Product Manual

CYTAG® CGH Labeling Kit

for preparation of Cyanine 3- and Cyanine 5-labeled DNA
for hybridization to Agilent & other arrays

Instruction Manual

ENZ-42671-K010

10 reactions for labeling with Cyanine 3-dUTP and 10 reactions for labeling with Cyanine 5-dUTP

ENZ-42671-K100
100 reactions for labeling with Cyanine 3-dUTP and 100 reactions for labeling with Cyanine 5-dUTP

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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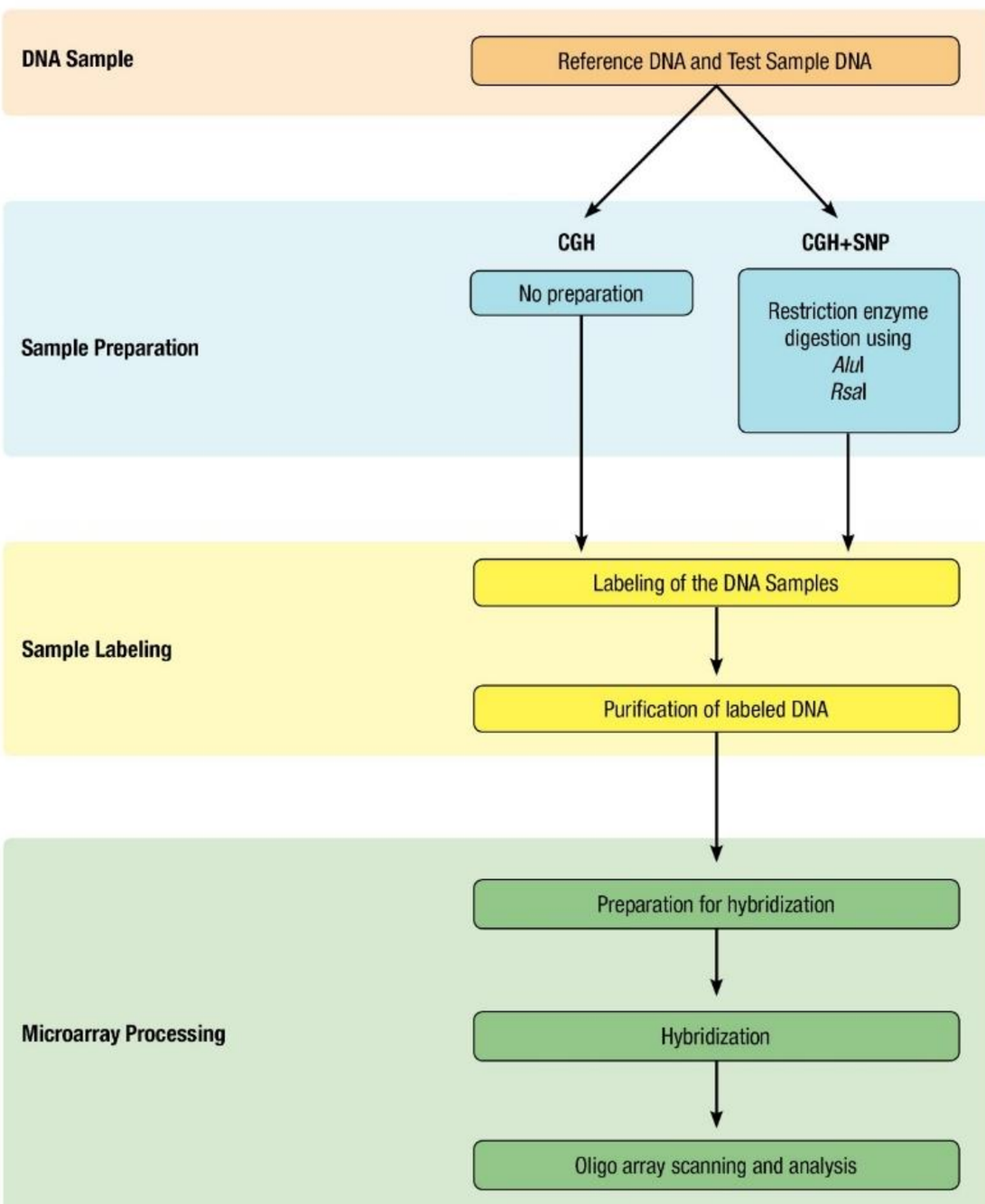
INTRODUCTION

Enzo Life Sciences' **CYTAG® CGH Labeling Kit** has been optimized to label DNA for dual sample comparative genomic hybridization (CGH) assays on Agilent or other arrays. Our CGH kits are not compatible with Affymetrix and other arrays that do not detect Cyanine 3 and Cyanine 5. All required reagents are provided including fully optimized nucleotide mixes that contain either Cyanine 3-dUTP or Cyanine 5-dUTP.

