cDNA Synthesis, Labeling and Microarray Hybridization Protocol[1]

9 Oct 2007
Daniel F. Simola
Laboratory of Junhyong Kim, University of Pennsylvania
http://kim.bio.upenn.edu/web/wetlab/KimLab_yeast_microarray_protocol.pdf

A. RNA harvest

Follow Qiagen Rneasy protocol for extraction of Yeast total RNA via Mechanical Lysis with glass beads.

B. cDNA Synthesis (~2.5 h)

Reagent	**Amount**
Total RNA	15 µg (maximum volume is 27.5 µl)
Anchored Oligo-dT (2.5 ug/µl)	1 µl
Alien Spike Mix	1 µl
Alien Spike [9] or [10] [500 pg/µl]	1 µl
DEPC H_20	to 30.5 µl
Total Volume	**30.5 µl**

1. Aliquot reagents listed above to a 200-µl PCR tube. You should have two reactions for each array, one for the experimental RNA sample, and one for the reference RNA sample. Spike [9] should be added to the experimental sample (which will be labeled with the green Alexa 555 Dye). Spike [10] should be added to the reference sample (which will be labeled with the red Alexa 647 Dye).
2. Heat tube at 70°C for 5 minutes – cool on ice for at least one minute.
3. Prepare a Master Mix sufficient for the number of reactions plus a little extra (10%) to ensure sufficient volume. Multiply quantities below by the number of reactions:

Reagent	**µl per Reaction**	**µl for X Reactions**
5X First Strand Buffer	10.0	
Invitrogen 10 mM aa-dNTP mix (Custom 12.5mM mix)	2.5 (2.0)	
0.1 M DTT	5.0	
RNasOut (40 U/µl)	0.5	
Superscript III RT (400 U/µl)	1.0	
DEPC H_20 (if custom NTP)	0.5 (1.0)	
Total Volume	**19.5**	

Keep Master Mix at room temperature and continue.

4. Add 19.5 µl of Master Mix to each sample. Mix gently and spin down.
5. Incubate at 46°C for 3–4 hours. *NB, 3 hour incubation improves yield 20–30% over 2 h.*
6. Add 20 µl 1N NaOH.
7. Heat 70°C 10 minutes; Spin down brief to neutralize pH.
8. Add 20 µl 1N HCl. *You can stop here and store cDNA at –20°.*

[1] This protocol is adapted from the Functional Genomics Core (Peter White) "Labeling and Hybridization with the PancChip Amino-allyl Indirect Labeling of RNA for Microarrays" protocol, with help from Chantal Francis.

C. Purify cDNA using Invitrogen module (20 min)

1. Transfer the 90 µl from above to a new 1.5 ml eppendorf.
2. Add 700 µl of Binding Buffer. Vortex briefly.
3. Transfer 790 µl to Invitrogen spin cartridge.
4. Centrifuge at 3300 x *g* in a microcentrifuge for 1 min. Remove collection tube and discard flow through.
5. Place spin cartridge in the same collection tube and add 600 µl of Wash Buffer to the column.
6. Centrifuge at max speed for 30 sec. Remove collection tube and discard flow through.
7. Place spin cartridge in the same collection tube and centrifuge at max speed for 30 sec to remove residual Wash Buffer. Remove collection tube and discard.
8. Place spin cartridge into a new amber collection tube.
9. Add 19 µl of pure water to the center of the spin cartridge and let stand at room temp. for 30 min. *DNA is concentrated next so elute in larger volume to improve recovery.*
10. Centrifuge at max speed for 1 min. **The eluate contains 18 µl purified cDNA.**

N.B., the sample can be stored at -20° C for up to one week prior to hybridization. Avoid freeze/thawing.

D. Fluorescent Dye Coupling (Alexa Fluor Reactive Dye Decapacks) (~1 h 40 min)

Do not use dyes if the desiccant packet in the pouch the dyes come in is blue, which indicates a humid environment. To minimize risk of dye oxidation, perform in dark, esp. if doing several slides
Thaw DMSO and 2x Coupling Buffer. If working solo, also thaw PolyA DNA, tRNA, and salmon sperm DNA for later.

