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Robotic spotting of microarrays:

Library management

- Adding spotting buffer to the oligonucleotide library ([jump](#))
- Library dessication and rehydration ([jump](#))

Instrument optimisation

- Introduction to printing microarrays ([jump](#))
- Microarray instrument optimisation ([jump](#))

Microarray printing

- Printing microarrays with a BioRobotics MicroGrid II 600 or 610 spotter ([jump](#))
- Printing microarrays with a Genetix Qarray2 spotter ([jump](#))

Microarray processing

- Processing Full Moon Biosystems cDNA slides after spotting ([jump](#))
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Microarray quality control

- Quality control by staining with SYBR_555 ([jump](#))

Microarray spot tracking file formats

- A key to standard microarray spot identity tracking file formats ([jump](#))

FlyChip protocol for adding spotting buffer to 384-well printing plates

Overview

Oligonucleotide libraries are dispatched to us in 384-well microtitre plates. Spotting buffer is added to these plates using a Beckman Coulter Biomek NX^P Liquid Handling Robot (LHR).

Equipment and reagents

- Beckman Coulter Biomek NX^P Liquid Handling Robot (LHR)
- AP96 non-sterile P20 tips (Beckman Coulter; Cat. No. 717254)
- 70% Ethanol
- Spotting buffer
- Hettich Rotina 35 microtitre plate centrifuge
- Adhesive PCR Film (Abgene; Cat. No. AB-0558)
- Horizontal laminar flow work station (Jencons; Cat. No. 566-031)

Procedure

1. Remove the microtitre plates from the -80 °C freezer and leave to defrost
2. Once defrosted, centrifuge all plates at 2000 rpm for 2 minutes
3. Clean the exterior and interior of the LHR using the Dyson vacuum cleaner and wipe with 70% Ethanol
4. Open the program "Hydrate_LIBRARY_YesWash" and home all instrument drives
5. Fill the in-flow wash tank with distilled water and prime the wash station, *e.g.*, for 3-5 min.
6. Load the instrument, as directed by the "Hydrate_LIBRARY_YesWash" program, *i.e.*, fresh box of AP96 P20 tips, reservoir filled with spotting buffer and the plate to be hydrated
 - ◆ Adhesive film should be removed just before the plate is loaded into the LHR
 - ◆ All plates should be loaded in the LHR with well A1 in the top-left corner
7. Start the program and watch to make certain the LHR is working correctly
8. Repeat steps 6 to 7 until all plates have been rehydrated: as each plate is finished, remove from LHR and affix an adhesive PCR film
9. Centrifuge all plates at 2000 rpm for 2 minutes and then incubate the plates at 37 °C for 2 hours to dissolve the probe DNA
10. Clean the LHR to make certain that it has been left ready for others to use
11. Centrifuge all plates at 2000 rpm for 2 minutes and store the plates at -80 °C

R. Auburn (24-02-2009).

Library desiccation and rehydration

Overview

Spotting solution in microtitre plates evaporates during printing. The buffer in the wells at the edge, and especially in the corners of each plate, usually evaporates more quickly than the from the wells in the centre. Evaporation therefore causes variations in probe concentration across the plate. These systematic variations can have a detrimental impact on microarray performance, *e.g.*, variable spot signals, variable spot diameters and variable spot morphologies. These problems are best overcome by desiccating and rehydrating plates between print-runs.

Equipment and reagents

- Beckman Coulter Biomek NX^P Liquid Handling Robot (LHR)
- AP96 non-sterile P20 tips (Beckman Coulter; Cat. No. 717254)
- MilliQ water (unmodified probe DNA) or 0.2 µm filtered distilled water (modified probe DNA)
- Hettich Rotina 35 microtitre plate centrifuge
- Adhesive PCR Film (Abgene; Cat. No. AB-0558)
- 70% ethanol
- Horizontal laminar flow workstation (Jencons; Cat. No. 566-031)

Procedure

Desiccation

1. Remove the microtitre plates from the -80 °C freezer and leave to thaw on a desk
2. Switch the laminar flow workstation on and leave for at least 30 minutes before putting the plates inside
3. Once defrosted, centrifuge all plates at 2000 rpm for 2 minutes
4. Remove the adhesive film from the plates and leave to desiccate in the laminar flow workstation, *e.g.*, four days
5. Once desiccated, the plates can then be resealed and stored at -80 °C

Whilst using the LHR

6. Remove the microtitre plates from the -80 °C freezer and leave to thaw
7. Once thawed, centrifuge all plates at 2000 rpm for 2 minutes
8. Clean the exterior and interior of the LHR using the Dyson vacuum cleaner and wipe with 70% Ethanol
9. Open the program "Rehydrate_LIBRARY_YesWash" and home the instrument drives
10. Fill the in-flow wash tank with distilled water and then prime the wash station by switching the FX Device Controller (HV 1) to 'manual', *e.g.*, for 3-5 min.
11. Load the instrument, as directed by the "Rehydrate_LIBRARY_YesWash" program, *i.e.*, fresh box of AP96 P20 tips, reservoir filled with water and the plate to be rehydrated:
 - ◆ The adhesive PCR film should be removed as the plate is being loaded into the LHR
 - ◆ All plates should be loaded in the LHR with well A1 in the top-left corner
12. Start the program and watch to make certain that the LHR is working correctly
13. Repeat steps 11 to 12 until all plates have been rehydrated: as each plate is finished, remove from LHR and affix an adhesive PCR film
14. Clean the LHR to make certain that it has been left ready for others to use

After using the LHR

15. Centrifuge all plates at 2000 rpm for 2 minutes and then incubate at 37 °C for 2 hours to redissolved the probe DNA
16. Clean the LHR to make certain that it has been left ready for others to use
17. Centrifuge all plates at 2000 rpm for 2 minutes and store the plates at -80 °C

An Introduction to printing spotted microarrays

Overview

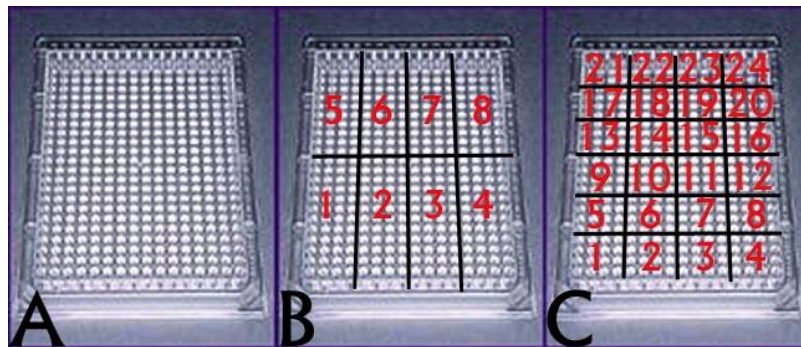
Microarrays are typically produced by transferring gene or transcript specific PCR amplified cDNA clones or long oligonucleotides in either high salt or other denaturing solutions from 384-well microtitre plates to chemically modified 25 x 75 mm glass microscope slides using robotic contact printing instruments. This process is sometimes termed robotic spotting or just spotting. Although robotic spotters were first described by Schena et al. (1995) they are also available commercially.

Printing microarrays

The spotting pins are a vital component because they are the only part of the instrument to contact the probe DNA and the slides. Spotting pins draw fluid into the pins by capillary action when a pin loading is performed. The pins enter adjacent wells of the 384-well microtitre plate because they have a fixed pitch of 450 μm in the print-head. The probe DNA within the microtitre plates therefore needs to be arranged accordingly for any given microarray layout to be printed correctly.

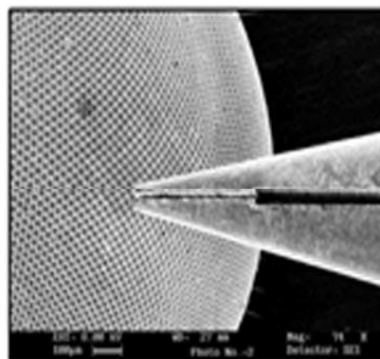
Example source visit layouts

A) A 384-well Genetix plate. B) 12x4 pin-tool source visit layout. C) 4x4 pin-tool source visit layout



When depositing a spot on the slide, surface tension interactions between the substrate surface and spotting buffer lead to spot formation when the pins pull away. Spot diameter is determined by a variety of parameters including the spotting buffer, substrate slide, temperature, relative humidity and the pin itself. Most spotting pins are blunt ended (50 to 100 μm) with a capillary or split and contain a storage reservoir. An example of one such pin is shown in the figure below.

Electron micrograph of a *BioRobotics MicroSpot 2500* pin



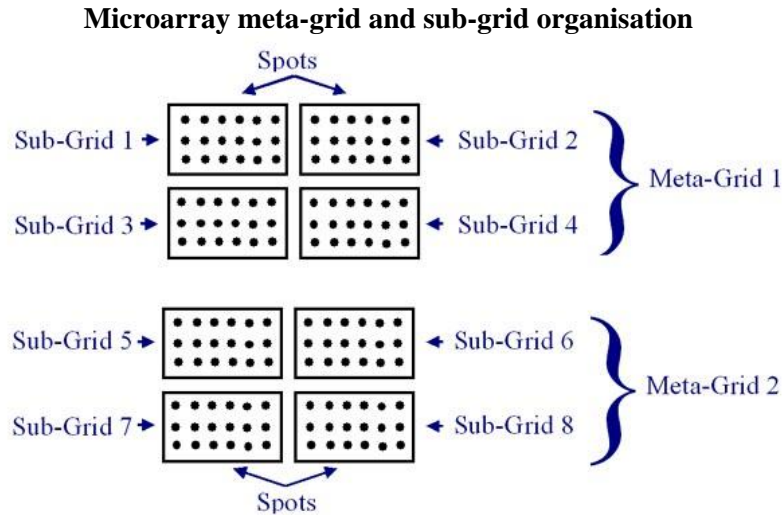
Electron micrograph of MicroSpot 2500 pin and the eye of a house fly

Spot density is determined by the diameter of the printed spots because this dictates the centre-to-centre spot distance that can be used during printing. Additionally, the accuracy and reliability of the robotic spotter will determine the reproducibility of the spot positioning within each microarray. This is sometimes referred to as pin or spot wobble. An imprecise instrument with lots of pin or spot wobble will require a greater

centre-to-centre spot distance, which will reduce the maximum spot density that can be achieved.

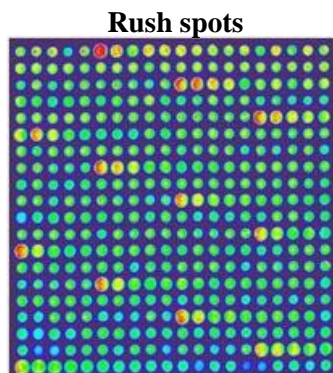
Sub-grids and meta-grids

Each pin within the print-head will produce a single pin-patch or a sub-grid of spots. The sum total of sub-grids printed by one pin-tool is called a meta-grid. A single microarray can consist of one or more meta-grids. The number of meta-grids that be printed per microarray is limited by the space available. FlyChip typically spots microarrays using 48 pins and therefore has 48 sub-grids per microarray but just one meta-grid. Other formats are also possible with our set-up and have indeed been produced.



Rush spots

The first few spots to be printed after a pin loading, the rush spots, are larger than any subsequently printed spots, as when a pin loading is performed some spotting solution will coat the outer surfaces of each pin. Until printing has exhausted this additional spotting solution the printed spots will not have a regular diameter. This problem can be overcome by discarding the first few slides after each pin loading or by using blotting slides that can then be discarded. The later option is preferable when printing arrays with an inter-spot distance that is less than the maximum spot size.



The above image is a close-up of 1 sub-grid printed with Cy3-labelled sonicated salmon sperm DNA using a BioRobotics MicroGrid II 600 spotter MicroSpot 2500 pins. The spotter started to print spots in the bottom left-hand corner and moved to the right and then up the image. 35 spots were printed per pin loading. This image clearly shows how the spot size initially decreases in diameter before reaching a uniform size. The number of slides to be discarded at the start of each print-run or the number of blotting spots to be printed should be defined in advance.

Spot morphology

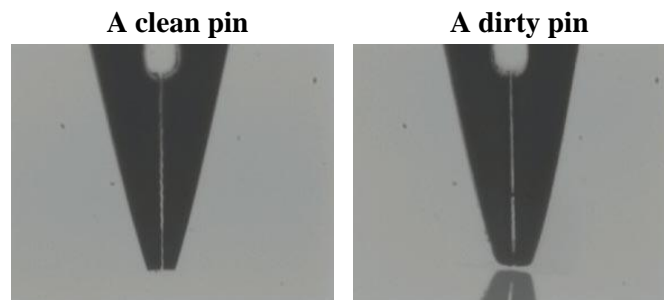
Key determinants of spot morphology have previously been described in the literature. These include substrate chemistry and hydrophobicity, spotter calibration and print settings, spotting buffer viscosity, pH and evaporation, probe DNA concentration, room temperature and relative humidity. Because of these complex interactions it is not possible to predict in advance which combination of conditions should be used for any given clone-set or spotter. For this reason, we routinely check and then optimise the spotting conditions for each of our clone-sets.

Wash cycles

Wash cycles are performed between each pin loading to ensure that the spotting solution from one loading does not contaminate any subsequent pin loadings. The number and length of wash cycles is a key determinant of how long any given print run will last for because the time taken to perform the pin loadings and spot depositions is minimal. Care must therefore be taken to ensure that that wash cycle is optimised for a fast print-run time and low probe DNA carry-over.

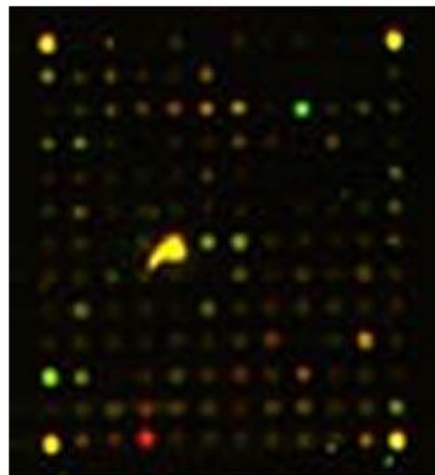
Cleanliness

Microarrays should be printed, stored, hybridised and scanned in a clean dust-free environment to ensure that arrays of the highest possible quality are produced. Spotting pins contain small capillaries and reservoirs that can be easily blocked by dust. Such blockages can lead to pins performing badly or being predisposed to further blockages. Poorly performing pins will either print inconsistently or not at all and a significant amount of potentially valuable data will be lost.



The biological samples being co-hybridised to the microarray are labeled with the Cy3 and Cy5 dyes respectively. Unfortunately, dust and other airborne contaminants are fluorescent under the Cy3 and/or Cy5 wavelengths. The complexities of image analysis mean that any fluorescent contaminant that lies within the spot will adversely affect the measured spot signal and hence, interpretation of the experimental result.

Dust particles



Spot tracking

Each microarray spot has a unique position and each spot corresponds to a specific probe DNA from a specific well of a microtitre plate. There are typically thousands to tens of thousands of probe DNAs distributed between tens of source plates and thousands to tens of thousands of spots on each microarray. A single microarray experiment can consist of a few or a hundred microarray hybridisations. Tracking where and what each probe DNA is on each microarray is therefore an important issue.

Most robotic spotters are supplied with a data tracking program that uses an input file to describe the positions of each probe DNA within the microtitre plates and another file that defines how the microarray was printed to produce a description of where each probe DNA is within each microarray. These spot identities can then be imported into a spot finding and quantification tool that will 'append' the fluorescence spot signal. These data are then analysed to determine what affect any given experimental condition or treatment has had on the gene expression of the samples being compared.

Summary

Many disparate factors need to be considered when printing microarrays. These affect the quality of the microarrays produced, the rate of production and the ease with which any downstream data analysis may be performed. Failure to fully appreciate these limitations will result in sub-standard microarrays being produced and experimental data that may be unreliable. Extreme care and consideration must therefore be taken to ensure that the best quality microarrays are produced.

R. Auburn (17-02-2006).

General introduction to optimising the performance of a robotic spotter

Overview

Many disparate factors need to be considered when printing microarrays. These affect the quality of the microarrays produced, the rate of production and the ease with which any downstream data analysis may be performed. Failure to fully appreciate these limitations will result in sub-standard microarrays being produced and hence experimental data that may be unreliable. Extreme care and consideration must therefore be taken to ensure that microarrays of the best quality are produced.

Loading the spotting pins with probe DNA

Two different approaches can be used to fill spotting pins with spotting solution. Firstly, one can repeatedly dip the pins into the spotting buffer with probe DNA. Secondly, one can use a slow speed single entry into the spotting solution followed by a brief pause. We have found that the second approach is more consistent and reduces the number of rush spots produced after each pin loading.

All of the pin tips need to enter far enough into the microtitre plate wells to ensure that they are all below the meniscus of the spotting solution. We will call this the source target depth and this needs to be determined experimentally. This can be achieved by systematically altering the source target depth and observing when the pins pass through the spotting solution meniscus. One should also then check to ensure that all pins are able print at least 60 spots per pin loading.

If the observed rush is proving to be problematic one could either switch to a different spotting buffer or reduce the source target depth height. If one or a few pins are not printing when all others are, one should check that the pins are all of equivalent length and that none are blocked. If in doubt contact the instrument supplier and pin manufacturer for further assistance.

We have not fully optimised the pin speed. However, a quick appraisal demonstrated that at 4 mm/s the pins would reliably fill with spotting solution without adversely effecting the print-run time. This pin speed therefore provides the best balance between the number of spots that can be printed per pin loading, rush duration and microarray throughput. Other pin types may vary and require different pin speeds.

Depositing the probe DNA on the substrate slide

We shall call the height at which all pins make contact with all slides in all slide positions the target height. Some robotic spotters enable the spotting pins to decelerate to a reduced pin speed at a specified height from the slide surface and we will these the the soft touch speed and soft touch distance. Each of these three parameters will need to be optimised if high quality microarrays are to be produced.

The slide trays or beds within most robotic spotters are not perfectly level and the pins themselves are not all the same length. In order to calculate the soft touch distance one firstly needs to determine the absolute difference between the lowest and highest slide position. Secondly, you then need to determine the difference in length between the pins. These values can then be added together and used as the soft touch distance. In order to confirm if this value has been calculated correctly, you just need to check that the longest pin is able to slow to the correct speed before contacting the highest slide position. Failure to do so will result in a poor and highly variable spot structure.

The target height is much easier to determine. Print one slide using a 10 x 15 sub-grid and systematically change the target height after each row of 10 spots has been printed. Use the height at which all pins print reliably plus the difference in height between the slide tray or bed position at which this test was performed and the lowest slide position to define the correct target height. This should enable the shortest pin to make contact with the lowest slide position. We have found that a pin speed of 4 mm/s provides the best balance between spot quality and microarray throughput.

The calculated settings are likely to vary between each instrument and print-head. Additionally, microarray slides from different commercial suppliers also vary in thickness. This means that the above settings need to be determine for each combination of print-head, instrument and substrate chemistry. It is therefore a good

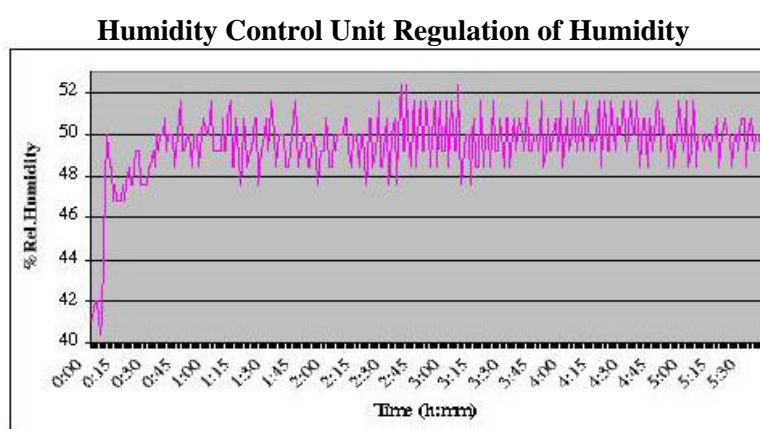
idea to document these settings, otherwise one risks printing microarrays with inappropriate spotter settings.

Selecting an appropriate substrate chemistry and spotting buffer

We have submitted a paper on this topic for publication. Once it has been accepted further details and a link to the abstract will be posted on this web page.

Printing temperature and relative humidity

High temperature and high humidity produces large ill-defined spots, whereas low temperature and low humidity produces small less distinct spots. FlyChip prints microarrays in an air-conditioned room using robotic spotters fitted with humidity control. This ensures that our microarrays are printed under controlled environmental conditions. We typically use a temperature range of between 19 to 21 °C and a relative humidity of 50 to 60%. We vary the humidity to match the slide manufacturers recommendations and are able to maintain the printing environment to within 2% of the target humidity with each of our spotters. We routinely record the temperature and humidity because these can have a profound influence on spot morphology and spot size.



Pin cleaning whilst printing and between print runs

Pins are cleaned between print-runs and between pin loadings to ensure that the spotting solution from one group of wells does not contaminate any subsequent pin loadings or spot depositions. If this were to happen it would effectively reduce the hybridisation stringency because each spot on the microarray would be comprised of the required probe DNA and a variable amount of all other probe DNAs. Each robotic spotter cleans pins in a different way and the amount of probe DNA carry-over between pin loading varies between spotting buffers. The number and length of wash cycles is a key determinant of how long a print run will last for, as the time taken to perform the pin loadings and spot depositions is often minimal.

Fortunately, it is quite straight forward to identify a good wash condition because all one needs to do to is to assess how much probe DNA is carried-over between one pin loading and the next. This can be achieved by using a microtitre plate with three pin loadings. The first and last should only contain spotting buffer, whereas the second should contain spotting buffer with labelled DNA. One can then print test slides using different wash conditions and measure the amount of carry-over between the second and third pin loading. The first can be used to correct for background fluorescence from the spotting buffer itself, e.g. 150 mM NaPO₄. These experiments should be performed using a full set of pins because the technical constraints of most spotters mean that pin number can effect how well pins are cleaned.

Microtitre plate storage and handling

Microtitre plate storage and handling is important because all spotting buffers evaporate and this evaporation is not uniform across the surface of each plate. Specifically, the spotting buffer within the wells at the corners and edge of the microtitre plate will evaporate more rapidly than within the wells towards the centre of each plate. This means that all microtitre plates should be treated with care and not stored uncovered. In fact, it is a good idea to use plate sealers to prevent evaporation leading to a reduction in the spotting buffer volume. One should also cover the plates with lids to prevent damage to the plastic

seals. The plates themselves can be stored in a freezer to further inhibit evaporation.

R. Auburn (17-02-2006).

Printing microarrays with a BioRobotics MicroGrid II 600 or 610 spotter

Overview

The following procedure is designed to minimize human error and maximize the performance of the MicroGrid II arrayer and the MicroSpot pins. This procedure assumes the 'Main Pump Unit' has been set to use both re-circulating wash tanks and the main wash station. If you are in any doubt about using the MicroGrid II ask the machines primary operator for assistance. FlyChip accepts no responsibility whatsoever for any damage incurred by following these instructions.

Equipment and reagents

- MicroGrid II 610 TAS spotter and print-head
- Substrate slides
- 70% Ethanol
- Ultrasonicator (Ultrawave; Cat. No. U100H)
- MilliQ water
- Surface-cleanse/930 (International Products Corporation; Cat. No. S-2001-12)
- Adhesive PCR Film (Abgene; Cat. No. AB-0558)
- Dyson DC05 Vacuum Cleaner

Procedure

Weekly and daily maintenance:

1. **Clean the Lab:** Use a Dyson vacuum cleaner to remove dust from the local work area and then wipe with a damp cloth. It is important to keep the working area and the laboratory in a generally clean state. This should be done every week.
2. **Cleaning the MicroGrid II 're-circulating wash tanks':** Turn the MicroGrid II on and start the 'TAS Application'. The MicroGrid II will zero itself. From within the 'Housekeeping Section' select 'Prime The Re-Circulating Baths' to fill the re-circulating wash tanks with Milton Sterilizing Solution (1 to 2 hours). From within the 'Housekeeping Section' select 'Fill the main wash station' (MWS) to fill with MWS 6-litre storage tank with Milton. Then rise each three times for 10 minutes using MilliQ water by following the same steps. Finally, wipe all accessible portions of the re-circulating wash tank and MWS pathway using 70% Ethanol. This should be done once a week.
3. **Cleaning the MicroGrid II interior:** Vacuum the arrayer using the Dyson vacuum cleaner to remove particulates from the arrayer. Wipe the interior of the arrayer using 70% Ethanol and then re-vacuum. This should be done before every print-run.

