# 11

# The Tetramethylammonium Chloride (TMAC) Method for Screening cDNA Libraries with Highly Degenerate Oligonucleotide Probes Obtained by Reverse Translation of Amino Acid Sequences

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## 1. Introduction

If an unknown protein is purified and available in relatively small amounts, it is possible to determine the sequences of short internal peptides *(1)*. In order to determine the whole sequence of the protein by cDNA cloning, one of the peptides of perhaps five to seven amino acids may be reverse translated into nucleotide sequence resulting in a 15–21 base long deoxyribonucleotide. Because of codon degeneracy, the number of possible oligonucleotides may be more than several hundred, which must be present in order to ensure that the correct sequence is represented. Only one of these oligonucleotides corresponds to the correct sequence. Traditionally long stretches of DNA are hybridized in buffered saline solution where physicochemical parameters affecting the annealing are well known *(2,3)*. However, for shorter DNA sequences, the melting temperature of each oligonucleotide depends on the G + C content, since G:C base pairs possessing three hydrogen bonds interact more strongly than A:T base pairs with two hydrogen bonds. The different oligonucleotides in a mixture will thus possess different melting temperatures. This means that in buffered saline solution, one usually chooses a melting temperature that is so low that the oligonucleotide with the lowest G + C content can hybridize. However, in doing so, it is possible that oligonucleotides with a higher G + C content may form stable hybrids with mismatches resulting in the cloning of artifact cDNAs. Even though this procedure has been used success-

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fully *(4–6)*, it is more convenient to use a different buffer type that contains tetramethylammonium chloride (TMAC), since it has been reported that this salt selectively binds to and stabilizes A:T base pairs so that their melting temperature becomes similar to that of G:C base pairs *(7–9)*.

The successful cloning of a large number of cDNAs shows that the technique works for 15- to 20-mer oligonucleotides with a degeneracy up to 512 and a G + C content between 27 and 61% in the cloned cDNA *(10)*. Although sequence-dependent stability of very short DNA sequences in TMAC have been reported recently *(11)*, such features might be averaged out in the longer oligonucleotides used here. Additionally, we have found that the TMAC technique works with oligonucleotides containing deoxyinosine as a neutral or slightly destabilizing base at highly ambiguous positions.

## 2. Materials

### 2.1. Plating of cDNA Library and Replica Lifts

1. Luria-Bertani (LB) medium: 10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, and LB-MM medium: LB medium supplemented with 2 g/L maltose and 2 g/L $MgCl_2$ for bacterial growth.
2. LB agar: LB medium with 15 g/L of agar and LB-MM top agar or agarose LB-MM medium with 7 g/L of agar or agarose for plating on screening plates
3. Saline magnesium (SM) buffer for diluting λ phages 100 m*M* NaCl, 10 m*M* $MgCl_2$, 50 m*M* Tris-HCl, pH 7.5.
4. 500 $cm^2$ Screening plates (cat. no. 240 835, Nunc, Roskilde, Denmark): 245 × 245 mm.
5. Nylon filters (Hybond-N, Amersham, Amersham, UK): 220 × 220 mm and MESH sheets to interpose between the filters in hybridization tubes.
6. Denaturation solution: 0.5*M* NaOH, 1.5*M* NaCl. Store at room temperature.
7. Renaturation solution: 0.5*M* Tris-HCl, 1.5*M* NaCl, pH 7 5. Store at room temperature.
8. 20X SSC: 3.0*M* NaCl, 0.3*M* sodium citrate. Store at room temperature

### 2.2. Labeling and Purification of Oligonucleotide

1. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 0.1 m*M* EDTA, pH 8.0. Store at 4°C.
2. T4 polynucleotide kinase (Amersham), and 10X kinase buffer: 500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$, 50 m*M* DTT, 1 m*M* spermidine HCl, 1 m*M* EDTA, pH 8.0. Store aliquots at –20°C.
3. [γ-$^{32}$P]Adenosine-5'-triphosphate (ATP) (ICN Radiochemicals, Irvine, CA), 7000 Ci/mmol, 167 μCi/μL, 24 pmol ATP/μL.
4. Formamide/dye mix: 10 m*M* EDTA, 0.5 g/L bromphenol blue, 0.5 g/L xylene cyanol in deionized formamide. Store at –20°C.
5. 10X TBE buffer: 1*M* Tris, 1*M* borate, 0.01*M* EDTA, pH 8.0. Store at room temperature.
6. 20% Polyacrylamide/7*M* urea: mix 25 mL of 40% polyacrylamide (380 g/L acrylamide, 20 g/L bis-acrylamide), 21 25 g urea, 5 mL 10X TBE buffer, and add water to 50 mL.

