybridization. Do not exceed this number, or low and variable signal will result.

Au: 3' ?

13. For a 23-mer, one mismatch is ~4°C, two mismatches is ~8°C. The author empirically found that 56–58°C is optimal for washing 23-mers. Use the higher temperature for less degenerate probes. Alternatively, test the washing conditions, using Southern blots and cloned sequences that should be detected by the probe.
14. This step exchanges sodium ions from the SSC for tetramethyl ammonium ions in the TMAC washing solution, which is important for effective washing.
15. An area of about 25 × 25 cm is adequate.
16. Doing the washing in a sealed bag is essential for proper temperature equilibration and control. In principle, use of a hybridization oven for these washing steps should be possible, but, in practice, it does not seem to work very well.
17. The type of film and intensifying screen used makes a very big difference for degenerate oligonucleotide screens (and other applications in which the signal strength is likely to be low). The combination of green-emitting screens and green sensitive film (e.g., Kodak BioMax MS screens and film) gives an approx eight-fold increase in signal over Kodak XAR-5 film (blue sensitive) and standard (blue-emitting) intensifying screens (e.g., Lightning Plus) and 2–3-fold increase in signal over a blue emitting film (e.g., XAR-5) and blue emitting rare-earth screens (e.g., Quanta III). Moreover, the BioMax MS filmscreen combination gives sensitivity comparable to using a Phosphorimager.

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