Pin care and maintenance:

4. **Cleaning the microspot pins:** These pins are best cleaned using ultrasonication. The pins should be sonicated in either 0.1 x SSC (sodium phosphate spotting buffers) or 2% Surface-cleanse/930 (all other spotting buffers) at room temperature (standard clean) or 65 °C (heavy clean). These sonications should last for between 5 minutes and 2 hours, depending on the level of cleaning required for the pins to function correctly.
5. **Rinsing the MicroSpot pins after cleaning:** After sonication with cleaning solution the pins need to be rinsed by sonicating 3 times for 2 minutes in MilliQ water. The pins can then be used in a print-run without carry-over from the cleaning solutions.
6. **Pin storage after cleaning:** If the pins will be used immediately they can be stored attached to the arrayer whilst the print-run is set-up. Otherwise they should be dried and stored in the BioRobotics pin-tool holder.

How to perform a print-run:

7. **Fill the arrayer with wash solution:** Turn the MicroGrid II on and start the 'TAS Application'. The MicroGrid II will zero itself. Using the 'Housekeeping Section' 'Prime The Re-Circulating Baths' option fill the left- and right-hand re-circulating wash tanks with MilliQ water. Fill the main wash

- tank with MilliQ water using the 'Fill 6-litre reservoir' option.
8. **Fill the 'Humidity Control Unit' with MilliQ water:** Fill the 'Humidity Control Unit' with MilliQ water following the manufacturers instructions. Click on the 'Climate' tab from the 'Run Preferences' section. Check the MicroGrid II is using the correct 'Humidity Control Unit' settings. Start the 'Humidity Control Unit' and set the display to update every 1 second. Periodically check the humidity level whilst the clone-set plates defrost.
 9. **Thawing the library:** Remove the library from the -80 °C freezer. Leave to defrost at room temperature in a safe place. Centrifuge all plates at 2000 rpm for 2 minutes in the 'Hettich Rotina 35' microtitre plate centrifuge. This will remove surface moisture.
 10. **Loading the arrayer:** Load the 'BioBank' with the library and the 'Slide Trays' with the slides. Make sure the plate seals are removed from the plates, the plates are in the correct orientation and the plates are in the correct order. Make sure the slides have been loaded correctly and the vacuum pump is able to keep them in place.
 11. **Loading the grid program:** Open the required print run parameters file from the 'My Gridding Runs' directory. Confirm the correct file has been opened. Confirm the settings are correct. If you are in any doubt ask the primary operator for assistance.
 12. **Confirm the correct layout will be printed:** Confirm the correct print-run settings file has been opened by comparing the set-up file of this print run with the standard file for the library to be printed. Do not start the run until the target humidity has been reached, otherwise the spots will be the wrong size and the library will rapidly evaporate.
 13. **Start the run:** Start the run and periodically observe the MicroGrid II during the print run to be certain everything is OK. If any problems occur get the MicroGrid II primary operator to take a look. The print-run time will depend on the print-run program being used. Please ask the MicroGrid II primary operator to determine how long the print-run will last for.

After the print-run has finished:

14. **End of the program:** Follow the 'on-screen' instructions to remove the printed slides, library and pin-tool. Store each in its correct location: pin-tool should be cleaned (above) and then stored (above); slides should be stored in a cool, dry and dust-free cupboard; plates should be sealed and put at -80 °C.
15. **Empty solution from the arrayer:** All solutions should be removed from the arrayer and the arrayer itself should then be cleaned using a cloth soaked in 70% ethanol. The re-circulating wash tanks, baths and pipework, the MWS, and the MWS inlet/outlet pipes, should also be cleaned in this way. This will prevent microbial growth within the arrayer, when used in conjunction with the weekly cleaning schedule (above).
16. **Complete all data tracking forms:** All data tracking forms should be completed in full to ensure a complete record of every slide and print run can be maintained. This will be used to track all slides produced by FlyChip that are either given to external groups, or used by FlyChip for external groups experiments.
17. **What's next:** The slides need to be processed and the print-run needs to be quality controlled before any of the slides that have just been printed can be used. Please refer to the appropriate protocols on this web site.

R. Auburn (17-02-2006).

Printing microarrays with a Genetix Qarray2 spotter

Overview

The following procedure is designed to minimize human error and maximize the performance of the Qarray2 arrayer and the aQu pins. This procedure assumes the stacker has a humidity control unit fitted. If you are in any doubt about using the Qarray2 ask the machines primary operator for assistance. FlyChip accepts no responsibility whatsoever for any damage incurred by following these instructions.

Equipment and reagents

- Qarray2 with humidity controlled stacker
- Substrate slides
- 80% Ethanol
- 70% Ethanol
- Ultrasonicator (Ultrawave; Cat. No. U100H)
- Water - distilled and MilliQ
- Surface-cleanse/930 (International Products Corporation; Cat. No. S-2001-12)
- Adhesive PCR Film (Abgene; Cat. No. AB-0558)
- Dyson DC05 Vacuum Cleaner

Procedure

Weekly and daily maintenance:

1. **Clean the Lab:** Use a Dyson vacuum cleaner to remove dust from the local work area and then wipe with a damp cloth. It is important to keep the working area and the laboratory in a generally clean state. This should be done every week.
2. **Cleaning the Qarray2 'wash tanks':** Turn the Qarray2 on then press the reset button and start the 'Qarray MicroArray' software. The Qarray2 will zero itself. Fill the water (distilled water) and ethanol (80% ethanol) bottles. From within the 'Robot Diagnostics' select 'Wash Head' and then 'Water' to rinse the wash tanks with water (repeat three times). Then, from 'Robot Diagnostics' select 'Wash Head' and then 'Ethanol' to rinse the wash tanks with ethanol. This should be done once a week.
3. **Cleaning the Qarray2 interior:** Vacuum the arrayer using the Dyson vacuum cleaner to remove particulates from the arrayer. Wipe the interior of the arrayer using 70% Ethanol and then re-vacuum. This should be done before every print-run.

Pin care and maintenance:

4. **Cleaning the aQu pins:** These pins are best cleaned using ultrasonication. The pins should be sonicated in either 0.1 x SSC (sodium phosphate spotting buffers) or 2% Surface-cleanse/930 (all other spotting buffers) at room temperature (standard clean) or 65°C (heavy clean). These sonications should last for between 5 minutes and 2 hours, depending on the level of cleaning required for the pins to function correctly.
5. **Rinsing the aQu pins after cleaning:** After sonication with cleaning solution the pins need to be rinsed by sonicating 3 times for 2 minutes in MilliQ water. The pins can then be used in a print-run without carry-over from the cleaning solutions.
6. **Pin storage after cleaning:** If the pins will be used immediately they can be stored attached to the arrayer whilst the print-run is set-up. Otherwise they should be dried and stored dry in a pin-tool holder.

How to perform a print-run:

7. **Fill the arrayer with wash solution:** Turn the Qarray2 on then press the reset button and start the 'Qarray MicroArray' software. The Qarray2 will zero itself. Fill the water (distilled water) and ethanol (80% ethanol) bottles. From within the 'Robot Diagnostics' select 'Wash Head' and then 'Water' to rinse the wash tanks with water. Then, from 'Robot Diagnostics' select 'Wash Head' and then 'Ethanol' to rinse the wash tanks with ethanol.

8. **Fill the 'humidifiers' with MilliQ water:** Fill the humidifiers with MilliQ water following the manufacturers instructions. Click on the 'Humidity' button to start the humidity control units. Periodically check the humidity level whilst the library to be printed defrosts.
9. **Thawing the library:** Remove the library from the -80 °C freezer. Leave to defrost at room temperature in a safe place. Centrifuge all plates at 2000 rpm for 2 minutes in the 'Hettich Rotina 35' microtitre plate centrifuge. This will remove surface moisture. The pins can be left printing water whilst this step is being performed.
10. **Loading the arrayer:** Load the 'Stacker' with the library plates and the 'Slide Bed' with the slides. Make sure the plate seals are removed from the microtitre plates, the microtitre plates are in the correct orientation and the plates are in the correct order. Make sure the slides have been loaded correctly and the vacuum pump is able to keep them in place.
11. **Loading the spotting script:** Open the required script file. Confirm the correct file has been opened. Confirm the settings are correct. If you are in any doubt ask the primary operator for assistance.
12. **Confirm the correct layout will be printed:** Confirm the correct print-run settings file has been opened by comparing the set-up file of this print run with the standard file for the library to be printed. Do not start the run until the target humidity has been reached, otherwise the spots will be the wrong size and the library will rapidly evaporate.
13. **Start the run:** Start the run and periodically observe the Qarray2 during the print run to be certain everything is OK. If any problems occur get the Qarray2 primary operator to take a look. The print-run time will depend on the print-run program being used. Please ask the Qarray2 primary operator to determine how long the print-run will last for.
14. **Refill the water wash bottles:** The Qarray2 water bottles do not store sufficient water for long print-runs. Ask the Qarray2 primary operator if this will be needed for this print-run and then seek his help when the bottles need to be refilled.

After the print-run has finished:

15. **End of the program:** Remove the printed slides, library and pin-tool. Store each in its correct location: pin-tool should be cleaned (above) and then stored (above); slides should be stored in a cool, dry and dust-free cupboard; plates should be sealed and put at -80 °C.
16. **Empty solution from the arrayer:** All solutions should be removed from the arrayer and the arrayer itself should then be cleaned using a cloth soaked in 70% ethanol. This will prevent microbial growth within the arrayer, when used in conjunction with the weekly cleaning schedule (above).
17. **Complete all data tracking forms:** All data tracking forms should be completed in full to ensure a complete record of every slide and print run can be maintained. This will be used to track all slides produced by FlyChip that are either given to external groups, or used by FlyChip in experiments for external groups.
18. **What's next:** The slides need to be processed and the print-run needs to be quality controlled before any of the slides that have just been printed can be used. Please refer to the appropriate protocols on this web site.

R. Auburn (17-02-2006).

Full Moon Biosystems (FMB) protocol for processing FMB cDNA slides:

Overview

After printing non-modified PCR amplified gene-specific cDNA clones the Full Moon Biosystems (FMB) cDNA slides are then processed to bind the denatured probe DNA to the slide and prevent non-specific hybridisation to the substrate. The outlined protocol is based on the method recommended by Full Moon Biosystems (<http://www.fullmoonbio.com/>).

Equipment and material

- Full Moon Biosystems cDNA slides (Full Moon Biosystems; Cat. No. AS 50)
- Slide staining rack (Philip Harris; Cat. No. B52651)
- Slide staining trough (Philip Harris; Cat. No. B52649)
- UV crosslinker (Ultraviolet products; CL-1000)
- Orbital shaker (Stuart Scientific; mini orbital shaker SO5)
- Hettich Rotina 35 microtitre plate centrifuge
- Microscope slide box (Merck EuroLab; Cat. No. 406/0286/00)
- Horizontal laminar flow work station (Jencons; Cat. No. 566-031)
- Bovine serum albumin (BSA) fraction V (Sigma; Cat. No. A-7906).
- Sodium dodecyl sulfate (SDS), molecular biology grade (Sigma; Cat. No. L-4390)
- Blocking Solution: 4xSSC, 0.1% SDS, 1% BSA
- MilliQ water
- Standard photographic air duster

Protocol

For best results, perform steps 4 onwards just before hybridisation.

1. After arraying, UV cross-link the slides with the cross linker set at 4000 ($x100\mu\text{J}$) = 400mJ
2. Allow slides to dry at room temperature for 30 minutes
3. Slides that are not needed for one month or two months can be stored at this stage:
 - ◆ Place the slides in a clean microscope slide box
 - ◆ Then place the microscope slide box in a pastic bag and seal this bag
 - ◆ Store the sealed bag at 2 to 8 °C for 3 to 6 months
4. Meanwhile prepare and preheat the blocking solution to 55 °C in a water bath
5. Pour the blocking solution into a slide staining trough
6. Transfer the slides to a slide staining rack and place the rack into the slide staining trough
7. Place box on shaker at 50 rpm for 20 minutes at room temperature
8. Remove rack, blot off excess solution by placing on a piece of tissue.
9. Place the slides in the staining rack in the plastic box filled with 2.5 L ultra pure water and put the lid on
10. Place the plastic box with the slides on the orbital shaker at 50 rpm for 15 minutes
11. Remove rack, blot off excess solution by placing on a piece of tissue.
12. Repeat steps 8 to 10 twice (three water washes in total)
13. Transfer slides from the rack to a microscope slide box with fresh tissue in the base
14. Centrifuge at 650 rpm for 15 minutes in a microtitre centrifuge to dry the slides
15. Remove any water droplets from the slide using an air duster
16. Store in a clean sealed slide box at room temperature and in the dark (one to two months) until ready to hybridise

R. Auburn (05-06-2006).

Full Moon Biosystems (FMB) protocol for processing FMB PowerMatrix (modified oligo) slides:

Overview

After printing amino-modified long oligonucleotides the Full Moon Biosystems (FMB) PowerMatrix slides are then processed to bind the single stranded probe DNA to the slide and prevent non-specific hybridisation to the substrate. The outlined protocol is based on the method recommended by Full Moon Biosystems (<http://www.fullmoonbio.com/>).

Equipment and material

- Full Moon Biosystems PowerMatrix slides for modified oligos (Full Moon Biosystems; Cat. No. PXP 50 M)
- Slide staining rack (Philip Harris; Cat. No. B52651)
- Slide staining trough (Philip Harris; Cat. No. B52649)
- Orbital shaker (Stuart Scientific; mini orbital shaker SO5)
- Hettich Rotina 35 microtitre plate centrifuge
- Microscope slide box (Merck EuroLab; Cat. No. 406/0286/00)
- Horizontal laminar flow work station (Jencons; Cat. No. 566-031)
- Bovine serum albumin (BSA) fraction V (Sigma; Cat. No. A-7906).
- Sodium dodecyl sulfate (SDS), molecular biology grade (Sigma; Cat. No. L-4390)
- Blocking Solution: 2xSSC, 0.2% SDS, 0.1% BSA
- Ultra pure water (do not use MilliQ water)
- Standard photographic air duster
- Air tight plastic box (30 x 30 x 18 cm) with lid

Protocol

For best results, perform steps 4 onwards just before hybridisation.

1. Incubate slides in a chamber with 65 to 75 % relative humidity overnight:
 - ◆ Within an air tight plastic box add 100 g solid sodium chloride to 50 ml water
2. Allow slides to dry at room temperature for 30 minutes
3. Slides that are not needed for one or two months can be stored at this stage:
 - ◆ Place the slides in a clean microscope slide box
 - ◆ Then place the microscope slide box in a pastic bag and seal this bag
 - ◆ Store the sealed bag at 2 to 8 °C for 3 to 6 months
4. Meanwhile prepare and preheat the blocking solution to 55 °C in a waterbath
5. Pour the blocking solution into a slide staining trough and then transfer the slides to a slide staining rack and place this rack in the staining trough
6. Place the trough containing the slides on the orbital shaker at 50 rpm for between 20 to 30 minutes at room temperature
7. Remove rack, blot off excess solution by placing on a piece of tissue.
8. Place the slides in the staining rack in the plastic box filled with 2.5 L ultra pure water and put the lid on
9. Place the plastic box with the slides on the orbital shaker at 50 rpm for 15 minutes
10. Remove rack, blot off excess solution by placing on a piece of tissue.
11. Repeat steps 8 to 10 twice (three water washes in total)
12. Transfer slides from the rack to a microscope slide box with fresh tissue in the base
13. Centrifuge at 650 rpm for 15 minutes in a microtitre centrifuge to dry the slides
14. Remove any water droplets from the slide using an air duster
15. Store in a clean sealed slide box at room temperature and in the dark (one to two months) until ready to hybridise

R. Auburn (17-02-2006).

Amersham protocol for processing CodeLink (modified oligo) slides:

Overview

After printing amino-modified long oligonucleotides, Amersham CodeLink slides are processed to bind the probe DNA to the slide and prevent non-specific hybridisation to the substrate. The outlined protocol is based on the method recommended by Amersham (<http://www1.amershambiosciences.com/>).

Equipment and material

- Amersham CodeLink slides (Amersham; Cat. No. 300011)
- Slide staining rack (Philip Harris; Cat. No. B52651)
- Slide staining trough (Philip Harris; Cat. No. B52649)
- Orbital shaker (Stuart Scientific; mini orbital shaker SO5)
- Hettich Rotina 35 microtitre plate centrifuge
- Microscope slide box (Merck EuroLab; Cat. No. 406/0286/00)
- Horizontal laminar flow work station (Jencons; Cat. No. 566-031)
- Blocking Solution: 50 mM ethanolamine, 0.1 M Tris, (pH 9.0), 0.1% SDS
- Wash solution: 4 x SSC, 0.1% SDS
- Ultra pure water (do not use MilliQ water)
- Standard photographic air duster
- Air tight plastic box (30 x 30 x 18 cm) with lid

Protocol

For best results, perform steps 4 onwards just before hybridisation.

1. Incubate slides in a chamber with 65 to 75 % relative humidity overnight:
 - ◆ Within an air tight plastic box add 100 g solid sodium chloride to 50 ml water
2. Allow slides to dry at room temperature for 30 minutes
3. Slides that are not needed for one or two months can be stored at this stage:
 - ◆ Place the slides in a clean microscope slide box
 - ◆ Then place the microscope slide box in a pastic bag and seal this bag
 - ◆ Store the sealed bag at 2 to 8 °C for 3 to 6 months
4. Meanwhile prepare and preheat the blocking solution to 50 °C in a waterbath
5. Prepare and preheat the wash solution to 50 °C in a waterbath
6. Pour the blocking solution into a slide staining trough and then transfer the slides to a slide staining rack and place this rack in the staining trough
7. Place the trough containing the slides on the orbital shaker at 50 rpm for between 20 to 30 minutes at room temperature
8. Remove rack, blot off excess solution by placing on a piece of tissue.
9. Transfer the slides to a staining trough containing RO water, then move up-and-down 30 times (to rinse the slides)
10. Repeat step 9
11. Pour the wash solution into a slide staining trough, then transfer the slides to this staining trough
12. Place the trough containing the slides on the orbital shaker at 50 rpm for between 20 to 60 minutes at room temperature
13. Remove rack, blot off excess solution by placing on a piece of tissue.
14. Transfer the staining rack to the plastic box filled with 2.5 L ultra pure water and put the lid on
15. Place the plastic box with the slides on the orbital shaker at 50 rpm for 5 minutes
16. Transfer slides from the rack to a microscope slide box with fresh tissue in the base
17. Centrifuge at 650 rpm for 15 minutes in a microtitre centrifuge to dry the slides
18. Remove any water droplets from the slide using an air duster
19. Store in a clean sealed slide box at room temperature and in the dark (one to two months) until ready to hybridise

R. Auburn (04-07-2005).

Quality control of printed microarrays by staining with SYBR 555

Overview

After printing, a random sample of microarrays is stained and then scanned so that we can assess the print quality and constancy. These checks include substrate defects, sub-grid and meta-grid positioning on the substrate, checking that all spots have been printed and spot morphology. Print batches that fail these quality control test are either used for teaching, or internal development.

Equipment and Reagents

- Between 2 and 8 microarrays from the printing batch to be tested
- 96% ethanol
- Slide staining rack (Philip Harris; Cat. No. B52651)
- Slide staining trough (Philip Harris; Cat. No. B52649)
- Hettich Rotina 35 microtitre plate centrifuge
- Microscope slide box (Merck EuroLab; Cat. No. 406/0286/00)
- Horizontal laminar flow work station (Jencons; Cat. No. 566-031)
- Paragon DNA microarray QC stain kit with SYBR 555 stain (Molecular Probes; Cat. No. P32930)
- Standard photographic air duster

Procedure (4 slides, per batch)

Prepare stain and wash buffers

1. Add 95 ml 96% ethanol to stain buffer component B
2. Add 190 ml 96% ethanol to wash buffer component C

Staining the slides

3. Add 27 ml stain buffer to stain tube (provided) and add 5 μ l SYBR_555, invert tube 5 times to mix
4. Place up to 4 slides in the staining tube, incubate for 5 minutes at room temperature in the dark
5. Remove slides from the stain and blot off excess solution
6. Place slides into a fresh staining tube containing 27 ml of wash buffer, invert once to wash and then remove slide
7. Place slides into a second staining tube containing 27 ml of fresh wash buffer and wash the slides for 5 minutes in an orbital shaker at 50 rpm in the dark
8. Remove slides from the stain and blot off excess solution, transfer to a microscope slide box with tissue in the base
9. Centrifuge at 1000 rpm for 5 minutes
10. Remove any water droplets from the slide using an air duster

Scanning the slides

11. Scan using the cy3-channel of a CCD or dual laser scanner

R. Auburn (17-02-2006).

A key to standard microarray spot identity tracking file formats

Overview

Each microarray spot has a unique position and each spot corresponds to a specific probe DNA from a specific well of a microtitre plate. There are typically thousands to tens of thousands of probe DNAs distributed between tens of source plates and thousands to tens of thousands of spots on each microarray. A single microarray experiment can consist of a few or a hundred microarray hybridisations. Tracking where and what each probe DNA is on each microarray is therefore an important issue.

Most robotic spotters are supplied with a data tracking program that uses an input file to describe the positions of each probe DNA within the microtitre plates and another file that defines how the microarray was printed to produce a description of where each probe DNA is within each microarray. These spot identities can then be imported into a spot finding and quantification tool that will 'append' the fluorescence spot signal. These data are then analysed to determine what affect any given experimental condition or treatment has had on the gene expression of the samples being compared.

For a definition of all terminology used below please refer to "Introduction to printing microarrays".

- [The microarray layout used in the following examples](#)
- [The microarray input file used in the following examples](#)
- [Example microarray spot identity file formats](#)
 - ◆ [BioRobotics 'TAM' format](#)
 - ◆ [Axon 'GAL' format](#)
 - ◆ [Molecularware 'MWBR' format](#)
 - ◆ [Applied Precision Instruments arrayWoRx 'REF' format](#)
 - ◆ [Quantarray file format](#)
 - ◆ [Imagene file format](#)
 - ◆ [Layout Map Xyxy](#)
 - ◆ [Layout Map XYyx](#)
 - ◆ [Layout Map YXxy](#)
 - ◆ [Layout Map YXyx](#)

The microarray layout used in the following examples

1. Meta-grid Layout:

A single 2x2 (X and Y axis) meta-grid printed on each slide
Numbers below refer to the pins that printed the sub-grids

01 02
03 04

Array position from bottom left of slide: 9.24 mm (X-axis), 34.19 mm (Y-axis)
Meta-grid has an array area of 6.10 mm (X-axis) by 6.10 mm (Y-axis)

2. Sub-grid Layout:

Each sub-grid has 4x4 (16) spots
Centre-to-centre spot distance is 400 microns
Numbers below refer to 'Source Visits'

13 14 15 16
09 10 11 12
05 06 07 08
01 02 03 04

Key to Source Visits
Source '1' to '16' = Imaginary clones

3. Comments:

This is a hypothetical microarray
The arrays contains 64 spots per slide

The microarray input file used in the following examples

The following is a small section of a file that describes a fictitious source plate. Well position is defined by source plate barcode (e.g. TST101), row (A to P) and column (1 to 24). CloneID is a cDNA clone accession code and UniqueID is a well-specific identifier. The columns do not need to be in the order shown. The example shown is a 'tab separated value' (TSV) text file.

CloneID	UniqueID	Row	Column
FC1234	TST101	A	1
FC1235	TST101	A	2
FC1236	TST101	A	3
FC1237	TST101	A	4
Empty	Empty	A	5
Empty	Empty	A	6
Empty	Empty	A	7
Empty	Empty	A	8
Empty	Empty	A	9
Empty	Empty	A	10
Empty	Empty	A	11
Empty	Empty	A	12
Empty	Empty	A	13
Empty	Empty	A	14
Empty	Empty	A	15
Empty	Empty	A	16
Empty	Empty	A	17
Empty	Empty	A	18
Empty	Empty	A	19
Empty	Empty	A	20
Empty	Empty	A	21
Empty	Empty	A	22
Empty	Empty	A	23
Empty	Empty	A	24
FC1238	TST101	B	1
FC1239	TST101	B	2
FC1240	TST101	B	3
FC1241	TST101	B	4
Empty	Empty	B	5
Empty	Empty	B	6
Empty	Empty	B	7

This input file was used to create a series of clone tracking files using the MicroGrid II so that the format structures could be explained using worked examples. These are shown in the following sections, an asterisk denotes an explanation rather than a component of the named file format.

