

Protocols - Location Analysis

The chromatin immunoprecipitation part of this protocol is based on a protocol obtained from the Nasmyth lab and one from Hecht et al. (1996). The Nasmyth protocol was optimized for use with W303 strains tagged with a Myc18 epitope inserted at the C-terminus of various transcription factors (strains obtained from Pia Cosma). The preparation of fluorescent targets (steps #5 and #6) was adapted from a protocol obtained from Oscar Aparicio (S. Bell lab / Univ. Southern California). The protocol for hybridization on glass slide arrays has been developed in the Young lab by C. Wilson and J. Wyrick. The gene specific PCR protocol was documented by F. Robert, scanning and scan analysis protocols were documented by J. Zeitlinger and N. Rinaldi, spectrophotometer analysis of samples was adapted from a protocol developed by the Corning / Whitehead Center for MicroArray Technology.

Location Analysis Protocols (Version 1.2, 08/2001)

Contents:

1. Preparation of cells, cross linking, cell washing and storing
2. Cell lysis, sonication, and immunoprecipitation
3. Bead washing, elution from beads and reversal of cross linking
4. DNA precipitation
5. Blunting DNA and ligation of blunt DNA to linker
6. Ligation-mediated PCR
7. Pre-hybridization, probe preparation, hybridization and wash
8. Scanning

Appendices:

1. Preparation of magnetic beads
2. Preparation of unidirectional linker
3. Solutions
4. Spectrophotometer analysis of LM-PCR products
5. Gene specific PCR
6. Shopping list

Preparation of Cells, Cross Linking, Cell Washing and Storing

Step 1 - Preparation of cells and cross linking

Inoculate fresh media from an overnight culture to OD600=0.1 and allow yeast to grow to OD600=0.6-1.0 (OD600=0.8 is commonly used).

- The experiments are usually done in triplicate, which means you need to put up 3 overnight cultures (inoculated with 3 independent colonies from the same plate).

Remove 50 ml cells and add to 50 ml Falcon tubes (cat #352070) containing 1.4 ml of Formaldehyde (37% Formaldehyde stock, final concentration 1%, J.T.Baker cat.#2106-01).

- Use the liquid dispenser for the formaldehyde and work in a fume hood.

Incubate for 20 minutes at room temperature on a rotating wheel.

- For some proteins, you may have to optimize the incubation time with formaldehyde. This may include transferring to 4°C and incubating overnight on a rotating wheel.

Step 1a - Preparation of beads

If you are planning to continue with the protocol the next day, you also need to incubate the magnetic beads with the anti-Myc antibody overnight (see Appendix 1).

Step 1b - Washing and storage of cells – should be done at 4°C

Spin 50 ml Falcon tubes for 5 minutes at speed 6 (~2800 rpm), temp 4°C in a tabletop centrifuge (Sorvall RT6000) to harvest the cells, and pour off the supernatant.

- Supernatant (medium + formaldehyde) should be treated as hazardous waste – do not pour down the sink

Wash 3 times with ~40 ml cold TBS.

- Add TBS, mix by inversion / shaking until the cells are resuspended, spin and pour off the supernatant.

After the last wash, resuspend the yeast pellet using any remaining liquid (add some, if necessary) and transfer to an Eppendorf tube.

Spin for 1 minute at maximum speed at 4°C and remove the remaining supernatant using a P-1000 pipette.

Snap freeze in liquid nitrogen and store at -80°C, or go directly to step 2.

Cell Lysis, Sonication, and Immunoprecipitation

Step 2 – Cell lysis

Thaw cell pellet on ice.

Resuspend in 700 μ l lysis buffer and transfer to a 1.5 ml Eppendorf tube (cat #2236320-4).

Add the equivalent of a 0.5 ml PCR tube (USA/Scientific Cat.#1405-4400) of glass beads (425-600 μ m, Sigma Cat.# G-8772).

Vibrax-VXR at setting 1800 for 2 hours at 4°C.

Pierce the bottom of the tube with a needle (Use Becton Dickinson Precision Glide 18G1 1/2) and set up over a 2 ml screw cap tube.

Spin 3-4 seconds (the material should be transferred to the 2 ml tube, while the beads stay in the 1.5 ml tube).

- Turn the centrifuge on, allow it to reach 7000rpm and then turn off.

Resuspend and transfer to a new 1.5 ml tube (be sure to have at least 700 μ l in each tube. Add lysis buffer to bring the volume up to 700 μ l, as necessary. Smaller volumes may splash out during sonication).

Step 2a - Sonication

Shear chromatin by sonicating 4 times for 20 seconds at power 1.5 using a Branson Sonifer 250 - use the 'Hold' and 'Constant Power' settings. (This should result in sheared DNA with an average size of 400 bp).

- *Note:* Sonication can be tricky. Keep samples on ice between each round of sonication. Immerse tip in sample first, turn the power on for 20 seconds, turn the power off and place sample back on ice. Wash the tip with water between sample types (it is not necessary to wash the tip between replicates from the same strain). Before and after use of the sonifier, rinse the tip with 98% EtOH.