ARRAY CGH WORKFLOW



REAGENTS PROVIDED AND STORAGE

Avoid repeated freeze/ thaw cycles.

The **CYTAG® CGH Labeling Kit** is shipped on dry ice. Upon receipt, store all reagents at -20°C in a non-frost-free freezer.

Avoid repeated freezing and thawing of the kit reagents. We recommend aliquoting the kit reagents into volumes convenient for your workflow during the first use. Cyanine 3- and Cyanine 5-labeled deoxynucleotide mixes are light sensitive. Protect from light exposure at all times. The product is stable for one year upon receipt when stored as recommended.

Reagent	Vial ID	Min. Volume Supplied	
		ENZ-42671-K010	ENZ-42671-K100
Primers/Reaction Buffer	1	400 µL	4 mL
Cyanine 3-dUTP Nucleotide Mix	2	2 x 50 µL	1 mL
Cyanine 5-dUTP Nucleotide Mix	3	2 x 50 µL	1 mL
Klenow DNA Polymerase	4	20 µL	200 µL
Stop Buffer	5	100 µL	1 mL
Nuclease-free Water	W	1 mL	10 mL

PRECAUTIONS

Protect from light

Handle with care

- To avoid photobleaching of cyanine-labeled nucleotides, perform all manipulations in amber microcentrifuge tubes or protected from light by other means.
- The Klenow DNA Polymerase contains dithiothreitol (DTT). DTT causes irritation to the skin, eyes and respiratory tract. It is harmful if swallowed or inhaled. Should the solution come in contact with skin or eyes, wash immediately with water.

OTHER MATERIALS REQUIRED BUT NOT PROVIDED

1. Reference DNA
    a. For CGH only arrays, we recommend Genomic DNA (human), male (Enzo Prod. No. ENZ-GEN106) or female (Enzo Prod. No. ENZ-GEN107).
    b. For CGH+SNP arrays, use genotyped HapMap DNA (e.g., from Coriell Institute) as recommended by array manufacturer.
2. Water baths, heating blocks, or incubators set at 37°C and 99°C or a PCR machine with heated-lid.
3. Ice bath or cold block (0° to 4°C).
4. DNA purification columns (PCR & Gel Clean-up Kit, Enzo Prod. No. ENZ-GEN100).
5. Optional: *Alu*I and *Rsa*I restriction enzymes and appropriate buffer (Enzo Prod. Nos. ENZ-GEN108 and ENZ-GEN109).
6. Optional: TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).
7. Optional: Additional water bath, heating block, or incubator set at 65°C or a PCR machine with heated lid.