Example microarray spot identity file formats

BioRobotics 'TAM' format

```
[FileInformation]
FileFormat=,1.0 *Version number of the file format
FormatName=,TAM *File format extension
GeneratedBy=,TAS2.1.5.16 *Arrayer software and version
BlockCount=,4 *Number of meta-grids
SpotSize=,180 *Estimated mean spot diameter

[Block1] *Sub-grid being examined
MetaGridX=,1 *Meta-grid X-axis co-ordinate
MetaGridY=,1 *Meta-grid Y-axis co-ordinate
OriginX=,9000 *Distance from top left edge of slide to centre of top left spot
OriginY=,35300 *Distance from top left edge of slide to centre of top left spot
BlockSizeX=,4 *Number of spots in each row
BlockSizeY=,4 *Number of spots in each column
SpacingX=,400 *X-axis centre-to-centre spot spacing
SpacingY=,400 *X-axis centre-to-centre spot spacing
*Information is repeated for each sub-grid on the slide

[mapping] *'comma separated value' spreadsheet that maps clones to wells
1,1,1,1,,1,9,4,"FC1269", "1036",1, {},FC1269,1036,TST101,I,4
```

```

1,1,1,2,,1,1,4,"FC1237","1004",1, {},FC1237,1004,TST101,A,4
1,1,1,3,,1,9,2,"FC1267","1034",1, {},FC1267,1034,TST101,I,2
1,1,1,4,,1,1,2,"FC1235","1002",1, {},FC1235,1002,TST101,A,2
1,1,2,1,,1,11,4,"FC1277","1044",1, {},FC1277,1044,TST101,K,4

```

*Spreadsheet abbreviated for brevity.
 *Each row of data relates to each spot on the microarray.
 *Origin is top left of slide for all measurements.

*Column 01: Meta-grid X-axis co-ordinate
 *Column 02: Meta-grid Y-axis co-ordinate
 *Column 03: Sub-grid Y-axis co-ordinate
 *Column 04: Sub-grid X-axis co-ordinate
 *Column 05: Plate Barcode
 *Column 06: Plate Number
 *Column 07: Row Number
 *Column 08: Column Number
 *Column 09: Sample Name
 *Column 10: Sample ID
 *Column 11: Block number
 *Column 12: Termination of additional fields
 *Column 13 to End: Source data from the operator (See Example Array Input File)

Axon 'GAL' format

```

ATF      1.0
10      5 *first term is the number of headers, not including this, the previous or
        the column header row. The second term is the number of columns
"Type=GenePix ArrayList V1.0" *Version number of the file format
"BlockCount=4" *Number of sub-grids
"BlockType=0"
"Block1=9000, 35300, 180, 4, 400, 4, 400" *Mapping information for sub-grid 1
"Block2=13500, 35300, 180, 4, 400, 4, 400" *Mapping information for sub-grid 2
"Block3=9000, 39800, 180, 4, 400, 4, 400" *Mapping information for sub-grid 3
"Block4=13500, 39800, 180, 4, 400, 4, 400" *Mapping information for sub-grid 4
"Supplier=BioRobotics" *Arrayer manufacturer
"ArrayerSoftwareName=TAS Application Suite (MicroGrid II)" *Arrayer software name
"ArrayerSoftwareVersion=2.1.5.16" *Arrayer software version
"Block" "Column" "Row" "ID" "Name"
*Header for clone mapping 'tab separated value' (TSV) spreadsheet
1      1      1      1036      FC1269
1      1      2      1004      FC1237
1      1      3      1034      FC1267
1      1      4      1002      FC1235
1      2      1      1044      FC1277
1      2      2      1012      FC1245
1      2      3      1042      FC1275
1      2      4      1010      FC1243
1      3      1      1052      FC1285
1      3      2      1020      FC1253
1      3      3      1050      FC1283
1      3      4      1018      FC1251
1      4      1      1060      FC1293
1      4      2      1028      FC1261
1      4      3      1058      FC1291
1      4      4      1026      FC1259
2      1      1      1040      FC1273
2      1      2      1008      FC1241
2      1      3      1038      FC1271
2      1      4      1006      FC1239
2      2      1      1048      FC1281
2      2      2      1016      FC1249
2      2      3      1046      FC1279
2      2      4      1014      FC1247
2      3      1      1056      FC1289
2      3      2      1024      FC1257

```

*Spreadsheet abbreviated for brevity.
 *Each row of data relates to each spot on the microarray.
 *Origin is top left of slide for all measurements.

*Block: Sub-grid identification
 *Column: Sub-grid Y-axis co-ordinate

*Row: Sub-grid X-axis co-ordinate
*ID: Sample ID (See Example Array Input File)
*Name: Sample Name (See Example Array Input File)

Molecularware 'MWBR' format

[FileInformation]

FileFormat=,1.0.2 *Version number of the file format
FormatName=,MwBr *File format extension
GeneratedBy=,TAS2.1.5.16 *Arrayer software and version
BlockCount=,4 *Number of sub-grids
SpotSize=,180 *Estimated mean spot diameter

[Source]

Comment=,Genetix384 *Source plate type
XWells=,16 *Number of wells in X-axis
YWells=,24 *Number of wells in Y-axis
XPitch=,4.5 *Well spacing in X-axis
YPitch=,4.5 *Well spacing in Y-axis
PlateCount=,1 *Number of source plates

[Tool]

Description=,2x2 (384 well) split pin *MicroSpot II pin number and type
PinsX=,2 *Number of MicroSpot pins in X-axis
PinsY=,2 *Number of MicroSpot pins in Y-axis
PinPitch=,4500 *Pin-to-pin spacing in the pin-tool

[Target]

TargetWidth=,25000 *Width of the glass microscope slide
TargetHeight=,75000 *Height of the glass microscope slide
LeftMargin=,9239 *Meta-grid distance from left of slide
RightMargin=,9239 *Meta-grid distance from right of slide
TopMargin=,34189 *Meta-grid distance from top of slide
BottomMargin=,34189 *Meta-grid distance from bottom of slide
XSpacing=,0 *Spacing between meta-grids in X-axis
YSpacing=,0 *Spacing between meta-grids in Y-axis
NumberOfCopies=,27 *Number of slides being printed

[slides] *Mapping of the slide the microarray was printed on

Slide1=,1,20000,243000, *List abbreviated to save space

[Block1] *Sub-grid being examined

MetaGridX=,1 *Meta-grid X-axis co-ordinate
MetaGridY=,1 *Meta-grid Y-axis co-ordinate
OriginX=,9000 *Distance from top left edge of slide to centre of top left spot
OriginY=,35300 *Distance from top left edge of slide to centre of top left spot
BlockSizeX=,4 *Number of spots in each row
BlockSizeY=,4 *Number of spots in each column
SpacingX=,400 *X-axis centre-to-centre spot spacing
SpacingY=,400 *X-axis centre-to-centre spot spacing
*Information is repeated for each sub-grid on the slide

[mapping]*As for TAM format expect column 3 and 4 are in the reverse order

1,1,1,1,,1,9,4,"FC1269","1036",1, {},FC1269,1036,TST101,I,4
1,1,1,2,,1,1,4,"FC1237","1004",1, {},FC1237,1004,TST101,A,4

*Spreadsheet abbreviated for brevity.

*Each row of data relates to each spot on the microarray.

*Origin is top left of slide for all measurements.

Applied Precision Instruments arrayWoRx 'REF' format

#ArrayWoRx Reference File *File Format

#Tag 0x00FF040C

#Version: 2.10 *File format version

#GridOrigin: 34190 9660

#GridRotation: 0

#GridType: Irregular

#GridColumnsRows: 64 1

#GridWidthHeight: 6100 6100 *Array size: X-axis, Y-axis

#NomSpotSpacingColumnRow: 400 400 *X-axis Y-axis centre-to-centre spot distance

#OddRowOffset: 0

```

#Number_UniqueID_Types: 2
#UniqueID_Type 1: 0
#UniqueID_Type 2: 0
#Column 1: Spot number *Key to the clone spreadsheet
#Column 2: Spot name 1 [gene name] *Key to the clone spreadsheet
#Column 3: Spot name 2 [chromosome] *Key to the clone spreadsheet
#Column 4: Spot type [1=experiment; 2=ratio control] *Key to the clone spreadsheet
#Column 5: Unique ID 1 *Key to the clone spreadsheet
#Column 6: Unique ID 2 *Key to the clone spreadsheet
#Column 7: X coordinate *Key to the clone spreadsheet
#Column 8: Y coordinate *Key to the clone spreadsheet
#Column 9: Spot size 1 [width in um] *Key to the clone spreadsheet
#Column 10: Spot size 2 [height in um] *Key to the clone spreadsheet
#Column 11: Bounding box width [ROI about the spot] *Key to the clone spreadsheet
#Column 12: Bounding box height [ROI about the spot] *Key to the clone spreadsheet
#Column 13: Reserved column 1 *Key to the clone spreadsheet
#Column 14: Reserved column 2 *Key to the clone spreadsheet
#Column 15: Reserved column 3 *Key to the clone spreadsheet
#Column 16: Reserved column 4 *Key to the clone spreadsheet
#Column 17: Reserved column 5 *Key to the clone spreadsheet
#Column 18: Reserved column 6 *Key to the clone spreadsheet
#Column 19: Reserved column 7 *Key to the clone spreadsheet
#Column 20: Reserved column 8 *Key to the clone spreadsheet
#Column 21: Include flag [0=exclude; 1=include] *Key to the clone spreadsheet
#Column 22: Description [optional] *Key to the clone spreadsheet

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*Tab separated value (TSV) clone spreadsheet

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1 FC1262 unknown 1 1029 Unknown 34190 9660 180 180 200 200 * * * * * * * * 1 Plate 1, Well H1
2 FC1294 unknown 1 1061 Unknown 34590 9660 180 180 200 200 * * * * * * * * 1 Plate 1, Well P1
3 FC1264 unknown 1 1031 Unknown 34990 9660 180 180 200 200 * * * * * * * * 1 Plate 1, Well H3
4 FC1296 unknown 1 1063 Unknown 35390 9660 180 180 200 200 * * * * * * * * 1 Plate 1, Well P3

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*Spreadsheet abbreviated for brevity.

*Each row of data relates to each spot on the microarray.

*Origin is bottom left of slide for all measurements.

Quantarray file format

*'Tab separated value' (TSV) text file

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1      1      1      1      1036
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1      1      2      2      1012
1      1      3      2      1042
1      1      4      2      1010
1      1      1      3      1052
1      1      2      3      1020
1      1      3      3      1050
1      1      4      3      1018

```

*Spreadsheet abbreviated for brevity.

*Each row of data relates to each spot on the microarray.

*Origin is top left of slide for all measurements.

*Column 01: Meta-grid X-axis co-ordinate

*Column 02: Meta-grid Y-axis co-ordinate

*Column 03: Sub-grid Y-axis co-ordinate

*Column 04: Sub-grid X-axis co-ordinate

*Column 05: 'UniqueID' (See [Example Array Input File](#))

