Preface

The observation that neuropeptide Y (NPY) is the most abundant peptide present in the mammalian nervous system and the finding that it elicits the most powerful orexigenic signal have led to active investigations of the properties of the NPY family of hormones, including peptide YY (PYY) and pancreatic polypeptide (PP). Nearly two decades of research have led to the identification of several NPY receptor subtypes and the development of useful receptor selective ligands. Moreover, these investigations have implicated NPY in the pathophysiology of a number of diseases, including feeding disorders, seizures, memory loss, anxiety, depression, and heart failure. Vigorous efforts are therefore continuing, not only to understand the biochemical aspects of NPY actions, but also toward developing NPY-based treatments for a variety of disorders. To facilitate these efforts, it was decided to produce the first handbook on NPY research techniques as part of the Methods in Molecular Biology Series.

In compiling *Neuropeptide Y Protocols*, I have gathered contributions on techniques considered critical for the advancement of the NPY field from experts in various disciplines. Each chapter starts with a brief introduction, with Materials and Methods sections following. The latter sections are presented in an easy to follow step-by-step format. The last section of the chapter, Notes, highlights pitfalls and the maneuvers employed to overcome them. This information, not usually disseminated in standard research publications, may prove extremely useful for investigators employing these techniques in NYP research.

Neuropeptide Y Protocols contains a total of 18 chapters divided into five parts. Part I describes various cloning techniques in six chapters, including genomic DNA isolation, expression cloning, classical techniques, PCR cloning, construction of hybrid receptors, and homology-based cloning. Production of transgenic and knockout models is described in Part II. Four chapters in Part III illustrate the use of antisense technology to define the receptors and the signal transduction pathways mediating NPY actions. Various qualitative and quantitative techniques used to study tissue mRNA distribution are described in a total of five chapters spanned across Parts IV

and V. A chapter on radioligand binding is also included in Part V. The techniques described here could easily be extrapolated to study any peptide hormone. Therefore, *Neuropeptide Y Protocols* should benefit all investigators involved in polypeptide hormone research.

I wish to express my appreciation to all the authors for their excellent contributions, and particularly for meeting their deadlines. Appreciation is also expressed to my colleague, Sulaiman Sheriff, for the chapter and his help in selecting the contents of this volume. The series editor, John Walker, has contributed to the success of this volume in many ways, and is appreciated very much. Finally, I am grateful to Koti Sreekrishna, Senior Scientist, Procter and Gamble, Inc., Cincinnati, OH, for his expert help in editing these chapters.

Ambikaipakan Balasubramaniam

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Human Y1/Y5 Receptor Gene Cluster

Isolation and Characterization

Herbert Herzog

1. Introduction

The methods used to isolate genomic clones of a particular gene have changed significantly over the years. In particular, the characterization of genes with many exons, separated by large introns, increased the need for clones with large inserts. The average length of an insert contained within one of the classical used λ cloning vectors (λgt10 or EMBL3) ranges from 10 to 15 kb. This insert size might be large enough to contain entire genes that contain no or only a few introns, but it is likely that such clones harbor only parts of larger genes. In many cases several overlapping λ clones must be identified and isolated by repeated screening ("chromosomal walking") of a library with end fragments isolated from the first clone. This significantly increases the time and effort involved in isolating a gene. In addition, the isolation of λ DNA is a laborious and time-consuming exercise that yields only low amounts of DNA.

Genomic libraries constructed in cosmid vectors have greatly improved the isolation of genes ***(1,2)***. This is mainly because of their capacity for harboring larger inserts of up to 40 kb and also because DNA can be conveniently isolated as a plasmid. However, to characterize and map very large genes or gene clusters it is desirable to have even larger inserts.

P1 and PAC/BAC vectors fulfil that criterion ***(3,4)***. Such clones contain high-molecular-weight inserts (75–100 or 120+ kb), about four to six times larger than λ clones, and two to three times larger than cosmids. In addition, the low copy number of the P1, PAC, or BAC vector (together with culture conditions such as growing them in restriction- and recombination-deficient *Escherichia coli* hosts), provides strongly improved stability of these clones.

