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Keck Foundation Biotechnology Resource Laboratory at Yale University  
**Microarray Resource**

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Ambion Illumina® TotalPrep™ cDNA Synthesis  
P/N AMIL1791

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Please do not make copies of or distribute this protocol.

A. Required reagents:

- Nuclease-free Water
- \*T7 Oligo(dT) Primer
- \*10X First Strand Buffer
- \*dNTP Mix
- \*RNase Inhibitor
- \*ArrayScript
- \*10X Second Strand Buffer
- \*DNA Polymerase
- \*RNase H

\*Reagents Supplied with Illumina® TotalPrep™ RNA Amplification kit (Ambion P/N AMIL1791).

B. Equipment and supplies:

- Microcentrifuge with 1.5 ml rotor
- 1.5 ml microcentrifuge tubes
- Micropipettors
- Aerosol-barrier tips
- Thermal cycler at 42°C
- Thermal cycler at 16°C
- Heatblock at 55°C
- Vortex mixer
- Powder-free gloves
- 0.2 ml RNase-free microcentrifuge tubes
- Lab Tracking Sheets

I. FIRST STRAND cDNA SYNTHESIS:

\* Starting material: High quality total RNA in Nuclease-free water. RNA should be isolated

by using RNeasy kit (Qiagen). If RNA is isolated by any other method, perform cleanup using RNeasy kit.

Recommended amount of input RNA	
Recommended mass amount of total RNA	500 ng
Minimum mass amount of total RNA	50 ng
Maximum Volume of RNA	11 ul

\* Assess the quality of total RNA by gel electrophoresis. The intact RNA indicates that there is no contamination of RNase, which could affect cRNA synthesis in the next step. If the RNA does not appear to be intact, do not proceed with cDNA reaction until the investigator has confirmed to proceed.

\* Briefly spin down all enzymes before use. Keep enzymes on ice block during master mix preparation.

\* Log sample names and concentrations in Lab Tracking Sheets.

\* Recommended batch size is 24. For larger batches, stagger the incubation times by ~30 min.

### I. FIRST STRAND cDNA SYNTHESIS:

1. Place a maximum volume of 11 ul of total RNA (50-500 ng) into an RNase-free 0.2 ml BioRad microcentrifuge tube.
2. Add Nuclease-free water to bring the samples to 11 ul.
3. At room temperature, prepare Reverse Transcription Master Mix in a 1.5 ml nuclease-free tube. Be sure to prepare n+1 overage to cover pipetting error.

Reverse Transcription Master Mix			
Amount for 1 RXN	13 RXNS	26 RXNS	Component
1 ul	13 ul	26 ul	T7 Oligo(dT) Primer
2 ul	26 ul	52 ul	10X First Strand Buffer
4 ul	52 ul	104 ul	dNTP Mix
1 ul	13 ul	26 ul	RNase Inhibitor
1 ul	13 ul	26 ul	ArrayScript

4. Mix well by gently vortexing. Centrifuge to collect master mix at bottom of the tube and place on ice.
5. Transfer 9 ul of Reverse Transcription Master Mix to each RNA sample. Mix thoroughly by pipetting up and down 2-3 times, cap tubes, flick the tube 3-4 times. Centrifuge briefly to collect the reaction at the bottom of the tube.
6. Place the samples in a 42°C thermal cycler. Incubate reactions for 2 hours at 42°C. Log time of incubation start in LTS. During this incubation, keep dNTPs on ice. Begin thawing Second Strand buffer to room temperature after ~~1.5 hrs~~ of 42C incubation.

12:15 10/11

- ✓. After the incubation, centrifuge briefly to collect the reaction mixture at the bottom of the tube and place on ice. Log end time of incubation on LTS.

8. Proceed immediately to Second Strand Synthesis.

## **II. SECOND STRAND cDNA SYNTHESIS:**

1 **On ice block**, prepare the Second Strand Master Mix in a 1.5 ml nuclease-free tube (or larger if needed) in the order listed below. Be sure to prepare  $n+1$  overage to cover pipetting error.

Second Strand Master Mix			
Amount for 1 RXN	13 RXNS	25 RXNS	Component
63 ul	819 ✓	1575	Nuclease-free Water <i>Put</i>
10 ul	130 ✓	250	10X Second Strand Buffer
4 ul	52 ✓	100	dNTP Mix
2 ul	26 ✓	50	DNA Polymerase
1 ul	13 ✓	25	RNase H

2. Mix well by gently vortexing. Centrifuge briefly.
  3. Transfer 80  $\mu$ l of Second Strand Master Mix to each sample. Mix thoroughly by pipetting up and down 2-3 times, cap tubes, flick the tube 3-4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.
  4. Place tubes in the 16°C thermal cycler. Incubate for 2 hours. Log incubation start time in LTS.

\*If the lid temperature cannot be adjusted to match the 16°C block temperature, cover the tubes with the lid turned off, or if the lid cannot be turned off-do not cover the tubes with it.

5. After the 2 hour incubation, place the reactions on ice, log end of incubation time in LTS and proceed to Illumina® TotalPrep™ cDNA cleanup. Do not freeze reactions at this point unless in an emergency situation.

12.75      2.401      12.40  
12.15      12.40      12.20  
11.30      12.20      12.05  
11.50      12.20      12.05

-00/ to room temperature before use.

\*Before using the Wash Buffer for first time, add 24 ml of 100% Ethanol.

\*Recommended batch size is 12 at a time. If doing multiple batches, do not leave reactions on ice for more than an hour.