Imagene file format

*'Tab separated value' (TSV) text file

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1      1      1      3      1034
1      1      1      4      1002

```

1	1	2	1	1044
1	1	2	2	1012
1	1	2	3	1042
1	1	2	4	1010
1	1	3	1	1052
1	1	3	2	1020
1	1	3	3	1050
1	1	3	4	1018

*Spreadsheet abbreviated for brevity.
 *Each row of data relates to each spot on the microarray.
 *Origin is top left of slide for all measurements.

*Column 01: Meta-grid X-axis co-ordinate
 *Column 02: Meta-grid Y-axis co-ordinate
 *Column 03: Sub-grid X-axis co-ordinate
 *Column 04: Sub-grid Y-axis co-ordinate
 *Column 05: 'UniqueID' (See [Example Array Input File](#))

Layout Map XYxy

*'Tab separated value' (TSV) text file

1	1	1	1	1036
1	1	1	2	1004
1	1	1	3	1034
1	1	1	4	1002
1	1	2	1	1044
1	1	2	2	1012
1	1	2	3	1042
1	1	2	4	1010
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1	1	3	2	1020
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 *Column 03: Sub-grid X-axis co-ordinate
 *Column 04: Sub-grid Y-axis co-ordinate
 *Column 05: 'UniqueID' (See [Example Array Input File](#))

Layout Map XYyx

*'Tab separated value' (TSV) text file

1	1	1	1	1036
1	1	2	1	1004
1	1	3	1	1034
1	1	4	1	1002
1	1	1	2	1044
1	1	2	2	1012
1	1	3	2	1042
1	1	4	2	1010
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1	1	2	3	1020
1	1	3	3	1050
1	1	4	3	1018

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 *Column 03: Sub-grid Y-axis co-ordinate
 *Column 04: Sub-grid X-axis co-ordinate
 *Column 05: 'UniqueID' (See [Example Array Input File](#))

Layout Map YXxy

*'Tab separated value' (TSV) text file

1	1	1	1	1036
1	1	1	2	1004
1	1	1	3	1034
1	1	1	4	1002
1	1	2	1	1044
1	1	2	2	1012
1	1	2	3	1042
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1	1	3	2	1020
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*Column 03: Sub-grid X-axis co-ordinate

*Column 04: Sub-grid Y-axis co-ordinate

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Layout Map YXyx

*'Tab separated value' (TSV) text file

1	1	1	1	1036
1	1	2	1	1004
1	1	3	1	1034
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1	1	1	2	1044
1	1	2	2	1012
1	1	3	2	1042
1	1	4	2	1010
1	1	1	3	1052
1	1	2	3	1020
1	1	3	3	1050
1	1	4	3	1018

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*Column 03: Sub-grid Y-axis co-ordinate

*Column 04: Sub-grid X-axis co-ordinate

*Column 05: 'UniqueID' (See [Example Array Input File](#))

R. Auburn (17-02-2006).

Gene expression sample processing:

Making the spike control mix:

- *In vitro* transcription of spike controls from 5'-T7 and dT₁₅₋₃ PCR Products ([jump](#))

RNA extraction and quality control:

- Small scale RNA extraction using TRIzol ([jump](#))
- Medium scale RNA extraction using TRIzol ([jump](#))
- Large scale RNA extraction with TRIzol ([jump](#))
- RNA quality control before labelling ([jump](#))

Reverse transcription, labelling and amplification:

- Reverse transcription and direct labelling for cDNA and oligo arrays ([jump](#))
- Reverse transcription and indirect labelling for cDNA and oligo arrays ([jump](#))
- Klenow labelling of double stranded DNA derived from 3 to 5 µg total RNA ([jump](#))
- Anti-sense strand amplification and direct labelling for cDNA microarrays ([jump](#))
- Anti-sense strand amplification of RNA and indirect labelling of amino-allyl RNA for oligo microarrays (1 round) ([jump](#))
- Anti-sense strand amplification of RNA and indirect labelling of amino-allyl RNA for oligo microarrays (2 rounds) ([jump](#))
- Measuring (nucleic acid concentration and) dye incorporation rates

Hybridisation:

- Hybridisation of labelled material to cDNA microarrays using a Genomic Solutions hybridisation station with Ambion Hybridisation Buffer #1([jump](#))
- Hybridisation to amino-modified long oligonucleotide microarrays using a Genomic Solutions hybridisation station with the Biosolutions hybridisation buffer([jump](#))

Image acquisition:

- Operating instructions for the Genepix 4000B dual laser scanner ([jump](#))

In vitro transcription of spike controls from T7-dT PCR Products

Outline

To make RNA from each of the spike control clones, specific primer pairs were designed for each spike to PCR amplify between 0.3 to 1.5 kb of each. The primers were 5' end modified to contain the T7 promoter sequence and 3' end modified to contain a 15mer poly T tail. The T7-dT amplified DNA for each spike was then transcribed into RNA using the Ambion MEGAscript T7 kit ([Ambion](#)). These spikes were then mixed and added to each labelling reaction.

PCR Amplification with 5'-T7 and dT₁₅-3' primers

PCR primers:

Please note that '[T7]' refers to the nucleotide sequence 'TAATACGACTCACTATAGGGAGA'.

Clone	CloneID	5'-T7 primer	3'-dT ₁₅ primer	RNA length (bp)
Arizona 4	M90509	[T7]-gaaccagtgataggtttcttg	(T) ₁₅ atagcatgctcgatgtgcaa	510
Arizona 6	U74610	[T7]-tcctctctctcaacctcg	(T) ₁₅ acaacggaagcaaatcttattg	942
Incyte 4	ATU18126	[T7]-tcaaaagcttcgaatctggc	(T) ₁₅ aaggtttgcaggttattcttc	517
Incyte 5	L22585	[T7]-agctcaatggttcactatgatg	(T) ₁₅ cgctaggcatgcttaataacc	489
AIMS 1	AB007987	[T7]-agatgcttctctctctc	(T) ₁₅ tgttgatgaggttaccgc	1151
AIMS 4	AF117335	[T7]-agtggatgaggttaataggagc	(T) ₁₅ taccatactggatccttccc	1540
AIMS 5	AF168390	[T7]-gatattcccgtgttctctc	(T) ₁₅ tgaccataagccactgcatc	1157
AIMS 9	AF372915	[T7]-agatcatctcatagggcagatg	(T) ₁₅ aagcgaagaagctctgggc	1102
AIMS 10	Y18469	[T7]-agtgtgctacttactggg	(T) ₁₅ tgagataactagagaaggctcc	1405
AIMS 11	Z49777	[T7]-actaaacatggcgacggag	(T) ₁₅ aaactagcgcgcatggtgg	987
AIMS 19	X644464	[T7]-tgggtaaagctggctgcaagg	(T) ₁₅ accgcaaatagcaatccgacc	775
Weed 1	O82258	[T7]-taaagtggaacctccgatgc	(T) ₁₅ gaagagctcatgccgatac	514
Weed 3	Q9LJQ4	[T7]-ttctcacaactcgcaattcaa	(T) ₁₅ gcaactgatgaccaggaaga	402
Weed 4	Q9XIB8	[T7]-aagacgagggcagatcttca	(T) ₁₅ tgttcttccagagtgcaaatg	396
Weed 6	O04600	[T7]-ttgagtaccaacggtttcagc	(T) ₁₅ tatcatcggtttgcctttgc	370
Weed 7	Q9LZJ2	[T7]-tcatgtgaacatacaacgcaat	(T) ₁₅ gggtctattgggggtggaatc	404
Weed 8	Q9LVF8	[T7]-tcaacctatcattctccatt	(T) ₁₅ gcctattgaggattgttgctt	394
Weed 9	O49366	[T7]-agcttgagaacataggccaca	(T) ₁₅ tggcatcggtgtctctgta	343
Weed 10	O81842	[T7]-agcatcctaaatccaaccaa	(T) ₁₅ ttcgattccgcagattatcc	361
Weed 13	Q9LU32	[T7]-tccaatatgatttggttgga	(T) ₁₅ tgtatgcttgcactcgatga	330
Weed 14	O04513	[T7]-agggcatttggttcatggt	(T) ₁₅ atagcatgctcgatgtgcaa	306

PCR reaction mix:

- 10 µl 10 x Stratagene Yield Ace reaction buffer
- 2 µl 10 mM dNTP
- 84 µl MilliQ water
- 1 µl Stratagene Yield Ace DNA polymerase
- 1 µl plasmid DNA

- 2 μ l 25 pmol / μ l of 5'-T7 and dT₁₅-3' primer pairs

PCR cycle:

All PCR reactions were performed in 0.2 ml microfuge tubes with a Dyad thermal cycler with the following PCR cycle.

1. 94 °C for 3 minutes
2. 94 °C for 30 seconds
3. 60 °C for 30 seconds
4. 72 °C for 4 minutes
5. Repeat steps 2 to 4 34 times
6. 72 °C for 10 minutes
7. 4 °C cold storage before unloading

The PCR products were purified by QIAquick spin columns and checked by agarose gel electrophoresis.

***In vitro* transcription reaction from 5'-T7 and dT₁₅-3' PCR templates**

Protocol for the Ambion MEGAscript T7 kit:

1. Prepare the following *in vitro* transcription reaction mix:
 - ◆ 7 μ l Ambion nuclease-free water
 - ◆ 2 μ l dATP
 - ◆ 2 μ l dUTP
 - ◆ 2 μ l dGTP
 - ◆ 2 μ l dCTP
 - ◆ 2 μ l 10 x Reaction Mix
 - ◆ 1 μ l DNA Template (5'-T7 and dT₁₅-3' PCR product)
 - ◆ 2 μ l T7 polymerase
2. Incubate at 37 °C for 2 to 4 hours
3. Perform a DNase treatment:
 - ◆ Add 1ul DNase
 - ◆ Mix with a pipette
 - ◆ Pulse spin to collect contents to bottom of tube
 - ◆ Incubate 37 °C for 15 minutes
4. Stop Reaction and precipitate RNA:
 - ◆ 30ul Nuclease-free water (from kit)
 - ◆ 25ul Lithium Chloride solution (from kit)
 - ◆ Mix and freeze at -20 °C for at least 30 minutes
 - ◆ Spin at 13,000 rpm (RT or 4 °C) for 15 minutes to pellet RNA
 - ◆ Wash pellet in 1 ml 70% ethanol (made with DEPC water)
 - ◆ Spin for 5 minutes at 13000 rpm
 - ◆ Resuspend RNA in DEPC water

Quality control and making the spike mix:

All *in vitro* transcribed RNA was then checked by both agarose gel electrophoresis and the Nanodrop. The RNA concentration was then adjusted so that each spike RNA concentration was approximately 1 μ g / μ l. The RNA was then aliquotted and stored at -80 °C

Each *Arabidopsis* RNA was then mixed and this mixture is spiked into each reverse transcription and labelling reaction performed by FlyChip.

R. Auburn (17-02-2006).

Small scale extraction of total RNA from *Drosophila melanogaster*

Overview

The RNA that is to be labelled must be of high quality. It must be undegraded and contain no genomic DNA contamination. Several extraction methods have been tested for use with *Drosophila* samples. However, extraction using TRIzol gives consistent, reliable results and is considerably cheaper than kit-based products and is therefore our method of choice.

Poly A+ mRNA constitutes approximately 2% of total RNA from a *Drosophila* embryo. Labelling of 50 µg total RNA using an oligo(dT) primer gives similar results to approximately 1 µg poly A+ RNA and it is therefore unnecessary to purify poly A+ RNA from the total RNA prep.

This protocol is based on a method from Kevin White's web site (<http://quantgen.med.yale.edu/>). This protocol has been optimised to extract 1 to 10 µg total RNA. Please ensure that you have a sufficient amount of tissue before sending us your samples ([recommended tissue amounts](#)).

Equipment and reagents

- TRIzol (Gibco/BRL; Cat. No. 15596-018)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- 1.5ml disposable Polypropylene Pellet Pestle with microtube (Anachem; Cat. No. K-749520-0000). Autoclave in DEPC-treated water to ensure that RNase-free
- Chloroform, (BDH; Cat. No. 100775A)
- Isopropanol (BDH; Cat. No. 102246L)
- DEPC-treated MilliQ water
- 70% ethanol/DEPC MilliQ water
- RNAlater (Ambion; Cat. No. 7020)
- Micro 20 centrifuge, Hettich
- GeneElute Linear Polyacrylamide (Sigma; Cat. No. 5-5675)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

1. For adult flies, imaginal discs and other tissues, transfer tissue to a 1.5 ml microfuge tube. For embryos, dechorionate first, rinse thoroughly with water and blot off excess before weighing (do not fix!). If samples are ready to be homogenised immediately, skip to step 2. If samples are not yet ready for processing, then either:
 - ◆ flash freeze tube in liquid nitrogen then store in -80 °C freezer until ready to homogenise. Thaw on ice before continuing with step 2, or;
 - ◆ Add 5 volumes of RNAlater. The tissue can be stored safely at 25 °C for a couple of days, at 4 °C for up to a week, and at -20 °C or -80 °C for at least a month. When ready to continue, remove RNAlater before continuing with step 2.
2. Place sample on ice and add 300 µl TRIzol.
3. Homogenise using an RNAase-free polypropylene pellet pestle. Avoid making sample hot.
4. At this point the sample can be stored at -80 °C until ready to be sent to us on dry ice.
5. Thaw sample on ice. Add 0.2 µl GeneElute Linear Polyacrylamide (25 µg / µl)
6. Centrifuge at 13,000 rpm in a microcentrifuge for 10 minutes to pellet debris such as the chorion, vitelline membrane, cuticle etc.
7. Transfer supernatant to a fresh 1.5 ml tube.
8. Add 0.2 volumes chloroform, vortex for 60 seconds.
9. Centrifuge at 13,000 rpm for 15 minutes.

10. Remove upper phase to a new RNase-free tube, being careful not to touch the interface.
11. Add 0.8 volumes of isopropanol, invert and then incubate for 1 hour at -20 °C.
12. Pellet the RNA by centrifugation at 13,000 rpm for 15 minutes.
13. Discard the supernatant and wash the RNA pellet with 500 µl 70% ethanol/DEPC MilliQ water.
14. Air dry the pellet briefly (leave on work bench). Resuspend in an appropriate volume of DEPC MilliQ water, e.g. 5 µl. The RNA will dissolve more readily if the DEPC MilliQ water is preheated to 55 °C.
15. Verify the quality of RNA by gel electrophoresis of 0.5 µl. Stain the gel using SYBR Gold instead of Ethidium Bromide. Do not quantify control the RNA using the Nanodrop, if you do there won't be enough total RNA for the amplification.
16. The remaining 4.5 µl of total RNA can now be used for amplification.

R. Auburn (10-10-2006).

Standard protocol for the extraction of total RNA from *Drosophila melanogaster*

Overview

The RNA that is to be labelled must be of high quality. It must be undegraded and contain no genomic DNA contamination. Several extraction methods have been tested for use with *Drosophila* samples. However, extraction using TRIzol gives consistent, reliable results and is considerably cheaper than kit-based products and is therefore our method of choice.

Poly A+ mRNA constitutes approximately 2% of total RNA from a *Drosophila* embryo. Labelling of 50 µg total RNA using an oligo(dT) primer gives similar results to approximately 1 µg poly A+ RNA and it is therefore unnecessary to purify poly A+ RNA from the total RNA prep.

This protocol is based on a method from Kevin White's web site (<http://quantgen.med.yale.edu/>). We only require 50 µg total RNA per labelling reaction and this protocol has been optimised to extract this amount of total RNA. Please ensure that you have a sufficient amount of tissue before sending us your samples ([recommended tissue amounts](#)).

Equipment and reagents

- TRIzol (Gibco/BRL; Cat. No. 15596-018)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- 1.5ml disposable Polypropylene Pellet Pestle with microtube (Anachem; Cat. No. K-749520-0000). Autoclave in DEPC-treated water to ensure that RNase-free
- Chloroform, (BDH; Cat. No. 100775A)
- Isopropanol (BDH; Cat. No. 102246L)
- DEPC-treated MilliQ water
- 70% ethanol/DEPC MilliQ water
- RNAlater (Ambion; Cat. No. 7020)
- Micro 20 centrifuge, Hettich

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

1. For adult flies, imaginal discs and other tissues, transfer tissue to a 1.5 ml microfuge tube and weigh on microbalance. For embryos, dechorionate first, rinse thoroughly with water and blot off excess before weighing (do not fix!). If samples are ready to be homogenised immediately, skip to step 2. If samples are not yet ready for processing, then either:
 - ◆ flash freeze tube in liquid nitrogen then store in -80 °C freezer until ready to homogenise. Thaw on ice before continuing with step 2, or;
 - ◆ add 5 volumes of RNAlater. The tissue can be stored safely at 25 °C for a couple of days, at 4 °C for up to a week, and at -20 °C or -80 °C for at least a month. When ready to continue, remove RNAlater before continuing with step 2.
2. Place sample on ice and add 300 µl TRIzol.
3. Homogenise the sample for 30-60 seconds using a disposable polypropylene pellet pestle and microtube. Avoid making sample hot.
4. At this point the sample can be stored at -80 °C until ready to be sent to us on dry ice.
5. Thaw sample on ice. Depending on the amount of tissue add up to 700 µl of TRIzol. Centrifuge at 13,000 rpm in a microcentrifuge for 10 minutes at 4 °C to pellet debris such as the chorion, vitelline membrane, cuticle etc. Transfer supernatant to a fresh 1.5 ml tube.
6. Add 0.2 volumes chloroform, shake vigorously for 15 seconds and incubate at room temperature for 2-3 minutes.

7. Centrifuge at 13,000 rpm for 15 minutes at 4 °C.
8. Remove upper phase to a new RNase-free tube, being careful not to touch the interface. Discard tube with lower phase and interface.
9. Add 0.7 volumes of isopropanol to precipitate the RNA. Incubate at room temperature for 5 minutes or 1 hour at -20 °C and then centrifuge at 13,000 rpm for 15 minutes at 4 °C.
10. Discard the supernatant and wash the RNA pellet with 1 ml 70% ethanol/DEPC MilliQ water. Centrifuge at 13,000 rpm for 10 minute at 4 °C.
11. Air dry the pellet briefly (leave on work bench). Resuspend in an appropriate volume of DEPC MilliQ water, e.g. 20 to 50 µl. The RNA will dissolve more readily if the DEPC MilliQ water is preheated to 55 °C.
12. Verify quality of RNA according to the RNA quality control / assessment protocol.

R. Auburn (10-10-2006).

Large scale extraction of total RNA from *Drosophila melanogaster*

Overview

The RNA that is to be labelled must be of high quality. It must be undegraded and contain no genomic DNA contamination. Several extraction methods have been tested for use with *Drosophila* samples. However, extraction using TRIzol gives consistent, reliable results and is considerably cheaper than kit-based products and is therefore our method of choice.

Poly A+ mRNA constitutes approximately 2% of total RNA from a *Drosophila* embryo. Labelling of 50 µg total RNA using an oligo(dT) primer gives similar results to approximately 1 µg poly A+ RNA and it is therefore unnecessary to purify poly A+ RNA from the total RNA prep.

This protocol is based on a method from Kevin White's web site (<http://quantgen.med.yale.edu/>). This particular protocol has been optimised to extract total RNA from very large samples, e.g. 0.4 g adult flies to make 2.5 mg of total RNA.

We only require 50 µg total RNA per labelling reaction. Please ensure that you have a sufficient amount of tissue before sending us your samples ([recommended tissue amounts](#)).

Equipment and reagents

- TRIzol (Gibco/BRL; Cat. No. 15596-018)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- Chloroform (BDH; Cat. No. 100775A)
- Isopropanol (BDH; Cat. No. 102246L)
- Phenol:Chloroform:Isoamyl alcohol (125:24:1 mixture, pH 4.3) (Fisher BioReagents; Cat. No. UN2821)
- DEPC-treated MilliQ water
- 70% ethanol/DEPC MilliQ water
- RNAlater (Ambion; Cat. No. 7020)
- Ultra-Turrax T8 homogeniser, Labortechnik
- RC-55 refrigerated superspeed centrifuge with SS-34 rotor, Du Pont instruments
- Jouan GR422 Centrifuge
- 50 ml Falcon tube (Falcon; Cat. No. 352070)
- Clear glass corex tube (Du Pont Instruments; Cat. No. 00152)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

1. For adult flies, imaginal discs and other tissues, transfer tissue to a 50 ml Falcon tube and weigh on microbalance. For embryos, dechorionate first, rinse thoroughly with water and blot off excess before weighing (do not fix!). If samples are ready to be homogenised immediately, skip to step 2. If samples are not yet ready for processing, then either:
 - ◆ flash freeze tube in liquid nitrogen then store in -80 °C freezer until ready to homogenise. Thaw on ice before continuing with step 2, or;
 - ◆ add 5 volumes of RNAlater. The tissue can be stored safely at 25 °C for a couple of days, at 4 °C for up to a week, and at -20 °C or -80 °C for at least a month. When ready to continue, remove RNAlater before continuing with step 2.
2. Place sample on ice and add 1 ml of TRIzol per 50 to 100 mg of tissue.
3. Homogenise the sample for using a Ultra-Turrax T8 homogeniser set to full speed. Avoid making sample hot.

4. Centrifuge at 4000 rpm in a Jouan GR422 centrifuge for 10 minutes at 4 °C to pellet debris such as the chorion, vitelline membrane, cuticle etc. Transfer the supernatant to an autoclaved corex tube.
5. Add an equal volume of Phenol:Chloroform:Isoamyl alcohol to the supernatant and mix by vortexing.
6. Centrifuge for 15 minutes at 13,000 rpm at 4 °C using an SS-34 rotor within the RC-55 refrigerated superspeed centrifuge.
7. Transfer the upper phase to a fresh autoclaved corex tube without touching the interphase or the side of the tube.
8. Add 0.2 volumes chloroform and vortex
9. Centrifuge at 13,000 rpm for 15 minutes at 4 °C using an SS-34 rotor within the RC-55 refrigerated superspeed centrifuge.
10. Transfer the upper phase to a fresh autoclaved corex tube without touching the interphase or the side of the tube.
11. Add 0.8 volumes of isopropanol to precipitate the RNA. Incubate at -20 °C for at least 1 hour.
12. Leave the sample at room temperature for a few minutes and then centrifuge at 13,000 rpm for 15 minutes at 4 °C using an SS-34 rotor within the RC-55 refrigerated superspeed centrifuge.
13. Discard the supernatant and wash the RNA pellet with 1 ml 70% ethanol/DEPC MilliQ water per 1 ml of the original TRIzol volume and centrifuge at 13,000 rpm for 10 minute at 4 °C using an SS-34 rotor within the RC-55 refrigerated superspeed centrifuge.
14. Air dry the pellet for a few minutes (leave on work bench). Resuspend in an appropriate volume of DEPC MilliQ water that has been pre-heated to 55 °C for 3 minutes
15. Transfer to a 1.5 or 2 ml microfuge tube
16. Verify quality of RNA according to the RNA quality control / assessment protocol.

R. Auburn (10-10-2006).

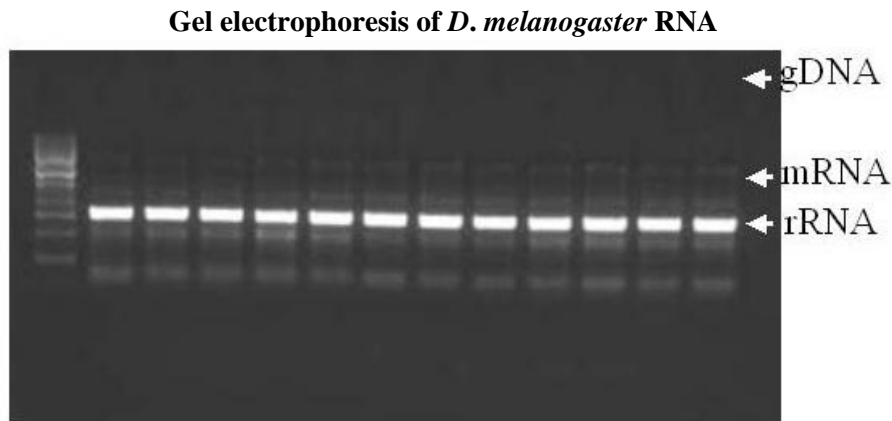
RNA quality control

Outline

Any extracted RNA must be devoid of contaminants such as salt, protein, solvents and genomic DNA. Poor quality RNA will lead to problems when performing the reverse transcription and labelling and might affect data quality. We quality control our extracted RNA using gel electrophoresis and optical density measurements. Gel electrophoresis is used to check for Genomic DNA contamination and RNA decay. Optical density is used to assay the RNA yield and to check for contamination by salt, solvent, protein, *etc.*

Gel electrophoresis to check for genomic DNA:

Genomic DNA from *D. melanogaster* will be visible as a tight DNA band of high molecular weight. Whereas rRNA will be visible as two sharp bands half way down the gel the mRNA is the smear in the background. Presence or absence of genomic DNA is easy to detect. mRNA decay is inferred from fuzzy rRNA bands and the presence of low molecular weight smearing (from the rRNA band and below). The following is an example of good quality *D. melanogaster* RNA.



Fermentas 1 kb Generuler mass ladder (Cat. No. SM0311)
(250 bp band is not visible on this this gel.)

Protocol - ethidium bromide (Medium to large-scale RNA extractions)

1. Make a 1% agarose gel and add 5 μ l ethidium bromide (10 mg / ml) per 100 ml of gel
2. Load 0.5 μ l RNA extract with 4.5 μ l water and 1 μ l 6 x loading buffer
3. Run the gel at 80 V until the fastest dye has moved 2/3 of the gel length
4. Visualise the gel using a UV transilluminator and then take a digital photograph

Protocol - SYBR Gold (Small-scale RNA extractions)

When following this protocol please note that the size ladder should be diluted 1:20 - otherwise it will be too bright.

1. Make a 0.8% agarose gel, then load 0.5 μ l RNA extract with 4.5 μ l water and 1 μ l 6 x loading buffer
2. Run the gel at 80 V until the fastest dye has moved 2/3 of the gel length
3. Add 30 μ l SYBR Gold (Molecular Probes; Cat. No. S-11494) to 300 ml 1 x TAE (1:10,000 dilution)
4. Remove gel from the gel tank, immerse in SYBR Gold and leave to stain for 30 minutes (with an orbital shaker, in the dark)
5. Destain the gel by washing in 1 x TAE for 10 minutes (with an orbital shaker, in the dark)
6. Visualise the gel using a UV transilluminator and then take a digital photograph

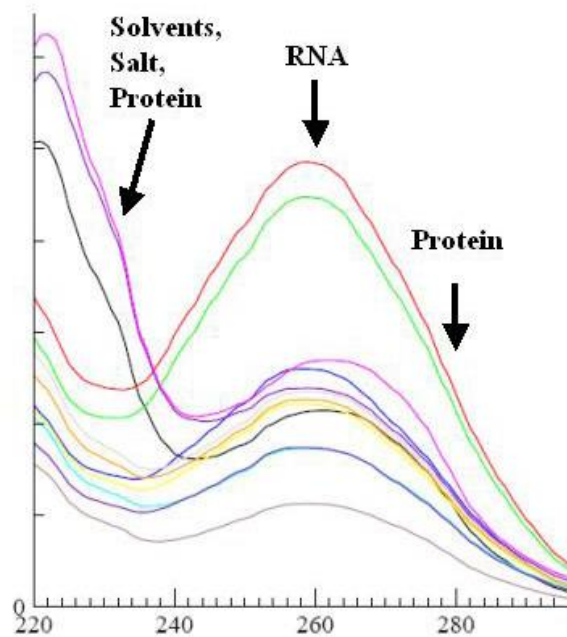
Optical density measurements:

Optical density measurements are made using a Nanodrop ND-1000 spectrophotometer (<http://www.nanodrop.com>) and have been calibrated using dilutions of RNA type III from *S. cerevisiae*

(Sigma; Cat. No. R-7125). This calibration showed that our linear range is within the reading range of 10 to 1000 ng/μl. Therefore, our samples are first diluted to fall within this range. Any measurement taken from outside of this range will be inaccurate.

Good quality RNA will have an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater. This is because nucleic acid is detected at 260 nm, whereas protein, salt and solvents are detected at 230 and 280 nm. A high OD 260/280 and OD 260/230 ratio therefore indicates that you have extracted RNA devoid of any of these contaminants. This is shown in the following graph that includes examples of good samples and samples with a poor OD 260/230 ratio.

Optical density measurements of *D. melanogaster* RNA



Protocol

1. Open the Nanodrop icon and select 'Nucleic Acid Measurements'.
2. Add 2 μl of the solvent the RNA has been dissolved in ("the solvent"); the instrument will then initialise.
3. After each and all subsequent measurements clean the pedestal by wiping with a dry lint-free tissue.
4. Add 2 μl of solvent and press 'Blank'
5. Repeat the blanking until there is a stable baseline, close to zero
6. Confirm that the baseline is correct by measuring 2 μl of solvent, as if it were your first sample by pressing 'Measure'
7. Add 2 μl of the first sample making sure to add the sample ID (or name) to the 'Sample ID' field and then press 'Measure'
8. Repeat step 3 and then 7 for all samples
9. Confirm that the baseline is correct after taking all measurements by measuring 2 μl of solvent, as if it were your last sample by pressing 'Measure'
10. Each of the measurements is automatically saved by the instrument and these can then be calibrated using the in house script

R. Auburn (07-06-2004).

Reverse transcription and direct labelling of total RNA for cDNA and oligo arrays