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Also, the isolation of super-coiled plasmids still follows standard plasmid purification protocols. Microgram quantities of plasmid DNA can be generated without any host genome contamination, which is always a problem with yeast artificial chromosomes (YACs), the only method available for cloning extremely large DNA (>1 million bp).

A further advantage of P1 and PAC/BAC clones is the fact that a relative low number of clones is required to cover a entire genome. For example, 10^5 clones with an average insert size of 100 kb theoretically represent three times the human genome. This is normally a large enough number of independent clones to identify a positive signal successfully. This relative low number of clones is amenable to semiautomated screening procedures. Master filter sets on which individual P1 or PAC/BAC clones are spotted at a very high density can be generated, allowing the quick screening of only a few filters. Each DNA spot on the filter corresponds to a location on a microtiter plate that contains the library. Clones from putative hybridization signals can then be recovered from these microtiter plates and used for further analysis.

An additional advantage of this method is the direct identification of a single clone without going through second and third screening rounds. Positive signals can also easily be distinguished from false hybridization signals, because every clone is spotted twice in a special pattern within each small square so that true-positive clones always give two signals. The filters can also be screened many times (we have successfully used one filter set 18 times); this saves money and avoids repetitive plating of the library, which is often accompanied by an inherited decrease in titer.

A wide range of very different ligands (including neurotransmitters, neuropeptides, polypeptide hormones, and other bioactive molecules) transduce their signal to the intracellular environment by specific interaction with a class of receptors that relies on interaction with intracellular guanosine-5'-triphosphate (GTP)-binding protein (G-proteins). Molecular cloning has led to the identification of several hundred discrete G-protein-coupled receptors, and it has been demonstrated that around 80% of known hormones and neurotransmitters, including the neuropeptide Y (NPY) family, mediate their signal by activating G-protein-coupled receptors ***(5)***. This makes the G-protein-coupled receptor superfamily, with its common structural and functional features, the largest single class of eukaryotic receptor, having an estimated 2000–3000 individual members. The structure of families of receptors exemplified by those coupled to G-proteins shows they are products that have evolved from an ancestral gene by duplication ***(6)***. Many of these receptor genes lack introns, supporting the proposition that they were created via RNA-mediated transpositional events ***(7)***. However, other mechanism of gene amplification may have occurred as well.

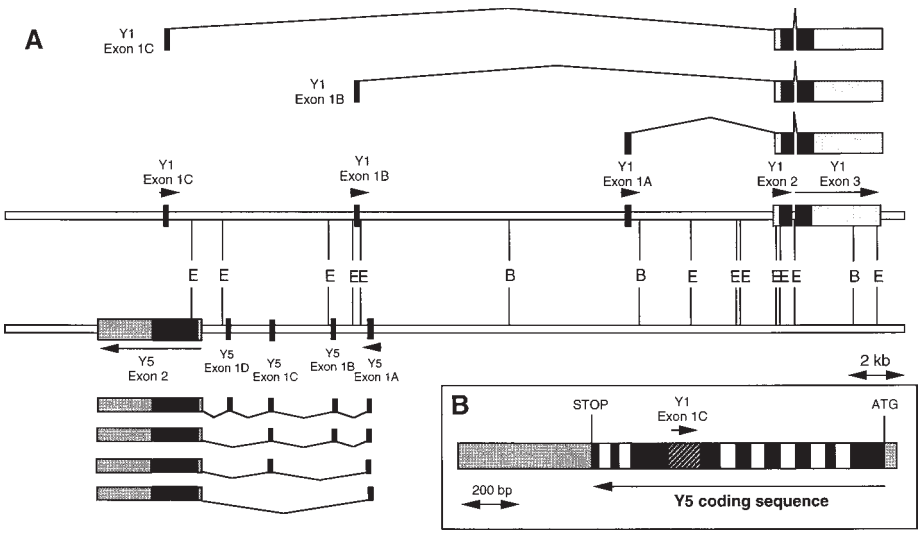

Fig. 1. The human Y1 and Y5 gene cluster. **(A)** A complete restriction map for the enzymes BamHI and EcoRI of the 30-kb region containing the genes for Y1 and Y5. The coding regions of both genes are shown as black boxes, noncoding regions are hatched, and intron sequences are white. The exact locations and direction of transcription of the exons is indicated by arrows in the upper strand for the Y1 gene and in the lower strand for the Y5 gene. The alternatively spliced forms of both genes are shown above and below the two strands. **(B)** Schematic representation of exon 2 of the human Y5 gene. White boxes, putative transmembrane domain coding regions; hatched box, position of exon 1C of the Y1 gene.