1. Add 20 µl of 2x Coupling Buffer to purifed cDNA from above.
2. Prepare dyes just before use by cutting the plastic tab.
3. Dissolve one vial of the Alexa Reactive Dye (555 or 647) in 2 µl of high quality DMSO. Vortex 15 sec to ensure that the dye is completely dissolved. Once the reactive dye has been dissolved, the reaction should be performed immediately. Dissolved dye cannot be saved for another day.
4. Transfer all 2 µl of the dissolved dye solution to the resuspended cDNA + coupling solution in the amber tube. Vortex and spin briefly to ensure that the reaction is well mixed.
5. Leave the reaction in a dark chamber at room temperature for 1 hour (up to overnight). *Cover tube rack with a lid wrapped with aluminum foil, and place into drawer.*

E. Purify cDNA using Invitrogen module (20 min)

1. If spotting 2 channel slides, combine 2 cDNA samples, 40 µl each, 80 µl total.
2. Add 700 µl of Binding Buffer to the 40 or 80 µl of dye-coupled cDNA in the amber tube.
3. Mix by vortexing or pipeting, then transfer sample to an Invitrogen spin cartridge.
4. Centrifuge 1 min at 3,300 *g* in a microcentrifuge. Discard flow through.
5. Place spin cartridge in the same collection tube and add 600 µl of Wash Buffer to the column.
6. Centrifuge at max speed for 30 sec. Remove collection tube and discard flow through.
7. Centrifuge at max speed for 30 sec to remove residual Wash Buffer. Discard collection tube. Place spin cartridge into a new amber collection tube.
8. Add 20 µl of pure water (heated to 45° C) to the center of the spin cartridge and let stand at room temp. for 5–30 min. *N.B. Can leave samples here in dark to incube, while proceeding with Pre-Hybridization. This will maximize eluted cDNA yield and minimize the time your slides dry after Pre-Hyb.*
9. Centrifuge at max speed for 1 min. **The eluate contains 18–19 µl purified cDNA.**

N.B., the sample can be stored at -20° C for up to one week prior to hybridization, protected from light! Avoid freeze/thawing.

F. Pre-Hybridization I: Preparation

NB if working solo, perform steps leading up to incubating cDNA probe at 95º while the cDNA is incubating with the dyes. This follows the Corning Pronto! Universal Microarray Kit manual, section "Background Reduction and Pre-Hybridization", substituting our Pre-Hyb and wash solutions for theirs. We use only their Background Reduction Kit, i.e. Pre-Soak. If < 7 arrays, only need 60ml of the Pre-Soak and Pre-Hybridization solutions, as you will pour them into Coplin jars.

1. ***Prepare Carrier Cocktail shown in table below.*** *Distribute 5 µl into 200 µl PCR tubes, and store at 4º C. Do not add 2x hyb buffer.*

Reagent	**1 slide**	**5 slides**	**10 slides**
Salmon Sperm DNA (1 µg/µl)	0.7 µl	3.5	7.0 µl
tRNA (1µg/µl)	1.4 µl	7.0	14.0 µl
PolyA DNA (1µg/µl)	2.9 µl	14.5	29 µl
Total	**5 µl**	**25 µl**	**50 µl**

N.B., prepare a tube of Salmon sperm DNA, tRNA, and polyA DNA separately in a .2 ml tube (total = 5 µl), then add 18.0ul cDNA to this tube.

2. Heat 200 mL of Pre-Hybridization solution and 198 mL of Pre-Soak solution at 42º C for at least 30 min.
3. Warm 2X hybridization buffer (50% formamide, 10X SSC, and 0,2% SDS) to 42°C.
4. ***Prepare coverslips.*** *To clean each coverslip, set up one Coplin jar with ddH_2O and another with 100% EtOH. Dip coverslip 3x in H2O, then 3x in EtOH. Use vacuum line or N2 gas to dry both sides of the coverslip. Store each coverslip in a Falcon tube, with rough side facing the frosted white of tube for easy placement onto the array slide later.*
5. ***Prepare slide chambers.*** *Pipet 14 µl ddH_2O into the two small holes in each chamber bottom.* This helps maintain a humid environment during hybridization.
6. *Dilute 2 mL of liquid borohydride into 198 mL (1:100 dilution) of 42º C Pre-Soak solution. Swirl gently to mix. Use the resulting solution within 30 minutes of preparation to maximize its effectiveness.* If using 60 ml of Pre-Soak, add only 600 µl of borohydride.

