METHODS:

2.1 Media and Solutions

All % shown are on a w/v basis (unless mentioned otherwise)

GPK : 0.5% Glucose, 0.5% Peptone, 0.3% KH$_2$PO$_4$.

YPD : 2% Glucose, 2% Peptone, 1% Yeast Extract

YPD Agar : YPD broth with 2% agar

LB : 1% Tryptone; 1% Sodium chloride, 0.5% Yeast Extract;
    pH adjusted to 7.5 with NaOH.

LB Agar : LB with 1.5% agar (Hi-Media, Bombay).

Important: For phage growth agar varieties from Hi-Media were not suitable; only Bacto-agar (Difco) was used for growth of phages.

Media with Ampicillin/Maltose:

    LB or LB agar when indicated contained
    50 µg ampicillin/ml medium
    Maltose concentration in the medium was 0.4%

Top Agar: LB containing 0.7% Bacto-agar (Difco)

Endo.R : 6x = 30% Ficoll 400, 60 mM EDTA, pH 8, 0.6% SDS,
        0.06% bromophenol blue.

SM, SSC, SSPE, Denhardt's Solution: Composition and preparation as described in Maniatis et al. (1982).

Phenol : Glaxo Laboratories Excel AR Grade was redistilled at 180° and stored frozen at -20° in small parts.

Laemmli Buffer : 3x = 0.1875 M Tris-Cl pH 6.8, 15% (v/v) β-mercaptoethanol, 6% SDS, 30% Sucrose, 0.006% bromophenol blue.
2.2 Breakage of \textit{C. albicans} Cells

1. Large scale—for purification: Weight of the cell pellet was determined and equal volume of the breakage buffer (50 mM K-phosphate pH 7.5, 1 mM each of β-ME, EDTA, and PMSF) was added. Equal volume of dry glass beads (washed with HCl and rinsed thoroughly with water and dried) were added to the cell suspension. Cell suspension with glass beads was cooled on ice and were vortexed vigorously in 1 min bursts at room temperature and then cooling on ice intermittently. Each tube was vortexed for 5 min (5x1 min bursts). The cell homogenate was spun at 5000g for 10 min at 4°C, and supernatant was recovered. Cell debris with the glass beads was reextracted with about 20-30 ml homogenizing buffer and pelleted as above.

2. Small scale—For enzyme assay and SDS-PAGE analysis: Small volume of cells was homogenized in microfuge tubes as described (Carter et al., 1987) using buffer containing 60 mM Tris-Cl pH 6.8, 1 mM each of β-ME, EDTA, PMSF.

2.3 Deaminase assay

Deaminase was assayed using a coupled enzyme system as described (White and Pasternak, 1975) with some modifications.
In a final volume of 1 ml, the following components were added at the indicated concentrations: 40 mM K-phosphate buffer pH 7.6, 1 mM D-glucosamine-6-phosphate (free acid, Sigma), 0.2 mM NADP (Sodium salt, Sigma), 8 units phosphoglucone isomerase (Sigma), 3 units glucose-6-P dehydrogenase and the sample. After all the components were added, crude extracts, or enzyme from various steps of purification were added and reaction initiated. The amount of sample used in the assay was controlled such that the change in absorbance at 340 nm was within 0.5. 1 ml disposable polystyrene cuvettes (Sigma) were used for the assay and the course of the reaction was monitored by increase in the absorbance at 340 nm due to the formation of NADPH. Reaction was done at room temperature between 25-30°. For calculation of NADPH concentration from the A_{340} values, the following relation was used: 1 µmole NADPH at 340 nm gives 6.22 absorbance units.

2.4 Protein Estimation

Protein was estimated by coomassie blue dye-binding method (Bradford, 1976) using bovine gamma globulin (BioRad) as standard. A commercial dye reagent (BioRad) was used when specific activity of deaminase had to be determined. But for routine work, a home-made reagent was used containing
coomassie blue G250 (Merck, Germany) as described (Bradford, 1976).

2.5 pH and Conductivity Measurement

pH values were measured either in a Beckman pH meter equipped with an automatic temperature compensation probe (Beckman) or in a Globe pH meter, using pH standard buffers (Beckman).

Conductivity of KCl gradient fractions were measured in a conductivity meter using KCl standards prepared in same buffer as sample. From a plot of conductivity of standard KCl and concentration, the KCl concentration in the sample was estimated.

2.6 Electrophoresis of Proteins

2.6.1 SDS-PAGE: Electrophoresis were done using a discontinuous buffer system, essentially as described by Laemmli (1970). The concentrations of TEMED and ammonium persulfate were adjusted such that gels polymerized in about 30 min. Gel stock solution contained 30% (w/v) acrylamide and 0.8% (w/v) bisacrylamide.

2.6.2 Non-denaturing PAGE: Polyacrylamide gel electrophoresis under non-denaturing conditions was done as per Chrambach (1985). Ammediol buffer system was used with
trailing phase pH 10.5 at 0°C and a trailing ion mobility of 0.064.

4x leading phase buffer = 5.4 g ammediol (2-amino-2-methyl-1,3-propanediol, Sigma) per 100 ml solution. pH was adjusted to 8.2 at 25°C with HCl.

Gel composition: **Separating Gel** = 7.5% (w/v) total acrylamide (stock 40% Total acrylamide, 5% (w/v) cross-linker, Bis-acrylamide), 1xleading phase buffer, 0.06% (v/v) TEMED, 1.8% (w/v) ammonium persulfate and appropriate volume of water. **Stacking gel** = 4% (w/v) Total acrylamide (Stock 40% (w/v) Total acrylamide, 20% cross-linker, bis-acrylamide), 1xleading phase buffer, 0.1% (v/v) TEMED, 1.8% (w/v), ammonium persulfate and appropriate volume of water. **Catholyte** = 2.06 g gamma-amino butyric acid, 100 ml 1 N KOH per litre, Adjust pH at 25°C = 10.5 **Anolyte** = 2.1 g ammediol, 10 ml 1N HCl per litre, Adjust pH to 8.8 using KOH at 25°C. 20 µl purified deaminase (1 mg/ml) TSEM pH 5.7) was mixed with equal volume of catholyte and then glycerol was added to 10% (v/v) and bromophenol blue to 0.02% (w/v) and loaded onto the gel. When the dye front reached three-quarters the length of the gel, run was terminated. One lane with deaminase, that was meant for staining was cut out, was stained with a rapid gel stain (Reisner, 1984). A parallel lane containing deaminase was aligned to the
stained gel and the gel corresponding to the stained bands were cut out and gel pieces soaked in TSEM pH 5.7 overnight at 4°C. Deaminase was assayed in the supernatant.

2.6.3 Isoelectric focussing: IEF was done as described (Pharmacia, Isoelectric focussing manual) using 3.5-10 ampholine. Gel solution contained 5% (w/v) T and 15% (w/v) cross-linker (DATD was used in place of bis-acrylamide). Anolyte was 40 mM (DL)-Aspartic acid and catholyte was 0.1 M NaOH. Focussing was done at 10 Watts constant power. Most of the gel was stained and destained (Pharmacia, Isoelectric focussing manual). For determining pH gradient, gel was sliced into 1 cm portions from an unstained gel, allowed to diffuse into 3 ml 10 mM KCl and pH was measured. From a plot of mobility versus pH, the pI of stained deaminase was estimated.

2.7 Purification of Glucosamine-6-phosphate Deaminase

Requirements:
1. Homogenizing buffer: 0.05 M Potassium phosphate pH 7.6, 1 mM EDTA, 1 mM β-mercaptoethanol and 1 mM PMSF.

2. 4 M Acetic acid pH 3.5: 4M acetic acid was prepared by diluting 11.4 ml of stock glacial acetic acid (17.5 M) to 50 ml and pH was adjusted to 3.5 at 30°C with KOH.
3. TSEM buffer pH 5.7 = 0.015 M succinate, 1 mM EDTA, 1 mM \( \beta \)-mercaptoethanol. pH was adjusted to 5.7 at 5\( ^\circ \) with Trizma-base. For three litres buffer, 1.1g Na\(_3\)EDTA was dissolved in water and 5.3g succinic acid and 9.8g Trizma-base were added and dissolved. After equilibrating the buffer at 5\( ^\circ \) pH was checked and if needed it was adjusted to 5.7 with either solid succinic acid or Trizma-base. pH meter was calibrated as described (Section 2.5). Just before use \( \beta \)-mercaptoethanol was added to 1 mM.

4. PEG 8000 (Sigma): PEG was dissolved in TSEM pH 5.7 to give a 50\% (w/v) solution.

5. TEM buffer pH 8.8 = 0.02 M Tris-Cl, 1 mM EDTA, 1 mM \( \beta \)-mercaptoethanol. To make four litres, 5.3g Trizma-base, 5.7g Trizma-HCl and 1.4g Na\(_3\)EDTA were dissolved. Buffer was equilibrated to 5\( ^\circ \) and pH adjusted to give 8.8 at 5\( ^\circ \). pH meter calibration is described elsewhere (Section 2.5). \( \beta \)-mercaptoethanol was added to 1 mM before use.

All steps were carried out below 10\( ^\circ \), either on ice or in a cold room.