NPY receptors also belong to this large superfamily of G-protein-coupled receptors; various cloning techniques have so far identified five receptor subtypes (Y1, Y2, Y4, Y5, and Y6) ***(8)***. Sequence analyses of the cloned receptors reveal a substantial divergence in primary sequence between the receptor subtypes. The Y1 subtype was the first Y receptor to be cloned, and full-length cDNAs have been isolated from several species including human ***(9)***, rat ***(10)***, mouse ***(11)***, and frog ***(12)***. The Y1 receptors display a highly conserved structure, with overall identities of 94% or higher between mammalian species.

To determine the molecular organization and regulation of the human Y1 receptor gene, we isolated several genomic clones, subcloned and sequenced the exons and exon/intron boundaries, and mapped the transcription start sites (**Fig. 1**) ***(13)***. In the course of this work, we identified several alternatively spliced 5' exons and also (surprisingly) an open reading frame in the opposite orientation within the most 5' exon, 1C ***(14,15)***. The amino acid sequence showed significant homology to G-protein-coupled receptors, with the highest

similarity (37%) to the Y1-receptor, suggesting that this new gene encodes a new Y-receptor subtype, the Y5 receptor. The Y5-receptor probably evolved by a gene duplication event from the Y1-receptor gene and is now encoded in the opposite orientation 23 kb upstream of exon 2 from the Y1 gene ***(15)***. The transcription of both genes from opposite strands of the same DNA sequence suggests that transcriptional activation of one gene will have an effect on the regulation of expression of the other gene. As both Y1 and Y5 receptors are thought to play an important role in the regulation of food intake, coordinate expression of their specific genes may be important in the modulation of NPY activity ***(16)***.

2. Materials

1. [α-^{32}P]dCTP (Amersham #CA025687).
2. Agarose (Ultrapure; Life Technologies BRL #540-5510UA.
3. Cloning vectors: pBluescript (Stratagene) and pZero (Invitrogen).
4. Denhardt's solution (100X): 2% (wt/vol) Ficoll type 400 (Sigma [Castle Hill, NSW, Australia] #F2637); 2% (wt/vol) polyvinylpyrrolidene (Sigma PVP; P5288); 2% (wt/vol) bovine serum albumin (fraction V; Sigma #A7030) made up in RNase-free distilled H_2O. Filter through a Millipore 0.2-µm filter, and store in 10-mL aliquots at –20°C.
5. Ethylenediaminetetraacetic acid (EDTA; 0.5 *M*): make a 0.5 *M* solution (Sigma #E5134) in RNase-free distilled H_2O, stir and dissolve by adjusting to pH 8.0 with NaOH (10 *M*), and then autoclave.
6. Ethanol: absolute, analytical grade.
7. Ethidium bromide (EtBr) solution: make a 10-mg/mL solution (Sigma #E7637) in distilled H_2O; store at 4°C in a sealed, dark bottle protected against light (very hazardous/carcinogen).
8. [γ-^{32}P]ATP: NEN (Easytides™ #502211268).
9. Genomic library: Genome Systems, peripheral blood genomic P1 library (#FP1-2285).
10. Hexanucleotide mix: 0.06 OD_{260} U/µL (Boehringer Mannheim #1277081) in 500 m*M* HEPES, pH 6.6, stored at –20°C.
11. Hybond N+: Amersham #RPN 203B.
12. Hybridization solution (1X): 6X standard saline citrate (SSC), 5X Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS). Warm solution to 65°C, and add denatured ssDNA to a final concentration of 100 µg/mL.
13. Klenow DNA polymerase: Boehringer Mannheim #1008404.
14. Kinase buffer (10X): 250 m*M* Tris-HCl, 50 m*M* $MgCl_2$, 25 m*M* 1,4-dithiothreitol (DTT) and 25% glycerol. Adjust pH to 9.5.
15. Lysis buffer: stock solution contains 50 m*M* D-glucose (Merck [Kilsyth, Victoria, Australia] #1-117.3Y), 10 m*M* EDTA (Sigma E5134) and 25 m*M* Tris-HCl. Adjust pH to 8.0, and store at 4°C. Important: Add 50 mg/mL lysozyme (Boehringer Mannheim #1243004) just prior to use.