METHODS AND PROCEDURES

AMOUNT OF DNA REQUIRED

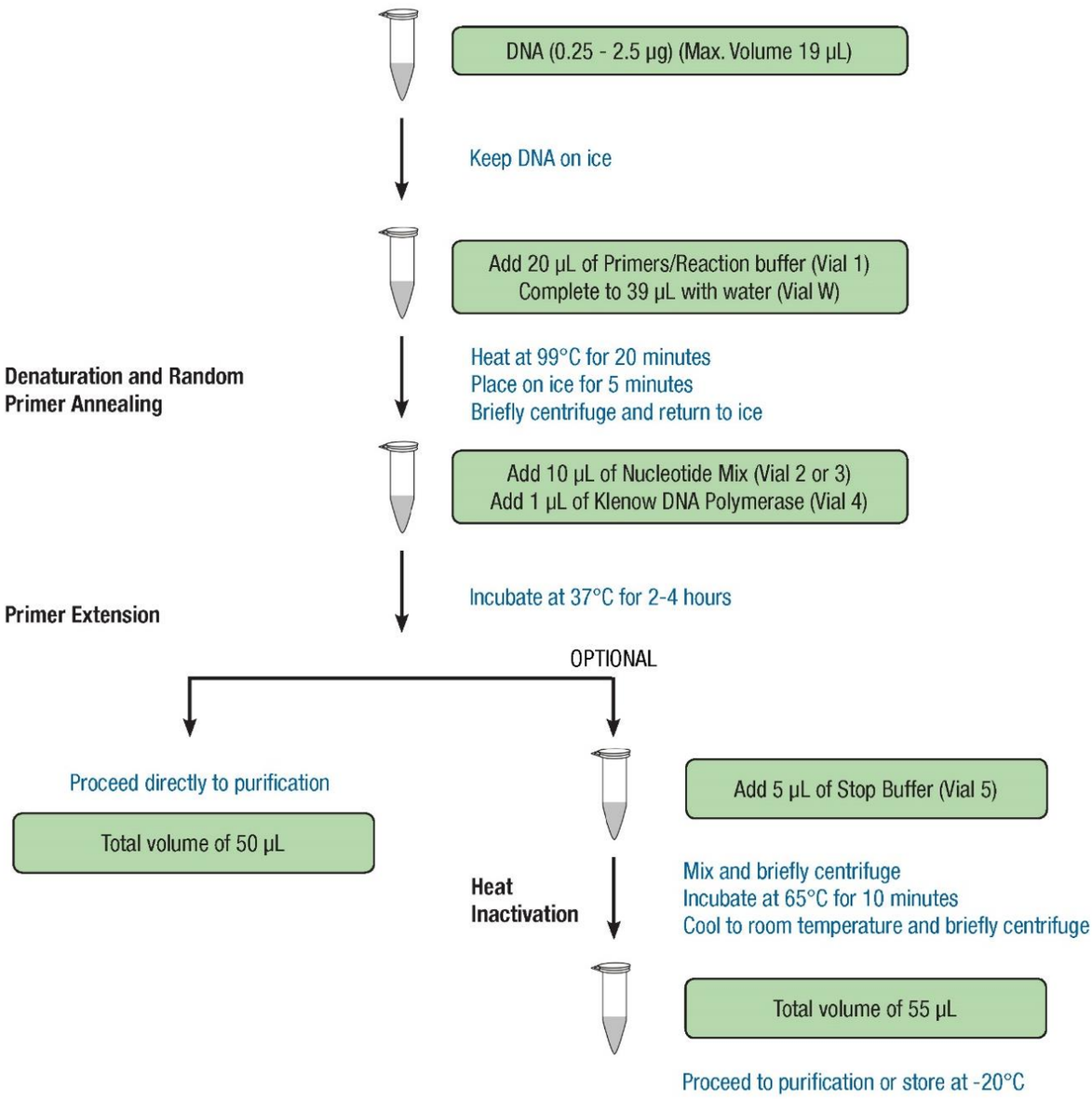
- Enzo's **CYTAG® CGH Labeling Kit** is optimized for use with 0.25 - 2.5 µg genomic DNA as starting material for Agilent or other arrays.
- In general, 0.5 µg of input genomic DNA should be sufficient for labeling and hybridization to all Agilent array formats.

ISOLATING GENOMIC DNA

- Isolate genomic DNA using an established protocol or commercially available kit.
- Determine concentration and purity of the genomic DNA by measuring the absorbance at 260 nm and 280 nm. We recommend the use of the NanoDrop ND-1000 UV-VIS Spectrophotometer or equivalent. The A_{260}/A_{280} ratio should be approximately 1.8. Significant deviation from this ratio suggests the presence of contaminants and indicates that the sample should be re-purified.
- DNA can be used directly for cyanine labeling and array hybridization without restriction digestion.

For labeling DNA for CGH+SNP arrays go to page 8, otherwise, proceed.

CGH DNA LABELING WORKFLOW



CGH DNA LABELING PROCEDURE (not CGH+SNP)

DNA is enzymatically labeled by the incorporation of cyanine dye labeled nucleotides according to the procedure summarized in **Table 1** and described below. For each pair of genomic DNAs to be compared, label one sample with Cyanine 3 and the other with Cyanine 5. If further validation is desired, the labels can be swapped in a parallel or subsequent experiment.

