



Spotting Libraries

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Spotting libraries

Making the cDNA libraries:

- Our cDNA libraries were PCR amplified before spotting in 96-well microtitre plates
- Sonicated salmon sperm DNA was random prime labelled and used as a landmark ([jump](#))
- PCR amplification of the Arabidopsis spike controls ([jump](#))
- Linearisation of *Drosophila melanogaster* control vectors and PCR of *Drosophila melanogaster* control genes ([jump](#))

Making the oligonucleotide libraries:

- Oligonucleotide synthesis was performed by a commercial supplier
- Adding spotting buffer to the oligonucleotide library ([jump](#))

Random prime labelling of sonicated salmon sperm DNA with Cy3/Cy5

Overview

Sonicated salmon sperm DNA is labelled with Cy3 or Cy5 dCTP and then mixed to produce a Cy3- and Cy5-labelled pool of probe DNA that can be used as a landmark for cDNA of gDNA arrays. The landmark is printed within every sub-grid of a microarray and is used to assist with the spot-finding, principally sub-grid recognition.

Equipment and reagents

- Sonicated salmon sperm DNA (Amersham; Cat. No 27-4565-01)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No dNTP-100A)
- Cy3 dCTP (Amersham; Cat. No PA 53021)
- Cy5 dCTP (Amersham; Cat. No PA 55021)
- MilliQ water
- BioPrime DNA labelling kit (GibcoBRL; Cat. No. 18094-011)
 - ◆ 2.5 X Random Primer Reaction Buffer: 125 mM Tris-HCL (pH 6.8), 12.5 mM MgCl₂, 25 mM 2-mercaptoethanol, 750 µg/ml oligodeoxyribonucleotide primers
 - ◆ Klenow Fragment: 40 U/µl Klenow in 50 mM Potassium Phosphate (pH 7.0), 100 mM KCL, 1 mM DTT, 50% Glycerol
 - ◆ Stop buffer: 0.5 M Na₂EDTA (pH 8.0)
- Hot-block, Grant QBT2
- Micro 20 centrifuge, Hettich
- AutoSeq G-50 column (Amersham; Cat. No. 27-5340-01)

Procedure

1. Denature the sonicated salmon sperm DNA (sssDNA):
 - ◆ 2 µl 1µg/µl sssDNA
 - ◆ 19 µl MilliQ water
 - ◆ 20 µl 2.5 X Random Primer Reaction Buffer
 - ◆ Incubate at 100 °C for 5 minutes in a hotblock, then place on ice
2. Prepare a concentrated stock of 10 X 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
3. Perform the random prime labelling:
 - ◆ 5 µl 10 X low-C dNTP mix
 - ◆ 3 µl Cy3/Cy5 dCTP
 - ◆ 1 µl Klenow
 - ◆ Incubate at 37 °C for 2 hours
 - ◆ Stop the reaction by adding 5 µl stop buffer
4. Purify the Cy3/Cy5 labelled sssDNA:
 - ◆ 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.
 - ◆ 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 for 1 min to remove the buffer (see information supplied with the columns for calculating centrifugation speed). Blot the tip of the column dry using a clean paper towel.
 - ◆ Remove the top cap and place column in a new 1.5 ml tube. Pipette 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.

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- ◆ Spin for 1 min 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.
- 5. Make up to 100 μ l with MilliQ water and then store in aliquotes at -20 °C in the dark
- 6. Before use, make dilutions and empirically determine which dilution to use for printing

R. Auburn (17-02-2006).

Amplification of *Arabidopsis* spike controls

Outline

The spike controls were amplified by PCR from double stranded cDNA clones and by PCR from *Arabidopsis columbia* genomic DNA. The double stranded cDNA clones were obtained from:

- Arabidopsis Biological Resource Centre (Aims) (<http://aims.cps.msu.edu/>)
- Incyte Genomics (Incyte) (<http://www.incyte.com/>)
- Functional Genomics of Plant Stress Tolerance (Arizona) (<http://www.stress-genomics.org/>)

PCR amplification from double stranded cDNA clones

The double stranded cDNA clone inserts were amplified using a 1 in 100 dilution of a Qiagen purified plasmid midi prep. M13 forward and reverse primers were used for all Incyte, Aims and Arizona clones.