16. Phenol/chloroform (1:1) saturated with Tris borate-EDTA (TBE) buffer.
17. Phosphate buffered saline (PBS; 0.1 *M*): 10 m*M* Na_2HPO_4 (Sigma S3264), 3 m*M* KCl (Sigma P9541), 1.8 m*M* KH_2PO_4 (Sigma P9791), 140 m*M* NaCl (Sigma S3014). Adjust pH to 7.4 and autoclave. Stable for 2–3 weeks at 4°C.
18. Reaction buffer (10X): 500 m*M* Tris-HCl, 50 m*M* $MgCl_2$, 500 µ*M* dATP, 500 µ*M* dGTP, 550 µ*M* dTTP, 50 µ*M* dCTP, 1% (vol/vol) mercaptoethanol. Adjust pH to 8.0, and store in 5-µL aliquots at –20°C.
19. Restriction enzymes: Boehringer Mannheim.
20. RNaseA solution: dissolve ribonuclease A (RNase A; bovine pancreas; Sigma #R5503; stock stored –20°C) in distilled H_2O at a concentration of 25 mg/mL. Store aliquots at –20°C. Boil aliquot for 1 min to remove DNase activity, and use at a final concentration of 25 µg RNase/mL.
21. Salmon sperm sonicated ssDNA: Sigma #D9156; stored as a 10-mg/mL solution at –20°C. Just before use, denature this solution at 100°C for 5 min, and then rapidly cool on ice.
22. SDS: 20% solution; Sigma #L4522.
23. Sephadex slurry: equilibrate Sephadex G25 powder (Sigma #G-25-80) in 1X TE buffer (pH 8.0) overnight, store slurry at 4°C.
24. SSC (20X): 3 *M* sodium chloride (Sigma #S3014), 0.3 *M* Tri-sodium citrate (Sigma #C8532), in distilled H_2O, adjust PH to 7.0, and autoclave. Stable for 2–3 wk at room temperature.
25. T4 polynucleotide kinase: (Boehringer Mannheim #174645).
26. Tris-buffered saline (TBS); (10X): 1.5 *M* NaCl, 1 *M* Tris-HCl. Adjust pH to 7.5 with HCl.
27. TBE (20X): 0.9 *M* Tris-borate, 0.08 *M* EDTA.
28. X-ray film: X-Omat AR film (Kodak CAT 1651512).

3. Methods

3.1. Probe Isolation and Labeling

Y-receptor-specific probes are normally isolated by polymerase chain reaction (PCR) using subtype-specific oligo nucleotides as primers and cDNA-carrying plasmids as templates (*see* **Note 1**).

*3.1.1. Y1 Receptor Probe Labeling (*see ***Note 2****)*

1. Place 25–50 ng of template DNA into a microcentrifuge tube and add 5 µL of hexanucleotide primer solution and the appropriate volume of water to give a total volume of 50 µL (*see* **Note 3**).
2. Denature the double-stranded DNA by heating for 5 min in a boiling water bath or 95–100°C heating block.
3. Spin the tube briefly in a microcentrifuge to combine the solution again.
4. Place tube on ice and add 10 µL of the reaction buffer, 5 µL of the appropriate radiolabeled α^{32}P-dNTP and 2 µL of Klenow enzyme (5 U/mL). Mix gently by

pipetting the solution up and down, and briefly spin in a microcentrifuge (*see* **Note 4**).
5. Incubate tube at 37°C for 10–30 min (*see* **Note 5**).
6. Stop the reaction by heating the sample for 5 min at 95–100°C, and then place on ice.

3.1.2. Y1 Receptor Probe Purification via Spun Column Procedure

See **Note 6**.