Overview

The samples or samples and controls to be compared are each labelled with a different fluorescent dye and then subjected to paired competitive hybridisations. The described protocol for reverse transcription and direct labelling is based on the method recommended by BioRobotics (<http://www.genomicsolutions.com/>).

Removal of RNase

All materials should be autoclaved and only handled using gloves to avoid RNase contamination. Glassware should be baked at 180 °C overnight. MilliQ water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If possible, keep a set of pipettes purely for RNA work.

Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Oligo(dT)₂₃ anchored (Sigma; Cat. No. 04387)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- DEPC-treated MilliQ water
- Cy3 dCTP (Amersham; Cat. No. PA 53021)
- Cy5 dCTP (Amersham; Cat. No. PA 55021)
- RNAsin (Promega; Cat. No. N2115)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- EDTA (BDH; Cat. No. 100935V)
- NaOH, (BDH; Cat. No. 102525P)
- Tris-HCl solid (BDH; Cat. No. 443864E)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- AutoSeq G-50 column (Amersham, Cat. No. 27-5340-01)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac

Procedure

Reverse transcription and direct labelling reaction:

1. Prepare a concentrated stock of low-C dNTP mix:
 - ◆ 25 µl of 100 mM dATP
 - ◆ 25 µl of 100 mM dGTP
 - ◆ 25 µl of 100 mM dTTP
 - ◆ 10 µl of 100 mM dCTP
 - ◆ Make to 500 µl with DEPC-treated MilliQ water
 - ◆ Store in small aliquots at -20 °C
2. Mix together 25-50 µg total RNA, DEPC MilliQ water and spike mix to a total volume of 28 µl in an RNase-free 1.5 ml tube. Add 1 µl of 500 ng/µl oligo (dT)₂₃ anchored primer.
3. Incubate at 65 °C for 10 minutes in a hot-block to denature RNA tertiary structure, then place on ice.
4. Mix together the following to make a master mix:
 - ◆ 8 µl of 5x first strand buffer
 - ◆ 2 µl of conc. low-C dNTP mix
 - ◆ 2 µl of 1 mM Cy3 or Cy5 dCTP
 - ◆ 2 µl of 0.1 M DTT
 - ◆ 0.5 µl of RNAsin
 - ◆ 2 µl of Superscript III reverse transcriptase
5. Add 16.5 µl master mix to each tube of RNA/MilliQ water mixing carefully to avoid bubbles. Do not expose samples to light any more than necessary, ie. wrap in foil when possible.
6. Incubate at 42 °C for 1-2 hours.

Hydrolysis and neutralisation:

7. Hydrolyse the remaining RNA by mixing equal volumes of 0.5 M EDTA and 1 M NaOH. Then add 20 μ l of this mix to the reaction and incubate at 65 °C for 15 minutes.
8. Bring samples to room temperature and add 25 μ l of 1 M Tris-HCl (pH 7.5) to neutralise. If required, the labelled probe can be stored at -20 °C in the dark at this point.

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

9. Reduce volume of probe to approximately 25 μ l, by placing in a speed vac with medium heat. With our machine, this takes about 30 mins. Then combine the Cy3- and Cy5-labelled probe (sample and control) into one 1.5 ml microfuge tube.
10. Resuspend the resin in the G-50 column by vortexing gently.
11. Loosen the cap a quarter turn and snap off the bottom closure.
12. Place the column in a 1.5 ml tube.
13. Pre-spin column at 5,000 rpm for 1 minute to remove the buffer. Blot the tip of the column dry using a clean paper towel.
14. Remove the top cap and place column in a new 1.5 ml tube. Pipette half of the sample onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
15. Spin for 1 minute at 5,000 rpm. The unincorporated dye and nucleotides should be retained by the column and the purified labelled probe should pass through into the support tube. Discard the column.
16. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
17. Reduce volume of probe to between 2 to 5 μ l by placing in a speed vac with medium heat
18. Add 2 μ l of 10 mg / ml sonicated salmon sperm DNA

The two samples (i.e. sample and control) have been combined together for hybridisation to a microarray and the blocking agent, sonicated salmon sperm DNA, has been added. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (07-06-2004).

Reverse transcription and indirect labelling of total RNA for cDNA and oligo arrays

Overview

The samples to be compared are each labelled with a different fluorescent dye and then subjected to paired competitive hybridisations. Indirect labelling involves two steps, firstly, incorporation of amino-allyl dUTP by reverse transcription, and then attachment of the fluorescent dyes.

The following is an adaptation of a protocol from the Ajioka group within the Department of Pathology, University of Cambridge ([Ajioka](#)). This protocol can be used to label as little as 3 µg of total RNA. However, the following is for up to 30 µg of total RNA. For each microarray slide you should have two target samples; one labeled with Cy3, the other with Cy5.

Microcon YM-30 column steps are approximate, and need to be optimized for your particular centrifuge. If you centrifuge for too long and the pellet is dry, reload waste and re-centrifuge.

Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Amino-allyl dUTP (Sigma; Cat. No. A0410)
- Oligo (dT)₂₃ anchored (Sigma; Cat. No. 04387)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- DEPC-treated MilliQ water
- Cy3 and Cy5 monofunctional dyes, pre-aliquoted (Amersham; Cat. No. RPN5661)
- RNasin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- EDTA (BDH; Cat. No. 100935V)
- Sodium hydroxide, (BDH; Cat. No. 102525P)
- Tris-HCl solid (BDH; Cat. No. 443864E)
- Sodium bicarbonate (Sigma; Cat. No. S-7277)
- Microcon YM-30 concentrators (Millipore; Cat. No. 42410)
- MinElute PCR Purification Kit (Qiagen; Cat. No. 28004)
- Hydroxylamine (Sigma; Cat. No. H-2391)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac

Removal of RNase

All materials should be autoclaved and only handled using gloves to avoid RNase contamination. Glassware should be baked at 180 °C overnight. MilliQ water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If possible, keep a set of pipettes purely for RNA work.

Procedure

Reagents to mix and aliquot:

1. Make a 50 x dNTP mix:
 - ◆ 10 µl dATP (100 mM stock)
 - ◆ 10 µl dCTP (100 mM stock)
 - ◆ 10 µl dGTP (100 mM stock)
 - ◆ 4 µl dTTP (100 mM stock)
 - ◆ 6 µl amino-allyl dUTP (100 mM)

Then mix together and add to the master mix as below

2. Master Mix for 1 sample (total 14.6 μ l)
 - ◆ 6 μ l First strand buffer (comes with Superscript III)
 - ◆ 0.6 μ l 50x dNTP mix made above
 - ◆ 3 μ l 0.1 M DTT (provided with Superscript III)
 - ◆ 0.25 μ l RNAsin
 - ◆ 2.75 μ l RNase-free water
 - ◆ 2 μ l Superscript III

You can make a pre-mix without Superscript III and make aliquots before storing at -20 °C. Add Superscript III before use.

Reverse transcription for amino-allyl dUTP incorporation:

3. RNA is isolated and purified using our standard protocol
4. Adjust the volume of RNA to 14.5 μ l with DEPC treated water and add to a 1.5 ml RNase/DNase free microfuge tube
5. Add 1 μ l of anchored Oligo dT primer to each RNA sample
6. Place the tubes at 65 °C for 10 minutes
7. Place the tubes immediately on ice for 5 minutes
8. Add 14.6 μ l of the master mix to each tube (made above) and incubate at 42 °C for 2 hours
9. Remove the tubes from hot block, and add 10 μ l 0.5 M EDTA and 10 μ l of 1 M sodium hydroxide. Place at 65 °C for 15 minutes
10. Remove from heat and place at room temperature for 2 minutes
11. Add 25 μ l of 1 M Tris-HCl (pH 7.5), mix
12. Add 450 μ l of DEPC water to each sample and add each sample to a microcon-YM30 concentrator. Add sample without touching the membrane.
13. Centrifuge at room temperature at 13,000 rpm for about 7 minutes (or until 50 μ l of water left in reservoir), empty waste, and then add another 400 μ l of water.
14. Centrifuge again at room temperature at 13,000 rpm for 7 minutes
15. Empty waste and add 400 μ l water for a third time
16. Centrifuge tube at room temperature at 13,000 rpm for 8 minutes (until volume left reached 10 μ l).
17. Invert the column into new 1.5 ml collection tube.
18. Centrifuge at RT at 3,000 rpm for 4 minutes.
19. Place the tubes in the speed vac to dry 10 minutes on high heat. Can be stored at -20 °C.

Dye attachment:

20. Resuspend the pellet in 4.5 μ l DEPC water
21. Resuspend one aliquot of Cy3 or Cy5 dye in 4.5 μ l of 0.1 M Sodium Bicarbonate (pH in range of 8.5-9.0).
22. Mix resuspended dye with resuspended pellet.
23. Place in the dark for 1 hour at 23 °C (in a hot block).

Dye quenching and removal of unincorporated dye:

24. Add 4.5 μ l of 4 M hydroxylamine to each sample to stop reaction and incubate in the dark for 15 minutes at 23 °C.

Clean up samples using Qiagen MinElute PCR Purification Kit:

25. Add 35 μ l of 3 M sodium acetate (pH 5.2) to each reaction, and mix both samples (Cy3 and Cy5) in a new 1.5 ml microfuge tube
26. Add 5 volumes (500 μ l) of buffer PB and mix.
27. Place a MinElute column in a provided 2 ml collection tube.
28. To bind DNA, apply the sample to the MinElute column and centrifuge for 1 minute at 13,000 rpm.
29. Discard flow through, and add 750 μ l of Buffer PE to the column.
30. Centrifuge for 1 minute at 13,000 rpm.
31. Repeat steps 29 and 30.
32. Discard flow through and place MinElute column back in same tube. Centrifuge column for 1 minute at 13,000 rpm.
33. Place column into a new 1.5 ml microfuge tube and add 10 μ l elution buffer (DEPC water).

34. Incubate at room temperature for 1 minute in dark.
35. Centrifuge for 1 minute at 13,000 rpm.
36. Add another 10 μ l of DEPC water to column and centrifuge for 1 minute at 13,000 rpm. Collect in same tube.
37. Dry in the speed vac to a volume of 2 to 5 μ l (about 10 minutes).
38. Add 2 μ l sonicated salmon sperm DNA (from 10 mg / ml stock).

The two samples (i.e. sample and control) have been combined together for hybridisation to a microarray and the blocking agent, sonicated salmon sperm DNA, has been added. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (17-02-2006).

Klenow labelling of double stranded DNA derived from 3 to 5 µg total RNA

Outline

Klenow labelling of double stranded DNA can be used when the amount of biological material is limiting. With this method, amplification by *in vitro* transcription of the sample can be avoided. The RNA samples are reverse transcribed to cDNA and second strand synthesis is then performed to obtain double stranded DNA (dsDNA). Fluorescent dyes are incorporated using Klenow fragment and the labelled samples are then subjected to paired competitive hybridisations.

Equipment and reagents

- Oligo(dT)23 anchored primer (Sigma; Cat. No. 04387)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- RNAsin (Promega; Cat. No. 18064-014)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Second strand buffer (Invitrogen; Cat. No. 10812-014)
- DNA Polymerase I (Invitrogen; Cat. No. 1801-025)
- RNaseH (New England Biolabs; Cat. No. M0297S)
- *E. coli* DNA ligase (GE Healthcare Bio-Sciences AB; Cat. No. E70020Z)
- Phenol:Chloroform:Isoamylalcohol (Sigma; Cat. No. P2069)
- Phase Lock Gel (Helena Bioscience; Cat. No. 0032 007.953)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB, Cat. No. 27-5340-01)
- Sodium Chloride (VWR; Cat. No. 102414P)
- Gene Elute LPA (Sigma; Cat. No. 56575)
- 100% Ethanol
- 70% Ethanol
- DEPC - Diethyl pyrocarbonate (Sigma; Cat. No. D 5758)
- DEPC-treated MilliQ water
- Bioprime DNA Labeling System (Invitrogen; Cat. No. 18094-011)
- Cy3 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 53021)
- Cy5 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 55021)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

Preparing the dNTP mixes

Making the 10mM dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
 - ◆ 100 µl 100mM dNTA
 - ◆ 100 µl 100mM dNTT
 - ◆ 100 µl 100mM dNTG
 - ◆ 100 µl 100mM dNTC
2. Then make up to 1 ml using DEPC-water
3. Aliquot the dNTP mix and then store at -20 °C

Making the 10 X low-C dNTP mix:

1. Make a large 10 X low-C dNTP mix for the labelling reaction (5 mM A-,G-,T-dNTPs and 3mM C-dNTP)
 - ◆ 25 µl 100mM dNTA
 - ◆ 25 µl 100mM dNTT
 - ◆ 25 µl 100mM dNTG
 - ◆ 15 µl 100mM dNTC
2. Then make up to 500 µl using DEPC-water
3. Aliquot the 10 X low-C dNTP mix and then store at -20 °C

Reverse Transcription reaction:

The following steps are performed in 200 µl PCR tubes and the PCR block

1. Take between 3 to 5 µg of the extracted RNA (small scale RNA extraction protocol), add 1 µl of Oligo(dT)23 anchored primer (500 ng/µl) and then make up to a total volume of 11 µl with DEPC-water
2. Incubate at 65 °C for 10 min
3. Snap freeze on ice
4. Make up a premix for the RT reaction:
 - ◆ 4 µl Superscript Buffer (from superscript kit)
 - ◆ 2 µl 0.1M DTT (from superscript kit)
 - ◆ 1 µl 10 mM dNTP mix
 - ◆ 0.25 µl 40 U/µl RNasin
 - ◆ 0.75 µl 200 U/µl Superscript III
5. Add 8 µl to each sample
6. Incubate at 46 °C for 2 hours
7. Incubate at 65 °C for 15 minutes
8. Snap freeze on ice

Second strand synthesis:

The following steps are performed in 200 µl PCR tubes and the PCR block

9. Make up a premix for the second strand synthesis:
 - ◆ 9.15 µl DEPC-water
 - ◆ 7.5 µl Second Strand Buffer
 - ◆ 0.75 µl 10 mM dNTP mix
 - ◆ 2 µl 10 U/µl DNA Polymerase I
 - ◆ 0.1 µl 5 U/µl RNaseH
 - ◆ 0.5 µl 10 U/µl *E. coli* Ligase
10. Add 20 µl to each sample
11. Incubate at 16 °C for 2 hours

Purification of dsDNA:

The following steps are performed in 1.5 ml microfuge tubes

12. Make the sample up to 100 µl (add 60 µl DEPC-water)
13. Immediately before use pellet Phase Lock Gel (PLG) tube at 13,000 rpm for 30 seconds
14. Add the cDNA and an equal volume (100 µl) of (Phenol/Chloroform/Isoamylalcohol pH 8.0) to the PLG tube and shake for 15 seconds (do not vortex)
15. Centrifuge for 5 minutes at 13000 rpm
16. Transfer upper phase to a new 1.5 ml microfuge tube
17. Reduce volume of sample to approximately 30 µl, by placing in a speed vac with medium heat
18. Prepare the G50 columns:
 - ◆ Resuspend the resin in the G-50 column by vortexing gently
 - ◆ Loosen the cap a quarter turn and snap off the bottom closure
 - ◆ Place the column in a 1.5 ml tube
 - ◆ Pre-spin column at 5,000 rpm (2000 x g) for 1 min to remove the buffer

- ◆ Remove the top cap and place column in a new 1.5 ml tube
- 19. Pipette the sample to the G50 columns onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
- 20. Centrifuge for 1 minute at 5000 rpm, discard the column
- 21. Adjust the volume to 100 µl with DEPC-water
- 22. Precipitate the dsDNA:
 - ◆ 3.5 µl 5M Sodium Chloride
 - ◆ 0.5 µl LPA
 - ◆ 220 µl 100% Ethanol
- 23. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
- 24. Centrifuge for 30 minutes at 13000 rpm
- 25. Remove supernatant and wash pellet with 70% Ethanol, centrifuge for 2-3 minutes
- 26. Dry pellet and dissolve in 10 to 20 µl DEPC-H₂O
- 27. Measure concentration on Nanodrop

Klenow labelling:

The following steps are performed in 200 µl PCR tubes and the PCR block

- 28. Take up to 1 µg double stranded DNA and make up to a total volume of 13 µl with DEPC-water
- 29. Add 10 µl 2.5x Random Primer Reaction Buffer (supplied in the Bioprime Labelling System Kit)
- 30. Incubate at 100 °C for 5 minutes
- 31. Snap freeze on ice
- 32. Mix together the following to make a master mix:
 - ◆ 0.5 µl 10 X low-C dNTP mix
 - ◆ 1 µl Cy3 or Cy5 dCTP
 - ◆ 0.5 µl 40U/µl Klenow
- 33. Add 2 µl to each sample and mix by pipetting up and down
- 34. Incubate at 37°C for 2 to 3 hours
- 35. Stop the reaction by adding 2.5 µl Stop Buffer (supplied in the Bioprime Labelling System Kit)
- 36. Combine the Cy3 and Cy5 pairs

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

- 37. Prepare the G50 columns (need 2 columns per combined sample):
 - ◆ Resuspend the resin in the G-50 column by vortexing gently
 - ◆ Loosen the cap a quarter turn and snap off the bottom closure
 - ◆ Place the column in a 1.5 ml tube
 - ◆ Pre-spin column at 5,000 rpm (2000 x g) for 1 min to remove the buffer
 - ◆ Remove the top cap and place column in a new 1.5 ml tube
- 38. Pipette half the sample to the G50 columns onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
- 39. Centrifuge for 1 minute at 5000 rpm
- 40. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
- 41. Reduce volume of sample to between 2 to 5 µl by placing in a speed vac with medium heat
- 42. Add 2 µl of sonicated salmon sperm DNA

The samples have now been labelled and combined together for hybridisation to a microarray with the blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (03-11-2008).

Anti-sense strand amplification of RNA using RT-PCR and Ambion MEGA script T7 and direct labelling for cDNA microarrays

Outline

Amplification is sometimes to produce sufficient RNA for labelling when the amount of biological material is limiting. The method we use is based on a protocol from Gertrud Woerfel (<http://www.gen.cam.ac.uk/Research/russell.htm>). The samples or samples and controls to be compared are each then labelled with a different fluorescent dye and subjected to paired competitive hybridisations.

Equipment and reagents

- (dT)-T7 primer: GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA-(T)_{n=21}-[A or G or C]
- T4 Gene 32 protein (Amersham; Cat. No. E70029Z)
- Second strand buffer (Invitrogen; Cat. No. 10812-014)
- DNA Polymerase I (Invitrogen; Cat. No. 1801-025)
- RNaseH (New England Biolabs; Cat. No. M0297S)
- *E. coli* DNA ligase (Amersham; Cat. No. E70020Z)
- T4 DNA polymerase (Roche Diagnostics; Cat. No. 1004786)
- Microspin columns with Bio-Gel P6 in Tris (Bio-Rad; Cat. No. 732-6221)
- Phenol:Chloroform:Isoamylalcohol (Fisher Scientific; Cat. No. BPE 1754 I-100)
- Sodium Chloride (VWR; Cat. No. 102414P)
- Gene Elute LPA (Sigma; Cat. No. 56575)
- 100% Ethanol
- MEGA-Script T7 kit (Ambion; Cat. No. 1334)
- Sodium Acetate
- Phenol, saturated pH 4.3 (Fisher Scientific; Cat. No. BPE 1751 I-100)
- 70% Ethanol
- RQ1 RNase-free DNase (Promega; Cat. No. M6101)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Random primer (Promega; Cat. No. C1181)
- DEPC - Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- DEPC-treated MilliQ water
- Cy3 dCTP (Amersham; Cat. No. PA 53021)
- Cy5 dCTP (Amersham; Cat. No. PA 55021)
- RNasin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- EDTA (BDH; Cat. No. 100935V)
- NaOH, (BDH; Cat. No. 102525P)
- Tris-HCl solid (BDH; Cat. No. 443864E)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- AutoSeq G-50 column (Amersham, Cat. No. 27-5340-01)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

RNA amplification

Making the dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
 - ◆ 100 μ l 100mM dNTA
 - ◆ 100 μ l 100mM dNTT
 - ◆ 100 μ l 100mM dNTG
 - ◆ 100 μ l 100mM dNTC
2. Then make up to 1 ml using DEPC-water
3. Aliquot the dNTP mix and then store at -20 °C

Reverse Transcription reaction:

The following steps are performed in 200 ml PCR tubes and the PCR block

1. Take up to 4.5 μ l of the extracted RNA (small scale RNA extraction protocol), add 0.5 μ l of Primer T7-dT (200 ng/ μ l) and then make up to a total volume of 5 μ l with DEPC- water
2. Incubate at 65 °C for 10 min
3. Then snap freeze on ice
4. Make up a premix for the RT reaction:
 - ◆ 2 μ l Superscript Buffer (from superscript kit)
 - ◆ 1 μ l 0.1M DTT (from superscript kit)
 - ◆ 1 μ l 10 mM dNTP mix
 - ◆ 0.8 μ l T4 Gene 32 protein
 - ◆ 0.5 μ l RNasin
 - ◆ 1 μ l Superscript III
5. Add 6.3 μ l to each sample
6. Incubate at 42 °C for 2 hours
7. Incubate at 65 °C for 15 minutes
8. Then snap freeze on ice

Second strand synthesis:

The following steps are performed in 200 ml PCR tubes and the PCR block

9. Make up a premix for the second strand synthesis:
 - ◆ For 1 reaction:
 - ◆ 45 μ l DEPC-water
 - ◆ 15 μ l Second strand buffer
 - ◆ 1.5 μ l 10 mM dNTP mix
 - ◆ 4 μ l DNA Polymerase I
 - ◆ 0.2 μ l RNaseH
 - ◆ 1 μ l *E. coli* Ligase
10. Add 66.7 μ l to each sample
11. Incubate at 16 °C for 2 hours
12. Add 2.0 μ l T4 DNA polymerase
13. Incubate at 15 °C for 15 minutes, then 70 °C for 10 minutes
14. Prepare Microspin columns with Bio-Gel P6 in Tris:
 - ◆ Invert columns sharply 2-3 times
 - ◆ Snap off the bottom and remove the lid
 - ◆ Let the column drain by gravity flow
 - ◆ Shortly before use spin for 2 minutes at 2500 rpm
15. Phenolisation: add 75 μ l PCI (Phenol/Chloroform/Isoamylalcohol) to the sample and vortex
16. Centrifuge for 10 minutes at 13000 rpm
17. Transfer upper phase to the Microspin columns with Bio-Gel P6 in Tris columns and centrifuge for 4 minutes at 2500 rpm (1000 x g)

Precipitate cDNA:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

18. Transfer the second strand synthesis product to 1.5 ml microfuge tubes and then add
 - ◆ 3.5 µl 5M Sodium Chloride
 - ◆ 0.5 µl LPA
 - ◆ 220 µl 100% Ethanol
19. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
20. Centrifuge for 30 minutes at 13000 rpm
21. Remove supernatant and wash with 70% Ethanol, centrifuge for 2-3 minutes
22. Dry pellet and dissolve in 8 µl DEPC-H₂O

In vitro transcription using Ambion Megascript T7 Kit:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

23. Make a premix (per sample) using the Ambion Megascript T7 Kit
 - ◆ 2 µl Txn buffer
 - ◆ 2 µl 75 mM ATP
 - ◆ 2 µl 75 mM CTP
 - ◆ 2 µl 75 mM GTP
 - ◆ 2 µl 75 mM UTP
 - ◆ 2 µl Enzyme Mix
24. Add 12 µl of the premix to each sample
25. Incubate at 37 °C for 9-16 hours
26. Add 1 µl RQ1 RNase-free DNase
27. Incubate 1 hour at 37 °C
28. Take 1 µl and analyse on a 1% agarose gel (RNA quality control protocol)

Purification of the now amplified RNA:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

29. To the Ambion Megascript T7 processed material add:
 - ◆ 20 µl 3M Sodium Acetate
 - ◆ 16 µl DEPC-water
 - ◆ 200 µl Phenol (pH4.5)
30. Vortex and centrifuge for 10 minutes at 13000 rpm
31. Transfer upper phase to a fresh 1.5 ml microfuge tube
32. Add 600 µl 100% EtOH
33. Incubate at -20 °C for at least 30 minutes (or 10 minutes at -80 °C)
34. Centrifuge for 30 minutes at 13000 rpm
35. Wash pellet with 500 µl 70% Ethanol
36. Dry pellet and dissolve in 10 µl DEPC-water
37. Measure concentration on NanoDrop (RNA quality control protocol)

Reverse transcription and direct labelling reaction:

38. Prepare a concentrated stock of low-C dNTP mix:
 - ◆ 25 µl of 100 mM dATP
 - ◆ 25 µl of 100 mM dGTP
 - ◆ 25 µl of 100 mM dTTP
 - ◆ 10 µl of 100 mM dCTP
 - ◆ Make to 500 µl with DEPC-treated MilliQ water
 - ◆ Store in small aliquots at -20 °C
39. Mix together up to 10 µg amplified RNA, DEPC MilliQ water and spike mix to a total volume of 28 µl in an RNase-free 1.5 ml tube. Add 1 µl of 500 µg/ml random primer.
40. Incubate at 65 °C for 10 minutes in a hot-block to denature RNA tertiary structure, then place on ice.
41. Mix together the following to make a master mix:
 - ◆ 8 µl of 5x first strand buffer

- ◆ 2 µl of conc. low-C dNTP mix
 - ◆ 2 µl of 1 mM Cy3 or Cy5 dCTP
 - ◆ 2 µl of 0.1 M DTT
 - ◆ 0.5 µl of RNAsin
 - ◆ 2 µl of Superscript III reverse transcriptase
42. Add 16.5 µl master mix to each tube of RNA/MilliQ water mixing carefully to avoid bubbles. Do not expose samples to light any more than necessary, ie. wrap in foil when possible.
43. Incubate at 42 °C for 1-2 hours.

Hydrolysis and neutralisation:

44. Hydrolyse the remaining RNA by mixing equal volumes of 0.5 M EDTA and 1 M NaOH. Then add 20 µl of this mix to the reaction and incubate at 65 °C for 15 minutes.
45. Bring samples to room temperature and add 25 µl of 1 M Tris-HCl (pH 7.5) to neutralise. If required, the labelled probe can be stored at -20 °C in the dark at this point.

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

46. Reduce volume of probe to approximately 25 µl, by placing in a speed vac with medium heat. With our machine, this takes about 30 mins. Then combine the Cy3- and Cy5-labelled probe (sample and control) into one 1.5 ml microfuge tube.
47. Resuspend the resin in the G-50 column by vortexing gently.
48. Loosen the cap a quarter turn and snap off the bottom closure.
49. Place the column in a 1.5 ml tube.
50. Pre-spin column at 5,000 rpm for 1 minute to remove the buffer. Blot the tip of the column dry using a clean paper towel.
51. Remove the top cap and place column in a new 1.5 ml tube. Pipette half of the sample onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
52. Spin for 1 minute at 5,000 rpm. The unincorporated dye and nucleotides should be retained by the column and the purified labelled probe should pass through into the support tube. Discard the column.
53. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
54. Reduce volume of probe to between 2 to 5 µl by placing in a speed vac with medium heat
55. Add 2 µl of sonicated salmon sperm DNA

The samples have now been amplified and combined together for hybridisation to a microarray with the blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (07-06-2004).

Anti-sense strand amplification of RNA and indirect labelling of amino-allyl RNA for oligo microarrays (1 round)

