**UW Medicine - Pathology**

400-11-01-16

**Labeling of Genomic DNA with Random Priming for Array CGH+SNP**

**Using Agilent Dual-Color DNA Labeling Kit**

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| Adopted Date: November. 12, 2011Review Date: November. 21, 2011Revision Date: Nov. 2011, Jan. 2012, June 2012Under Revision: November 2012 |

PURPOSE

### To label the gDNA samples and the normal control reference DNA in different fluorochromes using a Agilent Dual-Color DNA Labeling Kit through random priming with Cy dUTP

PROCEDURE

### SPECIMEN REQUIREMENTS

### 500 ~750 ng patient gDNA

### 500~750 ng normal control gDNA (gender matched, #5190-4370 for male, #5190-4371 for female)

### Material and Equipment

1. Spectrophotometer NanoDrop 2000c
2. Heat block capable of temperatures to 98°C
3. MJ Research Thermal Cycler
4. Microcentrifuge (12,000 x g capability)
5. SpeedVac Thermo Savant
6. Vortex mixer
7. Pipettmen p10, p20, p200, p1000
8. Aerosol-resistant sterile pipette tips
9. Sterile microcentrifuge tubes, 2.0 ml,1.5 ml, 0.6 ml, 0.2 ml
10. Ice buckets
	1. **REAGENTS**

SureTag DNA Labeling Kit (Agilent #5190-3400)\*

1. DNase/RNase-free distilled water
2. Klenow Fragment (3'->5' exo-) 50U/μL
3. 5x Reaction Buffer
4. Random Primer
5. Cyanine 3-dUTP
6. Cyanine 5-dUTP
7. 10x dNTPs
8. Alu I (10 U/μL)‡
9. Rsa I (10 U/μL)‡, which includes
10. 10× Restriction Enzyme Buffer C
11. Acetylated BSA (10 mg/μL)
12. Amicon Ultra-0.5, Ultracel-30 Membrabe, 30 kDa Purification Column (50 units)

 Additional Reagents:

1. Human Genomic DNA
2. 1 × TE (pH 8.0), Molecular grade (Fish BP2473-100)

### SAFETY PRECAUTIONS:

1. **Wear gloves** and take precautions to avoid sample contamination.
2. Cy dyes are **light sensitive**. Be sure to minimize light exposure of the dyes during use and store in the dark immediately after use.
3. Cy dyes are **ozone sensitive**. Take the necessary precautions to keep atmospheric ozone levels below 20ppb (parts per billion).
4. Cy dyes are **humidity sensitive**. Take the necessary precautions to keep humidity levels below 40%.

### Procedures

1. **Restriction Digestion with the Suretag DNA Labeling Kit**

***Note:*** a DNA concentration step is required before the restriction digestion, you must avoid carrying over high amounts of salt, EDTA, and contaminants to the restriction digestion reaction.

1. Equilibrate heat blocks or water baths to 37°C and 65°C or use a thermal cycler.
2. Thaw 10× Restriction Enzyme Buffer and BSA (included in the SureTag DNA Labeling Kit). Flick the tube to briefly mix, and spin in a microcentrifuge.
3. Store all reagents on ice while in use and return promptly to -20°C.
4. Label PCR tubes. For each reaction, add the amount of gDNA to the appropriate nuclease-free tube and add enough DNase/RNase-free distilled water to bring to the final volume to 20.8 μL (full reaction) or 10 μL (for half reaction), which is listed in Appendix D on page ?.
5. Prepare the Digestion Master Mix by mixing the components in **Table 1**, on ice in the order indicated. Mix well by pipetting up and down.
6. Add 5.8 μL for a full reaction or 3 μL for an half -reaction of Digestion Master Mix to each reaction tube containing the gDNA to make a total volume of 26 μL (for full reaction) or 13 μL (for half reaction) . Mix well by pipetting up and down.

**Table 1**. Digestion Master Mix (2x and 4x arrays)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Component** | **Per full reaction****(μL)** | **× 8 rxns (μL)****(including****excess)** | **Per half reaction****(μL)** | **× 8 rxns (μL)****(including****excess)** |
| Nuclease-Free Water | 2 | 18 | 1.1 | 9.9 |
| 10× Restriction EnzymeBuffer C | 2.6 | 23.4 | 1.3 | 11.7 |
| Acetylated BSA (10ng/ μL) | 0.2 | 1.8 | 0.1 | 0.9 |
| Alu I (10U/ μL) | 0.5 | 4.5 | 0.25 | 2.25 |
| Rsa I (10U/μL) | 0.5 | 4.5 | 0.25 | 2.25 |
| **Final volume of Master mix** | 5.8 | 52.5 | 3 | 27 |

1. Incubate the samples:
2. Transfer sample tubes to a circulating water bath or heat block at 37°C. Incubate at 37°C for 2 hours.
3. Transfer sample tubes to a circulating water bath or heat block at 65°C. Incubate at 65°C for 20 minutes to inactivate the enzymes.
4. Move the sample tubes to ice.
5. Or instead of a-c, transfer sample tubes or plates to a thermal cycler. Program the thermal cycler according to **Table 2** and run the program:

**Table 2**. Digestion program

|  |  |  |
| --- | --- | --- |
| **Step** | **Temperature** | **Time** |
| Step 1 | 37°C | 2 hours |
| Step 2 | 65°C | 20 minutes |
| Step 3 | 4°C | Hold |

1. ***Optional***. Take 2 μL of the digested gDNA and run on a 1.2% agarose gel stained with SYBR Gold or EB to assess the completeness of the digestion. The majority of the digested products should be between 200bp in length.
2. Proceed directly to Sample labeling on page 15 or store digested gDNA for up to a month at -20°C
	1. **Fluorescent Labeling of DNA**

***Note:*** Cyanine 3-dUTP and cyanine 5-dUTP are light sensitive and are subject to degradation

by multiple freeze thaw cycles. Minimize light exposure throughout the labeling

 procedure.

1. Equilibrate heat blocks or water baths to 95°C, 37°C and 65°C, or use a thermal cycler.
2. Spin the samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
3. Add Random Primer:
	1. For **FULL** reaction, add 5 μL of Random Primer to each reaction tube containing 26 μL of gDNA to make a total volume of 31 μL (or 24 μL of gDNA to make a total volume of 29 μL if the optional agarose gel step on page ? was done). Mix well by pipetting up and down gently.
	2. For **HALF** reaction, add 2.5 μL of Random Primer to each reaction tube that contains 13 μL of gDNA to make a total volume of 15.5 μL (or 11 μL of gDNA to make a total volume of 15.5 μL if the optional agarose gel step on page ? was done). Mix well by pipetting up and down gently.
4. Transfer sample tubes to a thermal cycler. Incubate at 95°C for 3 minutes (hot start, with restriction digestion) or 10 minutes (without restriction digestion), then move to ice and incubate on ice for 5 minutes.

**Table 3.** DNA denaturation and fragmentation using a thermal cycler

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temperature** | **Time (with restriction digestion)** | **Time (without restriction digestion)** |
| Step 1 | 95°C | 3 minutes | 10 minutes |
| Step 2 | 4°C | Hold | Hold |

***Note***: For a wide variety of samples, high quality microarray data is achieved when the restriction digestion step is used before the labeling step. But you can also achieve high quality data if you replace the restriction digestion step by a longer incubation at 95°C after you add the random primers in the labeling reaction step. See “Step 1. Fluorescent Labeling of gDNA” on 12 SurePrint G3 CGH+SNP microarrays can only be processed with restriction digestion and not by heat fragmentation.

1. Spin the samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
2. Add Labeling Master Mix as table 4 for 2-pack and 4-pack microarrays:
	1. Mix the components in Table 4 on ice in the order indicated to prepare one cyanine 3 and one cyanine 5 Labeling Master Mix.
	2. Add 19 μL (or 21 μL) of Labeling Master Mix to each reaction tube containing the gDNA to make a total volume of 50 μL. Mix well by gently pipetting up and down

 **Table 4.** Labeling Master Mix (2x and 4x arrays)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Component** | **1 x FULL reaction (μL)** | **× 8 rxns (μL)****(including excess)** | **1 x HALF reaction (μL)** | **× 8 rxns (μL)****(including excess)** |
| Nuclease-Free Water | 2.0\* | 17\* | 1\* | 10\* |
| 5× Reaction Buffer | 10.0 | 85 | 5 | 50 |
| 10× dNTPs | 5.0 | 42.5 | 2.5 | 25 |
| Cyanine 3-dUTP *or*Cyanine 5-dUTP | 3.0 | 25.5 | 1.5 | 15 |
| Exo (-) Klenow | 1.0 | 8.5 | 0.5 | 5 |
| **Final volume of Labeling****Master Mix** | **19.0 or 21.0\*** | **161.5 or 178.5\*** | **9.5 or 10.5** | **95 or 105** |

 **\**Note***: Do *not* add Nuclease-Free Water if you skipped the optional agarose gel step (step 7 on page ).

1. Transfer sample tubes to a thermal cycle. Program the thermal cycler according to Table 5 and run the program

 **Table 5.** DNA labeling using a thermal cycler

|  |  |  |
| --- | --- | --- |
| **Step** | **Temperature** | **Time** |
| Step 1 | 37°C | 2 hours |
| Step 2 | 65°C | 10 minutes |
| Step 3 | 4°C | hold |