1. Plug a sterile 1-mL syringe barrel with glass fiber, packing it in place with the syringe plunger.
2. Slowly pipet 1 mL of Sephadex slurry into the column, taking care to avoid air bubbles.
3. Place the column in a screw-capped microcentrifuge tube, and spin this setup in a 15-mL disposable centrifuge tube at >1100*g* for 5 min.
4. Repeat the pipetting and spinning (**steps 2** and **3**) until the column content stabilizes at 1 mL of Sephadex G25.
5. Add a known volume (e.g., 100 µL) of water to the top of the Sephadex bed, place the column in a fresh microcentrifuge tube, and repeat **step 3**. The volume recovered should be equal to that added to the top of the column. Then the column is ready for use.
6. Add 50 µL of water to the labeled sample, and load the combined 100 µL onto the column. Place the column in a fresh microcentrifuge tube, and spin for 5 min at >1100*g*.
7. Measure the cpm values of the sample in a β-scintillation counter. The average cpm range of a sample prepared like this is about $2–5 \times 10^6$ cpm.
8. Store sample at –20°C (*see* **Note 7**).

3.2. Library Screening (17)

3.2.1. Checking for Crossreactivity of the Y1 Receptor Probe

To screen a high density P1 or BAC library successfully, it is important to check for crossreactivity of the probe first. Testing the probe against human genomic DNA, the P1 or BAC vector DNA, and a small test grid is recommended. Standard Southern blot analysis of restriction-digested human genomic DNA and restriction-digested P1 vector is also a good method. Restriction enzymes with a six-base recognition site such as *Eco*RI, *Bam*HI, or *Hind*III work well. If a high crossreactivity of the probe with the vector DNA or a high background in the genomic blot is observed, a smaller probe or a probe from a different portion of the gene should be considered. Alternatively, one can try to block the repeat elements with genomic and/or Cot-1 DNA competition. A

slight background, barely revealing the individual spots on the test grid, is acceptable and can actually assist in the positioning of positive signals.

3.2.2. Prehybridization

1. For hybridizing eight filters (20 × 20 cm), make up 225 mL of hybridization solution consisting of:
   6X SSC
   5X Denhardt's solution
   0.5% SDS
   100 mg/mL ssDNA.
   Denature this solution just before use by heating it at 100°C for 5 min, and then rapidly cool on ice.
2. Heat the solution to 65°C in a suitable container able to fit the filters properly. A shaking water bath or an incubation oven with a shaking platform is used for this purpose.
3. Add the filters singly, avoiding trapping air bubbles between them, and incubate for 2 h at 65°C (*see* **Note 8**).

3.2.3. Hybridization

1. Take the filters out of the hybridization solution, and add ^{32}P-labeled probe that has just been denatured by heating to 5 min at 100°C (*see* **Note 9**). For eight filters, a total of 400,000–600,000 cpm/mL is recommended, which normally equals two standard labeling reactions.
2. Add the filters back into the hybridization solution, again avoiding the trapping of air bubbles, and incubate overnight.
3. To remove nonspecifically bound probe, the following washes are carried out:
   300 mL of 2X SSC, 0.5% SDS at room temperature for 5 min.
   300 mL of 2X SSC, 0.1% SDS at room temperature for 15 min.
   300 mL of 0.1X SSC, 0.5% SDS at 37°C for 30 min.
   300 mL of 0.1X SSC, 0.5% SDS at 65°C for 30 min.
4. Place filters on Whatman filter paper to dry. Do not allow filters to dry completely. Otherwise, it will be difficult to carry out further washes (if necessary).
5. For autoradiographic detection, place filters between two pieces of plastic wrap in a cassette with two intensifying screens, and place X-ray film on top. Expose overnight at –80°C.
6. Store filters at –20°C (*see* **Notes 10** and **11**).

3.3. Clone Identification

As mentioned earlier, positive clones are clearly identifiable by the appearance of two discrete signals, oriented in a special way, within one square. A transparent replica of the grid is normally supplied by the manufacturer of the filters to assist in identification of the clone position on the autoradiogram (*see* **Note 12**). Two clones are normally enough for further analysis.

