Van Andel Institute
Laboratory of Microarray Technology
2007

Procedure 2.3 – Agilent One-Color Gene Expression Analysis

Date: ___________________ **Experiment ID: 2.3-**yyyymmdd

Technician: ___________________

Project: ___________________

Client Signature: ___________________

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1. Prepare One-Color Spike Mix

1.1 Assign stock solution LMT number (Appendix 7.3). ________________

1.2 Dilute the stock solution according to the following table.

Starting amount of RNA		Serial dilution			
Total RNA (ng)	Maximum volume of RNA (µL)	First	Second	Third	Spike-Mix volume to be used in each labeling reaction (µL)
200	8.3	1:20	1:25	1:10	2
300	7.3	1:20	1:25	1:10	3
400	6.3	1:20	1:25	1:10	4
500	5.3	1:20	1:25	1:10	5
600	7.3	1:20	1:25	1:5	3
700	6.8	1:20	1:25	1:5	3.5
800	6.3	1:20	1:25	1:5	4
900	5.8	1:20	1:25	1:5	4.5
1000	5.3	1:20	1:25	1:5	5

Assign all dilutions LMT numbers and store as described in Procedure 1.2

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2. Prepare Labeling Reactions (Agilent LRILAK Kit) ________________

2.1 Combine total RNA, spike mix, and T7 promoter primer according to the following table.

(spike mix) ________________

(nuclease-free water)________________

Total RNA input (ng)	Max RNA volume (µL)	Third Dilution of Spike-Mix volume (µL)	T7 Promoter primer (µL)	Total volume (µL)
200	8.3	2	1.2	11.5
300	7.3	3	1.2	11.5
400	6.3	4	1.2	11.5
500	5.3	5	1.2	11.5
600	7.3	3	1.2	11.5
700	6.8	3.5	1.2	11.5
800	6.3	4	1.2	11.5
900	5.8	4.5	1.2	11.5
1000	5.3	5	1.2	11.5

2.2 Place in thermocycler and run <LRILAK1>

2.3 Prepare a first strand master mix of the following components by multiplying the volumes by n.5, where n is the number of reactions.

5X First Strand Buffer	4.0ul	(Prewarm: 80C, 5 minutes)
0.1M DTT	2.0ul	
10mM dNTP mix	1.0ul	
MMLV-RT	1.0ul	
RnaseOut	0.5ul	

2.4 Add 8.5ul first strand master mix to each sample. Mix.

2.5 Place in thermocycler and run <LRILAK2>

2.6 Prepare a transcription master mix of the following components by multiplying the volumes by n.5, where n is the number of reactions.

Nuclease-free water	**15.3ul**		________________
4X Transcription Buffer	**20.0ul**		
0.1M DTT	**6.0ul**		
NTP mix	**8.0ul**		
50% PEG	**6.4ul**	**(Prewarm: 40C, 1 minute)**	
RnaseOut	**0.5ul**		
Inorganic Pyrophosphatase	**0.6ul**		
T7 RNA Polymerase	**0.8ul**		
Cyanine 3-CTP	**2.4ul**		

2.7 Add 60ul of transcription master mix to each sample. Mix.

2.8 Place in thermocycler and run <LRILAK3>

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3. <u>Purify Labeled cRNA using Qiagen RNeasy Mini.</u> ________________

3.1 Add the following to each sample and mix well.

Nuclease-free water	**20 ul**	________________
Buffer RLT	**350 ul**	
100% EtOH	**250 ul**	________________

3.2 Transfer to an RNeasy mini column in a 2ml collection tube.

3.3 Centrifuge at 4C for one minute at 500rcf, then at 13,500rcf for 30 seconds. Discard the flow-through.

3.4 Add 500ul Buffer RPE to the column and centrifuge at 13,500rcf for 30 seconds at 4C. Discard the flow-through.

3.5 Repeat 3.4

3.6 Add 30ul Rnase-free water to the column and incubate at room temperature for one minute.

3.7 Centrifuge at 13,000rcf for one minute to elute the purified cRNA.

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4. cRNA Quantitation

4.1 Quantitate using the NanoDrop as described in Appendix 7.1

Save NanoDrop report as 2.3 _yyyymmdd_NanoDrop in the appropriate SOP Reports folder

4.2 Calculate specific activity of CyDye and add to NanoDrop data.

(concentration of CyDye) / (concentration of aRNA) *1000 = specific activity

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5. Prepare the 10X Blocking Agent **(Stock)________________**

5.1 Add 500 ul nuclease-free water to the vial containing lyophilized 10X Blocking Agent. ________________

5.2 Heat the solution at 37C for five minutes.

5.3 10X Blocking Agent can be stored at -20C for 2 months. Assign the blocking agent an LMT number (Appendix 7.3). ________________

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6. Prepare Hybridization Samples

6.1 Combine the following in a PCR tube or plate.

Cyanine 3-Labeled cRNA	**600 ng**
10X Blocking Agent	**5 ul**
25X Fragmentation Buffer	**1 ul**
Nuclease-free Water	**Bring Volume to 25 ul**

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6.1 Incubate at 60C for exactly 30 minutes.

6.3 Scan microarray barcodes into an Excel sheet and record corresponding sample identifications. Save file as dnamicroarray\Procedure_2.3\2.3_yyymmdd_microarrays ________________

6.4 Add 25ul 2X GEx Hybridization Buffer. Mix. Use Immediately. ________________

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7. Prepare the Hybridization Assembly

7.1 Load a clean gasket slide into the SureHyb chamber.

7.2 Dispense 40ul of the hybridization sample(s) into the gasket well(s). ________________

7.3 Slowly place the microarray onto the gasket slide so that the *numeric* barcode is facing up.

7.4 Place the SureHyb cover onto the sandwiched slides and slide the camp onto both pieces. Hand-tighten the clamp.

7.5 Place assembled slide chamber in rotisserie in a hybridization oven set to 65C. Set rotation speed at 10 RPM.

7.6 Hybridize at 65C for 17 hours.

7.7 Warm Wash Buffer 2 at 37C overnight. ________________

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8. <u>Microarray Wash</u>

8.1 Disassemble SureHyb chambers and carefully remove gasket slides under wash buffer 1.

8.2 Place microarrays in steel rack in staining chamber containing wash buffer 1. ________________

8.3 Place on rotator in ozone cabinet. Set speed at 3 and rotate in the dark for one minute.

8.4 Transfer slides to a new staining dish containing prewarmed wash buffer 2. Set speed at 3 and rotate in the dark for one minute. ________________

8.5 Centrifuge slides at 500 RPM for 5 minutes to dry.

8.6 Scan Arrays as described in Appendix 7.4