Spin for 5 minutes at maximum speed at 4°C and transfer the supernatant to another tube on ice (Supernatant = yeast whole cell extract (yWCE)).

Step 2b - Immunoprecipitation

Set up a new tube on ice containing: 500 μ l of yWCE and 30 μ l of a suspension of washed magnetic beads pre-bound to anti-Myc antibody (see Appendix 1). Set aside 5 μ l of WCE in a separate tube (to label as a control later) and store it and the rest of the yWCE at -20°C.

- Vortex the beads well before removing each 30 μ l aliquot to ensure equal amounts of beads are added to each tube and that the beads remain in suspension.

Incubate overnight on a rotating platform at 4°C.

Bead Washing, Elution from Beads and Reversal of Cross Linking

Step 3 - Bead Washing

***** Work in the Cold Room *****

Wash beads using appropriate device (e.g. MPC-E magnet, Dynal), as follows:

- Put the first 6 tubes into magnet, invert the tubes once, open the tubes and aspirate the supernatant using a vacuum (also aspirate what is left in the cap), add the appropriate washing solution, close the tubes and put them back on the rotating platform. Proceed with the next 6 tubes and so on. Don't forget to turn the rotator on while you are aspirating the supernatant from the next set of tubes etc.
- For this step, you don't need to add protease inhibitors to the lysis buffer.

Wash 2 times with 1 ml lysis buffer.

Wash 2 times with 1 ml lysis buffer containing an additional 360 mM NaCl

- 720 μ l of 5 M NaCl in 10 ml lysis buffer - the final concentration of NaCl is 500 mM.

Wash 2 times with 1ml wash buffer.

Wash once with 1 ml TE.

After you have removed the TE by aspiration, spin the tubes for 3 minutes at 3000 rpm and remove any remaining liquid with a pipette.

Step 3a - Elution from beads and reversal of cross links

Add 50 μ l elution buffer (NOT Qiagen buffer EB, see Appendix), vortex briefly to resuspend the beads and incubate at 65°C for 10 minutes. Vortex briefly every 2 minutes during the incubation.

*****The next steps should be done at room temperature*****

Spin for 30 seconds at maximum speed and transfer 30 μ l of supernatant to a new tube. Discard the rest (unless have a special reason to keep it).

Add 120 μ l of TE/SDS to the supernatant in the new tube in order to reverse the crosslinks. Also add 95 μ l of TE/SDS to 5 μ l of yWCE (prepare one yWCE for each IP).

Incubate overnight at 65°C in an incubator.

DNA Precipitation

Step 4 - Precipitation of DNA

Add 150 μ l of "proteinase K mix" (see Appendix) to each sample.

Incubate for at least 2 hours at 37°C in the warm room.

Extract 2 times with 1 volume of phenol (Sigma Cat. P-4557; OK to use at 4°C). Spin for about 5 minutes at room temperature for each extraction.

Extract once with 1 volume of chloroform/isoamyl alcohol (Sigma Cat.C-0549).

Add NaCl to 200 mM final (use 8 μ l of 5 M stock for 200 μ l of sample).

Add 2 volumes of cold EtOH and vortex briefly.

Incubate at -20°C for at least 15 minutes.

Spin at 14,000 rpm for 10 minutes at 4°C.

Pour off the supernatant, add 1 ml cold 75% EtOH, vortex briefly and spin at 14,000 rpm for 5 minutes at 4°C.

Pour off the supernatant, spin briefly and remove the remaining liquid with a pipette.

Let the pellet dry for a couple of minutes and resuspend the pellet in 30 μ l TE containing 10 μ g RNaseA (add 33 μ l of 10 mg/ml RNaseA to 1 ml of TE).

Incubate for at least 1 hour at 37°C in the warm room.

Purify using Qiagen PCR purification kit. Elute with 50 μ l of 10 mM Tris pH 8.0. (Qiagen Buffer EB)

If you are proceeding to step 5 and/or gene-specific PCR (see Appendix 5), place samples on ice.

Store samples / remainder of samples at -20°C

Blunting DNA and Ligation of Blunt DNA to Linker

Step 5 - Blunting DNA

Transfer 40 μ l of each immunoprecipitated DNA and 1-2 μ l of each whole cell extract DNA plus 39 μ l ddH₂O to separate PCR tubes. Place on ice. Save the remaining DNA at -20°C for gene specific PCR analysis.

- *Note:* If you are going to do a "WCE vs WCE control" (recommended), make 2 extra samples with 1-2 μ l whole cell extract DNA + 39 μ l ddH₂O, using the same whole cell extract DNA for each.