3.3.1. P1 Plasmid DNA Isolation

P1 clones are provided by the manufacturer as a bacterial stab culture. To isolate single clones, the bacteria must be streaked onto an agar plate containing 25 µg/mL kanamycin for selection and grown overnight at 37°C. P1 plasmids are normally maintained at one copy per cell; therefore a larger culture volume is required for the isolation of sufficient amounts of plasmid DNA.

1. Inoculate 30 mL of LB medium containing 25 µg/mL kanamycin with a single colony of bacteria, and incubate overnight at 37°C in a shaking incubator.
2. Collect the bacteria by centrifuging the culture at 10,000*g* for 5 min. Discard the supernatant.
3. Resuspend the bacteria in 1 mL of lysis buffer, transfer suspension to a 15-mL Falcon polypropylene tube, and incubate for 5 min at room temperature.
4. Add 2 mL of freshly prepared 0.2 *N* NaOH/1% SDS, seal the tube, invert the tube several times until the mixture becomes clear (do not vortex), and then place on ice for 5 min.
5. Add 1.5 mL of 3 *M* KAc pH 5.7, mix gently, and place on ice for 5 min.
6. Centrifuge for 5 min at 10,000*g*.
7. Transfer the supernatant to a new 15-mL Falcon tube, and add an equal amount of phenol/chloroform (1:1). Mix by repeated inversion, and centrifuge at 10,000*g* for 5 min.
8. Repeat step 7 twice.
9. Remove the aqueous phase, avoiding any contamination with the phenol/ chloroform, and place into a new falcon tube. Add precooled (–20°C) absolute ethanol to a final concentration of 70%, mix by inversion and centrifuge at 10,000*g* for 20 min at 4°C.
10. Remove the supernatant carefully, and wash the pellet with 3 mL of 70% ethanol. Centrifuge for 5 min at 10,000*g*.
11. Carefully discard the supernatant, and vacuum-dry the pellet until completely dry.
12. Resuspend the pellet in 100 µL of water containing 50 mg/mL of DNase-free RNase A. Transfer the DNA into a 1.5-mL microcentrifuge tube, and store at –20°C until further use.

3.4. Subcloning and Mapping

Direct sequencing of P1 plasmid DNA only works well using cycle sequencing. For exon/intron border determination and mapping, subcloning smaller fragments into a more suitable vector (e.g., pBluescript, pZero) is therefore recommended. This can easily be achieved by standard "shotgun" cloning of restriction enzyme-digested P1 plasmid DNA into the appropriate site of pBluescript or pZero. Six-base pair recognizing restriction enzymes such as *Eco*RI, *Hind*III, or *Bam*HI can be used to generate a small library of overlapping fragments. Cloning into the ampicillin-resistant pBluescript or the zeocin-resistant pZero vector avoids the survival of re-ligated kanamycin-resistant P1

plasmids. Individual white colonies can be picked and plasmid DNA isolated from them as described in **Subheading 3.3.1.** Alternatively, bacteria can be streaked out and screened with standard colony screening protocols, using specific probes (e.g., oligonucleotides) for a particular part of the cDNA, to obtain only those subclones that contain the portion of interest. Individual subclones are sequenced using standard techniques, and the sequences are compared with the cDNAs to determine the exon/intron borders.

3.5. Restriction Mapping With Oligonucleotide Probes

In this method the restriction map of large fragments subcloned from the P1 clone is determined by first digesting the plasmid with a rare cutting enzyme such as *Not*I or *Sfi*I to linearize the DNA, followed by partial digestion of the subsequent fragment with a more frequent cutting enzyme. The series of digestion products are then separated on an agarose gel, transferred to nitrocellulose, and probed with an oligonucleotide probe corresponding to one of the ends of the insert. The estimated difference in size of the hybridizing bands then corresponds to the size of the joining fragments. Maps for several different restriction enzymes can be obtained by this method; then they can be combined and verified by sequencing of the various identified fragments.