2.7.1 Large-scale Growth and Induction

\textit{C. albicans} SC5314 was inoculated into 1\% (w/v) glucose, 0.5\% (w/v) peptone, 0.3\% (w/v) KH\(_2\)PO\(_4\) medium and was grown
<table>
<thead>
<tr>
<th>Strain/Vector</th>
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<tr>
<td>C. albicans SC5314</td>
<td>a leu2-3 leu2-112 his4-519 can1</td>
<td>DR Kirsch, USA</td>
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<td>S. cerevisiae AH22</td>
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<td>Y. Koltin, Israel</td>
<td>Rosenbluh et al., 1985</td>
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</tbody>
</table>
for 18 h at 30° with shaking at 200 rpm. Then the inoculum was transferred to growth medium (0.5% (w/v) glucose, 0.5% peptone and 0.3% (w/v) KH₂PO₄) to give about 2% (v/v) final concentration of the inoculum. From a starting OD₅₉₅ 0.08-0.14, the culture reached an OD₅₉₅ of 1.4-1.5 in 8-10h. Routinely large batches of 9-15 litres were cultivated in a fermentor (BioEngineering, Sweden) with stirring at 300 rpm and an aeration of 2 litres/min. Cells were harvested in a continuous flow centrifuge (CEPA). Cell pellet was directly resuspended in the induction medium (0.5% (w/v) GlcNAc, 0.3% (w/v) KH₂PO₄) at 1/5 the culture volume dispensed in 500 ml erlenmeyer flasks and rotated at 30°, 3h. After induction cells were pelleted, washed with 0.3% (w/v) KH₂PO₄ solution and cell pellets were stored at -20° until use. Typical yield of cells was about 3g wet weight cells per litre of culture.

2.7.2 Crude Extract

C. albicans SC5314 cells induced with GlcNAc were then homogenized with glass beads (Section 2.2). The homogenate was first sedimented at low speed 5000xg, 10 min, 4°C and supernatant was aspirated. The pellet containing debris and glass beads was reextracted with about 20 ml homogenizing buffer and centrifuged as described above. The combined supernatants were then centrifuged at 15000xg for 30 min.
2.7.3 Acid Precipitation

Beaker containing crude extract (pH 6.4) was kept on ice-water bath and constantly stirred using a magnet; pH was adjusted to about 4.7-4.8 by drop wise addition of 4M acetic acid (acetic acid pH adjusted to 3.5 with KOH). Localized changes in pH of the extract was avoided by continuous stirring. The extract was then incubated in the water bath for 30 min to let the precipitation of proteins to go to completion. Then it was spun at 15,000xg, 20 min to pellet the coagulated proteins. The supernatant containing deaminase was recovered.

2.7.4 PEG Precipitation

To the supernatant from step 2, PEG 8000 was added to give a final concentration of 5% (w/v), mixed and incubated on ice for 30 min. Precipitate was spun at 15,000xg, 20 min. In a pilot experiment it was established that > 95% deaminase was recovered in the supernatant. The 5% (w/v) PEG supernatant was then made up to 25% (w/v) by adding 50% (w/v) PEG solution and incubated on ice. Deaminase in the precipitate was collected by centrifugation at 15,000xg, 20 min. The viscous supernatant was discarded. At this stage, a very sticky, reddish-yellow colored pellet was obtained. It was gently resuspended in TSEM with a glass rod and it
went into solution in about 1-2 h. At this stage, the sample was clarified again by centrifugation as above. The supernatant was collected and from the pellet residual enzyme was extracted again as described and the two supernatants were combined.

2.7.5 CM-Cellulose Chromatography

CM-cellulose was equilibrated to TSEM, pH 5.7 by combining with 3 volumes of 0.2M Tris-succinate, pH 5.7 and then equilibrated with at least 5 column volumes of TSEM after packing in a glass column (2.6x20 cm bed). About 400 mg of protein from step 3 was applied to the column and was washed with TSEM until no more protein was eluted. About three column volumes of TSEM was passed such that all unbound and non-specifically adsorbed proteins are washed off. The bound proteins were eluted with a linear gradient of 0 to 0.2 M KCl in TSEM (300 ml each) total about at a flow rate of 20 ml/h. Five ml fractions were collected and A$_{280}$ nm was found. Fractions were assayed for deaminase (Section 2.3). Salt concentration of fractions were determined by measuring conductivity of the fractions (Section 2.5). Fractions showing deaminase activity were pooled.
2.7.6 Dialysis of CM-cellulose Fractions

The pooled fractions were dialyzed initially against approx. 20 volumes of TEM pH 8.0 with constant stirring, for about 10 h; then two changes each of 10 volumes of TEM, pH 8.8 were made and dialyzed for about 5 h each. pH of the samples after dialysis was 8.2 at 4°. Then the sample pH was adjusted to 8.8 at 4° by adding solid Trizma base.

2.7.7 DEAE-Sepharose Column Chromatography

DEAE-sepharose (Pharmacia) was equilibrated in 10xTEM buffer, pH 8.8, packed (2.6 x 20 cms bed) and then equilibrated with 5-6 column volumes of 1xTEM buffer, pH 8.8. The dialyzed sample from the above step, usually about 50 ml (Fig. 5) was loaded at a flow rate of 15 ml/h. The conditions for adsorption of deaminase to DEAE-Sepharose has been optimized such that the enzyme is just bound and eluted early in the salt gradient (at 30 mM KCl). Routinely, a pilot small-scale DEAE-Sepharose column (2-3 ml) equilibrated as above was run with a small part of dialyzed sample, washed with TEM, pH 8.8, and eluted with 0.2 M KCl. Assays of loaded sample, wash and 0.2 M KCl eluent were done. If the sample had adsorbed to the column, then the conditions were maintained exactly for a large scale chromatography. The sample volumes used for pilot-
scale and large-scale did not matter for adsorption of the protein. Then the column was washed with about one column volume of TEM and then deaminase was eluted with a linear 0-0.2 M KCl (in TEM) gradient. 2.5 column volumes each of high and low limit of the gradient was used at a flow rate of 25 ml/h. 5 ml fractions were collected; proteins were monitored by absorbance at 280 nm and fractions were assayed for enzyme activity. Salt concentration of the fractions were estimated as described (Section 2.5). Fractions containing deaminase, about 50 ml (Fig. 5), were pooled. After the gradient elution the column was washed with about 2 column volumes of 2 M KCl. The column was washed with TEM and stored in 20% ethanol in TEM. For reuse the column was equilibrated as described above; no further processing was needed.

2.7.8 Concentration of DEAE-Sepharose Fractions

pH of the pooled fractions was readjusted to about 5.6 by adding solid succinic acid and loaded onto a 2 ml CM-cellulose column (conditions used were same as that for step 4), at a flow rate of 10 ml/h and deaminase was eluted with 0.2 M KCl in TSEM pH 5.7. Active fractions were pooled and concentrated using a Centricon-10 as described by the manufacturer (Amicon Corporation, USA). Purified enzyme was
stored in TSEM pH 5.7 in refrigerator, for more than one year without any degradation.

2.8 Raising of Anti-Deaminase Antibody in Rabbit

About 200 ug purified deaminase in about 1 ml was emulsified with equal volume of Freund's complete adjuvant (Difco) by repeated passage through a syringe fitted with 18 gauge needle. After several passes, when the consistency of the mixture had changed, a drop was placed over water; if emulsion had formed, the drop would float on water and remain intact for several hours. Then using a syringe with 23 gauge needle, the emulsion was injected into 20-25 sites through intradermal/subcutaneous routes on the back of the rabbit.

After about three weeks, a booster was given as above with 100-150 ug deaminase emulsified with Freund's incomplete adjuvant. After two weeks a similar booster-injection was given. One week after the last booster, a small volume of blood was withdrawn using 26 gauge needle, through marginal or mid ear vein. The blood was allowed to clot and serum processed as described in Garvey et al. (1977). The titer of antibody was monitored using double immunodiffusion technique as described below. When sufficient titer was seen, large volume 20 ml blood was collected, serum
recovered and one part stored at 0 in presence of 0.1% (w/v) sodium azide). The rest of the serum was stored in small parts at -70°C.

**Double Immunodiffusion Technique:** Double immunodiffusion was performed as described (Garvey et al., 1977). About 5 ug deaminase in 10 ul was placed in the central well. 10 ul each of two-fold serially diluted antiserum was placed in the surrounding wells and incubated 8-10 h at 37°C in a humid environment. The precipitate line was viewed against light over a dark background.

**2.9 Affinity-Purification of Antibody**

About 0.075 mg purified deaminase was separated in a 10% SDS-PAGE and protein transferred to nitrocellulose membrane. Deaminase band on the blot was visualized by Ponceau S staining, and the band was cut out and destained. The antigen-bound nitrocellulose membrane was washed with TBST, and non-specific sites were blocked with 5% (w/v) milk in TBST for 60 min with shaking. The blot was washed thrice for 10 min with 1 ml TBST. Deaminase antiserum 125 ul was diluted 4-fold with TBS and the antigen-bound membrane was added and antigen-antibody reaction was allowed to proceed for about 8 h at 15-20°C.
Antiserum was recovered and the blot was washed thrice for 10 min with 1 ml TBST. Bound antibody was eluted with 0.2 ml 0.2M glycine-HCl pH 2.5 containing 0.2% (w/v) BSA by gentle shaking for 2 min. The eluent was recovered and combined with an equal volume of 1 M K-phosphate pH 9, containing 0.2% BSA. The blot was rinsed once again with elution buffer and the two pools combined. The filter was washed with 0.4 ml TBST, recovered and combined with the eluted antibody. Volume was made upto 1 ml using TBS and stored at 4°C after addition of sodium azide to 0.05% (w/v).