Add 70 μ l of:

| | |
|--------------|--|
| 11 μ l | (10X) T4 DNA pol buffer (NE Biolabs cat #B02035) |
| 0.5 μ l | BSA (10 mg/ml) (NE Biolabs cat #007-BSA) |
| 0.5 μ l | dNTP mix (20 mM each) |
| 0.2 μ l | T4 DNA pol (3U/ μ l) (NE Biolabs cat #203L) |
| 57.8 μ l | ddH ₂ O |
| <hr/> | |
| 70 μ l | total |

Mix by pipetting and incubate at 12°C for 20 minutes in a PCR machine

- The program name is "12/20", under "Main" in the 2 head PCR machine. Do not use the heated lid option.

Place on ice and add 12 μ l of:

| | |
|--------------|---|
| 11.5 μ l | 3M NaOAc (Sigma cat #S-7899) |
| 0.5 μ l | glycogen (20 mg/ml) (Roche Diagnostics cat #901393) |
| <hr/> | |
| 12 μ l | total |

Mix by vortexing, and add 120 μ l of phenol/chloroform/isoamyl alcohol (25:24:1, Sigma cat.P-3803).

Vortex to mix and spin 5 minutes at maximum speed.

Transfer 110 μ l to a new 1.5 ml Eppendorf tube and add 230 μ l cold EtOH (100%).

Vortex to mix and spin for 15 minutes at 4°C.

Pour off supernatant and wash pellet with 500 μ l cold 75% EtOH.

Spin for 5 minutes at 4°C.

Pour off supernatant, spin briefly and remove any remaining liquid with pipette. Allow to air dry briefly.

Resuspend pellet in 25 μ l ddH₂O and place on ice.

Blunting DNA and Ligation of Blunt DNA to Linker cont.

Step 5a - Ligation of blunt DNA to linker

Add 25 μ l of cold ligase mix:

| | |
|--------------|---|
| 8 μ l | ddH ₂ O |
| 10 μ l | 5X DNA ligase buffer (GibcoBRL cat #46300-018) |
| 6.7 μ l | annealed linkers (15 μ M) (see appendix #2) |
| 0.5 μ l | T4 DNA ligase (NEB cat #202L) |
| <hr/> | |
| 25.2 μ l | total |

Mix by pipetting and incubate overnight at 16°C.

Ligation-mediated PCR

Step 6 - Ligation-mediated PCR

Add 6 μ l of 3M NaOAc (pH 5.2) (Sigma cat #S-7899) to linker-ligated DNA. Mix by vortexing and add 130 μ l cold EtOH.

Mix by vortexing and spin for 15 minutes at 4°C.

Pour off supernatant and wash with 500 μ l 75% EtOH.

Spin for 5 minutes at 4°C.

Pour off supernatant, spin and remove any remaining liquid with a pipette.

Resuspend in 25 μ l ddH₂O and place on ice.

Add 15 μ l of PCR labeling mix:

| | |
|--------------|---|
| 4 μ l | 10X ThermoPol reaction buffer (NEB cat #007-TDP) |
| 5.75 μ l | ddH ₂ O |
| 2 μ l | low T mix (5 mM each dATP, dCTP, dGTP; 2 mM dTTP) |
| 2 μ l | Cy3-dUTP or Cy5-dUTP (use Cy5 for IP DNA and Cy3 for WCE DNA) |
| 1.25 μ l | oligo oJW102 (40 μ M stock) |
| 15 μ l | total |

Try to use Cy3 or Cy5 from the same batch i.e. avoid mixing batches.

Transfer to PCR tubes on ice, place in PCR machine and start program "Cy3" or Cy5" (the programs are stored under "Main" in our PCR machines or under "FR" in the tetrad PCR machine in the back room):

| Step | Time/Instruction | Temp | Notes |
|------|-----------------------------------|------|--|
| 1 | 4 min | 55°C | (make this longer if you have a lot of samples) |
| 2 | 5 min | 72°C | |
| 3 | 2 min | 95°C | |
| 4 | 30 sec | 95°C | |
| 5 | 30 sec | 55°C | |
| 6 | 1 min | 72°C | |
| 7 | Go to step 4 for X* more times | | *32 cycles (total) for Cy5 and 34 cycles for Cy3 |
| 8 | 4 min | 72°C | |
| 9 | Hold | 4°C | |

Ligation-mediated PCR cont.

Add 10 μ l of polymerase mix during step 1 of PCR:

| | |
|--------------|--|
| 8 μ l | ddH ₂ O |
| 1 μ l | 10X ThermoPol reaction buffer (NEB cat #007-TDP) |
| 1 μ l | Taq polymerase (5 U/ μ l) (Perkin Elmer: Use Cat. #N801-0060 i.e. regular Taq., do not use AmpliTaq Gold) |
| 0.01 μ l | PFU Turbo (2.5 U/ μ l) (Stratagene Cat #600250-51) |
| 10 μ l | total |

Run 5 μ l on a 1.5% agarose gel. (The PCR product should be a smear ranging from 200 bp to 600 bp with an average size of 400 bp).

Purify with Qiaquick PCR purification kit. Elute in 80 - 100 μ l (Qiagen buffer EB).