1. Setup and label 3 sets of tubes: (1) to transfer samples from strip tubes to 1.5 ml tubes; (2) filter column seated in collection tube; (3) 1.5 ml cDNA collection tubes for final elution.
2. Transfer entire sample from the 0.2 microcentrifuge tube to a 1.5 ml RNase-free microcentrifuge tube.
3. Add 250 ul of cDNA Binding Buffer to each sample, and mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times. Spin briefly.

\*Check that the cDNA Filter Cartridge is firmly seated in its wash tube.

4. Pipet the entire cDNA sample/cDNA Binding Buffer onto the center of the cDNA Filter Cartridge.
5. Centrifuge for 1 min, or until the mixture is through the filter. Repeat steps 4 and 5 once.  
*10,000 rpm flow thru over filter. Respin.*
6. Discard flow-through and replace cDNA Filter Cartridge in the wash tube.

\*Make sure that the ethanol has been added to the bottle of Wash Buffer before using.

7. Apply 500 ul Wash Buffer to each cDNA Filter Cartridge.
8. Centrifuge for 1 min, or until all the Wash Buffer is through the filter.
9. Discard flow-through and spin the cDNA Filter Cartridge for an additional minute to remove trace amounts of Wash Buffer.
10. Transfer cDNA Filter Cartridge to a cDNA Elution Tube.

\*It is important that the Nuclease-free water is at 50-55°C for the cDNA Elution. Colder or hotter water will be less efficient at eluting the cDNA and may result in reduced cRNA yield.

11. Apply 10 ul of the preheated Nuclease-free water to the center of the filter in the cDNA Filter Cartridge.
12. Incubate at room temperature for 2 min and then centrifuge for ~1.5 min, or until all the Nuclease-free Water is through the filter.
13. Apply a second aliquot of 10 ul of preheated Nuclease-free water and centrifuge for 2 min. The double stranded cDNA will now be in the elute (~17.5 ul).

\*Store purified cDNA at -20°C or proceed to Illumina® TotalPrep™ IVT to synthesize cRNA.

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Keck Foundation Biotechnology Resource Laboratory at Yale University

## Microarray Resource

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### Illumina® TotalPrep™ IVT to synthesize cRNA

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Please do not make copies of or distribute this protocol.

A. Required reagents:

← \*T7 10X Reaction Buffer (thawed and vortexed thoroughly)

\*T7 Enzyme Mix

↗ \*Biotin-NTP Mix

\*Reagents supplied with Illumina® TotalPrep™ cDNA Amplification Kit (Ambion P/N AM11791)

B. Equipment and supplies:

Microcentrifuge with 1.5 ml rotor

Micropipettors

Aerosol-barrier tips

Vortex mixer

Powder-free gloves

1.5 ml microcentrifuge tubes

0.5 nonstick tubes

Thermal cycler programmed for 14 hours @ 37°C/4C hold

**I. Illumina TotalPrep IVT to synthesize cRNA**

*\* This procedure should begin as late in the day as possible to reduce time samples sit @ 4C in thermal cycler.*

1. Carefully transfer each cDNA sample to a 0.2 ml labeled strip tube.
2. Ensure there is no visible precipitate in T7 10X Reaction Buffer prior to use. At room temperature, prepare an IVT Master Mix by adding the following reagents to a 1.5 ml nuclease-free tube in the order listed below. Be sure to prepare n+1 or n + 2 overage to cover pipetting error. Briefly spin down T7 Enzyme Mix and keep on ice block during master mix preparation.

IVT Master Mix for a single 25 ul reaction			
Amount for 1 RXN	13 RXNS	25 RXNS	Component
2.5 ul	32.5 ul	65.0 ul	T7 10X Reaction Buffer
2.5 ul	32.5 ul	65.0 ul	T7 Enzyme Mix
2.5 ul	32.5 ul	65.0 ul	Biotin-NTP Mix

3. Mix well by gentle vortexing, spin down briefly, and place the tube on ice.
4. Transfer 7.5 ul of IVT Master Mix to each cDNA sample (volume ~17.5). Mix thoroughly by pipetting up and down 2-3 times, cap tubes, flick the tube 3-4 times. Spin briefly.
5. Place tubes in preheated thermal cycler. Run program.
6. Stop the reaction by adding 75 ul of RT Nuclease-free water to each cRNA sample to bring the final volume to 100 ul. Mix thoroughly by gentle vortexing. - set 5

\*Proceed to Illumina® TotalPrep™ cRNA cleanup or store at -20°C.

12 samples for clean-up  
at a time → rest  
Samples go in -20 freezer  
& button door

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## Illumina® TotalPrep™ cRNA cleanup

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Please do not make copies of or distribute this protocol.

A. Required reagents:

Nuclease-free water (Ambion)

Ethanol (ACS grade or equivalent proof)

\*cRNA Binding Buffer

\*Wash Buffer

B. Equipment and supplies:

1.5 ml microcentrifuge tubes

Microcentrifuge with 1.5 ml tube rotor

Vortex mixer

Air incubator at 55°C

Micropipettors

Aerosol-barrier tips

Nonstick Microcentrifuge tubes

Vortex mixer

Powder-free gloves

\*cRNA collection tubes

\*cRNA filters

\*cRNA elution tubes

\*Reagents supplied with Illumina® TotalPrep™ RNA Amplification kit (Ambion P/N AM11791)

### I. Illumina® TotalPrep™ cRNA Cleanup

\* All centrifugations in this procedure should be done at 10,000 x g (~10,000 rpm) at room temperature.