3.5.1. Transfer of DNA onto Hybond+

1. First soak the gel in 0.15 M HCl for 10 min to fragment the DNA. This aids in efficient transfer of DNA onto nitrocellulose. Agitate gently to ensure even exposure of gel to HCl. Alternatively, expose to ultraviolet (UV) light for 1 min. Caution must be used with the UV light approach, since the efficiency of nicking can decrease over time due to solarization of the filter. Efficiency of transfer should always be monitored by staining the gel following transfer.
2. If HCl is used, rinse gel with distilled H_2O to remove excess acid.
3. Denature DNA by soaking gel in 1.5 *M* NaCl and 0.5 *M* NaOH for 30 min.
4. Neutralize by soaking for 30 min in 1 *M* Tris-HCl, pH 8.0, containing 1.5 *M* NaCl.
5. Place gel on 3–4 sheets of Whatman filter paper slightly larger than the gel. Saturate with 6X SSC. Briefly wet nitrocellulose with water, cut to the size of the gel, and then soak in 6X SSC. Place nitrocellulose and 1–2 sheets of saturated filter paper carefully on top of the gel followed by a stack of paper towels and a light weight to keep all layers compressed. Surround the gel with parafilm to prevent siphoning of transfer buffer onto paper towels.
6. Transfer should be complete in 8 h. The efficiency can be checked by restaining the gel after transfer in 100 mL H_2O, 1 mg/mL ethidium bromide.

3.5.2. Labeling of Oligonucleotide Probes

Label oligos with fresh γ-^{32}P-ATP. High-specific-activity γ yields the best results.

1. Combine reagents as follows and perform the polynucleotide kinase reaction for 30 min at 37°C.
   2 µL 10X kinase buffer
   2 µL 10 m*M* ATP
   1 µL oligonucleotide (100 ng)
   1 µL polynucleotide kinase (10 U)
   14 µL H_2O
2. Incubate at 68°C for 15 min to destroy the enzyme.
3. Unincorporated nucleotides can be removed with a spin column as described in **Subheading 3.1.1.**

3.5.3. Hybridization with Oligonucleotides

In general, the same conditions for the hybridization with oligonucleotides can be used as for double-stranded probes, with the exception of a reduced temperature. The hybridization temperature depends strongly on the length and the GC content of the oligonucleotide. Several formulas for calculating the theoretical "melting" temperature of the double strand complex of oligos have been developed and can be used to estimate the optimal temperature. However, experimental condition might have to be adjusted on a case by case basis. More information on all standard molecular biology techniques can be found in Sambrook et al. ***(18)***.

4. Notes

1. Alternatively, DNA fragments of any particular Y receptor can also be isolated by restriction enzyme digests of plasmid DNA carrying the cDNA fragment and isolating the fragment after separation on an agarose gel.
2. Particular caution has to be taken in handling, storing, and disposing of radioactive material. Always wear gloves and safety glasses.
3. The reaction volume can be scaled up or down if more or less than 25 ng of DNA is to be labeled.
4. Avoid vigorous mixing of the reaction mixture, as this can cause severe loss of enzyme activity.
5. DNA can be labeled to high specific activity within 10 min at 37°C but can be labeled for longer periods of up to 3 h at the same temperature if an even higher level of labeling is desired.
6. Although probe purification is not always necessary for membrane applications, the removal of unincorporated label is recommended to reduce the background in filter hybridization experiments. Also, the use of a spin column is a quick and efficient way to achieve this goal.
7. Labeled probes should not be stored for long periods (maximum 3 days at –20°C): because of the short half-life of ^{32}P, substantial probe degradation can occur.
8. Incubation for shorter periods is possible particularly when the probe showed no signs of crossreactivity. However, a minimum of 30 min should be allowed. New

filters should be thoroughly soaked in hybridization solution and prehybridized for at least 2 h.

9. Place the filters on plastic foil, and proceed quickly to avoid cooling of the solution and filters.
10. The filters can be reused for hybridization many times. We have successfully used one set 18 times. However, in order to achieve this, one has to reduce handling of the filters by not stripping the filter between hybridizations. Repetitive hybridizations with different probes will show new hybridizing clones at each step.
11. Filters should be wrapped in plastic wrap and stored at –20°C between hybridizations. Before reusing filters, thaw them completely at room temperature prior to removing the plastic wrap.
12. Although identification of the position of positive clones is straightforward, obtaining a second opinion from a colleague to confirm the exact grid position is recommended.

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