7. TEMED (20–25 µL) and 10% ammonium persulfate (APS) (200–250 µL).
8 Kodak X-ray films, Saran Wrap™, $NaBH_4$ (10%), plastic bags.

### 2.3. Hybridization of Oligonucleotide

1. TMAC (Fluka [Buchs, Switzerland] or Merck [Darmstadt, Germany]): 5*M* in water (*see* Note 1) Store at room temperature. Note that this chemical is hazardous with a strong odor and should only be handled in a fume hood.
2. Yeast RNA (Boehringer Mannheim, Mannheim, Germany). 100 mg/mL in water (*see* Note 2). Store at –20°C.
3. 100X Denhardt's solution: 20 g/L bovine serum albumin (BSA), 20 g/L Ficoll 400 (Pharmacia, Uppsala, Sweden), 20 g/L polyvinylpyrollidone. Store at –20°C.
4 Prehybridization buffer. 6X SSC, 5X Denhardt's solution, 20 m*M* sodium phosphate, pH 7 0, and 1.5 mg/mL yeast RNA. Store at 4°C.
5. Hybridization buffer: 3*M* TMAC, 5X Denhardt's solution, 50 m*M* sodium phosphate, pH 7.0, 1 m*M* EDTA, 0.6% sodium dodecyl sulfate (SDS) and 0.25 mg/mL yeast RNA. Store at room temperature.
6. Washing buffer 1: 3*M* TMAC, 50 m*M* Tris-HCl, pH 8 0, 0.2% SDS.
7 Washing buffer 2· 2X SSC, 0 1% SDS.
8 2X YT-medium: 16 g/L peptone, 10 g/L yeast extract, 10 g/L NaCl.
9. Petri dishes (90 mm in diameter, Nunc) with LB agar supplemented, after autoclaving and cooling to 45°C, with 100 µg/mL of ampicillin for growth of bacteria containing pBluescript plasmids with ampicillin resistance.

## 3. Methods

### 3.1. Plating of cDNA Library and Replica Lifts

The library used should contain the cDNA encoding the protein to be cloned. The first choice is a cDNA library made with RNA purified from the same cell line or tissue as has been used to sequence the protein, although other cDNA libraries may be used (*see* Note 3).

1. Plate the library (e.g., λZAP) at a density of up to $1.5 \times 10^5$ PFU/screening plate (245 × 245 mm with 0.25 L of LB agar) using an overnight culture of *Escherichia coli* XL-1-Blue or LE392 and 30 mL of LB-MM top agar or agarose, which is allowed to set for 30 min. The plates are then incubated at 37°C overnight.
2. On the next day, place the plates for 1 h at 4°C (*see* Note 4). Plaque transfer to filters, denaturation, and renaturation are performed largely as described in the blotting and hybridization protocols for Hybond™ membranes from Amersham. In detail, one nylon filter (first replica filter) is marked with a few lines with India ink defining a unique pattern in a band from the edges of the filter and about an inch to the center, and then placed on the plate for 1 min. The pattern of lines are then marked on the reverse side of the plate with India ink. The filter is carefully lifted off the plate, placed with the plaque side up at filter paper, and a second filter (second replica filter) is placed on the plate Now the line pattern from the plate is marked on the filter with India ink, and the filter is left on the

plate for 3 min or more. The filters are then placed, still with the plaque side up, on top of filter paper, lightly soaked with denaturation solution and left for 7 min. The nylon filters are then transferred to filter paper lightly soaked with renaturation solution for 3 min. This step is repeated, and finally the filters are washed in 2X SSC, air-dried, and crosslinked with UV light from a transilluminator for 1.5 min on each side. For long-term storage of agar plates, *see* Note 5

### 3.2. Labeling and Purification of Oligonucleotide

The amino acid sequence of the peptide is reverse translated to the fully degenerated nucleotide sequence, and a suitable oligonucleotide is selected. The principles for the selection of the best possible oligonucleotide are described in Note 6.

1. Label 20–100 pmol of oligonucleotide in a volume of 10 µL by mixing the oligonucleotide with 1 µL of 10X kinase buffer, 3–5 µL of [$\gamma$-$^{32}$P]ATP (500–835 µCi, 72–120 pmol), TE buffer to 10 µL, and finally 1 µL of T4 polynucleotide kinase (10 U) The mix is incubated at 37°C for 1 h (*see* Note 7)
2. The labeled oligonucleotide may be purified from the unlabeled on a polyacrylamide/7*M* urea gel Add 12 µL of formamide/dye mix to the labeling mix, then heat to 95°C for 3 min, and load on a 20% polyacrylamide/7*M* urea gel, which has been prerun with 1X TBE buffer at 18 W for 20 min prior to loading
3. Run the gel at a maximum 18 W for a few hours until the blue color disappears Dismount the gel from the glass plates and cover with Saran Wrap™ A few pieces of filter paper are taped to the gel and lightly soaked with 10% $NaBH_4$
4. Expose an X-ray film for 5–10 s and develop. One strong band that represents the labeled oligonucleotide should be present This band can then be cut out from the gel with a scalpel by superimposing the X-ray film, which contains marks from the filter paper soaked with $NaBH_4$
5. Place the gel piece in an Eppendorf tube with 1 mL hybridization buffer and shake. In order to extract the labeled oligonucleotide from the gel, change, save, and pool the buffer several times. The efficiency of the extraction can be checked with a monitor.