2.10 Western blot

After SDS-PAGE, gel was equilibrated in Towbin’s buffer (Towbin et al., 1979) containing 25 mM Tris-base, 192 mM Glycine and 20% (v/v) methanol, for 30 min. A pre-wet nitrocellulose membrane of required dimension was placed over the gel and transferred in a small-format electrotransfer apparatus (mini Transphor, Hoefer, USA) as suggested by the manufacturer using Towbin’s buffer for about 5 hours at 150 mA constant current at 15°C. After transfer, blot was removed and washed with water and proteins visualized by staining in Ponceau S as described.
2.11 Immunodetection with Deaminase Antibody

The procedure for immunodetection of the plaque lifts and of Western blots was basically similar, but for a few initial steps:

**Plaque lifts:** Nitrocellulose circles were washed with TBS, three times, 10 min each, with shaking to remove any agar sticking to the filters. They were then blocked as described below.

**Western blots:** After electro-transfer of proteins from SDS-PAGE to nitrocellulose, the filters were thoroughly washed with water, to remove any acrylamide pieces sticking to the filters. Then the transferred proteins were visualized by reversible staining of NC with Ponceau S (Salinovich and Monteharo, 1986) and destained in 1% (v/v) acetic acid. The position of marker proteins were marked on the blots and dye was stripped off the proteins by washing in TBS. (i) The filters were then blocked as follows: Blocking: Nitrocellulose filters were blocked routinely in 5% (w/v) non-fat dry milk (Amersham) in TBST plus 0.1% sodium azide, 8-10 h in a refrigerator.

(2) Blots were washed three times, 10 min each in TBST with sufficient volume to cover the filters. Use of an orbital shaker prevented filters from sticking to each other.
(3) Primary antibody (either deaminase crude antiserum or affinity-purified deaminase antibody at 1:5000 and 1:1000 dilution, respectively, in TBST) was added and incubated for 2-4 h at room temperature with gentle shaking.

(4) Primary antibody solution recovered (for important experiments, the antibody solution was not re-used) and the filters were washed thrice, 10-15 min each with TBST as above.

(5) Secondary antibody (anti-rabbit IgG labeled with alkaline phosphatase (Promega) was used at 1:7500 as recommended by the supplier. Incubation was at room temperature with gentle shaking for 2-3h. Antibody solution was recovered and was discarded. Filters were washed in TBST as above (step 4).

(6) Antigen-antibody complex were detected using NBT/BCIP in alkaline phosphatase buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl, 5 mM MgCl₂) as suggested by supplier. Sufficient substrate solution was used to cover the blots. Color development was done at room temperature, for 10-20 min depending on signal intensity.

(7) Once sufficient color had developed, the filters were transferred to 5-10 mM EDTA in water.
2.12 Immunoprecipitation

IgG from either preimmune or immune rabbit serum was immobilized to protein A-Sepharose 4B (Pharmacia). It was swollen in 0.5 M sodium phosphate, pH 8 and washed with 0.1 M sodium phosphate pH 8 and resuspended in 0.1 M sodium phosphate pH 8 (Ey et al., 1978). About 30 mg beads (=0.1 ml swollen beads) was mixed with 50 µl each preimmune or immune serum. It was gently shaken for about 3 h at room temperature. Beads were then spun down, washed twice with 1 ml of 0.1 M Na-Phosphate pH 8. Finally beads were washed twice with 1 ml each of IP buffer (10 mM Tris-Cl pH 7.4, 2 mM EDTA, 0.15 M NaCl, 1% (v/v) Tween-20) and suspended in 0.12 ml IP buffer. For immunoprecipitation of in vitro translated proteins, the translation reaction mixture was diluted five-fold with IP buffer. For immunoprecipitation of purified enzyme, 5 µg was diluted five-fold with IP buffer.

The in vitro translated proteins and the purified deaminase were processed in parallel. The diluted samples were then pre-cleared by reacting with pre-immune IgG-bound beads for 2 h at room temperature with constant mixing. The beads were then pelleted in a microfuge. Supernatants were collected and reacted with immune IgG-bound beads for 3-4 h at room temperature with constant mixing. At the end of
incubation, beads were pelleted and washed five-times with IP buffer. Finally the beads were washed once with 20 mM Tris-Cl pH 7.5, 1 mM EDTA and pelleted. The beads were resuspended in 20 μl 2X Laemmli buffer and heated in a boiling water bath for 4 min. The beads were again pelleted, and the supernatant was resolved in a SDS-PAGE. $^{35}$S-labeled proteins were visualized by fluorography.

2.13 Isolation of total RNA

RNA was isolated by the method of Chomczynski and Sacchi (1987) with some modifications. The original method designed for animal cells was modified for yeast cells.

Requirements:

(1) 1.25x Solution D = 5M Guanidine thiocyanate (BRL), 31.25 mM Sodium citrate, 0.625% (w/v) Sarkosyl, 0.125 M $\beta$-Mercaptoethanol. Solution D without $\beta$-Mercaptoethanol is stable at room temperature for at least 3 months. But with $\beta$-mercaptoethanol it is reported to be stable at room temperature for only one month. Solid guanidine thiocyanate accumulates an yellow impurity on long storage. So, solution D (light yellow solution) was routinely filtered through a 0.22 μm cellulose nitrate membrane filter, which traps the entire yellow impurity.
(2) 2M Sodium acetate, pH 4. Solution autoclaved and stored frozen.

(3) Redistilled Phenol saturated with distilled water.

Care: Since guanidine thiocyanate is a powerful chaotropic agent ribonucleases are inactivated rapidly (half life of ribonuclease A in 4M guanidine thiocyanate is approximately 4 seconds (Castellino and Barker, 1968; Chirgwin et al., 1979). Consequently, with this method no special care was taken, such as treatment of solutions and glasswares with diethyl pyrocarbonate.

Procedure:
1. **C. albidans** was grown in an appropriate medium and quickly harvested during log phase of the growth, by centrifugation at 3000xg, 5 min.

2. Cells were resuspended in chilled sterile water and pelleted as above. The cell pellets were then frozen in liquid nitrogen and stored at -70° until use.

3. For every gram wet weight of cells, 2.6 ml of 1.25x solution D and 3.6 g sterile glass beads were added to frozen cells and thawed at room temperature.

4. Cell suspension was vortexed at full speed for five min with one min bursts, to break the cells.
5. Then the following were added sequentially with thorough mixing each time: 0.36 ml of 2M Sodium acetate pH 4, 3.6 ml Phenol, 0.72 ml Chloroform: isoamyl alcohol (24:1). Vortexed briefly and incubated in ice for 15 min.

6. Spun at 10,000xg, for 20 min at 4°C.

7. Supernatant was collected and RNA precipitated with an equal volume of isopropanol. Precipitate appeared soon after addition of isopropanol. Incubated 30-60 min in ice, instead of the recommended -20°C incubation, to avoid co-precipitation of impurities.

8. RNA was sedimented at 10,000xg for 20 min at 4°C. Supernatant was completely drained.

9. When the pellet was still wet, 3 ml of 1x solution D per gram wet weight of initial cell pellet was added. Vortexed to resuspend. Once the pellet was dislodged (can be hastened by dislodging the pellet with a sterile micropipette tip) it was solubilized by heating at 65°C for 5-10 min.

10. If any insoluble matter remained, it was removed by pelleting in a centrifuge as above.

11. The resolubilized RNA was again precipitated with an equal volume of isopropanol as described in step 7.
12. RNA was pelleted, and the pellet was washed once with 70% ethanol containing 0.3M potassium acetate, pH 4.8, and finally was washed twice with 70% ethanol.

13. The supernatant was completely drained and ethanol removed by vacuum drying.

14. The RNA pellet was resuspended in 0.5 ml sterile water; RNA was estimated by UV absorbance at 260 nm (Maniatis et al., 1982). For details on storage, see poly(A) RNA isolation step 17. Typical yield: 1.5 mg total RNA from 1 g wet weight cells.

### 2.14 Isolation of poly(A)$^+$ RNA by oligo(dT)-cellulose Chromatography

Poly (A)$^+$ RNA was isolated for cDNA synthesis as per the procedure of Okayama et al. (1987).

Precautions: (i) All solutions prepared in double distilled water, were stored in plastic wares (Tarsons) and sterilized in an autoclave at 15 psi, 20 min. No DEPC-treatment was done at any stage.

(ii) Care was taken not to touch/contaminate the mouth of glass wares and plastic wares. Once sufficient care is taken not touch the areas where solutions would come in contact, there was no need to use protective gloves.
Column (BioRad Econo column 0.7 cm diameter), tips and eppendorf tubes were washed with detergent (Teepol) rinsed thoroughly, dried, and siliconized. After drying they were again thoroughly rinsed with distilled water, dried and sterilized in an autoclave as above. Siliconizing the glasswares and plastwares would be helpful in reducing losses by non-specific adsorption. It should also help in the removal of RNases, if any.

Requirements: (1) Oligo(dT)-Cellulose (Type 7, Pharmacia), (2) 1 M NaCl, (3) 2 M NaCl, (4) TE: (10 mM Tris-Cl pH 7.5, 1 mM EDTA), (5) TE/NaCl: 1:1 mixture of TE and 1 M NaCl (0.5 M NaCl in TE).

Procedure:

1. Oligo(dT)-cellulose was swollen in TE and packed to about 0.5-0.6 ml (capacity: 1g can bind ca. 5mg poly(A)⁺ RNA). It was washed thoroughly with 5 column volumes of TE and equilibrated with TE/NaCl.