\* Before beginning the cRNA purification, preheat Nuclease-free Water to 50-55°C for at least 10 min.

\*Before using the Wash Buffer for first time, add 24 ml of 100% Ethanol.

\* Recommended batch size is 12. If doing multiple batches, do not leave reactions on ice, store at -20°C.

1. Setup and label 3 sets of tubes: (1) to transfer samples from strip tubes to 1.5 ml tubes; (2) filter column seated in collection tube; (3) 1.5 ml cRNA collection tubes for final elution.

✓ 2. Carefully transfer each IVT reaction to a 1.5 ml tube. Check to make sure that each IVT reaction was brought up to 100 ul with Nuclease-free water.

3. Set 3 P1000s: 350 ul, 250 ul, and 800 ul. Add 350 ul of cRNA Binding Buffer to one cRNA sample in a nonstick tube.

\*Proceed to the next step immediately.

\*Do steps 3-6 for each sample individually before starting the next sample.

4. Add 250 of ACS grade 100% ethanol to one cRNA sample.

\*Proceed immediately to the next step.

5. Use the P1000 set to 800 ul to mix sample by pipetting the mixture up and down 3 times.  
**Do not vortex and do not centrifuge.**

\*Proceed immediately to the next step.

6. Transfer the sample onto the center of the filter in the cRNA Filter Cartridge. Repeat steps 3-6 for each additional sample in batch.

7. Spin 1 min, or until the mixture has passed through the filter. ~~10,000 RCF~~ 10 RCF

8. Reload flow-through. Spin 1 min.

9. Discard flow-through and return cRNA Filter Cartridge to the cRNA collection tube.

\*Make sure that ethanol has been added to the Wash buffer before use.

10. Apply 650 ul of Wash Buffer to each cRNA Filter Cartridge.

11. Spin 1 min, or until Wash Buffer is through the filter.

12. Discard flow-through and spin cRNA Filter Cartridge for an additional 1 min to remove trace amounts of Wash Buffer.

13. Transfer Filter Cartridge to a fresh cRNA collection tube.

14. To the center of the filter, add 50 ul of preheated Nuclease-free water.

15. Leave at room temperature for 2 min and then centrifuge for ~1.5 min or until the Nuclease-free water is through the filter. ~~10,000 R.P.M.~~

16. To the center of the filter, add 50 ul of preheated Nuclease-free water. Centrifuge for ~1.5 min.

17. The cRNA will now be in the cRNA collection tube in ~100 ul of nuclease-free water.

18. Use spectrophotometric analysis to measure cRNA yield. Take OD at 260 nm and 280 nm to determine sample concentration and purity. The A<sub>260</sub>/A<sub>280</sub> ratio should be above 1.8. Apply the convention that 1 OD at 260 equals 40 µg/ml RNA. Log cRNA concentrations in the LTS.

19. Assess the quality of cRNA by gel electrophoresis. The expected cRNA profile is a

istribution of sizes from 250 to 5500 nucleotides with a peak at 1000-1500 nt. The intact cRNA indicates that there is no contamination, which could affect sample performance during hybridization. Log cRNA q.c. gel name in the LTS.

20. Prepare samples to 150 ng/ul in a 0.2 ml strip tube at the appropriate volume using the table below:

Microarray	Amount of sample
6- sample chip	10 ul @ 150 ng/ul = 1500 ng total
8- or 12-sample chip	5 ul @ 150 ng/ul = 750 ng total

# Check Dewad Data

## Whole-Genome Gene Expression with IntelliHyb Seal

### Hyb BC

#### Materials:

- HYB
- HCB
- Hyb. Chamber gaskets
- Hyb. Chamber
- Hyb. Chamber insert
- 10X High-Temp Wash Buffer

#### Preparation:

- Preheat Hybridization oven to 58°C
- Take out HYB and HCB to thaw (-20°C)
- Fill in tracking sheet

#### 1. Prepare Samples

- ◆ For 6-sample chip, add 1.5 ug RNA. Bring up to 10 ul with RNase-free water. Vortex and spin down.
- ◆ For 8-sample chip, add 750 ng RNA. Bring up to 5 ul with RNase-free water. Vortex and spin down.

2. Leave samples at room temp. for 10' to resuspend cRNA
3. Place HYB and HCB in 58°C hyb oven for 10'
4. Allow HYB and HCB to cool to room temp and mix thoroughly before using.
5. Add HYB
  - ◆ For 6-sample chip, add 20 ul HYB to sample
  - ◆ For 8-sample chip, add 10 ul HYB to sample
6. Vortex and spin down
7. Dispense 200 ul HCB into humidifying buffer reservoir.
8. Seal hyb chamber until ready for use
9. Place BC in hyb chamber insert

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## Whole-Genome Gene Expression with IntelliHyb Seal

### Hyb BC

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10. Incubate samples for 5' at 65°C ✓
  11. Vortex and spin down
  12. Allow samples to cool to room temp. before using
  13. Place hyb chamber insert into hyb chamber i.
  14. Dispense sample onto BC
    - ◆ For 6-sample chip, add 30 ul sample ✓
    - ◆ For 8-sample chip, add 15 ul sample l
  15. Seal hyb chamber
  16. Incubate in Hybridization Oven at 58°C for 16-20 hrs with rocker speed 5. ✓
- 

Prepare High-Temp wash buffer *Supplied by Illumina w/ chips*

1. Add 50 ml 10X stock to 450 ml RNase-free water
2. Place waterbath insert into heat block, add 500 ml prepped 1X High-Temp wash buffer.
3. Set heat block to 55°C. Close heat block and leave overnight.