Outline

Amplification can be used to produce sufficient RNA for labelling when the amount of biological material is limiting. The method we use is based on the Eberwine method. The samples and controls to be compared are each then labelled with a different fluorescent dye and subjected to paired competitive hybridisations.

Equipment and reagents

- (dT)-T7 primer:
GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA-(T)_{n=21}-[A or G or C]
- T4 Gene 32 protein (GE Healthcare Bio-Sciences AB; Cat. No. E70029Z)
- Second strand buffer (Invitrogen; Cat. No. 10812-014)
- DNA Polymerase I (Invitrogen; Cat. No. 1801-025)
- RNaseH (New England Biolabs; Cat. No. M0297S)
- *E. coli* DNA ligase (GE Healthcare Bio-Sciences AB; Cat. No. E70020Z)
- T4 DNA polymerase (Roche Diagnostics; Cat. No. 1004786)
- Microspin columns with Bio-Gel P6 in Tris (Bio-Rad; Cat. No. 732-6221)
- Phenol:Chloroform:Isoamylalcohol (Sigma; Cat. No. P2069)
- Sodium Chloride (VWR; Cat. No. 102414P)
- Gene Elute LPA (Sigma; Cat. No. 56575)
- 100% Ethanol
- MEGA-Script T7 kit (Ambion; Cat. No. 1334)
- 70% Ethanol
- RQ1 RNase-free DNase (Promega; Cat. No. M6101)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- DEPC - Diethyl pyrocarbonate (Sigma; Cat. No. D 5758)
- DEPC-treated MilliQ water
- RNasin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB; Cat. No. 27-5340-01)
- Phase Lock Gel (Helena Bioscience; Cat. No. 0032 007.953)
- Aminoallyl-UTP-sodium salt (Sigma; Cat. No. A-5660)
- RNeasy Mini Kit (Qiagen; Cat. No. 74104)
- Cy Dye Post-Labelling Reactive Dye Pack (GE Healthcare Bio-Sciences AB; Cat. No. RPN 5661)
- Sodium Bicarbonate (Sigma; Cat. No. S7277)
- Hydroxylamine hydrochloride (Sigma; Cat. No. H9876)
- Ammonium Acetate (VWR; Cat. No. 1.01116.0500)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

RNA amplification

Making the dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
 - ◆ 100 μ l 100mM dNTA
 - ◆ 100 μ l 100mM dNTT
 - ◆ 100 μ l 100mM dNTG
 - ◆ 100 μ l 100mM dNTC
2. Then make up to 1 ml using DEPC-water
3. Aliquot the dNTP mix and then store at -20 °C

Making the amino-allyl UTP stock:

1. For 75mM amino-allyl UTP stock, resuspend 1mg Aminoallyl-UTP-sodium salt in 24.7 μ l DEPC-water

Reverse Transcription reaction:

The following steps are performed in 200 μ l PCR tubes and the PCR block

1. Take up to 4.5 μ l of the extracted RNA (small scale RNA extraction protocol), add 0.5 μ l of Primer T7-dT (200 ng/ μ l) and then make up to a total volume of 5 μ l with DEPC- water
2. Incubate at 65 °C for 10 min
3. Then snap freeze on ice
4. Make up a premix for the RT reaction:
 - ◆ 2 μ l Superscript Buffer (from superscript kit)
 - ◆ 1 μ l 0.1M DTT (from superscript kit)
 - ◆ 1 μ l 10 mM dNTP mix
 - ◆ 0.8 μ l 5 μ g/ μ l T4 Gene 32 protein
 - ◆ 0.5 μ l 40 U/ μ l RNasin
 - ◆ 1 μ l 200 U/ μ l Superscript III
5. Add 6.3 μ l to each sample
6. Incubate at 46 °C for 2 hours
7. Incubate at 65 °C for 15 minutes
8. Then snap freeze on ice

Second strand synthesis:

The following steps are performed in 200 μ l PCR tubes and the PCR block

9. Make up a premix for the second strand synthesis:
 - ◆ For 1 reaction:
 - ◆ 45 μ l DEPC-water
 - ◆ 15 μ l Second strand buffer
 - ◆ 1.5 μ l 10 mM dNTP mix
 - ◆ 4 μ l 10 U/ μ l DNA Polymerase I
 - ◆ 0.2 μ l 5 U/ μ l RNaseH
 - ◆ 1 μ l 10 U/ μ l *E. coli* Ligase
10. Add 66.7 μ l to each sample
11. Incubate at 16 °C for 2 hours
12. Add 2.0 μ l 1 U/ μ l T4 DNA polymerase
13. Incubate at 15 °C for 15 minutes, then 70 °C for 10 minutes

Purification of ds DNA:

The following steps are performed in 1.5 ml microfuge tubes

14. Make the sample up to 100 μ l (add 20 μ l DEPC-water)
15. Prepare Microspin columns with Bio-Gel P6 in Tris:
 - ◆ Invert columns sharply 2-3 times and remove any air bubbles
 - ◆ Snap off the bottom and remove the lid
 - ◆ Let the column drain by gravity flow
 - ◆ Shortly before use spin for 2 minutes at 2500 rpm (1000 x g)

16. Immediately before use pellet Phase Lock Gel (PLG) tube at 13,000 rpm for 30 seconds
17. Add the cDNA and an equal volume (100 μ l) of (Phenol/Chloroform/Isoamylalcohol pH 8.0) to the PLG tube and shake for 15 seconds (do not vortex)
18. Centrifuge for 5 minutes at 13000 rpm
19. Transfer upper phase to the Microspin columns with Bio-Gel P6 in Tris columns and centrifuge for 4 minutes at 2500 rpm (1000 x g)
18. Precipitate the dsDNA:
 - ◆ 3.5 μ l 5M Sodium Chloride
 - ◆ 0.5 μ l LPA
 - ◆ 220 μ l 100% Ethanol
19. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
20. Centrifuge for 30 minutes at 13000 rpm
21. Remove supernatant and wash with 70% Ethanol, centrifuge for 2-3 minutes
22. Dry pellet and dissolve in 8 μ l DEPC-H₂O

In vitro transcription using Ambion Megascript T7 Kit:

The following steps are performed in 1.5 ml microfuge tubes and in a 37°C Incubator

23. Make a premix (per sample) using the Ambion Megascript T7 Kit
 - ◆ 2 μ l Txn buffer
 - ◆ 2 μ l 75 mM ATP
 - ◆ 2 μ l 75 mM CTP
 - ◆ 2 μ l 75 mM GTP)
 - ◆ 1.5 μ l 75 mM UTP
 - ◆ 0.5 μ l 75 mM Amino-allyl UTP
 - ◆ 2 μ l Enzyme Mix
24. Add 12 μ l of the premix to each sample
25. Incubate at 37 °C for 9-16 hours
26. Add 1 μ l 1 U/ μ l RQ1 RNase-free DNase
27. Incubate 1 hour at 37 °C

Purification of the amplified RNA using Qiagen RNeasy Mini Kit RNA cleanup protocol followed by precipitation:

The following steps are performed in 1.5 ml microfuge tubes

28. To the Ambion Megascript T7 processed material add:
 - ◆ 80 μ l DEPC-water
 - ◆ 350 μ l RLT buffer (add 10 μ l beta-mercaptoethanol to 1ml RLT buffer)
29. Mix thoroughly
30. Add 250 μ l ethanol (96-100%) and mix by pipetting
31. Apply sample (700 μ l) to an RNeasy mini column placed in a 2ml collection tube
32. Centrifuge at 13,000 rpm 15 seconds
33. Transfer RNeasy column into a new 2 ml tube and add 500 μ l Buffer RPE onto the column
34. Centrifuge at 13,000 rpm 15 seconds. Discard the flow-through
35. Add another 500 μ l Buffer RPE to the column
36. Centrifuge at 13,000 rpm for 2 minutes
37. Transfer RNeasy column into a new 2 ml tube and centrifuge at 13,000 rpm for 1 minute to dry the membrane
38. Transfer RNeasy column into a new 1.5 ml tube
39. Pipette 50 μ l DEPC-water directly onto membrane, incubate for 1 minute
40. Centrifuge at 13000 rpm for 1 minute to elute
41. Repeat step 39 and 40
42. Take 2.0 μ l aliquot and analyse on a 1% agarose gel (ethidium bromide)
43. Precipitate with:
 - ◆ 0.5 μ l LPA (25 μ g/ μ l)
 - ◆ 50 μ l 7.5M Ammonium acetate
 - ◆ 250 μ l 100% Ethanol
44. Mix and centrifuge immediately at 13,000 rpm for 30 minutes
45. Wash pellet with 70% Ethanol

46. Dry and resuspend in 5.0 μ l DEPC-water
47. Take 0.5 μ l aliquot and measure concentration on NanoDrop

Dye coupling:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

48. Resuspend 1 aliquot of monofunctional dye in 4.5 μ l 0.1M sodium bicarbonate (pH 8.5)
49. Mix with 4.5 μ l amplified amino-allyl RNA
50. Incubate at 23 °C for 1 hour in heating block
51. Quench reaction by adding 4.5 μ l 4M Hydroxylamine
52. Incubate at 23 °C for 15 minutes in heating block
53. Combine Cy3 and Cy5 sample pairs

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

54. Resuspend the resin in the G-50 column by vortexing gently.
55. Loosen the cap a quarter turn and snap off the bottom closure.
56. Place the column in a 1.5 ml tube.
57. Pre-spin column at 5,000 rpm for 1 minute to remove the buffer. Blot the tip of the column dry using a clean paper towel.
58. Remove the top cap and place column in a new 1.5 ml tube. Pipette half of the sample onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
59. Spin for 1 minute at 5,000 rpm. The unincorporated dye and nucleotides should be retained by the column and the purified labelled probe should pass through into the support tube. Discard the column.
60. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
61. Reduce volume of probe to between 2 to 5 μ l by placing in a speed vac with medium heat
62. Add 2 μ l of sonicated salmon sperm DNA

The samples have now been amplified and combined together for hybridisation to a microarray with the blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (18-05-2006).

Anti-sense strand amplification of RNA and indirect labelling of amino-allyl RNA for oligo microarrays (2 rounds)

Outline

Amplification can be used to produce sufficient RNA for labelling when the amount of biological material is limiting. The method we use is based on the Eberwine method. The samples and controls to be compared are each then labelled with a different fluorescent dye and subjected to paired competitive hybridisations.

Equipment and reagents

- (dT)-T7 primer:
GCATTAGCGGCCGCGAAATTAATACGACTCACTATAGGGAGA-(T)_{n=21}-[A or G or C]
- T4 Gene 32 protein (GE Healthcare Bio-Sciences AB; Cat. No. E70029Z)
- Second strand buffer (Invitrogen; Cat. No. 10812-014)
- DNA Polymerase I (Invitrogen; Cat. No. 1801-025)
- RNaseH (New England Biolabs; Cat. No. M0297S)
- *E. coli* DNA ligase (GE Healthcare Bio-Sciences AB; Cat. No. E70020Z)
- T4 DNA polymerase (Roche Diagnostics; Cat. No. 1004786)
- Microspin columns with Bio-Gel P6 in Tris (Bio-Rad; Cat. No. 732-6221)
- Phenol:Chloroform:Isoamylalcohol (Sigma; Cat. No. P2069)
- Sodium Chloride (VWR; Cat. No. 102414P)
- Gene Elute LPA (Sigma; Cat. No. 56575)
- 100% Ethanol
- MEGA-Script T7 kit (Ambion; Cat. No. 1334)
- 70% Ethanol
- RQ1 RNase-free DNase (Promega; Cat. No. M6101)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- DEPC - Diethyl pyrocarbonate (Sigma; Cat. No. D 5758)
- DEPC-treated MilliQ water
- RNAsin (Promega; Cat. No. 18064-014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB; Cat. No. 27-5340-01)
- Phase Lock Gel (Helena Bioscience; Cat. No. 0032 007.953)
- Aminoallyl-UTP-sodium salt (Sigma; Cat. No. A-5660)
- RNeasy Mini Kit (Qiagen; Cat. No. 74104)
- Cy Dye Post-Labelling Reactive Dye Pack (GE Healthcare Bio-Sciences AB; Cat. No. RPN 5661)
- Sodium Bicarbonate (Sigma; Cat. No. S7277)
- Hydroxylamine hydrochloride (Sigma; Cat. No. H9876)
- Ammonium Acetate (VWR; Cat. No. 1.01116.0500)
- Random Primer (Promega; Cat.No. C1181)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

Making the dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
 - ◆ 100 µl 100mM dNTA
 - ◆ 100 µl 100mM dNTT
 - ◆ 100 µl 100mM dNTG
 - ◆ 100 µl 100mM dNTC
2. Then make up to 1 ml using DEPC-water
3. Aliquot the dNTP mix and then store at -20 °C

Making the amino-allyl UTP stock:

1. For 75mM amino-allyl UTP stock, resuspend 1mg Aminoallyl-UTP-sodium salt in 24.7 µl DEPC-water

RNA amplification - first round

Reverse Transcription reaction:

The following steps are performed in 200 µl PCR tubes and the PCR block

1. Take up to 4.5 µl of the extracted RNA (small scale RNA extraction protocol), add 0.5 µl of Primer T7-dT (200 ng/µl) and then make up to a total volume of 5 µl with DEPC- water
2. Incubate at 65 °C for 10 min
3. Then snap freeze on ice
4. Make up a premix for the RT reaction:
 - ◆ 2 µl Superscript Buffer (from superscript kit)
 - ◆ 1 µl 0.1M DTT (from superscript kit)
 - ◆ 1 µl 10 mM dNTP mix
 - ◆ 0.8 µl 5 µg/µl T4 Gene 32 protein
 - ◆ 0.5 µl 40 U/µl RNasin
 - ◆ 1 µl 200 U/µl Superscript III
5. Add 6.3 µl to each sample
6. Incubate at 46 °C for 2 hours
7. Incubate at 65 °C for 15 minutes
8. Then snap freeze on ice

Second strand synthesis:

The following steps are performed in 200 µl PCR tubes and the PCR block

9. Make up a premix for the second strand synthesis:
 - ◆ For 1 reaction:
 - ◆ 45 µl DEPC-water
 - ◆ 15 µl Second strand buffer
 - ◆ 1.5 µl 10 mM dNTP mix
 - ◆ 4 µl 10 U/µl DNA Polymerase I
 - ◆ 0.2 µl 5 U/µl RNaseH
 - ◆ 1 µl 10 U/µl *E. coli* Ligase
10. Add 66.7 µl to each sample
11. Incubate at 16 °C for 2 hours
12. Add 2.0 µl 1 U/µl T4 DNA polymerase
13. Incubate at 15 °C for 15 minutes, then 70 °C for 10 minutes

Purification of double stranded DNA:

The following steps are performed in 1.5 ml microfuge tubes

14. Make the sample up to 100 µl (add 20 µl DEPC-water)
15. Prepare Microspin columns with Bio-Gel P6 in Tris:
 - ◆ Invert columns sharply 2-3 times and remove any air bubbles
 - ◆ Snap off the bottom and remove the lid

- ◆ Let the column drain by gravity flow
- ◆ Shortly before use spin for 2 minutes at 2500 rpm (1000 x g)
- 16. Immediately before use pellet Phase Lock Gel (PLG) tube at 13,000 rpm for 30 seconds
- 17. Add the cDNA and an equal volume (100 μ l) of (Phenol/Chloroform/Isoamylalcohol pH 8.0) to the PLG tube and shake for 15 seconds (do not vortex)
- 18. Centrifuge for 5 minutes at 13000 rpm
- 19. Transfer upper phase to the Microspin columns with Bio-Gel P6 in Tris columns and centrifuge for 4 minutes at 2500 rpm (1000 x g)
- 18. Precipitate the dsDNA:
 - ◆ 3.5 μ l 5M Sodium Chloride
 - ◆ 0.5 μ l LPA
 - ◆ 220 μ l 100% Ethanol
- 19. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
- 20. Centrifuge for 30 minutes at 13000 rpm
- 21. Remove supernatant and wash with 70% Ethanol, centrifuge for 2-3 minutes
- 22. Dry pellet and dissolve in 8 μ l DEPC-H₂O

In vitro transcription using Ambion Megascript T7 Kit:

The following steps are performed in 1.5 ml microfuge tubes and in a 37°C Incubator

- 23. Make a premix (per sample) using the Ambion Megascript T7 Kit
 - ◆ 2 μ l Txn buffer
 - ◆ 2 μ l 75 mM ATP
 - ◆ 2 μ l 75 mM CTP
 - ◆ 2 μ l 75 mM GTP
 - ◆ 2 μ l 75 mM UTP
 - ◆ 2 μ l Enzyme Mix
- 24. Add 12 μ l of the premix to each sample
- 25. Incubate at 37 °C for 9-16 hours
- 26. Add 1 μ l 1 U/ μ l RQ1 RNase-free DNase
- 27. Incubate 1 hour at 37 °C

Purification of the amplified RNA using Qiagen RNeasy Mini Kit RNA cleanup protocol followed by precipitation:

The following steps are performed in 1.5 ml microfuge tubes

- 28. To the Ambion Megascript T7 processed material add:
 - ◆ 80 μ l DEPC-water
 - ◆ 350 μ l RLT buffer (add 10 μ l beta-mercaptoethanol to 1ml RLT buffer)
- 29. Mix thoroughly
- 30. Add 250 μ l ethanol (96-100%) and mix by pipetting
- 31. Apply sample (700 μ l) to an RNeasy mini column placed in a 2ml collection tube
- 32. Centrifuge at 13,000 rpm 15 seconds
- 33. Transfer RNeasy column into a new 2 ml tube and add 500 μ l Buffer RPE onto the column
- 34. Centrifuge at 13,000 rpm 15 seconds. Discard the flow-through
- 35. Add another 500 μ l Buffer RPE to the column
- 36. Centrifuge at 13,000 rpm for 2 minutes
- 37. Transfer RNeasy column into a new 2 ml tube and entrifuge at 13,000 rpm for 1 minute to dry the membrane
- 38. Transfer RNeasy column into a new 1.5 ml tube
- 39. Pipette 50 μ l DEPC-water directly onto membrane, incubate for 1 minute
- 40. Centrifuge at 13000 rpm for 1 minute to elute
- 41. Repeat step 39 and 40
- 42. Take 2.0 μ l aliquot and analyse on a 1% agarose gel (ethidium bromide)
- 43. Precipitate with:
 - ◆ 0.5 μ l LPA (25 μ g/ μ l)
 - ◆ 50 μ l 7.5M Ammonium acetate
 - ◆ 250 μ l 100% Ethanol
- 44. Mix and centrifuge immediately at 13,000 rpm for 30 minutes

45. Wash pellet with 70% Ethanol
46. Dry and resuspend in 5.0 μ l DEPC-water
47. Take 0.5 μ l aliquot and measure concentration on NanoDrop

RNA amplification - second round

Reverse Transcription reaction:

The following steps are performed in 200 μ l PCR tubes and the PCR block

48. Take up to 1 μ g of the amplified RNA from the first round, add 0.5 μ l of 0.5 μ g/ μ l Random Primer and then make up to a total volume of 5 μ l with DEPC- water
49. Incubate at 65 $^{\circ}$ C for 10 min
50. Then snap freeze on ice
51. Make up a premix for the RT reaction:
 - ◆ 2 μ l Superscript Buffer (from superscript kit)
 - ◆ 1 μ l 0.1M DTT (from superscript kit)
 - ◆ 1 μ l 10 mM dNTP mix
 - ◆ 0.8 μ l 5 μ g/ μ l T4 Gene 32 protein
 - ◆ 0.5 μ l 40 U/ μ l RNasin
 - ◆ 1 μ l 200 U/ μ l Superscript III
52. Add 6.3 μ l to each sample
53. Incubate at 46 $^{\circ}$ C for 1 hour
54. Incubate at 65 $^{\circ}$ C for 15 minutes
55. Hold temperature at 37 $^{\circ}$ C and add 0.2 μ l 5 U/ μ l RNase H
56. Incubate at 37 $^{\circ}$ C for 30 minutes
57. Incubate at 95 $^{\circ}$ C for 2 minutes
58. Then snap freeze on ice
59. Add 0.5 μ l Primer T7-dT (200 ng/ μ l)
60. Incubate at 42 $^{\circ}$ C for 10 minutes

Second strand synthesis:

The following steps are performed in 200 μ l PCR tubes and the PCR block

61. Make up a premix for the second strand synthesis:
 - ◆ For 1 reaction:
 - ◆ 45 μ l DEPC-water
 - ◆ 15 μ l Second strand buffer
 - ◆ 1.5 μ l 10 mM dNTP mix
 - ◆ 4 μ l 10 U/ μ l DNA Polymerase I
 - ◆ 0.2 μ l 5 U/ μ l RNaseH
62. Add 65.7 μ l to each sample
63. Incubate at 16 $^{\circ}$ C for 2 hours
64. Add 2.0 μ l 1 U/ μ l T4 DNA polymerase
65. Incubate at 15 $^{\circ}$ C for 15 minutes, then 70 $^{\circ}$ C for 10 minutes

Purification of ds DNA:

The following steps are performed in 1.5 ml microfuge tubes

66. Make the sample up to 100 μ l (add 20 μ l DEPC-water)
67. Prepare Microspin columns with Bio-Gel P6 in Tris:
 - ◆ Invert columns sharply 2-3 times and remove any air bubbles
 - ◆ Snap off the bottom and remove the lid
 - ◆ Let the column drain by gravity flow
 - ◆ Shortly before use spin for 2 minutes at 2500 rpm (1000 x g)
68. Immediately before use pellet Phase Lock Gel (PLG) tube at 13,000 rpm for 30 seconds
69. Add the cDNA and an equal volume (100 μ l) of (Phenol/Chloroform/Isoamylalcohol pH 8.0) to the PLG tube and shake for 15 seconds (do not vortex)
70. Centrifuge for 5 minutes at 13000 rpm

71. Transfer upper phase to the Microspin columns with Bio-Gel P6 in Tris columns and centrifuge for 4 minutes at 2500 rpm (1000 x g)
72. Precipitate the dsDNA:
 - ◆ 3.5 µl 5M Sodium Chloride
 - ◆ 0.5 µl LPA
 - ◆ 220 µl 100% Ethanol
73. Incubate for 2 hours at -20 °C (or 15 minutes at -80 °C)
74. Centrifuge for 30 minutes at 13000 rpm
75. Remove supernatant and wash with 70% Ethanol, centrifuge for 2-3 minutes
76. Dry pellet and dissolve in 8 µl DEPC-H₂O

In vitro transcription using Ambion Megascript T7 Kit:

The following steps are performed in 1.5 ml microfuge tubes and in a 37°C Incubator

77. Make a premix (per sample) using the Ambion Megascript T7 Kit
 - ◆ 2 µl Txn buffer
 - ◆ 2 µl 75 mM ATP
 - ◆ 2 µl 75 mM CTP
 - ◆ 2 µl 75 mM GTP
 - ◆ 1.5 µl 75 mM UTP
 - ◆ 0.5 µl 75 mM amino-allyl UTP
 - ◆ 2 µl Enzyme Mix
78. Add 12 µl of the premix to each sample
79. Incubate at 37 °C for 9-16 hours
80. Add 1 µl 1 U/µl RQ1 RNase-free DNase
81. Incubate 1 hour at 37 °C

Purification of the amplified RNA using Qiagen RNeasy Mini Kit RNA cleanup protocol followed by precipitation:

The following steps are performed in 1.5 ml microfuge tubes

82. To the Ambion Megascript T7 processed material add:
 - ◆ 80 µl DEPC-water
 - ◆ 350 µl RLT buffer (add 10 µl beta-mercaptoethanol to 1ml RLT buffer)
83. Mix thoroughly
84. Add 250 µl ethanol (96-100%) and mix by pipetting
85. Apply sample (700 µl) to an RNeasy mini column placed in a 2ml collection tube
86. Centrifuge at 13,000 rpm 15 seconds
87. Transfer RNeasy column into a new 2 ml tube and add 500 µl Buffer RPE onto the column
88. Centrifuge at 13,000 rpm 15 seconds. Discard the flow-through
89. Add another 500 ul Buffer RPE to the column
90. Centrifuge at 13,000 rpm for 2 minutes
91. Transfer RNeasy column into a new 2 ml tube and entrifuge at 13,000 rpm for 1 minute to dry the membrane
92. Transfer RNeasy column into a new 1.5 ml tube
93. Pipette 50 µl DEPC-water directly onto membrane, incubate for 1 minute
94. Centrifuge at 13000 rpm for 1 minute to elute
95. Repeat step 93 and 94
96. Take 2.0 µl aliquot and analyse on a 1% agarose gel (ethidium bromide)
97. Precipitate with:
 - ◆ 0.5 µl LPA (25 µg/µl)
 - ◆ 50 µl 7.5M Ammonium acetate
 - ◆ 250 µl 100% Ethanol
98. Mix and centrifuge immediately at 13,000 rpm for 30 minutes
99. Wash pellet with 70% Ethanol
100. Dry and resuspend in 5.0 µl DEPC-water
101. Take 0.5 µl aliquot and measure concentration on NanoDrop

Dye coupling:

The following steps are performed in 1.5 ml microfuge tubes and the heating block

102. Resuspend 1 aliquot of monofunctional dye in 4.5 μ l 0.1M sodium bicarbonate (pH 8.5)
103. Mix with 4.5 μ l amplified amino-allyl RNA
104. Incubate at 23 °C for 1 hour in heating block
105. Quench reaction by adding 4.5 μ l 4M Hydroxylamine
106. Incubate at 23 °C for 15 minutes in heating block
107. Combine Cy3 and Cy5 sample pairs

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

108. Resuspend the resin in the G-50 column by vortexing gently.
109. Loosen the cap a quarter turn and snap off the bottom closure.
110. Place the column in a 1.5 ml tube.
111. Pre-spin column at 5,000 rpm for 1 minute to remove the buffer. Blot the tip of the column dry using a clean paper towel.
112. Remove the top cap and place column in a new 1.5 ml tube. Pipette half of the sample onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
113. Spin for 1 minute at 5,000 rpm. The unincorporated dye and nucleotides should be retained by the column and the purified labelled probe should pass through into the support tube. Discard the column.
114. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
115. Reduce volume of probe to between 2 to 5 μ l by placing in a speed vac with medium heat
116. Add 2 μ l of sonicated salmon sperm DNA

The samples have now been amplified and combined together for hybridisation to a microarray with the blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

R. Auburn (12-06-2006).

Measuring (nucleic acid concentration and) dye incorporation rates

Dye incorporation rates vary according to the dye being used (*e.g.*, Cy3 vs. Cy5) and the protocol employed (*e.g.*, direct vs. indirect). We can measure dye incorporation rates with the Nanodrop ND-1000 spectrophotometer (<http://www.nanodrop.com>). We do not routinely make such measurements because this would leave insufficient material for a hybridisation.

Protocol

1. Open the Nanodrop icon and select 'Microarray Measurement Mode'.
2. Add 2 μ l of solvent the labelled-sample has been dissolved in ("the solvent"); the instrument will then initialise.
3. After each and all subsequent measurements, clean the pedestal by wiping with a dry lint-free tissue.
4. Add 2 μ l of solvent and press 'Blank'
5. Repeat the blanking until there is a stable baseline, close to zero
6. Confirm that the baseline is correct by measuring 2 μ l of solvent, as if it were your first sample by pressing 'Measure'
7. Add 2 μ l of the first sample making sure to add the sample ID (or name) to the 'Sample ID' field and then press 'Measure'
8. Repeat step 3 and then 7 for all samples
9. Confirm that the baseline is correct after taking all measurements by measuring 2 μ l of solvent, as if it were your last sample by pressing 'Measure'
10. Each of the measurements is automatically saved by the instrument

Detection limits

Lower and upper detection limits for dye incorporation measurements of labelled-hybridisation extracts. The upper limit assumes the labelled-hybridisation extract has now been diluted.

Sample type (dye)	Lower limit (pmol/ μ l)	Upper limit (pmol/ μ l)
Cy3, Cy3.5, Alexa_555, Alexa_660	0.20	100
Cy5, Cy5.5, Alexa_647	0.12	60
Alexa_488, Alexa_594	0.40	215
Alexa_546	0.30	145

R. Auburn (20-02-2006).

Hybridisation of labelled material to cDNA microarrays using a Genomic Solutions Hybridisation Station with Ambion Hybridisation Buffer #1

Overview

Samples or samples and controls have been labelled with Cy3-dCTP or Cy5-dCTP and then mixed together with a blocking agent. We now need to add the hybridisation buffer and hybridise the labelled biological material to the cDNA microarrays. This protocol was based on a method from Genomic Solutions (<http://www.genomicsolutions.com>).

Equipment and reagents

- SlideHyb Glass Array Hybridisation Buffer #1 (Ambion; Cat. No. AM8861)
- 1 x SSC + 0.03% SDS
- 0.23 x SSC
- 0.06 x SSC
- 20 x SSC, pH 7.0
- Grant QBT2 hot-block
- Kenair air duster
- Hettich Rotina 35 microtitre plate centrifuge
- Microscope slide box (Merck EuroLab; Cat. No. 406/0286/00)
- Slide staining rack (Philip Harris; Cat. No. B52651)
- Slide staining trough (Philip Harris; Cat. No. B52649)

Procedure

How to set up the hybridisation cassettes:

1. Ensure slide covers are clean. If necessary, dab gently with a tissue soaked in 70% EtOH. Remove any dust by spraying with canned air and make sure the channels are free of liquid. Check that the red O-rings are in position.
2. Ensure that the metal plate is lying flat. If not, loosen screws with thumbnail and re-tighten with the plate in its proper position.
3. Fit black O-rings to slide cover. Do not stretch and avoid dust.
4. Spray slides with canned air and place slides on the black slide holder. Ensure slides are in the correct orientation.
5. Whilst holding the slide holder pointing slightly downwards and to the right, ensure that the slides are correctly positioned
6. Place the slide cover on top of the holder, without dislodging the slides.
7. Hold this 'slide cassette' together and place on the hybridisation station
8. Lower the clamp mechanism into position and screw until finger tight.