PCR primers:

Clone	CloneID	Vector	Antibiotic	5' primer	3' primer	PCR amplicon (kb)
Arizona4	M90509	pSK+	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.0
Arizona6	U74610	pSK+	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.1
Incyte4	ATU18126	pSTBlue-1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	0.6
Incyte5	L22585	pSTBlue-1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.0
AIMS 1	AB007987	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.3
AIMS 4	AF117335	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.7
AIMS 5	AF168390	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.5
AIMS 9	AF372915	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.3
AIMS 10	Y18469	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.6
AIMS 11	Z49777	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.2
AIMS 19	X644464	pZL1	Ampicillin	tgtaaacgacggccagt	caggaaacagctatgac	1.0

PCR reaction mix:

- 10 µl Sigma PCR Buffer (or ABgene ThermoStart standard buffer)
- 6 µl 25 mM Mg
- 2 µl 10mM dNTPs
- 2 µl 25 pmol / µl primers
- 78 µl MilliQ water
- 1 µl Sigma Taq polymerase (or ABgene ThermoStart DNA polymerase)
- 1 µl DNA template

PCR cycle:

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

1. 95 °C for 15 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

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7. 4 °C cold storage before unloading

PCR products were purified using Qiagen QIA quick columns or Millipore Multiscreen-PCR plates and checked by both agarose gel electrophoresis and the Nanodrop. The DNA concentration was then adjusted to make a final stock concentration for printing.

PCR amplification from *Arabidopsis columbia* Genomic DNA

PCR primers:

Clone	CloneID	Vector	Antibiotic	5' primer	3' primer	PCR amplicon (b)
Weed 1	O82258	pCR2.1-TOPO	Ampicillin	taaagtggaacctccgatgc	gaagagctcatcgccgatac	514
Weed 3	Q9LJQ4	-	Ampicillin	ttctcacaactcgtaattcaa	gcaaaactgatgaccaggaaga	402
Weed 4	Q9XIB8	pCR2.1-TOPO	Ampicillin	aagacgaggcgagatcttca	tgttccttcagagtgcaaatg	396
Weed 6	O04600	pCR2.1-TOPO	Ampicillin	ttgagtaccaacggttcagc	tatcatcggtttgcctttgc	370
Weed 7	Q9LZJ2	pCR2.1-TOPO	Ampicillin	tcatgtgaacatacaacgcaat	ggctattgggggtggaatc	404
Weed 8	Q9LVF8	pCR2.1-TOPO	Ampicillin	tcaacctatcattctctccatt	gcctattgaggatttggctt	394
Weed 9	O49366	pCR2.1-TOPO	Ampicillin	agcttgagaacataggccaca	tggcatcggttgctctgta	343
Weed 10	O81842	pCR2.1-TOPO	Ampicillin	agcatcmetaatccaaccaa	ttcgattccgcagattatcc	361
Weed 13	Q9LU32	pCR2.1-TOPO	Ampicillin	tccaatatgatttggttgga	tgtatgctgcactcgatga	330
Weed 14	O04513	pCR2.1-TOPO	Ampicillin	agggcatttggttcgatgt	atagcatgctcgatgtgcaa	306

PCR reaction mix:

- 10 µl Stratagene Yield Ace reaction buffer (or ABgene ThermoStart standard buffer)
- 2 µl 10mM dNTPs
- 2 µl 25 pmol / µl primers (spike specific 5'+3')
- 84 µl MilliQ water
- 1 µl Stratagene Yield Ace polymerase (or ABgene ThermoStart DNA polymerase)
- 1 µl DNA template

PCR cycle:

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

1. 95 °C for 15 minutes
2. 95 °C for 45 seconds
3. 60 °C for 40 seconds
4. 72 °C for 1 minute
5. Repeat steps 2 to 4, 34 times
6. 72 °C for 10 minutes
7. 4 °C cold storage before unloading

Spotting Libraries

PCR products were purified using Qiagen QIA quick columns or Millipore Multiscreen-PCR plates and checked by both agarose gel electrophoresis and the Nanodrop. The DNA concentration was then adjusted to make a final stock concentration for printing.