When using Enzo's CYTAG® CGH Labeling Kit, digestion with restriction enzymes is not recommended for CGH only arrays.

NOTE: *To prepare DNA prior to labeling for CGH+SNP Arrays, see CGH+SNP DNA LABELING PROCEDURE on page 8.*

Table 1. Procedure Overview for DNA Labeling for CGH Arrays

Step		Component/Condition	Amount
1. Add		DNA (0.25 - 2.5 µg)	up to 19 µL
2. Add		Primers/Reaction Buffer (Vial 1)	20 µL
3. Add		Water (Vial W)	to 39 µL
4. Incubate		99°C, 20 min	
5. Incubate		Ice, 5 min, briefly centrifuge, and place back into ice	
6. Add		Nucleotide Mix (Vial 2 or 3)	10 µL
7. Add		Klenow DNA Polymerase (Vial 4)	1 µL
8. Incubate		37°C, 2 - 4 hr	
Optional	Add	Stop Buffer (Vial 5)	5 µL
	Incubate	65°C, 10 min	
9. Purify		Purification using ENZ-GEN100	

NOTE: *It is critical that each step in this protocol be followed as indicated for successful labeling of DNA for array analysis. Any deviation from the stated procedure may lead to undesirable results.*

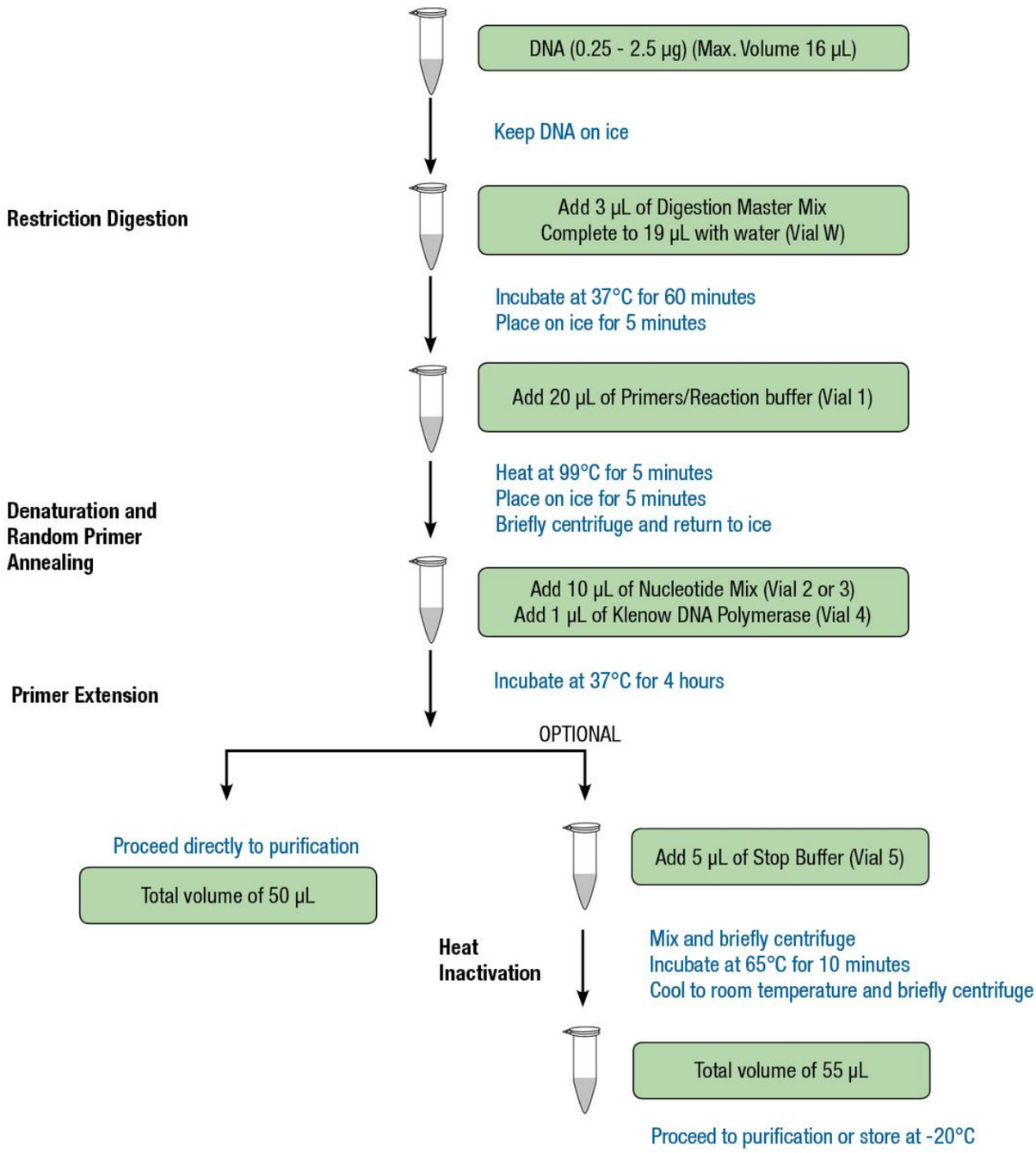
Denaturation / Heat Fragmentation of DNA and Random Primer Annealing.