2. Two mg total RNA in 1 ml TE was denatured at 65°C for 5 min, quickly chilled on ice and 1 ml 1M NaCl was added and mixed.

3. It was then loaded onto the column. Flow rate was kept constant around 5-6 ml/h throughout the procedure. The flowthrough was collected and reapplied to column.
4. Column was then washed thoroughly with approximately 4 column volumes of TE/NaCl, to wash off unbound and loosely bound RNA. The eluent was checked for RNA by RNA spot quantitation assay.

5. When the eluent did not show any fluorescence, bound poly(A)$^+$ RNA was eluted with TE. 0.2 ml fractions were collected in eppendorf tubes and RNA was checked in the eluted fractions by spot assay.

6. Fractions containing poly(A)$^+$ RNA was pooled, denatured as above, and made upto 0.5 M NaCl and rechromatographed on the same column that had been reequilibrated with about 4 column volumes of TE/NaCl.

7. The second cycle of chromatography was done exactly as the first cycle.

8. Poly(A)$^+$ RNA was detected in the fractions, pooled and RNA precipitated by adding NaCl to 0.4 M (from 2 M NaCl Stock) and 3 volumes of -20$^\circ$ ethanol. Precipitation was done at -20$^\circ$, for about 8-10 h in microfuge tubes.

9. Poly(A) RNA was pelleted (5 min, microfuge, 4$^\circ$) and the pellet was washed twice with chilled 70% ethanol by centrifugation as above. Ethanol was almost completely aspirated.
10. In a desiccator connected to a vacuum pump, poly(A) RNA pellet was dried briefly, such that ethanol (smell!) just disappears.

11. RNA pellet was dissolved in autoclaved water, and RNA was quantitated by ethidium fluorescence spot assay using yeast RNA (Pharmacia) as standard (Section 2.15). RNA was also estimated spectrophotometrically at 260 nm.

12. In the absence of a densitometer, the spot assay quantitation method is prone to subjective error, resulting from visual comparison of the fluorescence of the standards. For further work, estimate from spectrophotometric determination was used (since the amount of RNA was not limiting).

17. For long term storage, RNA was precipitated as above and stored in ethanol at -70°C in small parts. For short periods total RNA (5 mg/ml water) and poly(A)⁺ RNA (1 mg/ml water) was stored at -20°C.

2.15 RNA Spot Quantitation Assay (Okayama et al., 1987)

Quantitation of RNA: 1 mg/ml yeast RNA (Pharmacia) was diluted to obtain a set of standards.

Concentration of RNA (ng) per 5 ul: 1000, 800, 600, 400, 200, 100 and 50. 5 ul of standard RNA was mixed with 1 ul
of ethidium bromide solution (6 µg/ml) to, give ethidium bromide concentration of 1 µg/ml. RNA samples whose concentration is to estimated, was also prepared the same way as standards. The mixtures were then spotted onto Saran wrap and visualised with a transmitted uv light. The fluorescence intensity of the standards and the samples were visually compared and the concentration was determined. The sample volume was chosen to give a fluorescence intensity within the range of the standards used.

2.16 Single Stranded cDNA Probe Synthesis

cDNA probe was synthesized essentially as described (Maniatis et al., 1982).
1. In a reaction volume of 100 ul, the final composition of the components were :

0.1M Tris-Cl pH 8.3 (at 42°C), 0.05M KCl, 0.01M MgCl₂, 0.01M DTT, 10 µg oligo(dT)₁₂₋₁₈ (Pharmacia), 1mm each dATP, dGTP, dTTP, 25µM dCTP, 100 U RNasin (Pharmacia), 50uCi (α³²P) dCTP, 50 µg/ml Poly(A)⁺ RNA, 100 U Reverse Transcriptase (Molecular Genetic Resources, USA). The reaction was incubated at 42°C, 3 h.

2. To stop the reaction EDTA was added to 20 mM and mixed.

3. To hydrolyze the mRNA template, NaOH was added to a final concentration of 50 mM, mixed and incubated at 65°C for 1 h.
4. To neutralize the reaction mix, Tris-HCl, pH 8, was added to give a final concentration of 0.33 M and HCl was added to 50 mM.

5. The cDNA probe was then phenol extracted and was purified from free label through a Sephadex G-50 column (Maniatis et al., 1982).

2.17 Colony Blot Hybridization

1. *C. albicans* B792 genomic library constructed in plasmid YEpl3 was maintained in *E. coli* MC 1061 (provided by Y. Koltin). It was amplified and plated out on LB amp plates at high density (about 10-20 thousand colonies per 90 mm plate).

2. After incubation at 37°, when the colonies have grown to 1-2 mm, they were transferred to nitrocellulose and hybridized as described (Maniatis et al., 1982).

3. Dry nitrocellulose filters were overlaid on the colonies and orientation marks were made at asymmetric positions (by piercing the filter and agar together with a needle). After about 1-2 minutes, the filter was peeled off and placed colony side up on a fresh LB amp plate. Then a second nitrocellulose filter was overlaid on the same master plate, asymmetric marks placed and filter removed and placed onto fresh LB amp plate.

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4. Colonies were allowed to grow for 4-5 hrs and filters were transferred to LB plates containing ampicillin and chloramphenicol. The plates were incubated for about 17 h at 37°C for amplification of plasmids.

5. The colonies were lysed as follows:

Filters were placed colony side up serially on Whatman 3 MM paper saturated with 10% SDS for 3 min, 0.5 N NaOH-1.5 M NaCl for 7 min, 0.5 M Tris-Cl pH 8 - 1.5 M NaCl 7 min and 2xSSPE, 5 min.

6. The filters were air-dried for 1 h at room temperature and DNA fixed by baking at 80°C under reduced pressure for 2 h.

7. Filters were then pre-washed in a solution containing 50 mM Tris-Cl pH 8, 1M NaCl, 1 mM EDTA, 0.1% SDS at 42°C for 1-2 h.

8. Filters were then pre-hybridized in 50% (v/v) formamide, 5xDenhardt's solution, 5x SSPE, 0.1% SDS, 0.1 mg/ml denatured salmon sperm DNA for about 12 h at 42°C.

9. Glc- and GlcNAc-cDNA probes were denatured by heating in a boiling water bath for 5-10 min and quickly cooled in ice-water. Denatured single stranded cDNA probes (at 1X10^6 cpm/ml hybridization solution) were added to
prehybridization solution and incubated with shaking at 42°C, 36 h.

10. The first nitrocellulose replica was always used for hybridization with Glc cDNA probe and the second replica for GlcNAc cDNA hybridization; since more cells are transferred in the first replica and consequently more DNA would be bound to that filter, GlcNAc cDNA was hybridized to the second replica.

11. The filters were then washed sequentially in 2xSSC at room temperature for 45 min, 2xSSC at 68°C for 30 min, 0.1xSSC + 0.1% (w/v) SDS at 68°C for 30 min, 0.1xSSC at room temperature for 30 min.

12. Filters were air dried and autoradiographed at -70°C with intensifying screens.

13. The autoradiograms containing signals from Glc-cDNA probe and signals from GlcNAc-cDNA probe were aligned and compared to detect differentially hybridizing colonies.

14. Since it is a high density screening the lawn of cells in the area covering the signals were picked and resuspended in LB and streaked for single colonies.

15. Several single colonies from each signal giving clone were patched onto gridded nitrocellulose filters in duplicate and rescreened as for the high density screening.
16. After the third screening, clones consistently giving signals, were picked and maintained.

2.18 Preparation of Plasmid DNA

2.18.1 Mini-Preparation of Plasmid DNA (Promega K/RT sequencing manual)

1. Solution A: 50 mM Glucose, 10 mM EDTA, 25 mM Tris-Cl pH 8 autoclaved and stored frozen.

2. Solution B: 0.2 N NaOH, 1% (w/v) SDS made fresh from stock solutions.

3. Potassium Acetate: 5 M, pH 5.4. 60 ml 5M Potassium acetate (Sarabhai), 11.5 ml glacial acetic acid and 28.5 ml H₂O to a final volume of 100 ml is 3 M K⁺ and 5 M CH₃COO⁻ and pH of stock is 5.4 and when diluted to 0.3 M gives pH 4.8.

4. RNaseA: 10 mg/ml in 10 mM Tris-Cl pH 7.5, 15 mM NaCl Heated in boiling water bath, 15 min and allowed to cool at room temperature. Stored frozen.

5. NaCl 4 M: Autoclaved and stored at room temperature.

6. PEG 8000 (Sigma): 13% (w/v) in water. Autoclaved and stored in fridge.

7. TE pH 8: 10 mM Tris-Cl pH 8, 1 mM EDTA.
Plasmid DNA was isolated essentially as per the mini-prep protocol supplied by Promega Corporation (K/RT Sequencing Systems Manual). This preparation gave plasmid DNA that could be digested well with restriction enzymes (Promega claims that plasmid prepared by this method can actually used for double stranded DNA sequencing and other applications).

1. A single colony from LB amp plate was inoculated into 5-10 ml LB amp and incubated at 37°C, overnight, with vigorous shaking.

2. By three rounds of centrifugation, cells from 4.5 ml overnight culture was pelleted in a 1.5 ml microfuge tube. At this stage the rest of the culture could be stored frozen by combining 1 volume of 45% (v/v) glycerol with 2 volumes of culture and after snap freezing in liquid nitrogen.