Take spectrophotometer readings of each sample. Use OD260 and OD650 for Cy5, OD260 and OD550 for Cy3. See Appendix for procedure and calculations.

- Note: You should have at least 15pmol of incorporated dye in each channel (20 pmol is recommended) to continue with scanning a sample.

Mix Cy3 and Cy5 eluates for a given target. Use the same pmol of incorporated dye for each channel if possible.

Add 3M NaOAc (Sigma cat #S-7899) to a final concentration of 0.36M (12 μ l 3M NaOAc for 100 μ l eluate). Mix and add 2.5 volumes cold EtOH (260 μ l for 100 μ l eluate).

Mix and spin for 15 minutes at 4°C.

Pour off supernatant and wash with 500 μ l of 75% EtOH.

Spin for 5 minutes at 4°C.

Pour off supernatant, spin and remove any remaining liquid with a pipette.

Store PCR products at -20°C. Keep in a closed box to prevent exposure to light.

Pre-hybridization, Probe Preparation, Hybridization and Wash

Step 7 - Pre-hybridization

Incubate slide in 3.5X SSC, 0.1% SDS, 10 mg/ml BSA for 20 minutes at room temperature with agitation (use a shaker at 60 rpm) and then 20 minutes at 50°C using a pre-warmed solution (place Coplin jar in water bath; use a fresh solution).

Wash slide using RO water.

Blow-dry with nitrogen or by placing slides in a rack and spinning in a centrifuge for 1 min @ 2 krpm.

Step 7a - Probe preparation

During slide pre-hybridization, add 4 µl of ddH₂O to each sample to resuspend, then add 56 µl of 3X SSC, 0.1% SDS.

Add 4 µl of tRNA (8 mg/ml) and mix well by vortexing.

Boil for 5 minutes in a heat block.

Incubate for 5 minutes at 50°C.

Spin briefly.

Step 7b - Hybridization

Pipette 50 µl of probe onto slide and drop cover slip (use the big one so that it will cover the entire array) onto the liquid. Try to avoid bubbles as they exclude the hybridization solution.

Add 15 µl water to the holes in the hybridization chamber.

Assemble the chambers and submerge right side up in a 50°C water bath, hybridize for 20-24 hours.

Step 7c - Wash

Disassemble hybridization chambers with the right side up.

Remove coverslip and immediately place slide in 0.1X SSC, 0.1% SDS at room temperature for 8 minutes with agitation (use shaker at 60 rpm).

Transfer to 0.1X SSC for 5 minutes with agitation.

- *Note:* Transfer slide by slide (do not transfer the whole rack).

Repeat 0.1X SSC wash 2 more times.

Dry by placing slides in a rack and spinning in a centrifuge for 1 min @ 2 krpm and scan immediately or store in the dark until scanning.

Scanning

Step 8 - ScanArray Protocol

Open ScanArray, doubleclick on “SA 5000” under “Instruments”. Select the buttons in the tool bar labeled *1 and *3 to turn on the lasers for Cy5 and Cy3.

- The lasers need 15 minutes to warm up.

Insert the slide to be scanned into the opening in the front of the scanner, right side up. Push the slide in (a fair amount of pressure is needed) until only about 1/4” = 3/8” of the slide is still outside the aperture. Click on the button in the tool bar that looks like a side view of the scanner with something sticking out of it. This will draw the slide into the scanner.

Choose menu item “*Acquire / Start*” (or press Ctrl-a, or click the button in the toolbar labeled ►)

- The “Acquire Image” window will open.

Under “Protocol files”, select “all-IPs.sapr” in the folder “spot”

- (D:/MicroarrayData/ ScannerProtocols/all-IP.sapr).

Next, click the button labeled “Settings...”

- Opens the window “Adjust hardware settings”)

Click on the number next to the channel you wish to adjust, make sure the laser power is 100%, and change the PMT gain as appropriate

- Good starting values for PMT gain are 70 for Cy3 and 60 for Cy5

Click on the button labeled “OK”. This will return you to the “Acquire Image” window.

Make sure that in the box labeled “Resolution:”, you have “Quick scan” checked, as well as the bullet for “50 um”. Also, verify that the “Scan rate” is 100%.

Click on the button labeled “Acquire” to start the scan

- First you will get a dialog box, asking if you want to save changes to the settings. Click on “Yes”
- Two windows will open, one for each channel. You can stop the scan at any point by clicking on the button labeled ■

Once you see some spots in each channel, set the focus. Click on the rightmost button on the toolbar, the line with a yellow thing underneath it, then use the mouse to draw a line on one of your scans

- Draw the line through some spots that have a relatively high intensity – at least dark blue to green
- The focus window will open

Scanning cont.