 ***Note***: Reactions can be stored up to a month at -20°C in the dark.

1. **Clern-Up Labeled gDNA with Purification Column**
2. Spin the labeled gDNA samples in a centrifuge for 1 minute at 6,000 × g to drive the contents off the walls and lid.
3. Transfer sample to 1.6 ml microcentrifuge tube.
4. Add 430 μL of 1× TE (pH 8.0), Molecular grade to each reaction tube.
5. For each gDNA sample to be purified, place a column into a 2-mL collection tube and label the column appropriately. Load each labeled gDNA onto a column.
6. Cover the column with a cap and spin for 10 minutes at 13,000 × rpm in a microcentrifuge at room temperature. Discard the flow-through and place the column back in the 2-mL collection tube.
7. Add 480 μL of 1× TE (pH 8.0), Molecular grade to each column. Spin for 10 minutes at 13,000 × rpm in a microcentrifuge at room temperature. Discard the flow-through.
8. Invert the column into a fresh 2-mL collection tube that has been appropriately labeled. Spin for 1 minute at 1,000 × g (2600 rpm) in a microcentrifuge at room temperature to collect purified sample. The volume per sample will be approximately 20 to 32 μL.
9. Add 1× TE (pH 8.0), Molecular grade, or use a concentrator to bring the sample volume to that listed in Table 6. Do not excessively dry the gDNA because the pellets will become difficult to resuspend.

***Note***: Keep Cyanine-3 and Cyanine-5 labeled gDNA samples separated throughout this clean-up step.

1. Incubate the tube that contains gDNA sample on ice for 5 minutes, and then pipette the solution up and down 10 times.
2. Take 1.5 μL of each sample to determine yield and specific activity. See “To determine yield, degree of labeling or specific activity” on page 17. Refer to Table 7 on page 17 for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.
3. In a fresh 1.5 mL RNase-free Microfuge Tube combine test and reference sample using the appropriate cyanine 5-labeled sample and cyanine 3-labeled sample for a total mixture volume listed in Table 6. Use the appropriate container listed in Table 6.

***Note***: Labeled DNA can be stored up to one month at -20°C in the dark.

 **Table 6.** Sample volume and total mixture volumes

|  |  |  |  |
| --- | --- | --- | --- |
| **Microarray** | **Cy3 or Cy5 sample volume after purification** | **Total mixture volume after Nanodrop and combining** | **Container** |
| 2x array | 41 μL | 79 μL | 1.6 mL microfuge tube |
| 4x array | 21 μL | 39 μL | 1.6 mL microfuge tube |

1. **Determine Yield, Degreee of Labeling or Specific Activity**

1. From the main menu, select **MicroArray Measurement**, then from the **Sample Type** menu, select **DNA-50**.
2. Use 1.5 μL of 1× TE (pH 8.0), Molecular grade to blank the instrument.
3. Use 1.5 μL of purified labeled gDNA for quantitation. Measure the absorbance at A260nm (DNA), A550nm (cyanine 3), and A650nm (cyanine 5).
4. Calculate the Degree of Labeling or Specific Activity of the labeled gDNA:

 340 x pmol per μL dye

Degree of Labeling = ------------------------------------x 100%

 ng per μL gDNA x 1000

 pmol per μL of dye

Specific Activity\* = --------------------------------------

 μg per μL gDNA

\*pmol dyes per μg gDNA

The Specific Activity is Degree of Labeling divided by 0.034.

1. Record the gDNA concentration (ng/μL) for each sample. Calculate the yield as

 DNA Concentration (ng/μL) x Sample Volume (μL)

Yield (μg) = -------------------------------------------------------------------------

1. μg

Refer to **Table 7** for expected yield of labeled gDNA and specific activity after labeling and clean-up, when starting with high quality gDNA.

**Table 7.** Expected Yield and Specific Activity after Labeling and Clean-up with the SureTag DNA Labeling Kit

|  |  |  |  |
| --- | --- | --- | --- |
| **Input gDNA (μg)** | **Yield (μg)** | **Specific Activity of Cyanine-3 Labeled Sample (pmol/μg)** | **Specific Activity of Cyanine-5 Labeled Sample (pmol/μg)** |
| 0.2 | 3 to 5 | 20 to 25 | 15 to 25 |
| 0.5 | 8 to 11 | 20 to 35 | 20 to 30 |
| 1 | 9 to 12 | 25 to 40 | 1. to 35
 |

**Note:** If you replaced the restriction digestion step by a longer incubation at 95°C, the Specific Activity of Cyanine-3 and -5 Labeled Sample will be about 5 pmol/μg lower than the values indicated in Table 7. The Yield after labeling and the signal to noise of the microarrays will be the same. The cyanine 3 and cyanine 5 yields after labeling should be the same. If not, refer to “Troubleshooting” on page 95 of Agilent Arrays-based CGH for genomic DNA analysis protocol version 7.1 .

1. **REFERRENCE**

Agilent Arrays-based CGH for genomic DNA analysis protocol version 7.1 p. 33-62

Written By: Director Approval:

(Signature and Date) (Signature and Date)

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**UW Medicine - Pathology**

**Cytogenetics and Genomics**

**SIGNATURE PAGE FOR POLICIES AND PROCEDURES**

Procedure / Policy Title: **Labeling of Genomic DNA with Random Priming for Array CGH+SNP Using Agilent Dual-Color DNA Labeling Kit**

Procedure / Policy Number: 400-11-01-16

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