Starting the hybridisation:

9. Switch the hybridisation station on and when it has finished booting up insert the floppy disc with the hybridisation protocol.
10. Ensure that the wash solutions are full and the waste is empty and then connect each to the corresponding wash bottle
11. Press Start a run on the LCD field and select 'Floppy disk'. Scroll down to and then load the cDNA hybridisation protocol.
12. Select the slide positions for the run and press continue
13. The protocol will start automatically and prompt you to add the samples after 15 minutes
14. In the mean time prepare the samples that need to be loaded:
 - ◆ Preheat the SlideHyb Glass Array Hybridisation Buffer #1 to 65 °C for 15 to 30 minutes
 - ◆ Heat the labelled sample at 100 °C for 2 minutes on a hot-block
 - ◆ Add 140 µl SlideHyb Buffer to the sample
 - ◆ Centrifuge for 1 minute at 13,000 rpm
 - ◆ Add 135 µl of the labelled sample to the microarray, avoiding any precipitate

15. The instrument will tell you which sample it wants. To add it select the slide position and press probe - a tick should appear and the valve opens.
16. Add sample by pipetting slowly so as to avoid any bubbles. Put a plastic plug in the hole and press finish for this slide. The valve will then close.
17. Repeat procedure with second slide of the block, when finished press finished at the bottom of screen. The hybridisation station will now perform the hybridisation and washes. This will take 16 hours plus 20 minutes per block for the washes.

Hybridisation protocol

Step	Solution	Temperature	Duration	Agitation	Flow	Hold	Cycles
Hybridisation	Sample in SlideHyb	65 °C	16 hours	Yes	-	-	1

After the hybridisation:

18. Unscrew the clamp mechanism and remove the white plugs. Hold the cassette tightly together and then remove it from the hybridisation station.
19. Invert the sandwich and remove the slides by holding them by one edge and lifting away from the slide cover.
20. Now perform a manual wash as described below.

Manual wash:

21. Pre-heat wash solution 1 (0.2 x SSC; 0.2% SDS) and wash solution 2 (0.2 x SSC) to 55 °C using a water bath
22. Gently remove the slides submerged in pre-heated wash solution 1
23. Transfer the slides in a slide staining rack without letting the slides dry
24. Place the rack into a slide staining trough containing pre-heated wash solution 1
25. Mix on an orbital shaker at 50 rpm for 20 minutes at room temperature
26. Transfer the slides to a staining trough containing pre-heated wash solution 2
27. Gently dip the slides up and down for 3 seconds in wash solution 2
28. Transfer the slides to a second staining trough containing fresh pre-heated wash solution 2
29. Gently dip the slides up and down for 3 seconds in wash solution 2
30. Rinse the slides 3 times with fresh deionized water at room temperature (dip the rack in MilliQ water for 3 seconds)
31. Transfer the slides to a clean microscope slide box with tissue at the base and centrifuge at 1000 rpm for 5 minutes
32. The slides are now ready to be scanned.

Cleaning the hybridisation station:

33. Replace the hybridised slides with some blank slides and put the cassettes back on the hybridisation station
34. Place all of the in-flow tubes in 55 °C MilliQ water (helps to remove salt deposits)
35. Go to the main menu and select 'maintenance', followed by 'machine cleaning cycle'
36. Select the appropriate slide positions and start the cleaning cycle
37. Once the cleaning cycle has finished, remove the O-rings and place them in 200 ml boiling MilliQ water
38. Place the cassettes (not the metallic back plates) in a plastic beaker with warm MilliQ water, soak for a couple of minutes
39. Spray the cassettes with 70% ethanol and then leave them to dry at room temperature

B. Fischer (09-07-2008).

Hybridisation of labelled material to amino-modified long oligonucleotide microarrays using a Genomic Solutions Hybridisation Station with the Ocimum hybridisation buffer

Overview

Samples or samples and controls have been labelled with Cy3-dCTP or Cy5-dCTP and then mixed together with a blocking agent. We now need to add the hybridisation buffer and hybridise the labelled biological material to the amino-modified long oligonucleotide microarrays. This protocol was based on a method developed by FlyChip. The washes are based on a method recommended by Full Moon Biosystems (<http://www.fullmoonbio.com/>).

Equipment and reagents

- 10% SDS (Sigma; Cat. No. L4522)
- Ocimum hybridisation buffer (Biosolutions; Cat. No. 1180-200000)
- 20 x SSC, pH 7.0
- Grant QBT2 hot-block
- Kenair air duster
- Slide staining rack (Philip Harris; Cat. No. B52651)
- Slide staining trough (Philip Harris; Cat. No. B52649)
- Hettich Rotina 35 microtitre plate centrifuge
- Microscope slide box (Merck EuroLab; Cat. No. 406/0286/00)

Procedure

How to set up the hybridisation cassettes:

1. Ensure slide covers are clean. If necessary, dab gently with a tissue soaked in 70% EtOH. Remove any dust by spraying with canned air and make sure the channels are free of liquid. Check that the red O-rings are in position.
2. Ensure that the metal plate is lying flat. If not, loosen screws with thumbnail and re-tighten with the plate in its proper position.
3. Fit black O-rings to slide cover. Do not stretch and avoid dust.
4. Spray slides with canned air and place slides on the black slide holder. Ensure slides are in the correct orientation.
5. Whilst holding the slide holder pointing slightly downwards and to the right, ensure that the slides are correctly positioned
6. Place the slide cover on top of the holder, without dislodging the slides.
7. Hold this 'slide cassette' together and place on the hybridisation station
8. Lower the clamp mechanism into position and screw until finger tight.

Starting the hybridisation:

9. Switch the hybridisation station on and when it has finished booting up insert the floppy disc with the hybridisation protocol.
10. Press Start a run on the LCD field and select 'Floppy disk'. Scroll down to and then load the oligo hybridisation protocol.
11. Select the slide positions for the run and press continue
12. The protocol will start automatically and prompt you to add the samples after 15 minutes
13. In the mean time prepare the samples that need to be loaded:
 - ◆ Add 140 µl of Ocimum hybridisation buffer to the labelled mixture
 - ◆ Heat at 100 °C for 2 minutes on a hot-block
 - ◆ Centrifuge for 3 minutes at 13,000 rpm
 - ◆ Add 135 µl of the labelled sample to the microarray, avoiding any precipitate
14. The instrument will tell you which sample it wants. To add it select the slide position and press probe - a tick should appear and the valve opens.
15. Add sample by pipetting slowly so as to avoid any bubbles. Put a plastic plug in the hole and press finish for this slide. The valve will then close.

16. Repeat procedure with second slide of the block, when finished press finished at the bottom of screen. The hybridisation station will now perform the hybridisation, which will take 16 hours.

Hybridisation protocol

Microarray	Step	Solution	Temperature	Duration	Agitation	Flow	Hold	Cycles
<u>FL001</u>	Hybridisation	Sample in Hybridisation buffer	50 °C	16 hours	Yes	-	-	1
<u>FL002</u>	Hybridisation	Sample in Hybridisation buffer	51 °C	16 hours	Yes	-	-	1
<u>FL003</u>	Hybridisation	Sample in Hybridisation buffer	51 °C	16 hours	Yes	-	-	1

After the hybridisation:

17. Unscrew the clamp mechanism and remove the white plugs. Hold the cassette tightly together and then remove it from the hybridisation station.
18. Invert the sandwich and remove the slides by holding them by one edge and lifting away from the slide cover
19. Now perform a manual wash as described below.

Manual wash:

20. Pre-heat wash solution 1 (0.2 x SSC; 0.2% SDS) and wash solution 2 (0.2 x SSC) to 55 °C using a water bath
21. Place the slides in a slide staining rack without letting the slides dry
22. Place the rack into a slide staining trough containing pre-heated wash solution 1
23. Mix on an orbital shaker at 50 rpm for 20 minutes at room temperature
24. Transfer the slides to a staining trough containing pre-heated wash solution 2
25. Gently dip the slides up and down for 1 minute in wash solution 2
26. Repeat steps 24 to 25 twice with fresh wash solution 2
27. Rinse the slides 3 times with fresh deionized water at room temperature (dip the rack in MilliQ water for 3 seconds)
28. Transfer the slides to a clean microscope slide box with tissue at the base and centrifuge at 1000 rpm for 5 minutes
29. The slides are now ready to be scanned.

Cleaning the hybridisation station:

Failure to clean the hybridisation station cassettes correctly can result in high (apparently random) background signals.

30. Clean the cassettes by running under a hot water tap whilst rubbing the cassettes hybridisation surface with a clean paper tissue
31. Rinse the cassettes by running reverse osmosis water
32. Replace the hybridised slides with some blank slides and put the cassettes back on the hybridisation station
33. Place all of the in-flow tubes in 55 °C MilliQ water (helps to remove salt deposits)
34. Got to the main menu and select 'maintenance', followed by 'machine cleaning cycle'
35. Select the appropriate slide positions and start the cleaning cycle
36. Once the cleaning cycle has finished, remove the O-rings and place them in 200 ml boiling MilliQ water
37. Place the cassettes (not the metallic back plates) in a plastic beaker with MilliQ water, soak for a

couple of minutes

38. Spray the cassettes with 70% ethanol and then leave them to dry at room temperature

R. Auburn (16-07-2009).

Operating instructions for the Axon GenePix scanner

Preparation for scanning:

1. After power-on, give the scanner 15 minutes to warm up before acquiring any images
2. Load the slides upside down in the scanner
3. Start GenePixPro by clicking on the 'GenePixPro' icon

Preview scanning and defining the scan area:

4. Press 'Preview Scan' to start a preview scan
5. You can then press the 'Scan Area' button:
 - ◆ Move the mouse cursor to the top left of the features on the image
 - ◆ Hold down the mouse cursor and drag a rectangle around the region containing the features
6. Confirm that all features have been included within the scan area

Optimising the scanner settings:

Maximizing the dynamic range:

7. Open the 'Hardware Settings' dialog box
8. Start a 'Preview Scan' and change the 'PMT Gain' for each wavelength whilst scanning
9. You should aim to use the highest scan setting that does not lead to a significant level of saturation
10. Ensure that the image resolution has been set to 5 μm per pixel

Balancing the PMTs:

11. Start a 'Preview Scan' and 'Zoom' in on the features
12. Switch to the 'Histogram' tab and set the 'Min Intensity' and 'Max Intensity' fields to 500 and 65530
13. While scanning, adjust the PMT Gain in each channel until the ratio 'Count Ratio' is about 1.0
14. Save the 'Hardware Settings' by clicking the 'File' button and selecting 'Save Settings As'
15. Type in an appropriate file name and then press 'Save'

Performing a Data Scan and Saving an Image:

15. Press 'Data Scan' to start a full scan
16. To save the image, click on 'File' and select 'Save Images'
17. Navigate to the correct file location and select 'Single Image TIFF Files'
18. Type in an appropriate file name and then press 'Save'

Calibrate System (doesn't needed to be performed very often!):

19. Click 'Hardware Diagnostic' and select 'Calibrate System'
20. Check both the laser check boxes to calibrate both wavelengths
21. Click 'Start' and the wizard will then guide you through the procedure

R. Auburn (07-06-2004).

Chromatin immunopurification (ChIP) and DNA adenine methyltransferase identification (DamID) microarray:

ChIP

- Preparation of fixed chromatin from *Drosophila* embryos ([jump](#))
- Quality control of the fixed chromatin from *Drosophila* embryos ([jump](#))
- Chromatin immunoprecipitation (ChIP) from *Drosophila* embryos ([jump](#))
- Quality control of the chromatin immunopurification (ChIP) from *Drosophila* embryos ([jump](#))
- Ligation-mediated PCR of ChIP immunopurified genomic DNA ([jump](#))
- Measuring (nucleic acid concentration and) dye incorporation rates ([jump](#))

DamID

Coming soon!

Nimblegen microarray hybridisation

- Nimblegen processing of ChIP or DamID samples on 2.1M microarrays ([jump](#))

Affymetrix microarray hybridisation

Coming soon!

Preparation of fixed chromatin from *Drosophila* embryos

Overview

The aim of the method is to isolate fixed chromatin from *Drosophila* embryos. Essentially, the chromatin is fixed, the nuclei are then purified and lysed before the chromatin is fragmented by sonication. Our embryo collections are typically performed over a 16 hour time span, 1-2 grams of embryos were collected for each chromatin prep. This protocol was developed by [Rob White's](#) laboratory.

Equipment and reagents

- Sigma 4K10 bench top centrifuge
- Standard microfuge tube bench top centrifuge
- Formaldehyde (40% solution) 'Analar grade' (BDH; Cat. No. 101134A)
- n-Heptane 'Analar grade' (BDH; Cat. No. 103636C)
- 50 ml Falcon tubes
- 13.5 ml screw-capped conical base tubes (Bibby Sterlin; Cat. No. 144AS)
- 212-300 micron glass beads (Sigma; Cat No. G-1277)
- Heat Systems Ultrasonic Liquid Processor XL sonicator
- Wheaton Dounce homogeniser
- Pepstatin (Calbiochem; Cat. No. 516482)
- Aprotinin saline solution (Sigma; Cat. No. A6279)
- Leupeptin (Sigma; Cat. No. L2884)
- AEBSF* (Sigma; Cat. No. A8456)
- Nonidet P40 substitute (Sigma; Cat. No. 74385)

*See solutions and reagents

Solutions and reagents

10 x PBS, pH 7.4:

- 80 g Sodium chloride
- 2 g Potassium chloride
- 14.4 g Disodium hydrogen orthophosphate
- 2.4 g Sodium dihydrogen orthophosphate

Make to a total volume of 1 litre.

Crosslinking solution:

- 50 mM HEPES, pH 8.0
- 1 mM EDTA.Na₂
- 0.5 mM EGTA
- 100 mM NaCl

PBS/Triton:

1 x PBS containing 0.01% Triton X-100
Add 0.5 ml of 1% Triton stock to 50 ml 1 x PBS
(+ or - protease inhibitors)

PBS/Glycine/Triton:

1 x PBS/Triton containing 125 mM glycine.

Cell Lysis Buffer:

- 5 mM PIPES, pH 8.0
- 85 mM Potassium chloride

- 0.5% Nonidet P40 (NP40)
- + protease inhibitors

Autoclave without NP40 and then add appropriate amount of NP40 from 10% stock

Nuclear Lysis Buffer:

- 50 mM Tris.HCl, pH 8.1
- 10 mM EDTA.Na₂
- 1% SDS
- + protease inhibitors

Autoclave without SDS and then add appropriate amount of SDS from 10% stock.

Protease inhibitors:

Protease inhibitor	Stock concentration	Working concentration	Amount required per ml	Comments
Aprotinin	2.2 mg/ml	3.3 µg/ml	1.5 µl	Dissolve in water, store at 4 °C.
Leupeptin	10 mg/ml	10 µg/ml	1 µl	Dissolve in water, store at 4 °C.
Pepstatin	2 mg/ml	4 µg/ml	2 µl	Dissolve in methanol, store at -20 °C. Heat to 48 °C to dissolve before storage.
AEBSF, hydrochloride*	100 mM	1 mM	10 µl	Dissolve 100 mg in 4.18 ml water, store at 4 °C. This is a non-toxic alternative to PMSF

*8727;4-(-2-Aminoethyl) benzenesulfonyl fluoride, HCl

Protocol

1. Incubate embryos at 37 °C for 15 minutes to heat shock the embryos (omit this step if non-heat shocked embryos are required).
2. Dechorionate embryos for 3 minutes in a solution of weak bleach (5% w/w available chlorine) at room temperature.
3. Wash well with tap water and place onto filter paper. Transfer to fresh filter paper to weigh.
4. Transfer 1-2 g of embryos to a 50 ml Falcon tube and add 50 ml PBS/Triton to wash the embryos. Centrifuge for 1 minute at 500 x g (1680 rpm) to pellet embryos.
5. Discard supernatant and add 10 ml cross-linking solution, 487 µl 40% formaldehyde and 30 ml n-heptane. Shake vigorously (by hand works well...but tiring) at room temperature for 15 minutes.
6. Centrifuge for 1 minute at 500 x g (1680 rpm) to pellet embryos and discard supernatant.
7. Resuspend in 30 ml PBS/Glycine/Triton, best to resuspend in 3 ml of buffer and then add the rest. Then allow the embryos to sediment.
8. Remove supernatant and add 50 ml ice-cold PBS/Triton and resuspend. Best to add a small volume of the buffer to resuspend the embryos. Allow the embryos to sediment.
9. Remove supernatant and resuspend in 15 ml ice-cold PBS/Triton + protease inhibitors, again resuspend in a small volume and then add remainder. Dounce with a Wheaton homogeniser pestle B.
10. Centrifuge at 400 x g (1500 rpm) for 1 minute and transfer supernatant to a fresh tube.
11. Centrifuge at 1100 x g (2495 rpm) for 10 minutes at 4 °C and discard supernatant. Resuspend in 15 ml ice-cold Cell Lysis Buffer with protease inhibitors, best to resuspend in a small volume and then add remainder. Dounce with a Wheaton homogeniser pestle A. Transfer 2 equal aliquots into 13.5 ml screw-capped conical base tubes.
12. Centrifuge at 2000 x g (3365 rpm) for 4 minutes at 4 °C to pellet the nuclei.
13. Resuspend in 1 ml of ice-cold Nuclear Lysis Buffer with protease inhibitors, incubate for 20 minutes.
14. Add 2 ml ice-cold Nuclear Lysis Buffer with protease inhibitors and 0.3 g acid washed 212-300 micron glass beads.
15. Sonicate on ice following the regime below to produce chromatin fragments with an average size of 500 bp using a sonicator fitted with a microtip. This step should be calibrated for individual

sonicators to generate chromatin of an appropriate size.

- ◆ 1 x 30 seconds on level 3

- ◆ 5 x 30 seconds on level 4

- ◆ Between each sonication rest on ice for 90 seconds

16. Transfer to microfuge tubes and centrifuge at 16,000 x g (13000 rpm) in a microfuge for 10 minutes at 4 °C.

17. Take the supernatant (fixed sheared chromatin) and transfer to cryotubes, e.g., 200 µl aliquots, and flash freeze in liquid nitrogen. Store at -80 °C.

Version 1.2. R. Auburn. (25-08-2006)

Quality control of the fixed chromatin from *Drosophila* embryos

Overview

Before progressing to the chromatin immunopurification, it is important to quality control the chromatin itself. This will prevent valuable resources being wasted on sub-standard material, e.g., the extraction failed or, the chromatin is of the wrong size. The procedures outlined below can be used to assay chromatin batches.

Protocol - reversal of chromatin-protein crosslinks

To assay the quality and quantity of genomic DNA that has been purified, it is necessary to reverse the crosslinking, remove the protein with a proteinase K treatment and RNA with RNase.

1. To 50 μ l chromatin, add 2 μ l RNase A and 150 μ l nuclear lysis buffer (50 mM Tris.HCL, pH 8.1; 10 mM EDTA.Na₂; 1% SDS). Incubate at 67 °C for 4-5 hours (or overnight), to reverse the crosslinking. Store at -20 °C overnight.
2. Thaw out the samples and add 4.2 μ l proteinase K (Roche Diagnostics: recombinant PCR grade, cat. no. 3115887, 50 U/ml) and 0.8 μ l 10% SDS. Incubate at 45 °C for 2 hours.
3. Purify using Qiagen columns (QIAquick PCR purification kit) and elute in 50 μ l elution buffer supplied with the columns or water. Store at -20 °C.

Protocol - gel electrophoresis

After reversal of the chromatin-protein crosslinks genomic DNA from *D. melanogaster* will be visible as a smear between 200 bp and 2 kb, most will be between 500 bp and 1 kb.

1. Make a 1% agarose gel and add 5 μ l ethidium bromide (10 mg / ml) per 100 ml of gel.
2. To 5 μ l sample, add 1 μ l 6 x loading buffer. Load.
3. Run the gel at 80 V until the fastest dye has moved 2/3 of the gel length.
4. Visualise the gel using a UV transilluminator and photograph.

Protocol - optical density measurements

Optical density measurements are made using a Nanodrop ND-1000 spectrophotometer (<http://www.nanodrop.com>) that has been calibrated using dilutions of genomic DNA. This calibration showed that the linear range is 10 to 1000 ng/ μ l. The calibration curve is used to interpret the measurement using an in-house script.

Good quality DNA will have an OD 260/280 ratio of 1.8 to 2 and an OD 260/230 of 1.8 or greater. This is because nucleic acid is detected at 260 nm, whereas protein, salt and solvents are detected at 230 and 280 nm.

1. Open the Nanodrop icon and select 'Nucleic Acid Measurements'.
2. Add 2 μ l of solvent the sample has been dissolved in ("solvent"); the instrument will then initialise.
3. After each and all subsequent measurements clean the pedestal by wiping with a dry lint-free tissue.
4. Add 2 μ l of solvent and press 'Blank'.
5. Repeat the blanking until there is a stable baseline close to zero.
6. Confirm that the baseline is correct by measuring 2 μ l of the solvent, as if it were your first sample by pressing 'Measure'.
7. Add 2 μ l of the first sample making sure to add the sample ID (or name) to the 'Sample ID' field and then press 'Measure'.
8. Repeat step 3 and then 7 for all samples.
9. Confirm that the baseline is correct after taking all measurements by measuring 2 μ l of the solvent, as if it were your last sample by pressing 'Measure'.
10. Each of the measurements is automatically saved by the instrument and these can then be calibrated using the in-house script.

Chromatin immunoprecipitation (ChIP) protocol from *Drosophila* embryos

Overview

Target DNA binding protein that has been cross-linked to chromatin (which has been sheared) will be purified using antibodies raised against the specific target protein. *Staphylococcus aureus* cells (SAC) will be used to purify antibody/chromatin complex via protein A expressed on the bacterial cell surface. Protein A coupled to a support could be used but SAC is cheaper and it has a higher binding capacity than the equivalent amount of protein A beads that are commercially available.

Fixed chromatin solution will be precleared with SAC to remove non-specific binding of components before addition of antibody to the chromatin solution. After sufficient time for antibody/chromatin interaction this complex is purified using a fresh batch of SAC. The antibody/chromatin/SAC complex is washed and the antibody/chromatin complex is eluted using a SDS/low salt buffer. RNAase is added to remove any RNA and the cross linking is reversed by heating. Following an ethanol precipitation, protein is removed using proteinase K treatment and phenol/chloroform extraction. After another ethanol precipitation, a solution containing enriched DNA fragments (which were bound by the target protein) is produced.

This protocol was developed by Rob White's laboratory. This is a modification of the Farnham laboratory mouse protocol (<http://genomecenter.ucdavis.edu/farnham/farnham/>).

Unless otherwise stated, all centrifugations are at full speed in a standard microfuge.

Equipment and reagents

- Sigma 4K10 bench top centrifuge
- 10ml lyophilised Zysorbin (fixed and killed *S.aureus* Protein A positive strain) (Invitrogen; Cat. No. 10-1051-1)
- Bovine Serum Albumin (Fraction V), 50 mg/ml stock in water and filter sterilised (Sigma; Cat No. A-7906)
- 50 U/ml Proteinase K, recombinant PCR grade (Roche Diagnostics; Cat. No. 3115887)
- Phenol/Chloroform/Isoamyl alcohol (25:24:1) (BDH; Cat. No. 436732A)
- Glycogen, molecular biology grade (Roche Diagnostics; Cat. No. 901393)
- Pepstatin (Calbiochem; Cat. No. 516482)
- Aprotinin saline solution (Sigma; Cat. No. A6279)
- Leupeptin (Sigma; Cat. No. L2884)
- AEBSF* (Sigma; Cat. No.A8456)
- Nonidet P40 substitute (Sigma; Cat. No. 74385)

*See solutions and reagents

Solutions and reagents

Dialysis buffer

Reagent stock concentration	Working concentration required	Amount required / 50 ml
500mM EDTA.Na ₂ , pH 8	2 mM	200 µl
1M Tris.HCl, pH 8	50 mM	2.5 ml
Sarkosyl*	0.2%	100 mg
Water		Make to 50 ml

This buffer should be made up the day before as the detergents take a while to dissolve. Heating at 37 °C helps.

*Omit for monoclonal antibodies.

10 x PBS; pH 7.4

- 80 g Sodium chloride
- 2 g Potassium chloride
- 14.4 g Disodium hydrogen orthophosphate
- 2.4 g Sodium dihydrogen orthophosphate

Make to a total volume of 1 litre

PBS/SDS/BME solution

Reagent stock concentration	Working concentration required	Amount required / 10 ml
10X PBS	1X	1 ml
10% SDS	3%	3 ml
beta-mercaptoethanol	10%	1 ml
Water		Make to 10 ml

Nuclear lysis buffer

- 50 mM Tris.HCl, pH 8.1
- 10 mM EDTA.Na₂
- 1% SDS
- + protease inhibitors

Autoclave without SDS and then add appropriate amount of SDS from 10% stock.

Protease inhibitors:

Protease inhibitor	Stock concentration	Working concentration	Amount required per ml	Comments
Aprotinin	2.2 mg/ml	3.3 µg/ml	1.5 µl	Dissolve in water, store at 4 °C.
Leupeptin	10 mg/ml	10 µg/ml	1 µl	Dissolve in water, store at 4 °C.
Pepstatin	2 mg/ml	4 µg/ml	2 µl	Dissolve in methanol, store at -20 °C. Heat to 48 °C to dissolve before storage.
AEBSF, hydrochloride*	100 mM	1 mM	10 µl	Dissolve 100 mg in 4.18 ml water, store at 4 °C. This is a non-toxic alternative to PMSF

*4-(-2-Aminoethyl) benzenesulfonyl fluoride, HCl

IP Dilution Buffer

Reagent stock concentration	Working concentration required	Amount required / 50ml