3. Supernatant was completely drained and the pellet was resuspended in 300 ul ice-cold solution A.

4. Incubated at room temperature, 5 min.

5. Added 600 ul of fresh solution B, mixed and incubated on ice, 5 min.

6. Added 450 ul ice-cold potassium acetate, mixed and incubated on ice, 15-30 min.
7. Spun in a microfuge for 10 min to clarify the lysate.

8. The supernatant was divided into two microfuge tubes. Sometimes if the supernatant was contaminated with a white precipitate it was respun for 10 min and clarified.

9. RNase A was added to a final concentration of 20 μg/ml and incubated at 37°, 20 min.

10. An equal volume of TE-saturated phenol: Chloroform (1:1) mixture was added and vortexed. Spun for 5 min to separate phases.

11. The aqueous phases was transferred to a fresh tube and was extracted with an equal volume of chloroform: isoamylalcohol (24:1) and phases separated as above.

12. To the aqueous phase, in a fresh tube, 2.5 volumes of ethanol was added and incubated at room temperature for 15 - 30 min.

13. Then the precipitate was collected by centrifugation for 5 min and washed with 70% ethanol. Supernatant was completely aspirated and traces of ethanol was removed by mild vacuum drying.

14. Pellet was solubilized in 24 μl sterile water in two tubes. These were combined, 12 μl of 4M NaCl was added and
mixed. DNA was precipitated by adding 60 μl 13% (w/v) PEG 8000 solution. Mixed and incubated on ice, 20 min.

15. DNA pellet was collected by centrifugation for 10 min. Supernatant was aspirated and pellet was washed with 1 ml 70% ethanol. Pellet was dried as before and solubilized in 50 μl TE.

2.18.2 Medium Scale Preparation of Plasmid DNA

Plasmid DNA was also isolated by alkaline lysis (Birnboim and Doly, 1979) as described in Maniatis et al. (1982).

2.19 Binding of Plasmid DNA to Nitrocellulose

Plasmid DNA was first denatured and immobilized on nitrocellulose; this was used either for slot blot hybridization or for hybrid selection. Nitrocellulose can bind 1.6 μg DNA per 2 mm² slot.

1. Plasmid DNA solution in TE, pH 8, was incubated in a boiling water bath for 10 min.

2. It was quickly chilled in ice water and an equal volume 1 M NaOH was added and incubated at room temperature, 20 min.
3. During this time, a suitable piece of nitrocellulose membrane was cut, first saturated with water and then saturated with 6 x SSC. Nitrocellulose membrane along with a piece of Whatman 3M sheet pre-wet with 6 x SSC, was assembled in slot a blot apparatus (BRL). Vacuum was applied with a water aspirator, such that 100 ul passed through the membrane in about 10 min. The rinsed slots were rinsed with 6xSSC.

4. After incubation in alkali, the DNA solution was neutralized by adding 0.8 volume of neutralizing solution (2 volume 20 x SSC, 1 volume 1 N HCl, 1 volume 1 M Tris-Cl pH 8) and quick chilled on ice and applied to nitrocellulose immediately.

5. The slot was washed with 0.3 ml, 6x SSC and allowed to drain completely. The flow rate during binding and washing steps was controlled as mentioned above with a suction device.

6. Nitrocellulose was air dried for 1-2 h and baked at 80°, 2h.

2.20 Hybridization Selection of Specific mRNA

Hybrid-selection of specific mRNA (Ricciardi et al., 1979) was done as described by Maniatis et al. (1982) with
some modifications: 1. The baked nitrocellulose filter containing immobilized plasmid DNA (Section 2.19) was cut such that the slots corresponding to different clones were separated. Loosely bound DNA was removed by boiling the nitrocellulose pieces in water (Maniatis et al., 1982).

2. During hybridization and washing, all clones were processed together and at the stage of elution of mRNA, the filters containing individual clones were separated and individually eluted.

3. Hybridization solution: 1 mg total RNA from GlcNAc-grown cells were dried down from a solution in water. To the dried RNA, the following were added to the indicated concentrations: 65% (v/v) deionized formamide, 20 mM Pipes pH 6.4, 0.2% (w/v) SDS, 0.4 M NaCl, and volume was made up to 1 ml giving RNA concentration of 1 mg/ml hybridization solution. The rest of the steps were followed exactly as described (Maniatis et al., 1982).

4. RNA was recovered from hybridization solution by four-fold dilution with water and ethanol precipitation (Mason and Williams, 1985). The ethanol precipitate was washed with 70% (v/v) ethanol, dissolved in water and in vitro translated to check if RNA has remained intact during hybridization.
5. The eluted mRNA was ethanol precipitated, washed, dried and used directly for translation.

2.21 Rabbit Reticulocyte in vitro Translation System

Total, poly(A)\(^+\) and hybrid-selected specific mRNAs were translated in vitro in rabbit reticulocyte lysate as suggested by the manufacturer (Promega Corporation, USA).

**Fluorography (Chamberlain, 1979):** Polyacrylamide gels were stained with 0.25% (w/v) CBB R-250 in 25% methanol, 10% acetic acid and destained in the same solution without dye. The gel was then equilibrated in water and then transferred to 1M sodium salicylate (CDH) and allowed to equilibrate for 30-45 min. Gel was then dried in a gel dryer equipped with a vacuum pump and exposed to X-omat film (Kodak) without intensifying screen at -70\(^\circ\) for the desired time (exposure times are given in the respective figure legends).

2.22 *S. cerevisiae* Transformation

*S. cerevisiae* was transformed using modified lithium acetate method (Ito et al., 1982). Host strain *S. cerevisiae* AH 22 was transformed with miniprep DNA from GlcNAc-inducible clones, and transformants were selected for leucine prototrophy (YEpl3 has *S. cerevisiae* LEU2 gene).
Preparation of Competent Cells:

Requirements:

1. TE : 10 mM Tris-Cl, 1 mM EDTA, pH 7.5

2. 10xTE-Lithium acetate : 100 mM Tris-Cl pH 7.5, 10 mM EDTA, 1 M Lithium acetate (SRL). Filter sterilized and stored frozen.

3. PEG 4000 : 40% (w/v) PEG 4000 (Sigma) in 1xTE-Lithium acetate. PEG was dissolved in water and autoclaved; 10xTE-lithium acetate was added to give 40% (w/v) PEG.

4. 30% (v/v) glycerol: Glycerol (BRL) was diluted and autoclaved; 10xTE-Lithium acetate was added and volume made up with sterile water to give 30% (v/v) glycerol in 1 x TE-Lithium acetate.

5. Selective Plate (SD + his): 0.67% (w/v) Yeast Nitrogen Base (w/o amino acids), 2% (w/v) Glucose, 40 ug/ml L-Histidine, 1% (v/v) YPD, 100 u/ml Penicillin G and 2% (w/v) agar. Medium was autoclaved and then Penicillin G was added.

Procedure:

1. AH22 cells were grown overnight in YPD at 30° with shaking at 200 rpm.
2. About 2 ml of preculture was diluted into fresh 100 ml YPD and culture allowed to grow at 30° with shaking at 200 rpm until OD595 reached 1.0-1.2.

3. Cells were then pelleted in sterile screw-capped SS34 tubes at 5000 rpm, 5 min.

4. Cell pellet was washed once with TE pH 7.5 and then with 1xTE-lithium acetate and then resuspended in the same solution at 1/100 of the original culture volume.

5. Cell suspension was gently agitated at 30° for 1 h and then chilled on ice. At this stage, the cells rendered competent by lithium acetate can be used immediately for transformation, or stored for 2-3 days at 4° without much reduction in transformation efficiency or stored frozen at -70° after combining with an equal volume of 30% (v/v) glycerol in TE-lithium acetate and snap frozen in liquid nitrogen.

Transformation of AH22 Competent Cells:

Five µl (approx. 1µg) plasmid DNA (Miniprep), 5 µl (50 µg) Sheared Salmon Sperm Carrier DNA (10 µg/ul) and 100 ul competent cells + 100 ul TE-lithium acetate (if fresh) or 200 ul if from frozen cells. Mixed and incubated at 30° for 1 h.
2. Added 0.7 ml 40% (w/v) PEG 4000, mixed and incubated for 1 h at 30°.

3. Incubated for 5 min in a 42° water bath.

4. Spun down the cells (5-10 seconds in a microfuge) and resuspended in 200 ul TE pH 7.5.

5. 100 ul of this cell suspension was spread on a SD-his plate.

6. Transformants appeared after 3 days incubation at 30 °C.

7. Controls used in transformation experiments were:
   (a) - Plasmid DNA + Carrier DNA
   (b) + Control YEp13 DNA 1- 5 ug.

8. Using 2.5 ul miniprep DNA, about 1000-3500 transformants were obtained.

**Growth of S. cerevisiae and Analysis of Expression of Cloned Genes**

1. Single colonies were picked from the selective plate and the transformants were maintained as patches on SD his plates.

2. Cells were transferred from patches to SD his broth and allowed to grow for about 30 h at 30°. Growth rate of some transformants were poor.
3. Cells were pelleted and homogenized in 50 mM Tris-Cl pH 7.5, 1 mM each EDTA, ME and PMSF using glass beads as described (see basic Methods).