1. Combine 0.25 - 2.5 µg of genomic DNA (up to 19 µL in sterile water or TE) with 20 µL of Primers/Reaction Buffer (Vial 1).
2. Add enough Nuclease-free Water (Vial W) to bring the reaction mixture to 39 µL.
3. Heat at 99°C for 20 min and place on ice for 5 min.
4. Briefly centrifuge, and return to ice.

Primer Extension with Klenow DNA Polymerase.

1. While on ice, add 10 µL of the appropriate cyanine dye-labeled nucleotide mix (Vial 2 or 3) and 1 µL of Klenow DNA Polymerase (Vial 4) to the primer annealed DNA sample.
   ***NOTE**: Making a Labeling Master Mix is recommended. See Appendix A.*
2. Flick the tube gently to mix contents. Briefly centrifuge.
3. Incubate the reaction at 37°C for 2-4 hr.
   With good quality input DNA greater than 500 ng, incubation time may be shortened to 2 hours without significant reduction in yield, however, a decrease in specific activity may be observed.
4. Proceed to purification step as described on page 16 or if you wish to stop the protocol and continue purification later:
   a. Add 5 µL of Stop Buffer (Vial 5).
   b. Mix and briefly centrifuge.
   c. Heat for 10 min at 65°C to heat-inactivate the enzyme.
   d. Cool to room temperature and centrifuge briefly.
   e. Store labeled DNA at -20°C.
   f. The following day, proceed to purification step as described on page 12.

CGH+SNP DNA LABELING WORKFLOW



CGH+SNP DNA LABELING PROCEDURE

For CGH+SNP arrays, genomic DNA must be digested using a combination of restriction enzymes, *Alu*I and *Rsa*I. Genotyped HapMap DNA (e.g. from Coriell Institute) must be used as the reference DNA on Agilent's CGH+SNP microarray. Sample DNA must be isolated from a single individual (same gender as reference DNA) and can be genotyped by hybridization against reference DNA.

Digested DNA is enzymatically labeled by the incorporation of cyanine dye labeled nucleotides according to the procedure summarized in **Table 2** and described below. For each pair of genomic DNAs to be compared, label one sample with Cyanine 3 and the other with Cyanine 5. If further validation is desired, the labels can be swapped in a parallel or subsequent experiment.

Table 2. Procedure Overview of DNA Labeling for CGH+SNP Arrays

Step		Component/Condition	Amount
1. Add		DNA (0.25 - 2.5 µg)	up to 16 µL
2. Add		Digestion Master Mix (as shown in Table 3)	3 µL
3. Add		Water (Vial W)	to 19 µL
4. Incubate		37°C, 60 min	
5. Incubate		Ice, 5 min, briefly centrifuge, and place back into ice	
6. Add		Primers/Reaction Buffer (Vial 1)	20 µL
7. Incubate		99°C, 5 min	
8. Incubate		Ice, 5 min, briefly centrifuge, and place back into ice	
9. Add		Nucleotide Mix (Vial 2 or 3)	10 µL
10. Add		Klenow DNA Polymerase (Vial 4)	1 µL
11. Incubate		37°C, 4 hours	
Optional	Add	Stop Buffer (Vial 5)	5 µL
	Incubate	65°C,10 min	
12. Purify		Purification using ENZ-GEN100	

NOTE: *It is critical that each step in this protocol be followed as indicated for successful labeling of DNA for array analysis. Any deviation from the stated procedure may lead to undesirable results.*

Restriction Digestion of DNA and Random Primer Annealing.