## Whole-Genome Gene Expression with IntelliHyb Seal

### Wash and Stain BC

#### Materials:

- EIBC
- Block E1 Buffer
- Streptavidin-CY3
- ETOH ✓

#### Preparation:

- Take out Block E1 Buffer to thaw to room temp
- Turn on Bead Scanner
- Make Wash E1BC solution:
  - ◆ Add 7.5 ml E1BC buffer to 2.5 L RNase-free water  
*(3,3,1,5)*
- Add 1.5 L E1BC solution to pyrex dish
- Prepare Block E1 buffer with Streptavidin-CY3 (E1 + CY3)  
*FREZEX - 12 hours*
  - ◆ 2 ul Streptavidin-CY3 in 2 ml Block E1 Buffer (per chip) in a conical tube wrapped in foil to protect from light.

BC-ch

1. Remove Hyb chamber from oven
2. Remove BC, submerge in E1BC buffer solution in the pyrex container, placing it face up at the bottom of container
3. Carefully remove coverseal, be sure to keep BC submerged
4. Place rack in staining dish containing 250 ml Wash E1BC solution
5. Place BC in rack
6. Repeat for all BCs
7. Transfer wash rack to Hybex Waterbath insert containing High-Temp Wash buffer
8. Incubate 10' with lid closed ✓
9. Transfer rack to staining dish containing 250 ml fresh E1BC solution

*up / down 20X  
prep. night 6180R*

# Whole-Genome Gene Expression with IntelliHyb Seal

## Wash and Stain BC

shake up and down

10. Briefly agitate, then shake on orbital shaker 5' *Set shaker to 1*
11. Place rack to staining dish containing 250 ml ETOH
12. Briefly agitate, then shake on orbital shaker 10'
13. Place rack in staining dish containing 250 ml fresh E1BC solution
14. Briefly agitate, then shake on orbital shaker 2'
15. Pipette 4 ml Block E1 buffer into wash tray
16. Place BC face up in wash tray using tweezers
17. Rock 10' on rocker *between A & S set up*
18. Pipette 2 ml E1 + CY3 in wash tray *A set up balance for centrifuge*
19. Place BC face up in wash tray, add cover
20. Rock 10' on rocker
21. Using tweezers, transfer BC to wash rack in 250 ml fresh E1BC solution
22. Briefly agitate, then shake on orbital shaker 5'
23. Spin 4' at 275 rcf to dry BC

24. Scan

2.60

↓  
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Hi-Scan  
System

MBAK

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II

III 9030-xt

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↓  
Image Data

Gene expression

10.14.09 test

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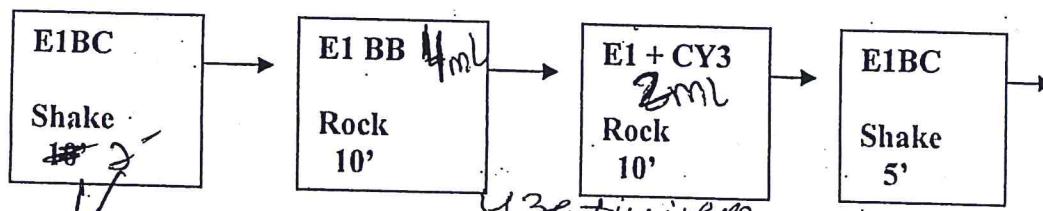
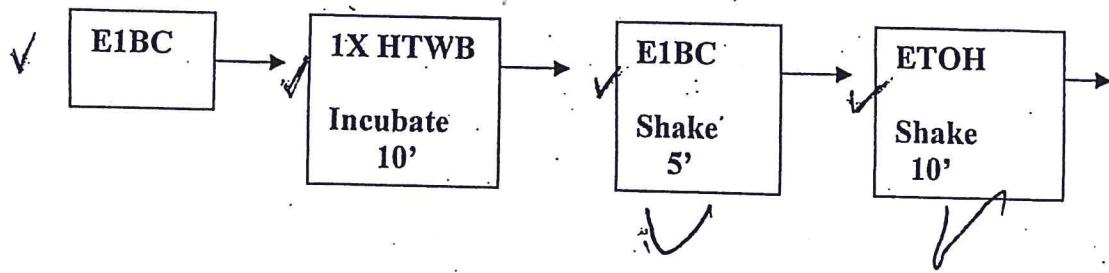
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I have to  
be green

# Whole-Genome Gene Expression with IntelliHyb Seal

## Wash and Stain BC



Spin  
275 ref  
4'

280  
≈ 4°

in centrifuge

In 5 min add Block El Gerber  
to chamber on rocker

Put chip in chamber Block  
El Gerber

*meier*

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**Ambion Illumina® TotalPrep™ cDNA Synthesis**  
P/N AMIL1791

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A. Required reagents:

- Nuclease-free Water
- \*T7 Oligo(dT) Primer
- \*10X First Strand Buffer
- \*dNTP Mix
- \*RNase Inhibitor
- \*ArrayScript
- \*10X Second Strand Buffer
- \*DNA Polymerase
- \*RNase H

\*Reagents Supplied with Illumina® TotalPrep™ RNA Amplification kit (Ambion P/N AMIL1791).