### 3.3. Hybridization of Oligonucleotide

1. Interpose the nylon filters between MESH sheets and incubate in prehybridization buffer for 1–2 h Replace the prehybridization buffer with fresh buffer and incubate for a further 1–2 h (*see* Note 8).
2. Wash the filters thoroughly four times for about 15 min in hybridization buffer and incubate overnight or longer in hybridization buffer together with the labeled oligonucleotide at the calculated hybridization temperature, which depends on the length of the oligonucleotide (*see* Note 9)
3. Pour off the hybridization buffer with labeled probe and save at –20°C (*see* Note 10), then wash the filters in washing buffer 1 at the hybridization temperature with four exchanges each for 15 min and then follow by 1–2 h of incubation.

4. Wash the filters two times for 2 min in washing buffer 2 at room temperature and seal in plastic bags. The marks on the filters are drawn up with radioactive ink, and autoradiography is performed at –70°C with intensifying screens for 24 h to a few weeks
5 After development of the films, they are aligned and stronger spots appearing on both replica films (usually strongest on the first replica) likely represent correct hybridizing clones. Cut out about 1 $cm^2$ or less of the agar/agarose and put in Eppendorf tubes for each positive clone. Add 300 µL of SM buffer, crush the agar/agarose with a glass rod, and shake the tube for 15 min. Dilute 1 µL from this solution with 1500 µL of SM buffer, and plate 1, 10, and 100 µL together with 300 µL of bacteria and 3 mL of LB-MM top agar on smaller Petri dishes Put nylon filters on these as previously described. It is usually not necessary to use replica filters, but it may be advantageous for the beginner.
6 Well-isolated positive plaques may be used for *in vivo* excision of pBluescript plasmids with the cDNA insert from the λZAP phages Cut out the plaque from the plate with a scalpel and place in an Eppendorf tube. Add 300 µL of SM buffer, crush the agar/agarose with a glass rod, and shake the tube for 15 min.
7 Perform self-excising of the pBluescript plasmid from the phage DNA as described by Stratagene by mixing 100 µL of the λZAP phages, 200 µL of XL-1 Blue bacteria, and 1 µL of the ExAssist helper phage in a 50-mL tube, incubated at 37°C for 15 min. Add 2X YT medium and grow the mixture in a shaking incubator at 300 rpm for 2–2.5 h. Heat the contents to 70°C for 20 min, centrifuge, and save the supernatant containing the pBluescript plasmid, packaged as filamentous phage particles, at 4°C. Mix 1 µL of the phage supernatant with 200 µL of SOLR bacteria incubated at 37°C for 15 min and finally plate on Petri dishes with agar containing 100 µg/mL of ampicillin. After overnight growth, the colonies may be transferred to Hybond-N filters in a similar way to that described for plaque transfer (Section 3 1., step 2). After the colony transfer, the Petri dishes should be grown again at 37°C for some hours until the colonies are again visible by the naked eye. Plasmid preparations may then be prepared by growing positive colonies in larger scales by standard procedures.

## 4. Notes

1. The TMAC is hygroscopic, so the water is added directly to the bottles to dissolve the powder The solution is then filtered to remove undissolved material, and water is finally added to bring the concentration to 5*M* by taking the weight as indicated on the bottles. We do not find it necessary to determine the actual concentration by measuring the refractive index of the solution as used in some protocols.
2 The yeast RNA blocking solution is made by dissolving 10 g RNA in 50 mL 0.3*M* sodium acetate (pH 6.0). This solution is extracted twice with 0.8 vol of phenol and twice with 0.8 vol of chloroform (note that the lower phase is the RNA-containing phase). Precipitation is performed by adding 2 vol of ethanol. The solution is centrifuged, the pellet dried, redissolved in 50 mL 0.3*M* sodium

acetate (pH 6.0), and 2 vol of ethanol are added for reprecipitation. After centrifugation, the pellet is washed several times with ethanol/water (80/20, v/v), dried, and dissolved in water at a concentration of 100 mg/mL as measured by the UV light absorbance at 260 nm.