4. It was then clarified by centrifugation in a microfuge for 5-10 min.

5. Supernatant was used for deaminase enzyme assays and about 100 ug protein was analyzed by Western blot as described.

2.23 Construction of λgt11 cDNA Expression Library

2.23.1 Double Stranded cDNA Synthesis for Cloning into λgt11.

Double stranded cDNA was synthesized essentially as described by Gubler and Hoffman (1983). For first strand cDNA synthesis, either oligo(dT)\(_{12-18}\) or random hexanucleotide primer can be used. The latter is reported to be useful if expression screening of the library is anticipated (Haymerle et al., 1986), since 5' and 3' non-coding regions of eukaryotic mRNA are reported to contain stop codons, use of random primer would give a more complete representation of all regions of mRNA. However in this work oligo(dT)\(_{12-18}\) was used as primer; it is being conventionally used with success even for expression
screening. The size of cDNA is also expected to be large and the chance of isolating full length or near full-length cDNA clones is much higher than if random primer is used. As it turned out (see Results and Discussion), a near full length cDNA clone has been obtained and also two smaller clones, probably one representing the 3' and the other more to the 5' end of the mRNA.

Outline procedure (which included modifications/deviations from protocol supplied by the manufacturer of the kit, λgt11 cDNA synthesis Plus (Amersham) is described):

1. Five μg poly(A)⁺ RNA was combined with four ug oligo(dT)₁₂-₁₈ primer and denatured at 70°C, 5 min and was allowed to cool slowly to room temperature to anneal primer to template.

2. Other components of the reaction mix was added to this tube at room temperature (to avoid precipitation of reaction mix).

3. The reaction mixture contained (in the order of additions): 5 μg Poly(A)⁺ RNA, 4 μg oligo(dT)₁₂-₁₈, first strand reaction mix, sodium pyrophosphate (to suppress fold back in the first strand), human placental RNase inhibitor, 100 units reverse transcriptase, 1 mM each dATP, dGTP, dTTP and 0.5 mM dCTP in a final reaction volume of 50 ul.
The ratio of poly(A) RNA to reverse transcriptase, and reaction volume was maintained as suggested by the supplier.

4. After all the components were added, 5 µl was drawn out and mixed with 2 µCi (α-32P) dATP (3000 Ci/mmol) in a separate tube, to follow the reaction. Reaction mixture was incubated at 42° in a covered water bath for 90 min and at the end of the incubation, tubes were stored on ice.

5. To the tracer reaction, 1 µl 0.2 M EDTA was added and volume made upto 20 µl with water. 5 µl of this was used for incorporation analysis (Section 2.32).

6. To the first strand reaction mixture stored on ice, second strand synthesis components were added: Second strand synthesis reaction buffer, 20 uCi(α-32P)dATP (as tracer), 4 units E. coli RNaseH (an endoribonuclease, that nicks the mRNA template), 115 units E. coli DNA polymeraseI and reaction volume made upto 225 µl with water.

7. Incubated for 60 min at 12°, 60 min followed by another incubation for 60 min at 22°. Reaction was terminated by heating at 70°, 10 min and stored on ice.

8. 2 µl of reaction mixture was diluted to 20 µl with water and 4 µl was used for incorporation analysis.
9. In a typical experiment,

incorporation into first strand = 3%
incorporation into second strand = 8%

From incorporation analysis data, the amount of cDNA synthesized was calculated as follows:

Amount of unlabeled dATP in 45 ul first strand reaction = 45 n moles
Amount of unlabeled dATP incorporated = 1.4 n moles
Total amount of dNTPs (four) incorporated = 5.6 n moles
Weight of cDNA synthesized = 2 μg

There was some discrepancy in the estimate based on incorporation data. It appeared that approx. 8.4 ug of ds cDNA was synthesized starting from 5 μg poly(A) RNA. So, an ethidium bromide-agarose plate assay was done as described (Christen and Montalbano, 1989) using DNA as standard. The estimate from this method was about 4 μg of ds cDNA; this value was used for further experiments.

2.23.2 Purification of ds cDNA by Phenol-Chloroform and Ethanol Precipitation.

1. The stopped cDNA synthesis reaction mixture was extracted with equal volume of phenol (equilibrated with 0.1M Tris-Cl pH 8, 0.1% hydroxyquinoline)-chloroform.
2. The aqueous phase was recovered and the organic phase was reextracted with 100 ul TE (pH 8) and aqueous phases were combined.

3. Then it was extracted with phenol-chloroform as above (no back extraction of organic phase).

4. The aqueous phase from step 3 was then extracted with equal volume of chloroform. Aqueous phase was recovered and organic phase was back extracted once with 50 ul TE.

5. To the combined aqueous phase, 0.36 volume of ammonium acetate (from 7.5 M stock) was added, mixed and two volumes of -20° ethanol was added and precipitated at -70° for 2-6 h. For rapid precipitation at -70°, an 80% (v/v) isopropanol slush maintained at -70° was used as described (Bird and Wu, 1989).

6. The ethanol precipitate was brought to room temperature, mixed and spun in cold room for 10 min.

7. Pellet was washed with 0.5 ml ammonium acetate-ethanol (1 vol 2 M NH₄ acetate plus 2 vol ethanol) and spun as above.

8. Pellet was washed once with -20° absolute ethanol and cDNA pelleted as above.
9. The supernatant was completely aspirated and ethanol was removed using a vacuum pump. The extent of drying is critical for quantitative resolubilization. Drying time was such that the ethanol (smell!) had just disappeared.

10. Pellet was resolubilised at 0.2 μg/μl, TE pH 8.

2.23.3 EcoRI Methylase Treatment of ds cDNA.

The reaction mixture contained: 1 μg ds cDNA, 100 μM S-adenosyl methionine (Promega), 1 mM EDTA, 100 mM Tris-Cl (pH8), 0.1 mg/ml nuclease-free BSA (Pharmacia) and 100 units EcoRI Methylase (Promega), in a reaction volume of 25 μl. Incubated at 37°C, 60 min and stopped the reaction at 65°C, 10 min. Control: DNA (1 μg) was treated with EcoRI methylase under similar conditions; Methylase-treated and untreated DNA was digested with 40 units of EcoRI (NEB) and products analyzed on agarose gel. The EcoRI Methylase reaction had completely protected 1 μg of DNA from EcoRI digestion; this indicated that methylase treatment of ds cDNA should have completely worked.

2.23.4 Repairing the Ends of ds cDNA:

To the same reaction mixture after methylase treatment (25 μl), 13 μl water, 0.1 mM each dNTP (A,G,C,T) mix, 10 mM MgCl₂ and 8 units T4 DNA Polymerase
were added, mixed and incubated at 37°, 15 min. To the reaction mix, 1 µl (7.5 u) Klenow polymerase was added and incubated at 22°, 15 min. To stop the reaction, EDTA was added to 20mM final, phenol-chloroform extracted and ethanol precipitated as described (Section 2.1.15.1). The cDNA pellet was dissolved in 20 ul TE pH 8.

2.23.5 EcoRI Linker Addition to Blunted ds cDNA:

Phosphorylated EcoRI linkers were ligated to the blunt ended ds cDNA using T4 DNA ligase as per the instructions of the supplier (Amersham, cDNA Cloning System, λgt11).

2.23.6 EcoRI Digestion and Separation of EcoRI Linkered cDNA:

The linker ligation reaction mixture was directly taken for EcoRI digestion and the EcoRI cohesive-ended cDNA was separated from linkers on a 3 ml gel permeation column as per the supplier’s instructions (Amersham, cDNA Cloning System λgt11). Since cDNA is 32p-labeled, the fractions containing cDNA were monitored by Cerenkov counting. cDNA was ethanol precipitated in presence of 0.3M sodium acetate pH 5.2 and 2.5 volumes of -20° ethanol at -70° for 8-10 h. cDNA was pelleted in a microfuge, washed once with -20° absolute ethanol, dried and redissolved in TE to give a concentration of approximately 50 ng/µl; the amount of TE
to be added to give the requisite cDNA concentration was decided by estimating the cDNA amount, from cerenkov counts of the ethanol precipitate.

2.23.7 Ligation of EcoRI-ended ds cDNA to λgt11 arms

EcoRI-ended cDNA was ligated to λgt11 arms (EcoRI cut, dephosphorylated; Amersham) as per the supplier's instructions; about 35 to 70 ng of cDNA was ligated to 1 μg λgt11 arms.

2.23.8 In vitro Packaging of Ligation Mixture

The entire ligation mixture containing the recombinant λgt11 was packaged in vitro as per Amersham instructions. The efficiency of packaging extracts was tested with wild type λgt11 vector that had been concatenated using T4 DNA ligase. The titre was about 6-9 x 10^7 pfu in comparison to 4.7 x 10^8 pfu, the data from the supplier.

2.23.9 Titration and Screening of λgt11 Recombinants

E. coli Y1090 was used for titration and screening. Growth of plating cells:

(1) A single Y1090 colony, from a freshly streaked LB amp plate, was inoculated into LB medium containing ampicillin
and maltose and preculture grown for 12 h at 37° with vigorous aeration (200-225 rpm).

(2) Preculture was diluted into fresh LB amp + maltose medium to 2% (v/v), and culture grown at 37° as above.

(3) As soon as OD600 of the culture reached 0.35-0.4, the cells were pelleted at 5000g, 10 min, 4°.

(4) Cell pellet was resuspended in chilled 8 mM MgSO4 (used about 3 ml MgSO4 for every 10 ml culture), and cells were stored on ice until use.

Note: For titration and screening of library, plating cells were prepared fresh; plating cells stored for 12 h resulted in about 30-50% drop in phage titer.