G. Pre-Hybridization II: Wash & Dry

1. Immerse arrays in above Pre-Soak solution and incubate at 42º C for 20 min.
2. Transfer arrays to Wash Solution 2 and incubate at ambient (22º to 25º C) temperature for 30 sec.
3. Repeat above step with fresh Wash Solution 2.
4. Transfer arrays to 42º C Pre-Hybridization solution and incubate for 15 min.
5. Transfer arrays to fresh Wash Solution 2 and incubate at ambient temp for 1 min.
6. Transfer arrays to Wash Solution 3 and incubate at ambient temp for 30 sec.
7. Repeat above step with fresh Wash Solution 3.
8. Incubate arrays 30 sec in nuclease-free water at ambient temperature (22º to 25º C), and dry by blowing high-purity nitrogen gas over the array (or by centrifugation at 2136 rpm (750 rcf) for 5 minutes (500 rcf may work better)).

H. Hybridization

N.B., If cDNA remains incubating in column, centrifuge at max 1min, then add each purified cDNA sample to a 200 µl PCR tube containing 5 µl carrier cocktail, for a total 23–24 µl probe solution.

1. Denature probe solution at 95° C for 5 min and spin down briefly.
2. Immediately add **23 µl 2X hyb buffer** *(do not mix)*. Total hybridization volume is 46 µl. *Instead of mixing, pipet solution 2–3 times in tube just before pipetting onto slide.*
3. During hybridization keep unused cDNA samples warm in 45º C dry bath, and keep covered to protect from light.
4. Immediately apply probe to slide (using your favorite technique). Cover with Lifterslip Coverslip, seal in chamber, and incubate 24–72 h (48 rec), submerged in 42°C waterbath.
5. Put wash buffer 1 and 2 in 42° C incubator in preparation for post-hyb washing.

The general procedure we have developed is the following. Work in a reasonably dark environment. Pipet the hyb solution in small drops along the short axis, towards one the end of the slide. Use your fingers to anchor one end of the coverslip on the edge with probe solution, and lower the other end slowly with foreceps. Use pointed tip foreceps to disperse bubbles. Refrain from tinkering after ~2 min. Replace slide in chamber, cover, seal, and position in the water bath. Make sure chamber appears level.

I. Morning after: Wash and Scan (30 min / 10 min per slide to scan)

1. Remove coverslip by placing slide into low stringency Wash buffer 1 (42°C) in a Coplin Jar until the coverslip falls off.
2. Coverslip may not fall freely, but if loose you can simply lift the slide out, and the coverslip will remain in solution. Using 2 Coplin jars accelerates this step.
3. Place into high stringency Wash buffer 2 42° C and shake on platform rotator for 5 minutes.
4. Place slide into Wash buffer 3 and shake for 5 minutes at room temperature.
5. Place slides into dd H2O (chilled at 4° C overnight) and dip 3 times. *NB excessive dipping may decrease fluorescence*
6. Dry with high-purity, filtered nitrogen gas and air vacuum line or centrifuge slide 5 minutes at 100 rcf in swinging bucket rotor or in Falcon tubes at 850 g.
7. Make sure container is secure in each rotor chamber or your container and slides may break)
8. Scan slides to obtain TIFF image files.

Appendix I: Reagents and Sources:

