

Gene Expression

Generated on 03/11/2008

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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](#))
- Klenow labelling of double stranded DNA ([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

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 acaacggaagcaaattctattg	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]-agcatccaaaatccaaccaa	(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

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- 2 µl 25 pmol / µl 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 µl Ambion nuclease-free water
 - ◆ 2 µl dATP
 - ◆ 2 µl dUTP
 - ◆ 2 µl dGTP
 - ◆ 2 µl dCTP
 - ◆ 2 µl 10 x Reaction Mix
 - ◆ 1 µl DNA Template (5'-T7 and dT₁₅-3' PCR product)
 - ◆ 2 µl 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 µg / µl. 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
- RNeasy (Qiagen; 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 RNeasy. 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 RNeasy 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.

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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.

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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.

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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).

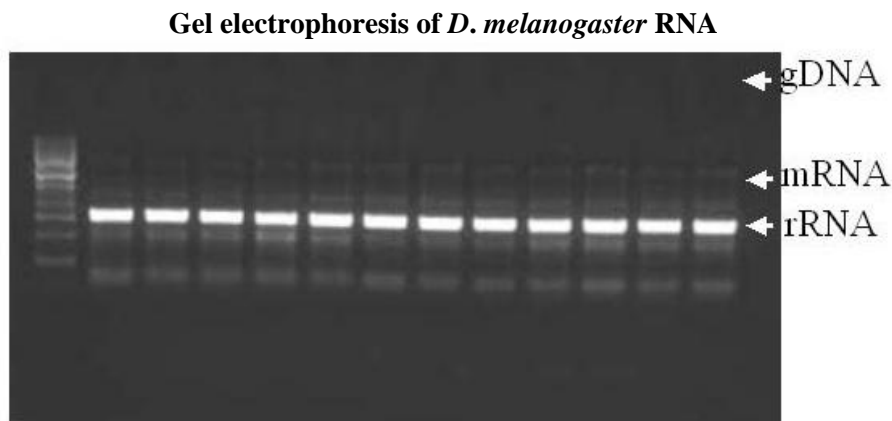
RNA quality control

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.



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:

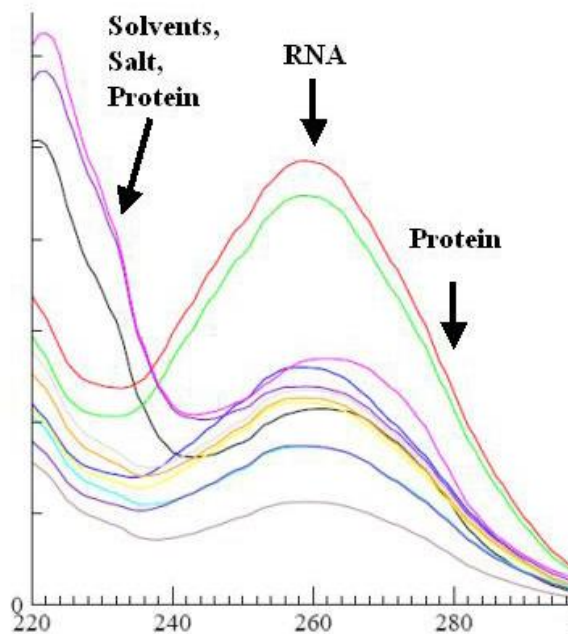
Optical density measurements are made using a Nanodrop ND-1000 spectrophotometer (<http://www.nanodrop.com>) and have been calibrated using dilutions of RNA type III from *S. cerevisiae* (Sigma; Cat. No. R-7125). This calibration showed that our linear range is within the reading range of 10 to 1000 ng/ μ l. Therefore, our samples are first diluted to fall within this range. Any measurement taken from

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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 μ l 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 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 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

Gene Expression

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).

Gene Expression

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

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- ◆ 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 μ l with DEPC-water
- 22. Precipitate the dsDNA:
 - ◆ 3.5 μ l 5M Sodium Chloride
 - ◆ 0.5 μ l LPA
 - ◆ 220 μ l 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 μ l DEPC-H₂O
- 27. Measure concentration on Nanodrop

Klenow labelling:

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

- 28. Take up to 1 μ g double stranded DNA and make up to a total volume of 13 μ l with DEPC-water
- 29. Add 10 μ l 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 μ l 10 X low-C dNTP mix
 - ◆ 1 μ l Cy3 or Cy5 dCTP
 - ◆ 0.5 μ l 40U/ μ l Klenow
- 33. Add 2 μ l 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 μ l 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 μ l by placing in a speed vac with medium heat
- 42. Add 2 μ l 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).

Klenow labelling of double stranded DNA

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.

Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB, Cat. No. 27-5340-01)
- 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)

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 μ l 100mM dNTA
 - ◆ 25 μ l 100mM dNTT
 - ◆ 25 μ l 100mM dNTG
 - ◆ 15 μ l 100mM dNTC
2. Then make up to 500 μ l using MilliQ water
3. Aliquot the 10 X low-C dNTP mix and then store at -20 °C

Klenow labelling:

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

1. Take up to 1 μ g double stranded DNA and make up to a total volume of 25 μ l with MilliQ water
2. Add 20 μ l 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:
 - ◆ 1 μ l 10 X low-C dNTP mix
 - ◆ 2 μ l Cy3 or Cy5 dCTP
 - ◆ 1 μ l 40U/ μ l Klenow (supplied in the Bioprime Labelling System Kit)
6. Add 4 μ l 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 5 μ l Stop Buffer (supplied in the Bioprime Labelling System Kit)
9. 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:

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10. 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 collection tube (supplied with the G-50 columns)
 - ◆ 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
11. 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.
12. Centrifuge for 1 minute at 5000 rpm
13. 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.
14. Reduce volume of sample to between 2 to 5 μ l by placing in a speed vac with medium heat
15. Add 2 μ l 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.

B. Fischer (09-07-2008).

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 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 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 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 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

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- ◆ 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)

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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

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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-Labeling 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 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

RNA amplification - first round

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 double stranded 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

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- ◆ 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 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

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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 °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 °C for 1 hour
54. Incubate at 65 °C for 15 minutes
55. Hold temperature at 37 °C and add 0.2 μ l 5 U/ μ l RNase H
56. Incubate at 37 °C for 30 minutes
57. Incubate at 95 °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 °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 °C for 2 hours
64. Add 2.0 μ l 1 U/ μ l T4 DNA polymerase
65. 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

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

Gene Expression

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 µl 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 centrifuge 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).