There are two ways to set the focus:

- Click on the button labeled “Auto Focus”
 - The scanner will choose the best focus settings. Once it has completed, the check for each channel, the “Focus position” column will give the position and say whether the scan passed or failed.
 - If it failed, go back and choose another line for scanning – signal is not high enough to check the focus in the failed channel.
 - If both channels passed, click on “OK” and go on to determine the optimal PMT gain settings.
- Manual focus:
 - Click on the number next to the channel you wish to adjust.
 - Click on “Start scan”
 - Move the focus position using the mouse (click on either end of the focus position bar) until the signal is maximal
 - Click on “Stop scan”
 - Repeat for the other channel
 - Click on “OK”, then go on to determine optimal PMT gain settings
- NOTE: If you see that the quality of your scan degrades up and down the slide, that indicates that the focus is not the same along the length of the array. There are two fixes for this. First, remove the slide from the scanner (click on the same button you used to draw it in, then pull the slide the remainder of the way out using both hands), then remove the front cover. Use compressed air to clean out any glass dust from the stage area. The second fix is to run the program called “flatcal” – seek assistance to do this if you are still having problems with focus.

Determine the upper value for PMT gain for the Cy3 channel using the following criteria:

- Background value ≤ 200
 - Check the background by clicking on one of the buttons on the right of the toolbar labeled with a circle with an arrow sticking out.
 - Use the mouse to draw a circle on a small section of background of your array.
 - The value of the background will appear in the middle of the gray bar at the bottom of the ScanArray window
- No spot is composed predominantly of saturated (white) pixels

Observe how Cy3 is being scanned. If the image does not pass the criteria, stop scanning and go back to “Acquire / Start”, “Settings...” and change the PMT gain accordingly.

Once the PMT gain for Cy3 passes the two criteria, adjust the PMT gain for Cy5 such that the intensities of the average spot are as similar as possible in the two channels.

- You can scan the Cy5 channel first by changing the channel order in the “Acquire Image” window – click on the “Move Down” or “Move Up” buttons
- You can scan Cy5 without Cy3 by selecting the protocol file “aCy5-IP.sapr” (D:/MicroarrayData/ ScannerProtocols/aCy5-IP.sapr) after opening the “Acquire Image” window

Scanning cont.

Once you are satisfied with the focus and PMT gain settings for each channel, open the “Acquire Image” window again, and change the “Resolution:” to 10um. Click on “Acquire” to scan the images.

Once the scans are complete, click on the image you want to save. Select “File / Save As” from the menu, and go to D:/MicroarrayData/ScannerOutput/YourFolder to save the image as TIFF file. (Example: “JZ 122200 1652-Cy3 int 100_65”). Document the scanning and its quality. Repeat for other images.

- **NOTE: Separate the constant portions of the file name (Initials, date and slide number) from the remainder (channel name, PMT gain) with a unique delimiter (i.e. space, hyphen, underscore). This is to simplify analysis with MolecularWare**

To remove the current slide and scan another, click again on the button you used to draw the slide into the scanner. The slide will be ejected, pull it the rest of the way out using both hands. Insert the next slide and repeat the steps described.

- While the focus doesn’t typically change much from slide to slide, it is a good idea to check it for each slide to make sure it is optimal

At the end of session, drag and drop your saved images into your folder on the lab server (Open “Network Neighborhood”, select “Entire Network”, select “Microsoft Windows Network”, select “Workgroup”, select “Youngserver”

- NOTE: To access your folder on the server, you must be logged into NT with your own username (first initial last name)

Delete the files from the computer!!!

Step 8 - GenePix Protocol

Double click on the GenePix shortcut, select your name from the list of users (type in your name if it’s not already in the list), then click “OK” to open the software

- If you get a message stating that the scanner was not found, make sure that it has been turned on, then click “Retry”. If you still get the message, try shutting down the scanner and computer and then turning everything back on.

Place your slide into the scanner. Slide the door to the left to open it, then open the bracket that holds the slide in place by lifting the locking tab in front of the slide holder. Place your slide into the slide holder FACE DOWN, pulling the clip that secures the slide gently to the left. Close the scanner door.

Open the hardware settings dialog box by clicking on the button labeled with a picture of the scanner – right hand side of the application window, second button from the bottom.

Select starting values for PMT voltage for the two channels

- Good starting values are 600 for Cy5 (635 nm) and 550 for Cy3 (532 nm)

Click on the topmost button on the right hand side, labeled with ►► to start the preview scan

- You can change the PMT voltage settings as the scan is occurring

Scanning cont.

Determine the value for PMT gain for the Cy3 channel using the following criteria:

- Background value ≤ 100
 - Check the background by moving the mouse to a background area of your scan.
 - The value of the background will appear on the left hand side of the window in the statistics for each channel as the value labeled “P:” (for pixel)
- No spot is composed predominantly of saturated (white) pixels

Once the PMT gain for Cy3 passes the two criteria, adjust the PMT voltage for Cy5

- The intensities of the average spot should be as similar as possible in the two channels.
- Click on the “histogram” tab at the top of the application. The histograms for the two channels should be as closely matched as possible

When you are satisfied with the PMT voltage settings for the scan, click on the button on the left hand side that has a picture of a black background with a single white box (Scan Area). This will display a re-sizeable box which you should stretch with the mouse to cover the area of the spots on your scan.