Reagent	Source	Catalog Number	Phone Number
SuperScript Plus Indirect cDNA Labeling Kit (contains the following reagents: Anchored Oligo-dT (2.5 ug/µl), 5X First Strand Buffer, 0.1M DTT, RNasOut (40U/µl), Superscript III RT (400U/µl), DEPC H_2O, Alexa 555 and 647 Dyes, DMSO)	Invitrogen	L1014-06 $612.00	(800) 955-6288, Option 3, x46029
SuperScript Plus Indirect cDNA labeling kit with AlexaFluor Dyes (no purification module) Contains all of the above minus the purification, which we don't use	Invitrogen	L1014-04	(800) 955-6288, Option 3, x46029
Alien mRNA Spikes	Stratagene	252561 through 252570	1-800-424-5444
Formamide (Highly-Deionized) 25 mL	Applied Biosystems	4311320 $29?	1-800-327-3002
MinElute Reaction Cleanup Kit (50 rxns)	Qiagen	28204	1-800-426-8157
dNTPs (dATP, dGTP, dCTP) bulk [100mM] 4*40µl	Promega (Cell Center)	Manufacturer No.:U1240, Cell Center Code: 4240 $193.18	N/A
5-(3-aminoallyl)-dUTP [50mM]	Ambion	8439 $121.00	1-800-888-8804
UltraPure™ DNase/RNase-Free Distilled Water (10 x 500 mL)	Invitrogen	10977-023 $109.00	(800) 955-6288, Option 3, x46029
UltraPure Glycogen (20ug/µl)	Invitrogen	10814-010 $53.00	(800) 955-6288, Option 3, x46029
Ethanol (100%)	Pharmco Products, Inc.	111000200CSPP	1-800-243-5360
Saline–Sodium Citrate (SSC), 20X Solution	Fisher Scientific	BP1325-1 $59.96	
10% SDS (4x100ml)	Invitrogen	15553-027 $48.00	(800) 955-6288, Option 3, x46029
Salmon Sperm DNA (10mg/ml)	Stratagene	201190-81	1-800-424-54444
tRNA (1ug/µl) (yeast)	Fisher Scientific	NC9648070	800 766 7000
PolyA DNA	Invitrogen	POLYA.GF $50.00	800 955 6288
Hybridization Chambers	Corning	?	?
Glass Coverslips	Erie Scientific Company	25x60I-2-4789	1·888·374·3724
Sodium Bicarbonate	Fisher Scientific	BP328-500 $25.12	1-800-766-7000

Appendix II: Solutions and Mixes

Solution	Reagents	Amounts (µl)		Instructions
25X aa dNTP mix	dATP [100mM]	62.5		Prepare 500 µl dNTP mix using Ambion amino-allyl dUTP. Aliquot into 5 tubes of 100 µl each and store at -80°C. A working stock can be kept at -20°C and is stable for a month, with several freeze/thaw cycles. Final concentrations of reagents, respectively: 12.5mM, 12.5mM, 12.5mM, 5mM,7.5mM
	dGTP [100mM]	62.5		
	dCTP [100mM]	62.5		
	amino-allyl dUTP [50mM]	50		
	dTTP [100mM]	37.5		
	H_2O	225		
	Total Volume	**500µl**		
2X Sodium BiCarbonate Labeling Buffer (225mM)	sodium bicarbonate	18.75 mg	937.5 mg	Add 25mg of sodium bicarbonate to 1 mL of nuclease-free H_2O and vortex the solution until the solid is completely dissolved. Store the labeling buffer at -20°C in single-use aliquots. When properly stored the labeling buffer should be stable for at least six months.
	UltraPure Water (nuclease-free)	1 mL	To 50 mL	
	Total Volume	**1 mL**	**50 mL**	
Pre-Hybridization Buffer (50ml) 5X SSC 0.1% SDS 1% BSA	20X SSC	25 ml	150 ml	Add SDS, SSC and BSA to water and dissolve in incubator or on heated stir plate. Filter sterilize the solution with a .22um CA (low protein binding) filter. *Dry BSA remains stable for 2.5+ years. Pre-hyb solution good for 1 month at 4°C after preparation.*
	10% SDS	1 ml	6 ml	
	Fraction V BSA	1 g	6 g	
	DiH20 (MilliQ)	74 ml	444 ml	
	Total Volume	**100 ml**	**600 ml**	
2X Hybridization Buffer (25mL) 50% formamide 10X SSC .2% SDS	Formamide, deionized	12.5 ml		Filter this solution with a .22um filter (CN or CA) and aliquot to smaller volumes to avoid repeated thawing and re-freezing.
	20X SSC	12.5 ml		
	10% SDS	.5 ml (.05 g)		
	Total Volume	**25 ml**		
Wash Buffer I: 2X SSC 0.1% SDS	20X SSC	100 ml	200 ml	Add water first, then add SDS and SSC. Filter with .22um filter (CN or CA).
	10% SDS	10 ml	20 ml	
	DiH20 (MilliQ)	890 ml	1780 ml	
	Total Volume	**1 L**	**2 L**	
Wash Buffer II: .2X SSC 0.1% SDS	20X SSC	10 ml	20 ml	Add water first, then add SDS and SSC. Filter with .22um filter (CN or CA).
	10% SDS	10 ml	20 ml	
	DiH20 (MilliQ)	980 ml	1960 ml	
	Total Volume	**1 L**	**2 L**	
Wash Buffer III: 0.2X SSC	20X SSC	10 ml	20 ml	Add water first, then add SSC. Filter with .22um filter (CN or CA).
	DiH20 (MilliQ)	990 ml	1980 ml	
	Total Volume	**1 L**	**2 L**	