B. Equipment and supplies:

- Microcentrifuge with 1.5 ml rotor
- 1.5 ml microcentrifuge tubes
- Micropipettors
- Aerosol-barrier tips
- Thermal cycler at 42°C
- Thermal cycler at 16°C
- Heatblock at 55°C
- Vortex mixer
- Powder-free gloves
- 0.2 ml RNase-free microcentrifuge tubes
- Lab Tracking Sheets

I. FIRST STRAND cDNA SYNTHESIS:

\* Starting material: High quality total RNA in Nuclease-free water. RNA should be isolated

by using Rneasy kit (Qiagen). If RNA is isolated by any other method, perform cleanup using RNeasy kit.

Recommended amount of input RNA	
Recommended mass amount of total RNA	500 ng
Minimum mass amount of total RNA	50 ng
Maximum Volume of RNA	11 ul

\* Assess the quality of total RNA by gel electrophoresis. The intact RNA indicates that there is no contamination of RNase, which could affect cRNA synthesis in the next step. If the RNA does not appear to be intact, do not proceed with cDNA reaction until the investigator has confirmed to proceed.

\* Briefly spin down all enzymes before use. Keep enzymes on ice block during master mix preparation.

\* Log sample names and concentrations in Lab Tracking Sheets.

\* Recommended batch size is 24. For larger batches, stagger the incubation times by ~30 min.

### I. FIRST STRAND cDNA SYNTHESIS:

1. Place a maximum volume of 11 ul of total RNA (50-500 ng) into an RNase-free 0.2 ml BioRad microcentrifuge tube.
2. Add Nuclease-free water to bring the samples to 11 ul.
3. At room temperature, prepare Reverse Transcription Master Mix in a 1.5 ml nuclease-free tube. Be sure to prepare n+1 overage to cover pipetting error.

#### Reverse Transcription Master Mix

Amount for 1 RXN	13 RXNS	26 RXNS	Component
1 ul	13 ul	26 ul	T7 Oligo(dT) Primer
2 ul	26 ul	52 ul	10X First Strand Buffer
4 ul	52 ul	104 ul	dNTP Mix
1 ul	13 ul	26 ul	RNase Inhibitor
1 ul	13 ul	26 ul	ArrayScript

4. Mix well by gently vortexing. Centrifuge to collect master mix at bottom of the tube and place on ice.
5. Transfer 9 ul of Reverse Transcription Master Mix to each RNA sample. Mix thoroughly by pipetting up and down 2-3 times, cap tubes, flick the tube 3-4 times. Centrifuge briefly to collect the reaction at the bottom of the tube.
6. Place the samples in a 42°C thermal cycler. Incubate reactions for 2 hours at 42°C. Log time of incubation start in LTS. During this incubation, keep dNTPs on ice. Begin thawing Second Strand buffer to room temperature after ~~1.5 hrs~~ of 42C incubation.

12 15 10

- ✓. After the incubation, centrifuge briefly to collect the reaction mixture at the bottom of the tube and place on ice. Log end time of incubation on LTS.

8. Proceed immediately to Second Strand Synthesis.

## II. SECOND STRAND cDNA SYNTHESIS:

- 1 On ice block, prepare the Second Strand Master Mix in a 1.5 ml nuclease-free tube (or larger if needed) in the order listed below. Be sure to prepare  $n+1$  overage to cover pipetting error.

Second Strand Master Mix			
Amount for 1 RXN	13 RXNS	25 RXNS	Component
63 ul	819 ✓	1575	Nuclease-free Water <i>Put</i>
10 ul	130 ✓	250	10X Second Strand Buffer
4 ul	52 ✓	100	dNTP Mix
2 ul	26 ✓	50	DNA Polymerase
1 ul	13 ✓	25	RNase H

2. Mix well by gently vortexing. Centrifuge briefly.
  3. Transfer 80  $\mu$ l of Second Strand Master Mix to each sample. Mix thoroughly by pipetting up and down 2-3 times, cap tubes, flick the tube 3-4 times, and centrifuge briefly to collect the reaction in the bottom of the tube.
  4. Place tubes in the 16°C thermal cycler. Incubate for 2 hours. Log incubation start time in LTS.

\*If the lid temperature cannot be adjusted to match the 16°C block temperature, cover the tubes with the lid turned off, or if the lid cannot be turned off-do not cover the tubes with it.

5. After the 2 hour incubation, place the reactions on ice, log end of incubation time in LTS and proceed to Illumina® TotalPrep™ cDNA cleanup. Do not freeze reactions at this point unless in an emergency situation.

12<sup>75</sup> 12<sup>15</sup> 11<sup>30</sup> 11<sup>50</sup> 2<sup>40</sup> 12<sup>40</sup> 12<sup>30</sup> 12<sup>25</sup>) 13<sup>40</sup> 13<sup>20</sup> 13<sup>05</sup> 12<sup>05</sup> 12<sup>05</sup> 12<sup>05</sup> 12<sup>05</sup> 12<sup>05</sup>

-00/ to room temperature before use.

\*Before using the Wash Buffer for first time, add 24 ml of 100% Ethanol.

\*Recommended batch size is 12 at a time. If doing multiple batches, do not leave reactions on ice for more than an hour.

1. Setup and label 3 sets of tubes: (1) to transfer samples from strip tubes to 1.5 ml tubes; (2) filter column seated in collection tube; (3) 1.5 ml cDNA collection tubes for final elution.
2. Transfer entire sample from the 0.2 microcentrifuge tube to a 1.5 ml RNase-free microcentrifuge tube.
3. Add 250 ul of cDNA Binding Buffer to each sample, and mix thoroughly by pipetting up and down 2-3 times, then flicking the tube 3-4 times. Spin briefly.