Scan the slide by clicking on the button second from the top on the right hand side, labeled ▶

- You can change the image you are viewing at the top left of the screen to view each channel separately, or the ratio of the two

When the scan is finished, click on the button on the right with a picture of a floppy disk and a folder to save your scan. Choose “Save images...” from the list that appears. The “Save file” window will open. Save the file under E:\Yourname as a multi-image TIFF file (Example: “JZ 122200 1652-550 Cy3 600 Cy5”). Document the scanning and its quality. Repeat for other images.

- **NOTE: Separate the constant portions of the file name (Initials, date and slide number) from the remainder (channel name, PMT gain) with a unique delimiter (i.e. space, hyphen, underscore). This is to simplify analysis with MolecularWare**
- **NOTE: If the filename ends with a number, the program will assume each subsequent file is incremented from the first, which can be a pain, so it's better to end the file name with an alphanumeric value (e.g. 600 Cy3 instead of Cy3 600)**
- **NOTE: Ensure that the “Save as type” value reads “Single-image TIFF file”**

Appendix 1: Preparation of Magnetic Beads

***** Prepare the day before use *****

Take 50 μ l of beads (4×10^8 beads/ml stock *i.e.* 2×10^7 beads per sample) and place in a 15 ml Falcon tube. Use Dynabeads M-450 - pre-coated with pan anti-mouse IgG, cat #110.23

Spin for 1 minute at speed 6 (~3000 rpm) in a tabletop centrifuge (Sorvall RT6000).

Remove supernatant with a pipette and resuspend in 10 ml PBS containing 5mg/ml BSA (make immediately before use from Sigma BSA powder, cat #A-3350).

Wash again with 10 ml PBS/BSA.

Incubate overnight (or at least 8 hours) with antibody on a rotating platform at 4°C (Add the appropriate amount of anti-Myc 9E11 antibody plus 250 μ l PBS + 5 mg/ml BSA per 50 μ l of beads).

- *Note:* The 9E11 antibody we are using has been purified from ascites and concentrated. The amount used has been determined empirically so that the beads are saturated. Batch date 03/28/2000 use 1 μ l per sample, batch date 04/17/2001 use 2 μ l per sample.

***** Day of use *****

Spin for 1 minute at speed 6 (~3000 rpm) in a tabletop centrifuge (Sorvall RT6000).

Remove supernatant with a pipette and resuspend in 10 ml PBS containing 5mg/ml BSA (make immediately before use, as above).

Wash again with 10 ml PBS/BSA.

Resuspend the beads in 30 μ l per sample of PBS containing 5mg/ml BSA.

Appendix 2: Preparation of Unidirectional Linker

Mix the following:

| | |
|--------------|---------------------------------|
| 250 μ l | Tris-HCl (1M) pH 7.9 |
| 375 μ l | oligo oJW102 (40 μ M stock) |
| 375 μ l | oligo oJW103 (40 μ M stock) |
| <hr/> | |
| 1000 μ l | total |

oJW102: GCGGTGACCCGGGAGATCTGAATTC

oJW103: GAATTCAGATC

- *NOTE:* Order these oligos dessicated, then resuspend in ddH₂O.

Make 50 or 100 μ l aliquots in Eppendorf tubes.

Place in a 95°C heat block for 5 minutes.

Transfer samples to a 70°C heat block (there should be water in the holes).

Place the block at room temperature and allow it to cool to 25°C.

Transfer the block to 4°C and allow to stand overnight.

Store at -20°C.

Appendix 3: Solutions

TBS (store at 4°C)

| 1X | 5X | for 1L of 5X |
|----------------------|------------------------|--------------|
| 20 mM Tris-HCl pH7.5 | 100 mM Tris-HCl pH 7.5 | 100 ml of 1M |
| 150 mM NaCl | 750 mM NaCl | 150 ml of 5M |

Lysis Buffer (add protease inhibitors just prior to use, store stock solution at 4°C)

| 1X | for 150 ml | for 5 ml |
|---------------------------------------|------------------|-----------------|
| 50 mM HEPES-KOH pH7.5 | 7.5 ml of 1 M | 250 µl of 1 M |
| 140 mM NaCl | 4.2 ml of 5 M | 140 µl of 5 M |
| 1 mM EDTA | 300 µl of 500 mM | 10 µl of 500 mM |
| 1% Triton X-100 | 15 ml of 10% | 500 µl of 10% |
| 0.1% Na-deoxycholate | 3 ml of 5% | 100 µl of 5% |
| 1 mM PMSF, 1mM Benzamidine | 1.5 ml of 100X | 50 µl of 100X |
| 10 µg/ml Aprotinin, 1 µg/ml Leupeptin | 1.5 ml of 100X | 50 µl of 100X |
| 1µg/ml Pepstatin | 1.5 ml of 100X | 50 µl of 100X |