(5) Phage dilutions were made in SM buffer. For 150 mm plates, 250 μl cell suspension was mixed with 100 ul plaque suspension. For 90 mm plate, 100 μl of cell suspension was infected with 100 μl of phage suspension. Phages and cells were mixed and allowed to adsorb at 37°, 15 min.

(6) Molten top agar (maintained between 47-50° was mixed and quickly poured onto LB amp plates on a level surface. LB amp plates were made fresh (used within one day of preparation) and prior to plating, was left open at 37° for 30 min. The LB amp plates were also prepared on a level
surface. This helps in uniform spreading of top agar, thereby resulting in more uniform growth of plaques.

(7) Once top agar had set, plates were covered and incubated top side down at $42^\circ$ until plaques were seen (routinely after about 3 h incubation).

(8) For screening, after the appearance of plaques fusion protein expression was induced by overlaying the plate with 10 mM IPTG-saturated and dried nitrocellulose circles.

(9) After about 3-4 h incubation at $37^\circ$, asymmetric alignment marks were made and the filter was peeled off and processed for immunodetection.

(10) At this stage, another replica filter was prepared by overlaying the plate with a fresh IPTG-saturated nitrocellulose filters and incubation continued at $37^\circ$ for about 8 h. The replica would serve as a back-up copy in case the immunodetection with the first filter did not go well, or could be used as a duplicate filter to see if the signals are consistent.

(11) Plates were sealed in plastic bag and stored top down in fridge until the screening was completed.
2.23.10 Picking the Positive Signal

(1) Positions of orientation marks and positive signals were traced onto a plastic sheet from wet nitrocellulose filters (if dry, the filter would shrink and it would be difficult to align to the master plate).

(2) The place on the agar plate encompassing the signal (in primary screening) or the positive plaque (in subsequent screenings) was cored out using a cut autopipette tip and phages were allowed to diffuse from agar plug into 0.5 ml SM buffer. 20-30 ul chloroform was added to each tube, to arrest bacterial growth.

Typical results:

(a) pfu/plaque from first screening = 9.2x10^6-2x10^8.
(b) pfu/plaque from subsequent screenings = 1.1x10^7-6.8x10^7.

2.24 Ethidium Bromide-Agarose Plate Assay (Christen and Montalbano, 1989)

Agarose was dissolved to 1% (w/v) in TE pH 8, cooled to 50°C and ethidium bromide was added to give a final concentration of 5 µg/ml. Gel was poured in a petridish and allowed to set. It was dried lid open for 2 h at room temperature and then left at 37°C (lid open) for another 2 h. DNA was used as standard. It was serially diluted to give 100, 50, 25,
12.5, 6.25, 3, 1.5 and 0.7 ng/ul. 1 μl of each standard and double stranded cDNA were spotted onto the dried plate and allowed the liquid to be absorbed. Fluorescence intensity of the cDNA sample was compared with that of standards and amount was estimated.

2.25 Generation of λgt11 Lysogens

Lysogens were made in E. coli Y1089 as described by Huynh et al. (1985).

(1) Cells were infected at a multiplicity of infection of 5, and diluted and plated on LB amp to give about 200 colonies/90 mm plate.

(2) Colonies were patched serially using sterile tooth picks onto two LB amp plates. The first plate was incubated at 42° and the second at 32° to test for lysogens.

(3) After about 16 h, patches that did not grow at 42° but grew at 32° were temperature-sensitive which is characteristic of lysogens and were taken for further work.

Fusion Protein Induction from Lysogens: Fusion proteins were induced from lysogens as described (Huynh et al., 1985; Miller, 1987).

(1) Lysogens were grown at 32° in LB amp to OD₆₀₀ = 0.5.
(2) An equal volume of pre-heated (to 65°) LB amp was added to raise the temperature of cultures rapidly to 42-45° and incubated at that temperature for 15 min with vigorous aeration to induce the lytic cycle.

(3) Cultures were then shifted to 37° and incubated with vigorous aeration.

(4) At this stage 1-2 ml cultures were separated and incubated in duplicate with aeration at 37°; to induce fusion proteins IPTG was added to a final concentration of 10 mM to one tube and was incubated with shaking for 30-60 min. A duplicate tube without IPTG served as a control.

(5) Cells were rapidly pelleted in a microfuge and pellets were resuspended in 1/20 culture volume of 20 mM Tris-Cl pH 6.8, 1 mM EDTA, 1 mM PMSF and frozen in liquid nitrogen.

(6) Cell suspensions were thawed and sonicated using a micro probe for 15 seconds at an amplitude of 12 microns; the tube was kept cool in ice-water bath during sonication.

(7) Cell debris were sedimented in a microfuge for 5 min.

(8) 5-10 ul of crude lysates from step 7 was combined with an equal volume of 2x Laemmli buffer and resolved on SDS-PAGE, blotted and immunodetected.
2.26 Preparation of DNA from \( \lambda \text{gt} \text{11} \) Clones

Small-scale isolation: (1) Phages were induced from lysogens as described in the fusion protein preparation, except that IPTG was not added and phages were allowed to accumulate within cells, by growing the cells at 37° with vigorous shaking for 2-4 h.

(2) Cells were pelleted, resuspended in TM buffer at 1/10 culture volume.

(3) Cells were lysed by adding 1/10 the suspension volume of chloroform and left shaking at 37° for 60 min.

(4) Debris were pelleted at 12,000g, 10 min.

(5) To the supernatant, DNase I to 20 \( \mu \)g/ml and RNase A to 25 \( \mu \)g/ml were added and incubated at 37°, 2-3 h and left overnight in fridge. The long incubation with RNase A seemed to digest the otherwise stubborn RNA. Even after this step, quite a bit of RNA remained.

(6) DNA was isolated from the phages as described for small-scale isolation (Maniatis et al., 1982).

(7) Yield of DNA was quantitated by agarose gel electrophoresis with serial dilution of purified DNA standards. DNA from small-scale preparations were used for
insert size determination and also for Southern blots (Section 2.30).

Medium scale isolation: Phages were prepared as per the procedure of Miller (1987) by infection of E.coli LE392 and purified on a CsCl gradient. DNA was isolated from phages as described for large scale isolations (Maniatis et al., 1982). Insert DNA isolated from this DNA preparation was used for making probes.

2.27 Agarose Gel Electrophoresis

Agarose gel electrophoresis of DNA fragments was done as described by Maniatis et al. (1982). Routinely 1xTAE buffer was used (Maniatis et al., 1982).

2.28 Determination of Insert Size

Phage DNA (from small-scale preparations) were digested with EcoRI to release the insert, end-labeled specifically at the EcoRI termini as described (Amersham, cDNA Cloning System \( \lambda \)gt11).

(1) A 30 µl reaction contained 150-200 ng phage DNA, 10x EcoRI buffer 3 µl, RNase A (1 mg/ml) 1.5 ul, EcoRI 10 Units. Incubated at 37°, 5 h.
(2) Reaction was stopped by heating at 70°, 10 min and then cooled on ice.

(3) End-labeling: To the EcoRI reaction mix, 0.5 uC (α²²P)dATP, and 1 Units of Klenow polymerase were added and volume made up to 35 μl. Incubated 60 min, at room temperature.

(4) At the end of reaction, the mixture was stored on ice.

(5) To 10 μl of labeled reaction mix, endo.R was added. Samples and standards were heated at 65°, 5 min and cooled in ice.

(6) Fragments were separated on 2% agarose gel in 1xTAE as described.

(7) When dye reached 3/4 the gel length, run was terminated, DNA was fixed in about 3-5 gel volumes of 7% (w/v) TCA, dried and autoradiographed.

2.29 Isolation of genomic DNA from *C. albicans* SC5314

*C. albicans* genomic DNA isolated by the method of Holm et al. (1986) was kindly provided by K. Ganesan and A. Banerjee.
2.30 Southern Transfer

(1) About 50 ng of EcoRI-digested clone DNA samples or about 1 μg C. albicans genomic DNA digested with various enzymes, were resolved in appropriate agarose gel concentrations.

(2) DNA was transferred from gel to Gene Screen Plus membrane (NEN) using the alkaline transfer method (Reed and Mann, 1985).

(3) At the end of electrophoresis, gel was soaked in 2-3 volumes of 0.25 N HCl with gentle agitation for 10-15 min to depurinate the DNA.

(4) The gel was then briefly rinsed and transfer was set up.

(5) Gene Screen Plus membrane was cut to the required size and used as suggested by the manufacturer. The membranes were then made wet with water and soaked in 0.4N NaOH (transfer solvent) for 5-10 min.

(6) DNA was transferred by capillary action using 0.4 N NaOH with the help of Whatman 3MM wick and a 3-4 inch stack of handicraft blotting sheets as described (Maniatis et al., 1982).

(7) Typically transfer was done for 12-16 h.
2.31 Random Primer Labeling (Feinberg and Vogelstein, 1984)

Template DNA: Denatured DNA was labeled within molten agarose. The recombinant DNA was cut with appropriate restriction enzyme to release the insert. The insert was resolved from the vector in a 2% (w/v) agarose gel containing 0.5 μg ethidium bromide per ml gel and 1xTAE (low melting agarose; Sea plaque, FMC, USA) in a cold room. Insert was visualized by UV fluorescence and the insert band was cut out. After trimming, the gel slice was added to pre-weighed microfuge tube and weight of the gel was calculated. At this stage gel piece could be stored frozen at -20°. For use, the agarose slice was molten at 70°, 10 min and was diluted with 2ml water per gram wet weight of agarose slice.