\*Check that the cDNA Filter Cartridge is firmly seated in its wash tube.

4. Pipet the entire cDNA sample/cDNA Binding Buffer onto the center of the cDNA Filter Cartridge.
5. Centrifuge for 1 min, or until the mixture is through the filter. Repeat steps 4 and 5 once.
6. Discard flow-through and replace cDNA Filter Cartridge in the wash tube.

\*Make sure that the ethanol has been added to the bottle of Wash Buffer before using.

7. Apply 500 ul Wash Buffer to each cDNA Filter Cartridge.
8. Centrifuge for 1 min, or until all the Wash Buffer is through the filter.
9. Discard flow-through and spin the cDNA Filter Cartridge for an additional minute to remove trace amounts of Wash Buffer.
10. Transfer cDNA Filter Cartridge to a cDNA Elution Tube.

\*It is important that the Nuclease-free water is at 50-55°C for the cDNA Elution. Colder or hotter water will be less efficient at eluting the cDNA and may result in reduced cRNA yield.

11. Apply 10 ul of the preheated Nuclease-free water to the center of the filter in the cDNA Filter Cartridge.
12. Incubate at room temperature for 2 min and then centrifuge for ~1.5 min, or until all the Nuclease-free Water is through the filter.
13. Apply a second aliquot of 10 ul of preheated Nuclease-free water and centrifuge for 2 min. The double stranded cDNA will now be in the elute (~17.5 ul).

\*Store purified cDNA at -20°C or proceed to Illumina® TotalPrep™ IVT to synthesize cRNA.

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Keck Foundation Biotechnology Resource Laboratory at Yale University

## Microarray Resource

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### Illumina® TotalPrep™ IVT to synthesize cRNA

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A. Required reagents:

- ← \*T7 10X Reaction Buffer (thawed and vortexed thoroughly)
- \*T7 Enzyme Mix
- \*Biotin-NTP Mix

\*Reagents supplied with Illumina® TotalPrep™ cDNA Amplification Kit (Ambion P/N AM11791)

B. Equipment and supplies:

Microcentrifuge with 1.5 ml rotor

Micropipettors

Aerosol-barrier tips

Vortex mixer

Powder-free gloves

1.5 ml microcentrifuge tubes

0.5 nonstick tubes

Thermal cycler programmed for 14 hours @ 37°C/4C hold

I. Illumina TotalPrep IVT to synthesize cRNA

*\* This procedure should begin as late in the day as possible to reduce time samples sit @ 4C in thermal cycler.*

1. Carefully transfer each cDNA sample to a 0.2 ml labeled strip tube.
2. Ensure there is no visible precipitate in T7 10X Reaction Buffer prior to use. At room temperature, prepare an IVT Master Mix by adding the following reagents to a 1.5 ml nuclease-free tube in the order listed below. Be sure to prepare n+1 or n + 2 overage to cover pipetting error. Briefly spin down T7 Enzyme Mix and keep on ice block during master mix preparation.

IVT Master Mix for a single 25 ul reaction			
Amount for 1 RXN	13 RXNS	25 RXNS	Component
2.5 ul	32.5 ul	65.0 ul	T7 10X Reaction Buffer
2.5 ul	32.5 ul	65.0 ul	T7 Enzyme Mix
2.5 ul	32.5 ul	65.0 ul	Biotin-NTP Mix

3. Mix well by gentle vortexing, spin down briefly, and place the tube on ice.
4. Transfer 7.5 ul of IVT Master Mix to each cDNA sample (volume ~17.5). Mix thoroughly by pipetting up and down 2-3 times, cap tubes, flick the tube 3-4 times. Spin briefly.
5. Place tubes in preheated thermal cycler. Run program.
6. Stop the reaction by adding 75 ul of RT Nuclease-free water to each cRNA sample to bring the final volume to 100 ul. Mix thoroughly by gentle vortexing. - set 5

\*Proceed to Illumina® TotalPrep™ cRNA cleanup or store at -20°C.

12 samples for clean - up  
 at a time → rest  
 Samples go in -20 freezer  
 & button door }  
 113

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Keck Foundation Biotechnology Resource Laboratory at Yale University  
**Microarray Resource**

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## Illumina® TotalPrep™ cRNA cleanup

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Please do not make copies of or distribute this protocol.

A. Required reagents:

- Nuclease-free water (Ambion)
- Ethanol (ACS grade or equivalent proof)
- \*cRNA Binding Buffer
- \*Wash Buffer

B. Equipment and supplies:

- 1.5 ml microcentrifuge tubes
- Microcentrifuge with 1.5 ml tube rotor
- Vortex mixer
- Air incubator at 55°C
- Micropipettors
- Aerosol-barrier tips
- Nonstick Microcentrifuge tubes
- Vortex mixer
- Powder-free gloves
- \*cRNA collection tubes
- \*cRNA filters
- \*cRNA elution tubes

\*Reagents supplied with Illumina® TotalPrep™ RNA Amplification kit (Ambion P/N AMIL1791)

### I. Illumina® TotalPrep™ cRNA Cleanup

**\* All centrifugations in this procedure should be done at 10,000 x g (~10,000 rpm) at room temperature.**

\* Before beginning the cRNA purification, preheat Nuclease-free Water to 50-55°C for at least 10 min.

\*Before using the Wash Buffer for first time, add 24 ml of 100% Ethanol.