Wash Buffer (store at 4°C)

| 1X | for 500 ml |
|-----------------------|-----------------------|
| 10 mM Tris-HCl pH 8.0 | 5 ml of 1 M |
| 250 mM LiCl | 25 ml of 5 M |
| 0.5% NP40 | 2.5 ml of <u>100%</u> |
| 0.5% Na-deoxycholate | 25 ml of 10% |
| 1 mM EDTA | 1 ml of 500 mM |

Elution buffer (make with ddH₂O, store at room temperature)

| 1X | for 100 ml |
|----------------------|----------------|
| 50 mM Tris-HCl pH8.0 | 5 ml of 1 M |
| 10 mM EDTA | 2 ml of 500 mM |
| 1% SDS | 10 ml of 10% |

Appendix 3: Solutions cont.

TE/SDS (make with ddH₂O, store at room temperature)

| | |
|----------------------|----------------|
| 1X | for 500 ml |
| 10 mM Tris HCl pH8.0 | 5 ml of 1 M |
| 1 mM EDTA | 1 ml of 500 mM |
| 1% SDS | 5 g |

Proteinase K mix (make fresh)

| For 1 sample | For 26 samples |
|--|----------------|
| 140 µl of TE | 3640 µl |
| 3 µl of glycogen (Boehringer cat# 901393) | 78 µl |
| 7.5 µl of proteinase K (20 mg/ml stock) (<u>Gibco 25530-049</u>) | 195 µl |

20X SSC

| | |
|--|-----------------|
| 20X | for 1L solution |
| 3 M NaCl | 175.32 g |
| 0.3M Na ₃ citrate·2H ₂ O | 88.23 g |
| pH'd to 7.0 with HCl | |

PMSF/Benzamidine mix 100X stock (aliquot and store at -20°C)

| | |
|------------------|----------------------------|
| 1X | For 10 ml of 100X |
| 1 mM PMSF | 0.1742 g |
| 1 mM Benzamidine | 0.1566 g |
| EtOH | Bring to a volume of 10 ml |

Aprotinin/Leupeptinin mix 100X stock (aliquot and store at -20°C)

| | |
|--------------------|----------------------------|
| 1X | For 10 ml of 100X |
| 10 µg/ml Aprotinin | 0.01 g |
| 1 µg/ml Leupeptin | 0.001 g |
| ddH ₂ O | Bring to a volume of 10 ml |

Pepstatin mix 100X (aliquot and store at -20°C)

| | |
|-------------------|----------------------------|
| 1X | For 10 ml of 100X |
| 1 µg/ml Pepstatin | 0.001 g |
| DMSO | Bring to a volume of 10 ml |

Appendix 4: Spectrophotometer analysis of LM-PCR products

Determination of picomoles of dye incorporated and specific activity

Use the spectrophotometer on the 4th floor which can accommodate a microcuvette. Make sure that the UV lamp is on for at least 30 minutes before you read your absorbances.

Select the “Nucleic Acids” protocol from the main spectrophotometer screen

Turn on “Background Correction”, change the three wavelengths read to 260nm, 550 nm and 650 nm. Turn off the “Concentration”.

Put 70-100 μ l of your elution buffer into the microcuvette, and click on “Blank”.

Rinse the cuvette with ddH₂O, then read the absorbance of each sample by clicking on “Read Sample”.

Remove the sample from the cuvette back into the Eppendorf tube, and rinse the cuvette 1-2 X with ddH₂O

- Read absorbance of the entire sample (70-100 μ l)
- Wash the microcuvette with ddH₂O between each sample. Be very careful about contamination in the cuvette as you will be recovering sample for hybridization

Amount of target cDNA:

$$A_{260} \times 37 \times \text{total volume of sample } (\mu\text{l}) = \text{ng of target}$$

pmoles of dye incorporated:

$$\text{Cy3: } A_{260} \times (\text{total volume of sample in } \mu\text{l}) / 0.15 = \text{pmoles of Cy3 dye incorporated}$$

$$\text{Cy5: } A_{260} \times (\text{total volume of sample in } \mu\text{l}) / 0.25 = \text{pmoles of Cy5 dye incorporated}$$

Frequency of incorporation: (# labeled nucleotides per 1000 nucleotides):

$$\text{f.o.i.} = \text{pmoles of dye incorporated} \times 324.5 / \text{amount of cDNA target (ng)}$$

Determination of sample volume to hybridize:

From the above calculations, determine the volume required from each sample to give at least 15 pmoles of incorporated dye for each channel.

$$\text{Total pmoles dye incorporated}/\mu\text{l sample} = \text{pmoles}/\mu\text{l}$$

$$\text{pmoles}/\mu\text{l} \times 20 = \mu\text{l target per array}$$

- Combine at least 15 pmoles of cy3 and cy5 and proceed with DNA precipitation as in Step 6

Appendix 5: Gene-specific PCR for Chromatin IP reaction

Use 1 μ l of immunoprecipitated DNA and series of dilutions of the input DNA (yWCE) (2 μ l, 1 μ l, 1 μ l (1:2) and 1 μ l (1:4) as template for the PCR.