R. Auburn (17-02-2006).

***Drosophila melanogaster* control vectors and genes**

Outline

A selection of commonly used *Drosophila* control vectors and genes were prepared for the FlyChip cDNA microarrays using the protocols defined below.

- Vectors: pBluescript, pOT2a, pUAST, pLacW and pGawB
- Genes: white, lacZ, gal4, P transposase, FLPase and GFP

Linearisation of vectors

DNA for the control vectors, pOT2a, pBluescript, pUAST, pLacW and pGawB was transformed by standard methods into XL Gold cells and plasmid containing colonies selected on either LB-Ampicillin or LB-Kanamycin plates.

Plasmid preps were prepared using Qiagen plasmid purification spin columns and the purified DNA was then linearised with the restriction enzyme Xba I.

Protocol:

1. Make the following restriction digestion mixture:
 - ◆ 20 to 40 µg DNA
 - ◆ 20 µl NEB Buffer 2
 - ◆ 20 µl 10 x BSA (1 mg / ml)
 - ◆ 4 µl XbaI enzyme (2U per µl)
 - ◆ Make up to 200 µl with MilliQ water
2. Incubate each restriction digestion at 37 °C for 2 hours
3. Heat denature enzyme at 65 °C for 20 minutes
4. Phenol:Chloroform extraction and ethanol precipitation of DNA
5. Centrifuge at 13000 rpm for 10 minutes, perform an ethanol wash, and then precipitate using 70% ethanol
6. Resuspend in MilliQ water

20 to 30 µg of linearised vector were generated from several Xba I digests. The DNA was then pooled and concentrated to make a stock of approximately 0.2 to 0.3 µg / µl.

PCR of Control Genes

The control genes white, lacZ, gal4, P transposase, FLPase and GFP were amplified by PCR from the following vectors using the specified gene-specific forward and reverse primer pairs:

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Gene	Template	Forward primer	Reverse primer	Annealing Temp.
white	pUAST	AAGTATCGCCATCCGGGATGCG	TAAGCGTCTCCAGGATGACCTTG	68 °C
lacZ	pLacW	TATCCCATTACGGTCAATCCGCC	ACCGTCGATATTCAGCCATGTGC	60 °C
gal4	pGawB	GTATCGATTGACTCGGCAGCTC	AGTTTGGTCCGTCCAACCAGG	68 °C
P transposase	p 25.7wc	TTGCTGCAAAGCTGTGACTGGAG	TTCGGACGGCTTAATAAGTCCG	60 °C
FLPase	phsFLP	CAGCAATCAAGAGAGCCACA	CTGCCACTCCTCAATTGGAT	60 °C
GFP	pUASmGFP6	GGAATTCATGAGTAAAGGAGAAGAAC	CTAGATCTCATCATTATTTGTATAGTTCATCC	60 °C
pOT2a	pOT2a	AATGCAGGTAAACCTGGCTTATCG	AACCGCGGCTACAATTAATACATAACC	68 °C
pBlueScript	pBlueScript	GGCGTAATCATGGTCATAGCTGTTTC	GAGTCGTATTACAATTCACTGGCCG	68 °C

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Protocol:

PCR Reaction mix:

- 10 μ l 10 x Stratagene Yield Ace reaction buffer (or ABgene ThermoStart standard buffer)
- 2 μ l 25 mM dNTP mix
- 2 μ l 25 pmol / μ l forward and reverse primer mix
- 84 μ l MilliQ water
- 1 μ l Stratagene Yield Ace polymerase (or ABgene ThermoStart DNA polymerase)
- 1 μ l DNA template

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. Annealing temperature for 30 seconds
4. 72 °C for 4 minutes
5. Cycle between steps 2 to 4, 34 times
6. 72 °C for 10 minutes
7. 4 °C cold storage before unloading

PCR products were purified using Qiagen QIA quick columns or Millipore Multiscreen-PCR plates and checked by both agarose gel electrophoresis and the Nanodrop. The DNA concentration was then adjusted to make a final stock concentration for printing.

R. Auburn (17-02-2006).

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).