\* Recommended batch size is 12. If doing multiple batches, do not leave reactions on ice, store at -20°C.

1. Setup and label 3 sets of tubes: (1) to transfer samples from strip tubes to 1.5 ml tubes; (2) filter column seated in collection tube; (3) 1.5 ml cRNA collection tubes for final elution.

2. Carefully transfer each IVT reaction to a 1.5 ml tube. Check to make sure that each IVT reaction was brought up to 100 ul with Nuclease-free water.

3. Set 3 P1000s: 350 ul, 250 ul, and 800 ul. Add 350 ul of cRNA Binding Buffer to one cRNA sample in a nonstick tube.

\*Proceed to the next step immediately.

\*Do steps 3-6 for each sample individually before starting the next sample.

4. Add 250 of ACS grade 100% ethanol to one cRNA sample.

\*Proceed immediately to the next step.

5. Use the P1000 set to 800 ul to mix sample by pipetting the mixture up and down 3 times.

**Do not vortex and do not centrifuge.**

\*Proceed immediately to the next step.

6. Transfer the sample onto the center of the filter in the cRNA Filter Cartridge. Repeat steps 3-6 for each additional sample in batch.

7. Spin 1 min, or until the mixture has passed through the filter. 10,000 RCF 10 RCF

8. Reload flow-through. Spin 1 min.

9. Discard flow-through and return cRNA Filter Cartridge to the cRNA collection tube.

\*Make sure that ethanol has been added to the Wash buffer before use.

10. Apply 650 ul of Wash Buffer to each cRNA Filter Cartridge.

11. Spin 1 min, or until Wash Buffer is through the filter.

12. Discard flow-through and spin cRNA Filter Cartridge for an additional 1 min to remove trace amounts of Wash Buffer.

13. Transfer Filter Cartridge to a fresh cRNA collection tube.

14. To the center of the filter, add 50 ul of preheated Nuclease-free water.

15. Leave at room temperature for 2 min and then centrifuge for ~1.5 min or until the Nuclease-free water is through the filter. 10,000 RPF

16. To the center of the filter, add 50 ul of preheated Nuclease-free water. Centrifuge for ~1.5 min.

17. The cRNA will now be in the cRNA collection tube in ~100 ul of nuclease-free water.

18. Use spectrophotometric analysis to measure cRNA yield. Take OD at 260 nm and 280 nm to determine sample concentration and purity. The  $A_{260}/A_{280}$  ratio should be above 1.8. Apply the convention that 1 OD at 260 equals 40  $\mu$ g/ml RNA. Log cRNA concentrations in the LTS.

19. Assess the quality of cRNA by gel electrophoresis. The expected cRNA profile is a

istribution of sizes from 250 to 5500 nucleotides with a peak at 1000-1500 nt. The intact cRNA indicates that there is no contamination, which could affect sample performance during hybridization. Log cRNA q.c. gel name in the LTS.

20. Prepare samples to 150 ng/ul in a 0.2 ml strip tube at the appropriate volume using the table below:

Microarray	Amount of sample
6- sample chip	10 ul @ 150 ng/ul = 1500 ng total
8- or 12-sample chip	5 ul @ 150 ng/ul = 750 ng total

# Check Readout Data

## Whole-Genome Gene Expression with IntelliHyb Seal

### Hyb BC

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#### Materials:

- HYB
  - HCB
  - Hyb. Chamber gaskets
  - Hyb. Chamber
  - Hyb. Chamber insert
  - 10X High-Temp Wash Buffer
- 

#### Preparation:

- Preheat Hybridization oven to 58°C
  - Take out HYB and HCB to thaw (-20°C)
  - Fill in tracking sheet
- 

#### 1. Prepare Samples

- ◆ For **6-sample chip**, add 1.5 ug RNA. Bring up to 10 ul with RNase-free water. Vortex and spin down.
- ◆ For **8-sample chip**, add 750 ng RNA. Bring up to 5 ul with RNase-free water. Vortex and spin down.

2. Leave samples at room temp. for 10' to resuspend cRNA ✓
3. Place HYB and HCB in 58°C hyb oven for 10' ✗
4. Allow HYB and HCB to cool to room temp and mix thoroughly before using. ✗
5. Add HYB
  - ◆ For **6-sample chip**, add 20 ul HYB to sample ✓
  - ◆ For **8-sample chip**, add 10 ul HYB to sample ✓
6. Vortex and spin down ✓
7. Dispense 200 ul HCB into humidifying buffer reservoir. ✓
8. Seal hyb chamber until ready for use ✓
9. Place BC in hyb chamber insert ✓

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## Whole-Genome Gene Expression with IntelliHyb Seal

### Hyb BC

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10. Incubate samples for 5' at 65°C ✓
  11. Vortex and spin down
  12. Allow samples to cool to room temp. before using
  13. Place hyb chamber insert into hyb chamber i.
  14. Dispense sample onto BC
    - ◆ For **6-sample chip**, add 30 ul sample ✓
    - ◆ For **8-sample chip**, add 15 ul sample l
  15. Seal hyb chamber
  16. Incubate in Hybridization Oven at 58°C for 16-20 hrs with rocker speed 5. ✓
- 

Prepare High-Temp wash buffer *Supplied by Illumina w/ chips*

1. Add 50 ml 10X stock to 450 ml RNase-free water
2. Place waterbath insert into heat block, add 500 ml prepped 1X High-Temp wash buffer.
3. Set heat block to 55°C. Close heat block and leave overnight.