1. Equilibrate heat blocks or water baths to 37°C, 65°C and 99°C or use a thermal cycler.
2. Thaw all the reagents except three enzymes (Klenow DNA Polymerase, *Alu*I, and *Rsa*I) at room temperature. Once thawed, flick the tube to briefly mix, spin in a microcentrifuge and then keep the reagents on ice. Also, briefly spin the tubes containing above mentioned enzymes in a microcentrifuge tube and return them to ice.
   NOTE: *Store all reagents on ice while in use and return promptly to -20°C*
3. Mix the components in **Table 3** on ice in the order indicated to prepare Digestion Master Mix.

Table 3. Preparation of Digestion Master Mix (example is shown for 10 reactions)

Component	Per reaction (µL)	x 11 reactions (µL) (including 10% excess)
10X Cutting Buffer	1.9	20.9
*Alu*I	0.38	4.18
*Rsa*I	0.38	4.18
Nuclease-free Water (Vial W)	0.34	3.74
Final Volume of Digestion Master Mix	3.0	33.0

4. Add 3 µL of Digestion Master Mix to each reaction tube containing 16 µL of genomic DNA solution. Mix well by gently pipetting up and down.
5. Incubate at 37°C for 60 min to digest the DNA and immediately place on ice for 5 min. Briefly centrifuge, and return the reaction tubes to ice.
   NOTE: *The majority of the digested DNA should be between 200 bp and 500 bp in length as determined by agarose gel electrophoresis.*
6. Add 20 µL of Primer/Reaction buffer (Vial 1) to each reaction. Mix well by pipetting up and down.
7. Heat at 99°C for 5 min and place on ice for 5 min.
8. Briefly centrifuge, and return to ice.

Primer Extension with Klenow DNA Polymerase.

1. While on ice, add 10 µL of the appropriate cyanine dye-labeled nucleotide mix (Vial 2 or 3) and 1 µL of Klenow DNA Polymerase (Vial 4) to the primer annealed DNA sample.
   ***NOTE**: Making a Labeling Master Mix is recommended. See Appendix A.*
2. Flick the tube gently to mix contents. Briefly centrifuge.
3. Incubate the reaction at 37°C for 4 hr.
4. Proceed to purification step as described on page 16 or if you wish to stop the protocol and continue purification later:
   a. Add 5 µL of Stop Buffer (Vial 5).
   b. Mix and briefly centrifuge.
   c. Heat for 10 min at 65°C to heat-inactivate the enzyme.
   d. Cool to room temperature and centrifuge briefly.
   e. Store labeled DNA at -20°C.
   f. The following day, proceed to purification step as described on page 12.

PURIFICATION WORKFLOW

Adjust DNA binding condition by adding 2 volumes (100 or 110 µL) of Binding Buffer

Place a PCR & Gel Binding column into 2 mL collection tube

DNA Binding to the Silica Membrane

Load labeled DNA sample

Centrifuge for 30 seconds at 11,000 x g
Discard flow-through

Wash Silica Membrane

Add 650 µL of 1X Wash Buffer*

Centrifuge for 30 seconds at 11,000 x g
Discard flow-through
Repeat this wash step to prevent salt carryover

Dry Silica Membrane

Dry silica membrane

Centrifuge for 2 minutes at 11,000 x g with lid open
Discard flow-through
Transfer column to clean tube

Elute DNA

Elute DNA by adding 25 µL of Elution Buffer

Incubate at room temperature for 1 minute
Centrifuge for 1 minute at 11,000 x g
Repeat the elution step by adding another 25 µL of Elution Buffer

Total volume of 50 µL

* Prepare Wash Buffer according to the procedure.