3 The first choice is to use a cDNA library made with mRNA purified from the tissue or cell line from which the amino acid sequencing results have been obtained. However, the same protein may be expressed in other cell lines or tissues as well The presence of a protein in a cell line or a tissue may be analyzed by two-dimensional gel electrophoresis, separating the proteins in the first dimension according to pI and in the second dimension according to molecular mass *(12)*. Two proteins from two different sources that migrate to the same position in the gel may indicate that they represent the same protein or are closely related proteins. This can be further confirmed by reaction with a specific antibody against the protein (immunoblotting) or cutting of the Coomassie brilliant blue-stained spot from the dried 2D gel for amino acid microsequencing *(1)*.

4. Bacterial plates, especially those made with top agar, are placed at 4°C in order to solidify the agar further Without this step, a part of the agar with plaques may stick to the nylon filter with the result that the plaques in that area are lost

5 The agar plates may be stored for a few days at 4°C For long-term storage (years), the plates can be put directly at –70°C. The filters can then be stored at –20°C and reused for screening with other oligonucleotides later or immediately if the probe is first stripped off the filters, e.g , by adding water that is boiled, slightly cooled, and brought to 0.1% SDS (blotting and hybridization protocols for Hybond™ membranes, Amersham). The filters should never dry out.

6. The oligonucleotide used to screen the library should in principle be as long as possible and as little degenerated as possible We have successfully used oligonucleotides with lengths from 15–20 bases and up to 23 for inosine-containing oligonucleotides *(10)*. In order to make the oligonucleotide as little degenerated as possible, it may be advantageous to terminate the oligonucleotide at codon position 2, since the third codon position usually is ambiguous. If possible, avoid oligonucleotides reverse translated from peptides with Leu, Arg, or Ser, since the codons for these amino acids are degenerated at two or all three positions. We have successfully used oligonucleotides with a degeneracy up to 512 *(10)*. If all oligonucleotides are equally represented in this mixture, the concentration of each different oligonucleotide will be <0.2% of the total concentration. If one or a few nucleotides are unintentionally preferred at the ambiguous positions during the synthesis, the mixture may become "biased" at some of the ambiguous positions, so that the correct oligonucleotide may be underrepresented in the mixture. This means that the concentration of the correct oligonucleotide may be even lower than 0.2% of total. It is important to keep in mind that the quality of the oligonucleotide preparation is crucial for a successful result. We have found that different batches of oligonucleotides that should recognize the same cDNA sequence may in fact hybridize to the plaques with very different strengths and some of them very poorly indeed. Thus, if one batch of oligonucleotides works

unsuccessfully, it may be worth trying one or two other batches for screening, perhaps from another supplier.

7. The equilibrium constant for the labeling reaction:

$$[\gamma\text{-}^{32}\text{P}]\text{-ATP} + 5'\text{-oligonucleotide} \leftrightarrow \text{ADP} + [^{32}\text{P}]\text{oligonucleotide} \quad (1)$$

is about unity *(13)* The specific activity of the labeled oligonucleotide can be increased by increasing the concentration of [γ-$^{32}$P]-ATP, whereas the amount of transferred [$^{32}$P] to the oligonucleotide can be increased by increasing the oligonucleotide concentration. The unlabeled oligonucleotide will hybridize as strong as the labeled one, and it is therefore necessary to purify the labeled oligonucleotide, e.g., on a denaturing polyacrylamide gel where the labeled oligonucleotide that contains a 5'-phosphate will move faster than the unlabeled oligonucleotide

8. The prehybridization buffer is used to block the filters with RNA in order to get a low background for the following autoradiography, which may be performed for several days or weeks. The blockage does not work sufficiently in TMAC and is therefore performed in saline sodium citrate.
9. The hybridization temperature of the oligonucleotide is determined as follows. First the irreversible melting temperature is calculated as described by Jacobs et al. *(9)*:

$$T_i = -682 \times (L^{-1}) + 97°\text{C} \quad (2)$$

where $L$ is the number of nucleotides in the oligonucleotide and $T_i$ is the irreversible melting temperature The hybridization temperature, $T_h$, is usually about 10°C below the $T_i$. Thus, for a 15-mer oligonucleotide, the $T_i$ is calculated to 52°C and $T_h$ is accordingly 42°C. If the oligonucleotide used contains inosines, these should be regarded as neutral or slightly destabilizing, e.g., a 20-mer oligonucleotide with three inosines is regarded as a 17-mer or shorter when calculating $T_h$

10. The oligonucleotide probe may be reused for the purification of plaques if performed within 1 or 2 wk. The half-life of the probe is 14 d.

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