You need to do multiple PCR reactions containing

1. One control primer pair (for a gene that should not be bound or for which the binding should not vary during the experiment)
2. 1 to 3 primer pairs for genes to be tested (this can include a positive control if such a gene is known). If you have more than 3 genes to test, use multiple primer mixes (Protocol B). Too many primers in a PCR reaction can interfere with each other.

Protocol A - One primer mix

Prepare the following master mix for n+2 samples:

| | |
|------------------------|--------------------------|
| 0.05 μ l | ³² P- dATP |
| 0.35 μ l | 10mM dNTPs |
| 1.5 μ l | 10X reaction buffer (PE) |
| 1.5 μ l | MgCl ₂ (PE) |
| 0.15 μ l each | Primers |
| 0.15 μ l | Taq polymerase (PE) |
| Complete to 14 μ l | ddH ₂ O |

Pipet DNA into PCR tubes (as mentioned above) and distribute 14 μ l of master mix to each. Mix by pipetting and place in PCR machine

Run the program "DMITRY" under "MAIN":

| Step | Temp / Instruction | Time |
|------|------------------------|--------------|
| 1 | 95°C | 1 min 30 sec |
| 2 | 95°C | 40 sec |
| 3 | 50°C | 40 sec |
| 4 | 72°C | 40 sec |
| 5 | Go to step 2, 20 times | |
| 6 | 4°C | hold |

During the PCR reaction, prepare a 6% acrylamide gel (add APS and TEMED just prior to pouring):

| | |
|-------------------|--|
| 3.75 ml | 40% acrylamide (30:1) solution (Sigma cat #A-9926) |
| 5 ml | 5X TBE |
| Complete to 25 ml | ddH ₂ O |
| 250 μ l | 10% APS |
| 25 μ l | TEMED (Sigma cat #T-7024) |

Mix and pour gel slowly. Each gel has 20 wells.

After PCR, spin briefly to collect sample at the bottom of the tube

Add 6 μ l of "orange/blue dye" (leave tips into tubes)

Set pipet to 10 μ l, mix by pipetting and load 10 μ l

Store the remaining at 4°C in a beta-secure container

Appendix 5: Gene-specific PCR for Chromatin IP reaction cont.

Protocol B - multiple primer mixes

Prepare the following master mix for 'n+2+number of mixes' samples:

| | |
|-------------------|--------------------------|
| 0.05 µl | ³² P- dATP |
| 0.35 µl | 10mM dNTPs |
| 1.5 µl | 10X reaction buffer (PE) |
| 1.5 µl | MgCl ₂ (PE) |
| 0.15 µl | Taq polymerase (PE) |
| Complete to 14 µl | ddH ₂ O |

Prepare the following primer mixes:

| | |
|--------------|---------------------|
| 0.15 µl each | Primers |
| 14 µl | Master mix total |

Pipet DNA into PCR tubes (as mentioned above) and distribute the appropriate amount of primer mix to each tube. Mix by pipetting and place in PCR machine. Follow the remainder of protocol A.

Appendix 6: Shopping list

| Component | Manufacturer | Catalog number | Amount |
|---|---------------------|-----------------------|-------------------------|
| 37% formaldehyde | J.T. Baker | 2106-01 | 500 ml |
| Glass beads | Sigma | G-8772 | 1 kg |
| Pan anti mouse IgG | Dynal | 110.23 | 10 ml |
| Magnetic beads | | | |
| Proteinase K | Gibco BRL | 25530-049 | 5 x 1 ml |
| Glycogen | Roche Diagnostics | 901393 | 1 ml |
| Phenol | Sigma | P-4557 | 100 ml |
| Chloroform / isoamyl alcohol | Sigma | C-0549 | 100 ml |
| PCR purification kit | Qiagen | 28106 | 250 spin columns |
| PCR purification kit | Qiagen | 28144 | 50 x 8-well strips |
| T4 DNA polymerase buffer | NEB | B02035 | 4 x 1.5ml |
| T4 DNA polymerase | NEB | 203L | 50 µl |
| BSA | NEB | 007-BSA | 1 ml |
| Ultrapure dNTPs | Amersham Pharmacia | 27-2035-01 | 250 µl (100mM)each dNTP |
| 3M NaOAc | Sigma | P-7899 | 100 ml |
| Phenol / chloroform / isoamyl alcohol (25:24:1) | Sigma | P-3803 | 100 ml |
| 5X ligase buffer | Gibco BRL | 46300-018 | 1 ml |
| T4 DNA ligase | NEB | 202L | 250 µl |
| Thermo Pol buffer | NEB | 007-TDP | 4 x 1.5 ml |
| Taq polymerase | Perkin Elmer | N808-0153 | 12 x 50 µl |
| | | N808-0167 | 6 x 50 µl |
| PFU Turbo | Stratagene | 600250-51 | 40 µl |