## Whole-Genome Gene Expression with IntelliHyb Seal

### Wash and Stain BC

#### Materials:

- EIBC
- Block E1 Buffer
- Streptavidin-CY3
- ETOH ✓

#### Preparation:

- Take out Block E1 Buffer to thaw to room temp ✓
- Turn on Bead Scanner
- Make Wash E1BC solution:
  - ◆ Add 7.5 ml E1BC buffer to 2.5 L RNase-free water  
*(3,3,1,5)*
- Add 1.5 L E1BC solution to pyrex dish
- Prepare Block E1 buffer with Streptavidin-CY3 (E1 + CY3)  
*FREZER-1°C min*
- 2 ul Streptavidin-CY3 in 2 ml Block E1 Buffer (per chip) in a conical tube wrapped in foil to protect from light.

BC-ch

1. Remove Hyb chamber from oven
2. Remove BC, submerge in E1BC buffer solution in the pyrex container, placing it face up at the bottom of container
3. Carefully remove coverseal, be sure to keep BC submerged
4. Place rack in staining dish containing 250 ml Wash E1BC solution *up/down 20X*
5. Place BC in rack
6. Repeat for all BCs *prep ap. night before*
7. Transfer wash rack to Hybex Waterbath insert containing High-Temp Wash buffer
8. Incubate 10' with lid closed ✓
9. Transfer rack to staining dish containing 250 ml fresh E1BC solution

# Whole-Genome Gene Expression with IntelliHyb Seal

## Wash and Stain BC

Shake up and down

10. Briefly agitate, then shake on orbital shaker 5' ~~Set shaker to 1~~
11. Place rack to staining dish containing 250 ml ETOH
12. Briefly agitate, then shake on orbital shaker 10'
13. Place rack in staining dish containing 250 ml fresh E1BC solution
14. Briefly agitate, then shake on orbital shaker 2' ~~Set shaker to 1~~
15. Pipette 4 ml Block E1 buffer into wash tray
16. Place BC face up in wash tray using tweezers
17. Rock 10' on rocker ~~between 1 & 5~~ set tray ~~set tray~~  
~~Set up balance~~  
~~for centrifuge~~
18. Pipette 2 ml E1 + CY3 in wash tray
19. Place BC face up in wash tray, add cover
20. Rock 10' on rocker
21. Using tweezers, transfer BC to wash rack in 250 ml fresh E1BC solution
22. Briefly agitate, then shake on orbital shaker 5'
23. Spin 4' at 275 rcf to dry BC
24. Scan 280

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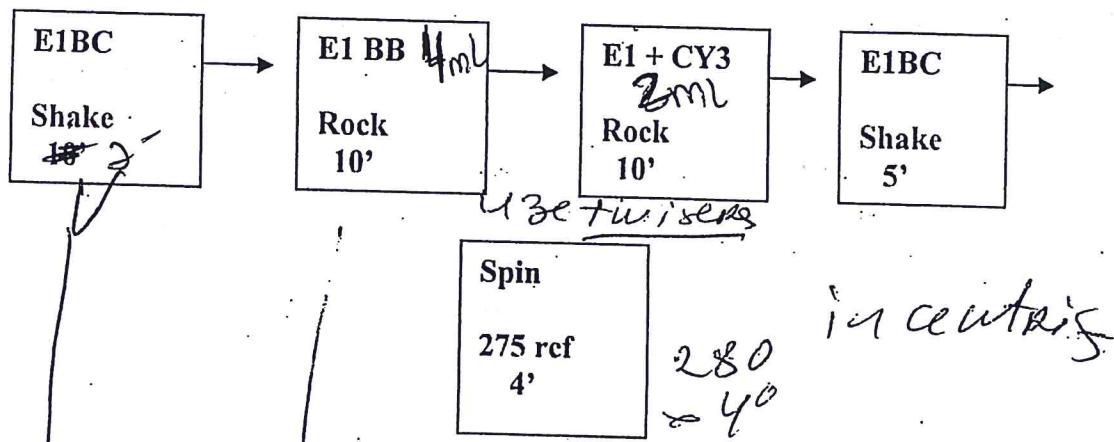
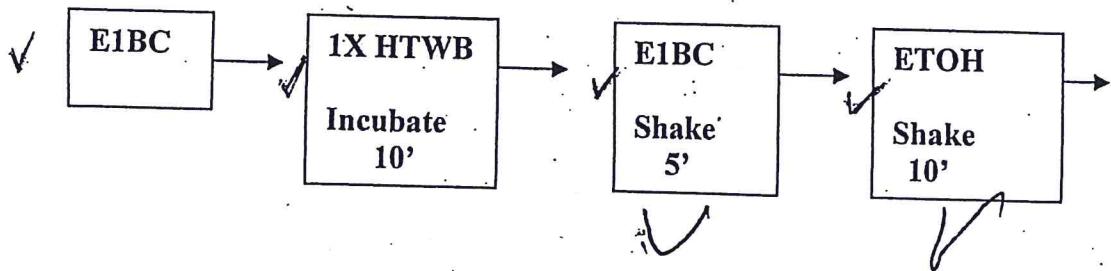
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I have to  
be green

# Whole-Genome Gene Expression with IntelliHyb Seal

## Wash and Stain BC



Handwritten instructions:

- In 5 min add Block El Beaker to chamber on rocker
- Put chip in chamber <sup>N</sup> <sub>Block</sub> <sub>Buffer</sub> <sub>Beaker</sub>