10% SDS	0.01%	50 µl
20% Triton X-100	1.1%	2.75 ml
500 mM EDTA.Na ₂ , pH 8	1.2 mM	120 µl
1M Tris.HCl, pH 8	16.7 mM	835 µl
4M NaCl	167 mM	2.09 ml
Water		Make to 50 ml

IP wash buffer

Reagent stock concentration	Working concentration required	Amount required / 50ml
1M Tris.HCl, pH 9*	100 mM	5 ml
1M LiCl	500 mM	25 ml
10% Nonidet P40 (NP40)	1%	5 ml
Deoxycholic acid	1%	0.5 g
Water		Make to 50 ml

This buffer should be made up the day before because the detergents take a while to dissolve. Heating at 37 °C helps.

*pH 8 for monoclonal antibodies

IP Elution buffer

Reagent stock concentration	Working concentration required	Amount required / 50ml
1M NaHCO ₃	50 mM	2.5 ml
10% SDS	1%	5 ml
Water		Make to 50 ml

TE buffer

Reagent stock concentration	Working concentration required	Amount required / 50ml
1M Tris.HCl, pH 8	10 mM	0.5 ml
500mM EDTA.Na ₂ , pH 8	1 mM	100 µl
Water		Make to 50 ml

5X PK Buffer

Reagent stock concentration	Working concentration required	Amount required / 50ml
1M Tris.HCl, pH 7.5	50 mM	2.5 ml
500mM EDTA.Na ₂ , pH 8	25 mM	2.5 ml
10% SDS	1.25%	6.25 ml
Water		Make to 50 ml

Protocol

Preparation of *Staphylococcus aureus* cells (SAC):

1. Resuspend Zymed Protein A reagent in 10 ml water.
2. Centrifuge at 5000 rpm (4400 x g) for 5 minutes at 4 °C in 15 ml Falcon tubes (or equivalent) in the Sigma 4K10 bench top centrifuge.
3. Discard supernatant and resuspend pellet in 10 ml dialysis buffer.
4. Repeat steps 2 and 3.

5. Repeat step 2, discard supernatant and resuspend in 3ml PBS/SDS/BME solution. Aliquot into 6 microfuge tubes and boil in water bath for 30 minutes.
6. Centrifuge for 5 minutes at room temperature.
7. Discard supernatant and resuspend in 1 ml Dialysis Buffer.
8. Repeat steps 6 and 7.
9. Discard supernatant and resuspend pellets in final volume of 4 ml Dialysis Buffer.
10. Store as 100 μ l aliquots at -20 $^{\circ}$ C.

Chromatin immunopurification procedure:

Day 1

11. Thaw out 100 μ l aliquot of SAC and add 20 μ l Bovine Serum Albumin to preblock SAC.
12. Incubate overnight at 4 $^{\circ}$ C on a roller (3 hours is fine).

Day 2

13. Centrifuge blocked SAC for 1 minute, discard supernatant and resuspend in 100 μ l of 1X dialysis buffer. Add 1ml of Dialysis Buffer and invert to mix.
14. Repeat step 13.
15. Centrifuge for 1 minute, discard supernatant and resuspend in 100 μ l of Dialysis Buffer. Store on ice until required.
16. To preclear chromatin, thaw out chromatin aliquot and add 15 μ l blocked SAC to each chromatin aliquot. Rotate on roller at 4 $^{\circ}$ C for 15 minutes.
17. Centrifuge for 1 minute and transfer supernatant to fresh tube.
18. Repeat step 17 and transfer supernatant to fresh tube.
19. To fresh tube add chromatin (for Heat Shock Factor experiments we used 20-25 μ l) to a total volume of 100 μ l (use nuclear lysis buffer to make the volume up). Add 200 μ l IP Dilution Buffer. Add serum (for Heat Shock Factor (HSF) we used 1 μ l of pre-immune or anti-HSF antiserum). Incubate overnight at 4 $^{\circ}$ C on roller.
20. Set up a fresh aliquot of SAC and block overnight, as per steps 11 to 12.

Day 3

21. For monoclonal antibodies only, add 1 μ g of an appropriate secondary antibody that binds to Protein A and incubate for an additional hour at 4 $^{\circ}$ C on roller. Only omit this step when working with a polyclonal antibody that binds to Protein A.
22. Process overnight blocked SAC, as per steps 13 to 15.
23. Add 10 μ l SAC (from step 22) to each IP reaction. Rotate for 15 minutes at room temperature.
24. Centrifuge for 1 minute, remove supernatant and discard. Pulse spin, then remove remaining residual fluid. Resuspend pellet in 200 μ l Dialysis Buffer using pipette tip. Add 1 ml Dialysis Buffer and invert to mix.
25. Incubate on roller at room temperature for 3 minutes.
26. Repeat steps 24-25 once.
27. Centrifuge for 1 minute, remove supernatant and discard. Pulse spin, then remove the remaining residual fluid. Resuspend pellet in 200 μ l IP Wash Buffer using pipette tip. Add 1 ml IP Wash Buffer and invert to mix.
28. Incubate on roller at room temperature for 3 minutes.
29. Repeat steps 27-28 three times.
30. Centrifuge for 1 minute, remove supernatant and discard.
31. Resuspend pellet in 150 μ l IP Elution Buffer and vortex at setting 3 for 15 minutes at room temperature.
32. Centrifuge for 1 minute and transfer supernatant to fresh tube.
33. Repeat step 31.
34. Centrifuge for 1 minute and pool supernatant with that of step 32. Centrifuge for 5 minutes and transfer to fresh tube.
35. Add 1 μ l RNase (stock 10 mg/ml, in water) and 22.5 μ l 4M NaCl (final concentration 0.3M). Incubate samples at 67 $^{\circ}$ C for 4-5 hours to reverse cross-linking.
36. Cool for 1-2 minutes on ice. Add 812 μ l ice-cold ethanol, mix by inversion and store at -20 $^{\circ}$ C overnight.

Day 4

37. Centrifuge at 4 °C for 20 minutes and remove supernatant.
38. Pulse spin and remove remaining residual fluid.
39. Air dry for 1 hour.
40. Resuspend pellet (quite large and white) in 100 µl TE.
41. Add 25 µl 5X PK buffer and 1.5 µl proteinase K. Incubate at 45 °C for 2 hours.
42. Add 175 µl TE to each sample. Add 300 µl phenol/chloroform/isoAmyl alcohol and vortex to mix.
43. Centrifuge for 1 minute and carefully remove upper aqueous layer to fresh tube.
44. Add 300 µl chloroform and centrifuge for 1 minute. Carefully remove upper aqueous layer to fresh tube.
45. Add 37.5 µl 4M NaCl, 10 µg (0.5 µl) glycogen, and 750µl ethanol (ice-cold). Store overnight at -20 °C.

Please note, instead of performing steps 42 to 48, it is possible to purify the genomic DNA using a QIAquick PCR purification kit (Qiagen; Cat. No. 28104), as per the manufacturers instructions. Elution is either performed with water, when the material is to be used for a microarray experiments, or else, the Qiagen supplied elution buffer can be used, e.g., when performing a quality control test. This modification to the method increases yield; hence reducing the background signal after hybridisation for both standard and quality control extractions.

Day 5

46. Centrifuge at 4 °C for 20 minutes and remove supernatant.
47. Pulse spin and remove remaining residual fluid.
48. Air dry pellet (small and white) for 1 hour and resuspend pellet in 30 µl water. Store at -20 °C.

Version 1.2. R. Auburn. (10-08-2006)

Quality control of the chromatin immunopurification from *Drosophila* embryos

Overview

Assay of ChIP for HSF by direct PCR-based detection of known heat shock factor binding fragments. This procedure is used to assess batch quality following chromatin immunopurification and before ligation-mediated PCR. (As the embryo chromatin preparation protocol activates HSF in the absence of heat-shock, this procedure can also be used to quality control non-heat-shocked chromatin preparations.)

Protocol

PCR primers

PCR amplicon	5' primer	3' primer
Heat shock element for heat shock protein 26	GCTGTTTCTTTTGCGCTCTT	TTGTTTGACTTGTAAGCAAAGGTT
3'-end of heat shock protein 26 (negative control)	CGCATCATTCAAATTCAGCAAGT	GGTGAACTATTTTCGGACACCAA

15 µl of PCR reaction was prepared:

- 3 µl immunopurified DNA
- 1 µl 100 pmol/µl primers
- 1.5 µl Buffer IV (ABgene)
- 1.2 µl 25 mM Mg²⁺
- 1.5 µl 2.5 mM dNTPs
- 1 µl 5U/µl ThermoStart Taq polymerase (ABgene; Cat. No. AB-0908a)
- 5.8 µl water

PCR cycle

1. 95 °C for 5 minutes
2. 95 °C for 1 minute
3. 57 °C for 1 minute
4. 72 °C for 1 minute
5. Repeat steps 2 to 4, 35 times
6. 72 °C for 10 minutes
7. 4 °C and hold

Assay products by agarose gel electrophoresis.

Version 1.2. R. Auburn. (08-09-2006)

Ligation-mediated PCR (LM-PCR)

Overview

Following ChIP, there is insufficient genomic DNA for labelling and hybridisation so we amplify with LM-PCR. We first repair the genomic DNA to make the amplicons blunt ended then ligate on the linkers. Afterwards, ligation-mediated PCR is performed using a single primer to ensure that the amplification is 'linear'. Random prime labelling is then performed using one of two commercial kits. Pre-immune and immune ChIP genomic DNA are then purified and mixed before hybridisation.

Warning:

The following procedures require pure DNA and the Qiagen column sometimes leaves a small white salt pellet in the microfuge tubes. Whenever a qiagen column is used, care must be taken to check whether a pellet is present. Since the pellets can rapidly dissolve, these must be removed immediately after centrifugation.

Protocol

Repairing the sheared genomic DNA after ChIP

Prepare the following reaction mix and incubate for 5 minutes at 37 °C

- 15 to 30 µl ChIP genomic DNA
- 1 µl T4 DNA polymerase (Promega; Cat. No. M4211)
- 5 µl 10X T4 polymerase buffer
- 2.5 µl 2 mM dNTP
- Make up to 50 µl with water

DNA is purified with a Qiagen MinElute PCR purification Kit (Cat. No.28004), as per the manufacturers protocol. Elute the repaired ChIP genomic DNA with a volume of water that is equal to the starting amount of ChIP genomic DN, *i.e.*, 15 to 30 µl. Remember to remove the white pellet, if present.

Linker preparation

Linker-1 (100 pmol/µl) 5'-AGA AGC TTG AAT TCG AGC AGT CAG-3'

Linker-2 (100 pmol/µl) 5'-CTG CTC GAA TTC AAG CTT CT-3'

Mix 2.5 µl of each linker with 45 µl water

- Incubate for 2 minutes at 94 °C
- Incubate for 5 minutes at 70 °C (To remove secondary structures)
- Incubate for 5 minutes at 55 °C (annealing)

Slowly cool down to room temperature to obtain 50 µl of 5 µM blunt linker. Add 200 µl water to get 1 µM linker and store at -20 °C.

Ligation of repaired ChIP genomic DNA to linker

15 µl of ligation reaction was prepared:

- 10.5 µl repaired ChIP genomic DNA
- 1 µl 1 µM linker
- 1 µl 5 U/µl T4 DNA ligase (Invitrogen; Cat. No. 15224-041)
- 2 µl 5X ligase buffer
- 0.5 µl Water

Mix and incubate overnight at 4 °C

Ligation-mediated PCR (LM-PCR)

Clean the overnight ligation reactions with the Qiagen MinElute PCR purification Kit (Cat. No.28004), as per the manufacturers protocol. Elute the ChIP genomic DNA with 17 µl water. Remember to remove the white pellet, if present. Keep the DNA on ice and prepare the following.

Reaction mix

- 15 µl Ligated DNA
- 10 µl 10X buffer 4 (no Mg²⁺) (ABgene)
- 8 µl 25 mM Mg²⁺
- 8 µl 2 mM dNTPs
- 1 µl Taq polymerase (ABgene; Cat. No. AB-0192)
- 1 µl 100 pmol/µl linker-2
- 57 µl water

PCR cycle

1. 55 °C for 2 minutes
2. 72 °C for 5 minutes
3. 94 °C for 5 minutes
4. 94 °C for 1 minute
5. 55 °C for 1 minute
6. 72 °C for 1 minute
7. Repeat cycles 4 to 6, 24 times
8. 72 °C for 5 minutes
9. 4 °C forever

DNA is purified with a Qiagen MinElute PCR purification Kit (Cat. No. 28004), as per the manufacturers protocol. Elute LM-PCR ChIP genomic DNA with 25 µl water. Remember to remove the white pellet, if present.

Random priming and dye labelling

Measure sample concentrations using the Nanodrop, as per the RNA quality control protocol for gene expression, i.e., follow this standard operating procedure but set the Nanodrop to assay DNA. We label 100 ng template using the BioPrime array CGH genomic labelling system (Invitrogen; Cat. No.18095-011).

BioPrime array CGH genomic labelling system

Make the following reaction mix and incubate for 5 minutes at 95 °C.

- 25 µl Purified PCR product (100 ng DNA, plus water to 25 µl)
- 20 µl 2.5X Random primer mix

Immediately cool for 5 minutes on ice. Then, add the following reagents, mix them together and incubate for 2-3 hours at 37 °C

- 1 µl Exo-Klenow Fragment
- 5 µl 10x dCTP or dUTP mix
- 3 µl Cy3 or Cy5

Stop reaction with 5 µl of stop buffer. 3 µl of the reaction is then checked by agarose gel electrophoresis. You should see a well defined smear between 200 to 600bp.

Probe clean-up

1. Add 45 µl TE, PH 8.0 to the labelling mixtures.
2. Add 400 µl Purification buffer A and vortex for 30 seconds.
3. Place the purification column in a 2 ml collection tube, load labelling mixture.
4. Centrifuge at 11,000 x g for 1 minute at room temperature and discard the flow-through.

5. Add 600 μ l of Purification buffer B, centrifuge at 11,000 x g for 1 minute at room temperature and discard the flow-through.
6. Add 200 μ l of Purification buffer B, centrifuge at 11,000 x g for 1 minute at room temperature and discard the flow-through.
7. Place the collection tube in a new sterile microfuge tube and add 50 μ l sterile water. Incubate at room temperature for 1 minute.
8. Centrifuge at 11,000 x g for 1 minute at room temperature. The flow-through contains your purified labelling mixture.
9. Measure the nucleic acid concentration and dye-incorporation with the Nanodrop ND-1000 spectrophotometer. Ensure samples are matched in concentration.
10. The two samples (e.g. pre-immune and immune) can now be combined together for hybridisation to a microarray. The combined samples are reduced in volume to between 2-10 μ l in a SpeedVac at medium heat and the blocking agent, sonicated salmon sperm DNA (2 μ l of 10 mg/ml), is added. This material should now be used immediately to prevent any decay. Refer to the appropriate hybridisation protocol.

Version 1.2. R. Auburn. (01-09-2006)

Measuring (nucleic acid concentration and) dye incorporation rates

Dye incorporation rates vary according to the dye being used (*e.g.*, Cy3 vs. Cy5) and the protocol employed (*e.g.*, direct vs. indirect). We can measure dye incorporation rates with the Nanodrop ND-1000 spectrophotometer (<http://www.nanodrop.com>).

Protocol

1. Open the Nanodrop icon and select 'Microarray Measurement Mode'.
2. Add 2 μ l of solvent the labelled-sample has been dissolved in ("solvent"); the instrument will then initialise.
3. After each and all subsequent measurements, clean the pedestal by wiping with a dry lint-free tissue.
4. Add 2 μ l of solvent and press 'Blank'.
5. Repeat the blanking until there is a stable baseline close to zero.
6. Confirm that the baseline is correct by measuring 2 μ l of the solvent, as if it were your first sample by pressing 'Measure'.
7. Add 2 μ l of the first sample making sure to add the sample ID (or name) to the 'Sample ID' field and then press 'Measure'.
8. Repeat step 3 and then 7 for all samples.
9. Confirm that the baseline is correct after taking all measurements by measuring 2 μ l of the solvent, as if it were your last sample by pressing 'Measure'.
10. Each of the measurements is automatically saved by the instrument.

Detection limits

Lower and upper detection limits for dye incorporation measurements of labelled-hybridisation extracts. The upper limit assumes the labelled-hybridisation extract has now been diluted.

Sample type (dye)	Lower limit (pmol/ μ l)	Upper limit (pmol/ μ l)
Cy3, Cy3.5, Alexa_555, Alexa_660	0.20	100
Cy5, Cy5.5, Alexa_647	0.12	60
Alexa_488, Alexa_594	0.40	215
Alexa_546	0.30	145

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Nimblegen Processing of ChIP or DamID DNA on 2.1M Arrays

Outline

Random primers are annealed to denatured DNA templates and extended by Klenow fragment while fluorescent dyes are incorporated. The labelled samples are then subjected to paired competitive hybridisations on Nimblegen 2.1M tiling arrays.

Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Bioprime DNA Labeling System (Invitrogen; Cat. No. 18094-011)
- Cy3 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 53021)
- Cy5 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 55021)
- Isopropanol (BDH; Cat. No. 102246L)
- NimbleGen Hybridization Kit, LS (Roche-Nimblegen; Cat. No. 05583934001)
- NimbleGen Wash Buffer Kit (Roche-Nimblegen; Cat. No. 05584507001)
- NimbleGen Hybridization System Accessory Kit (Roche-Nimblegen; Cat. No. 05327695001)
- NimbleGen Mixer HX1 (10) (Roche-Nimblegen; Cat. No. 05223741001)
- Sigma water (Sigma; Cat. No. W4502)
- Sodium Chloride (Sigma; Cat. No. S-3014)
- Hettich micro 20 centrifuge
- Hettich Rotina 35 microtitre plate centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)
- GenePix 4000B Microarray Scanner
- NimbleGen Hybridization System 4

Procedure

Making the 10 X low-C dNTP mix:

1. Make a large 10 X low-C dNTP mix for the labelling reaction (5 mM A-,G-,T-dNTPs and 3mM C-dNTP)
 - ◆ 25 µl 100mM dNTA
 - ◆ 25 µl 100mM dNTT
 - ◆ 25 µl 100mM dNTG
 - ◆ 15 µl 100mM dNTC
2. Then make up to 500 µl using MilliQ water
3. Aliquot the 10 X low-C dNTP mix and then store at -20 °C

Klenow labelling (Flychip protocol):

The following steps are performed in 200 µl PCR tubes and the PCR block. Perform 2 reactions per sample and control.

1. Take 1 µg DNA and make up to a total volume of 31.2 µl with Sigma water
2. Add 30 µl 2.5x Random Primer Reaction Buffer (supplied in the Bioprime Labelling System Kit)
3. Incubate at 100 °C for 5 minutes
4. Snap freeze on ice
5. Mix together the following to make a master mix:
 - ◆ 7.5 µl 10 X low-C dNTP mix
 - ◆ 4.5 µl Cy3 or Cy5 dCTP
 - ◆ 1.8 µl 40U/µl Klenow (supplied in the Bioprime Labelling System Kit)
6. Add 13.8 µl to each sample and mix by pipetting up and down
7. Incubate at 37 °C for 2 to 3 hours
8. Stop the reaction by adding 7.5 µl Stop Buffer (supplied in the Bioprime Labelling System Kit)
9. Combine the Cy3 and Cy5 pairs

Precipitation purification (as per Nimblegen protocol):

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides.

10. Combine the 2 same reactions into 1.5 ml microcentrifuge tubes
11. Add 17.25 μ l 5M NaCl-solution
12. Add 165 μ l Isopropanol
13. Vortex and incubate for 10 minutes at room temp (dark)
14. Centrifuge for 10 minutes at 13,000 rpm
15. Discard the supernatant and wash the DNA pellet with 500 μ l 80% ice-cold ethanol
16. Centrifuge for 2 minutes at 13,000 rpm and remove supernatant
17. Dry the DNA pellet for 5 minutes (dark)

Quantify samples (as per Nimblegen protocol):

18. Rehydrate DNA pellet in 50 μ l Sigma water and vortex
19. Briefly centrifuge and incubate for 5 minutes (dark), then vortex and centrifuge again
20. Measure concentration on Nanodrop - as Nucleic acid
21. Combine 34 μ g Test Sample with 34 μ g Reference Sample into a 1.5 ml tube. (Hybridisation can be performed with as little as 24 μ g.)
22. Dry contents in SpeedVac, protect from light
23. Store samples at -20 °C until ready for hybridisation (up to 1 month)

Hybridisation to 2.1M arrays (as per Nimblegen protocol):

The Alignment Oligo provided in the Nimblegen Hybridisation Kit are labeled with Cy dyes, which are sensitive to photobleaching and freeze-thawing. After thawing stock tubes for the first time, aliquot 1.2 μ l of the Alignment Oligo into PCR tubes and store at -20 °C (protect from light).

24. Set the Hybridisation System to 42 °C, allow at least 3 hours for the temperature to stabilise
25. Resuspend dried sample pair in 12.3 μ l of Sigma water and vortex
26. Prepare Hybridisation Master Mix:
 - ◆ 1.2 μ l Alignment Oligo
 - ◆ 11.8 μ l Hybridisation Component A
 - ◆ 29.5 μ l 2X Hybridisation Buffer
27. Add 31.7 μ l of Hybridisation Master Mix to each sample pair
28. Vortex and spin
29. Incubate at 95 °C for 5 minutes in hotblock
30. Incubate at 42 °C for at least 5 minutes or until ready to load sample
31. Vortex and spin before loading

Prepare Mixers (as per Nimblegen protocol):

32. Remove HX1 mixer from package
33. Open the Precision Mixer Alignment Tool (PMAT, supplied with the Hybridisation System)
34. Push back the plastic spring with a thumb; place the slide in the base of the PMAT so that the barcode is readable facing outward. Make sure the slide is positioned to the rightmost and closest to you; make sure the slide is lying flat against the PMAT. Gently use the AirDuster to remove any particles from the slide
35. Snap the mixer onto the two alignment pins on the lid of the PMAT, with the end of the mixer pointing towards the hinge and the adhesive gasket facing outward
36. Use forceps to remove the backing from the adhesive gasket of the mixer and close the lid of the PMAT
37. Lift the lid by grasping the long ends of the PMAT while applying pressure with a finger through the window in the lid to free the mixer-slide assembly from alignment pins
38. Remove the mixer-slide from the PMAT and place on clean smooth dark surface
39. Rub the Mixer Brayer (supplied in the Hybridisation System Accessory Kit) over the mixer to adhere the gasket and remove any bubbles, starting in the center and rub outwards. Gasket becomes clear when fully adhered to both surfaces
40. Place the mixer-slide in the slide bay of the Hybridisation System

Load and Hybridise Samples (as per Nimblegen protocol):

41. Using a Gilson P200 slowly load 41 μ l into the fill port until the sample starts to overflow from the vent port (avoid bubbles, keep pipette tip perpendicular, apply gentle pressure of the tip into the port to ensure a tight seal)
42. Dry sample overflow from the ports, e.g. using a cotton swap
43. Adhere a mixer port seal over both ports and press simultaneously to seal
44. Close the bay clamp
45. Turn on the Mixing Panel, set the mix mode to B, and press the mix button to start mixing. Check indicator light is green for each slide position
46. Hybridise for 20 hours at 42 °C

Wash Hybridised Arrays (as per Nimblegen protocol):

It is important to proceed through all the washing and drying steps without interruption. We only process two arrays at a time to minimise the time the arrays are stored until scanned.

47. Prewarm 10X Wash Buffer I at 42 °C for a couple of minutes as it can precipitate before use
48. Prepare 250 ml Wash I and for each array 25 ml of Washes I, II and III according to the following table:

Solution	Wash I (250 ml)	Wash I, II and III (25 ml)
RO-water	225 ml	22.5 ml
10X Wash Buffer I, II or III	25 ml	2.5 ml
1M DTT	25 μ l	2.5 μ l

49. Prewarm 250 ml of Wash I to 42 °C and pour warm solution into shallow dish, so that the Mixer Disassembly Tool (supplied in the Hybridisation System Accessory Kit) is completely covered
50. Insert Mixer Disassembly Tool into dish and load the mixer-slide assembly into the Tool
51. Carefully peel the mixer off the slide (keeping the Mixer Disassembly Tool flat)
52. Remove the slide from the Tool and agitate the slide for 10-15 seconds in the shallow dish
53. Transfer slide into a slide container that contains Wash I (room temperature)
54. Wash for 2 minutes, shaking the container at least 20 times every 10 seconds
55. Blot off excess buffer on tissue and transfer slide into a slide container that contains Wash II (room temperature)
56. Wash for 1 minute, shaking the container at least 20 times every 10 seconds
57. Blot off excess buffer on tissue and transfer slide into a slide container that contains Wash III (room temperature)
58. Wash for 15 seconds, shaking the container at least 20 times every 10 seconds
59. Transfer the slides to a clean microscope slide box with tissue at the base and centrifuge at 1000 rpm for 5 minutes
60. Proceed immediately to scanning

Two-Colour Array Scanning (as per Nimblegen protocol):

Keep arrays in the dark until you are ready to scan them.

61. Launch the GenePix software 10 minutes before scanning to allow lasers to warm
62. Place the slide in the slide carriage so that the array is facing down and the barcode end is closest to you
63. Open the Hardware Settings dialog box and select the following settings for scanning:
 - ◆ 532 PMT Gain = 650
 - ◆ 635 PMT Gain = 750
 - ◆ Power (%) = 100%
 - ◆ Pixel size (μ m) = 5
 - ◆ Lines to average = 1
 - ◆ Focus position (μ m) = 0
64. Press Preview Scan to start a preview scan of the entire slide

65. Then press the Scan Area button:
 - ◆ Move the mouse cursor to the top left of the features on the image
 - ◆ Hold down the mouse cursor and drag a rectangle around the region containing the features
 - ◆ Confirm that all features have been included within the scan area
66. Start a Scan of the array and zoom into a smaller region and adjust the 532 and 635 PMT gains until only few spots remain saturated (saturated spots are displayed white)
67. Zoom into as large a region as possible (exclude areas with high background) and select the Histogram tab:
 - ◆ Make sure that the Wavelength 532 and Wavelength 635 boxes are checked so both wavelength histograms are displayed
 - ◆ Make sure that the Log Axis box is checked
 - ◆ The red and green curves should be superimposed. If the ref curve is above the green, lower the red PMT setting or raise the green PMT setting on the Image tab.
 - ◆ The curve should end above $1e-5$ normalized counts at the 65,000 intensity level (saturation)
 - ◆ The histogram graphs only the region of the image viewable on-screen in the image tab
68. After the PMT settings are properly adjusted, stop the current scan (do not save image)
69. Restart the scan
70. Save the images for both channels (single image .tif files) as: NNNNNN_XXXXXX, where NNNNNN = barcode and XXXXXX = user defined text (sample name)

Proceed to NimbleScan data analysis as outlined in the Nimblegen Arrays User's Guide.

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