Labeling reaction: Just prior to use, the diluted DNA in agarose was denatured by heating in a boiling water bath for 7 min and immediately transferred to 37° water bath and held for 5-10 min and then the reaction pre-mix was added, mixed and incubated at room temperature which varied between 22-30°.

The reaction pre-mix contained:

50 mM Tris-Cl pH 8, 5 mM MgCl₂, 10 mMME, dNTP mix (dATP) 20 μM, 200 mM Hepes-NaOH pH 6.6, 0.3 mg/ml random
hexanucleotide primer [pd(N)₆; Pharmacia], 0.4 mg/ml nuclease free BSA (Pharmacia), 50 μCi (³²P)dATP (3000 Ci/m mol; BARC, Bombay), 0.17 μM dATP and 100 units/ml Klenow polymerase (Biolabs).

The reaction volume 100 μl contained approximately 3-10 ng template DNA, 34 p moles dATP. Reaction was allowed to proceed at room temperature for 24-36 hours. The kinetics of reaction was slow due to low concentration of template, and presence of agarose; during incubation, agarose had become semisolid. Routinely 60-80% incorporation was obtained after about 36 hours incubation. Probe specific activity was 5x10⁸ cpm/μg DNA.

Purification of probe: The reaction mix was heated at 70⁰, 10 min and extracted once with phenol:Chloroform (1:1). A thick interphase was reextracted with 50 ul TE pH 8. Aqueous phases were combined and salmon sperm carrier DNA added to 0.1 mg/ml and ethanol precipitated in presence of 1.5 M ammonium acetate (see purification of cDNA). Alternately, the reaction mixture, after phenol:chloroform extraction was passed through a 10 ml Sephadex G50 (Pharmacia Nucleic acid grade) column. Fractions were monitored for elution by Cerenkov counting and those give maximum counts in the void volume was used for hybridization after denaturing the probe.
2.32 Measurement of Incorporation

(i) In vitro translated $^{35}$S-labeled proteins. Briefly, the in vitro translation reaction mixture was diluted 10-fold in 1xLaemmli buffer and 5 ul of the diluted reaction mix was spotted onto Whatman 1mm filters, that was saturated in 5% (w/v) TCA, L-methionine, and dried. After spotting the filters were dried and washed at 0°C in 75ml 5% (w/v) TCA, L-methionine; then the filters were transferred to 75ml 5% (w/v) TCA at about 95°C and heated for 10-15 min to hybridize aminoacyl tRNAs; hydrogen peroxide 3% (v/v) final, was added to filters at 95°C in 5%TCA and further heated for 5-10 min to bleach the hemoglobin color; the filters were then transferred to 50ml 5% (v/v) TCA in ice and swirled for 10 min, TCA was discarded and rinsed once with ether and dried under infrared lamp and radioactivity determined by counting in Toluene-based Scintillation fluid (Cocktail "O", Spectrochem; 0.6% (w/v) PPO, 0.02% (w/v) POPOP in Toluene).

(ii) ($^{32}$P)dNTP-labeled nucleic acids:

About 1 ul reaction mixture was diluted to 100 ul 0.1 mg/ml sheared salmon sperm DNA; mixed and 10 ul was spotted onto GF/C and dried and used for determination total counts. to the rest 90ul, 1ml 10%(w/v) TCA/1%(w/v) sodium pyrophosphate was added and precipitated on ice for 20 min; the
precipitate was collected onto GF/C by vacuum filtration, washed each filter with 20 ml 10% TCA/1% sodium pyrophosphate; dried and the unprecipitated and the precipitated sample containing filters were then counted using Cocktail "O" in $^{32}$P channel. From the unprecipitated and precipitated counts, percent incorporation was calculated.

2.33 Agarose Gel Electrophoresis of Glyoxal-denatured RNA

Total RNA (20 µg) was denatured by glyoxal-DMSO method of Carmichael and McMaster (1980) as described by Maniatis et al. (1982).

(1) Briefly, 20 µg RNA samples in water were dried down and the following were added to give the indicated final concentrations in 16ul: 1M Glyoxal, 50% (v/v) DMSO (SRL), 10 mM Na-phosphate pH 6.5, and volume made up with sterile water. Double stranded DNA strands either DNA digested with Hind III, \( \Phi \)X174 digested with Hae III (BRL) or 1Kb ladder (BRL) were denatured as for RNA samples. Glyoxal was deionized as described (Maniatis et al., 1982). The pH of glyoxal solution (2-3) increased after deionization to about 5-6 and did not change even after repeated deionization. It was then stored in small parts at -20°.
(2) Incubation was at 50°, 60 min; chilled on ice (at this stage could be stored in cold for several hours).

(3) 4 μl of 5x loading buffer (50% glycerol, 10 mM Na-phosphate pH 6.5) was added and loaded onto 1.5% agarose gel prepared in 0.01 M Na-phosphate buffer, pH 6.5 and electrophoresed using 0.01 M Na-phosphate pH 6.5 at 80 volts.

(4) After 30 min of start, buffer was recirculated at about 1 litre/hour using a peristaltic pump.

(5) When the dye reached about 3/4 the length of gel (typically 3 h), run was terminated. Lanes meant for ethidium bromide staining was cut off the gel and RNA was transferred to Gene Screen Plus membrane using 10X SSC for about 24-30 h.

Note: As described by Carmichael and McMaster (1980), (i) Mobility of glyoxal-treated samples were about half that of untreated samples and (ii) acridine orange fluorescence of the glyoxal-treated samples were different from that of untreated samples. This indicated that glyoxal denaturation was working well under our conditions (K. Ganesan, personal communication).
Ethidium bromide staining of glyoxal-treated samples: Unless the glyoxalation is reversed, the glyoxal-treated samples do not stain well with ethidium bromide. So, for ethidium bromide staining of glyoxal treated RNA and DNA samples, the protocol of William and Mason (1985) was followed.

2.34 Agarose Gel Electrophoresis of Formaldehyde-Denatured RNA

Formaldehyde-denatured RNA gel (Lehrach et al., 1977) was performed as described by Maniatis et al. (1982). Formaldehyde (Glaxo, ExcelaR) pH 3-3.5, was used. Formamide was deionized (Bio Rad AG 501-X8) and stored in small parts at -70°C.

(1) RNA samples (upto 20 μg) were dried down and the denaturation premix was added.

(2) Denaturation premix: 1 μl, 10x MOPS buffer (autoclaved), 10 μl formamide, 3.5 μl Formaldehyde and 5.5 μl sterile water

(3) Mixed and incubated 15 min, 55°C. Chilled on ice.

(4) Added 2 μl 5x sterile loading buffer (50% glycerol, 1 mM EDTA; did not contain bromophenol blue for RNA samples).

(5) 1.5% (w/v) agarose gel containing 1x MOPS buffer and 2.2 M formaldehyde was used.
(6) Gel was placed in electrophoresis tank with 1x MOPS buffer and samples were loaded. It was allowed to stand for 45 min after loading samples before power was applied, to allow partial diffusion of formamide; this helps to prevent H-shaped bands.

(7) Voltage was set to 80 (50 mA) and run continued for about 3-4 h.

(8) After electrophoresis, gel was rinsed for 10 min in 10 volumes of water and RNA was partially hydrolyzed by soaking in 50 mM NaOH, 10mM NaCl for 45 min.

(9) The gel was then neutralized in 0.1M Tris-Cl pH 7.5 and RNA transferred to Gene Screen Plus membrane using 10 x SSC as described (Gene Screen Plus protocols, NEN), and baked for 2 h at 80° to reverse formaldehyde reaction.

(10) The load of RNA was estimated by ethidium bromide staining (Maniatis et al., 1982) of parallel lanes.

(11) The mobility of formaldehyde-treated DNA is not comparable to that of RNA. In the absence of RNA standards, size of RNA was estimated using glyoxal system. However, one of the advantages of formaldehyde system is that the transfer is more efficient and consequently transfer time is short (3-4 h).
2.35 Nucleic Acid Hybridization

Southern and Northern hybridizations were performed as suggested by the supplier of the Gene Screen Plus membrane (NEN Research Products, Du Pont).

(1) Blots were prehybridized (1M NaCl, 50% formamide, 10% dextran sulfate, 1% SDS) for 10-12h at 42° with gentle shaking.

(2) Probe (prepared by random primer method) was combined with sheared salmon sperm DNA (100 μg/ml hybridization solution) and denatured at 95° 10 min and quick cooled in ice water.

(3) Denatured probe was then added into prehybridization solution, mixed and incubated at 42°, 24 h.

(4) Prehybridizations and hybridizations were performed in sealed plastic bags (Davis et al., 1986).

(5) After hybridization, the blots were washed thrice in 2xSSC at room temperature.

(6) The blots were then washed in 2xSSC, 1% (w/v) SDS at 65° with three changes of 20 min each with shaking.

(7) Blots were then washed in 0.1xSSC with three changes for 20 min each at room temperature.
(8) Blots were not dried, but were sealed in plastic bags and exposed to X-OMAT AR films at \(-70^\circ\) with one intensifying screen.

(9) X-ray film was developed and if the background persisted, blots were rewashed at \(65^\circ\) with 0.25xSSC for 30 min.

Use of heterologous probe: *E. coli* deaminase gene (nag B) probe was hybridized using the same composition of prehybridization and hybridization solutions as described above except that the temperature was \(37^\circ\) and stringency washes were performed at \(42^\circ\) for 20 min, with three changes.