Corning Washes

Solution	Reagents	Amounts (µl)	Instructions
Wash Solution 2	ddH20 (MilliQ)	855 ml	Wash Solution A comes in the Pronto! Universal Microarray Hybridization Kit. Filter with .22um filter.
	Corning Pronto! Wash Solution A	45 ml	
	Total Volume	**900 ml**	
Wash Solution 3	ddH20 (MilliQ)	320 ml	Filter with .22um filter.
	Wash Solution 2 (above)	80 ml	
	Total Volume	**400 ml**	

Spike Mix

This spike mix contains Stratagene's Alien Spikes 1,2,3,5,6,7, and 8. It does not contain Alien Spike 4 because it cross-hybridizes.

Reagent	Dilution #1	Dilution #2	Volume (µl)	Final Concentration (pg/µl)
Alien Spike 1 [10000pg/µl]	2µl + 18µl diH20 [1000pg/µl]		20	500
Alien Spike 2 [10000pg/µl]	2µl + 18µl diH20 [1000pg/µl]		4	100
Alien Spike 3 [10000pg/µl]	2µl + 18µl diH20 [1000pg/µl]		2	50
Alien Spike 5 [10000pg/µl]	2µl + 198µl diH20 [100 pg/µl]		2	5
Alien Spike 6 [10000pg/µl]	1µl + 499µl diH20 [20pg/µl]		2	1
Alien Spike 7 [10000pg/µl]	1µl + 499µl diH20 [20pg/µl]		1	.5
Alien Spike 8 [10000pg/µl]	1µl + 999µl diH20 [10pg/µl]	4µl Dilution 1 + 36µl diH20 [1pg/µl]	2	.05
diH20			7	
Total Volume			**40µl**	

Stock solutions of spikes 9 and 10 should be diluted to a working cocentration of 500 pg/µl.

Hybridization schedule					
Date:					
Samples:					
Step	**Task (sample prep)**	**Duration**	**Actor**	**Day/Date**	**Time interval**
1	Prepare reverse transcription reaction	45 m			
2	RT incubation	2 h	NA		
3	MinElute cleanup of cDNA	1 h			
4	Ethanol precipitation of cDNA	45 m			
5	Prepare pre-hybridization buffer	20 m			
6	Dye coupling of cDNA setup	45 m			
7	Dye coupling incubation	1 h	NA		
8	MinElute cleanup of coupling reaction	30 m			
9	Make tRNA, polyA, salmon cocktail	15 m			
10	Combine cDNA, cocktail; heat; add hyb	10 m			
Step	**Task (wash/slide prep)**	**Duration**	**Actor**	**Day/Date**	**Time interval**
1	Confirm new slides in dessicator	5 m			
2	Make up new wash buffers	10 m			
3	Setup wash buffers in square glass jars	45 m			
4	Add 14 µl ddH20 to hyb chamber dimples	5 m			
5	Prepare coverslips	20 m			
6	Warm slides in prehyb buffer at 42° C	45 m	NA		
7	Wash slides	10 m			
8	Pipet sample onto slide, set in water bath	20 m			
9	Hybridization incubation	18–24 h	NA		
10	Wash/dry slides with 3 wash buffers	35 m			
11	Scan slides	10 m/slide			

SLIDE MAP| Scan Date: _____Hyb Duration: _____Authors: _____ Dyes: _____

ID	Barcode	Strain	Stage	*Ref Dye*	*Dye Swap*	*Cell sample*	*Cell ref*	*RNA sample*	*RNA ref*	*cDNA sample*	*cDNA ref date*
1											
2											
3											
4											
5											
6											
7											
8											
9											
10											
11											
12											
13											
14											
15											
16											
17											
18											
19											
20											