PURIFICATION OF LABELED DNA

NOTE: *Purify each labeling reaction in* ***separate*** *columns (combining the labeled sample and labeled reference DNA is not recommended prior to purification). Enzo's PCR & Gel Clean-up columns (Prod. No. ENZ-GEN100) are recommended with the following optimized protocol. For reference, the binding capacity of the columns for labeled DNA is approximately 10 µg.*

1. **Preparation of 1X Wash Buffer**
   1X Wash Buffer should be prepared before starting any Enzo PCR purification or gel extraction protocols: Add 96-100% ethanol to 5X Wash Buffer (supplied in ENZ-GEN100). For example, for ENZ-GEN100-0020, add 32 mL of 96-100% ethanol to the 8 mL 5X Wash Buffer to make a 40 mL 1X Wash Buffer.
2. **Bind DNA to the column**
   NOTE: *Binding Buffer contains chaotropic salt. Wear gloves and goggles.*
   a. For each sample, place a PCR & Gel Binding Column into a 2 mL Collection Tube (both supplied in ENZ-GEN100).
   b. Mix labeled sample with 2 volumes (100 µL or 110 µL, if Stop Buffer was used) of Binding Buffer by pipetting up and down a few times, one sample per tube at a time.
   c. Immediately add to a PCR & Gel Binding Column.
   d. Follow same steps for the remainder of samples.
   e. Centrifuge for 30 sec at 11,000 x g. Discard flow-through and place the PCR & Gel Binding Column back into the Collection Tube.
3. **Wash silica membrane**
   a. Add 650 µL 1X Wash Buffer.
   b. Centrifuge for 30 sec at 11,000 x g. Discard flow-through and place the PCR & Gel Binding Column back into the Collection Tube.
   c. Add another 650 µL 1X Wash Buffer and repeat Step b.
4. **Dry silica membrane**
   Centrifuge for 2 min at 11,000 x g with the lid open to remove Wash Buffer. The tip of the spin column should not come in contact with the flow-through while removing it from the centrifuge and the Collection Tube.
5. **Elute DNA**
   a. Place the PCR & Gel Binding Column into a clean 1.5 mL microcentrifuge tube (not provided). Add 25 µL Elution Buffer (supplied in ENZ-GEN100) and incubate at room temperature for 1 min to increase the yield.
   b. Centrifuge for 1 min at 11,000 x g.
   c. Add another 25 µL Elution Buffer and repeat Step b. to obtain a total volume of 50 µL.
   NOTE*: Based upon the requirements of your hybridization platform, volume reduction may be required.*

DETERMINATION OF YIELD AND INCORPORATION

If you would like to determine the yield & dye incorporation, we recommend using a NanoDrop ND-1000 UV-VIS Spectrophotometer in the Microarray Measurement Mode, using the dsDNA setting, to determine yield and dye incorporation. Blank the instrument using Elution Buffer from the purification kit and use 1.5 µL from each sample to measure.

For a typical labeling reaction, with an input of 500 ng of high quality genomic DNA, the expected yield should be at least 4 µg. This DNA should contain either at least 200 pmols of incorporated Cyanine 3 or at least 130 pmols of incorporated Cyanine 5.

EXPECTED DERIVATIVE LOG RATIO (DLR) SCORE

After hybridization to a 4x180 CGH array, typical DLR score for 500 ng of good quality input DNA should be ≤ 0.18.

TROUBLESHOOTING

1. **Low yield and incorporation.** Poor labeling often reflects low quality input DNA. This can be due to the presence of contaminants from the purification of genomic DNA.
2. **Poor signal to noise ratio despite good incorporation.** Low signal can be caused by inappropriate hybridization conditions (too stringent buffer or excessive temperature) or when the hybridization time is too short. High background can be caused by inadequate blocking and/or washing conditions. Excessive amounts of labeled DNA can also result in high background.
3. **Good DLRs in the CGH analytics QC report, but some hybridizations show whiskering or significant deviation from the expected $\log_2$ ratio**. Make certain the Cot DNA is added in sufficient quantities. Ensure to follow the Denaturation / Heat Fragmentation step on page 9 for 20 min at 99°C.
4. **Bright yellow array with reduced magnitude of expected changes.** This can be due to insufficient Cot DNA, blocking agents or hybridization and washing conditions that are not stringent enough.
5. **High DLR scores on the array.** High DLR scores can be caused by labeling of amplified DNA. If DNA is limiting, or of poor quality, amplified DNA can be used, but the results will not be of high quality. Labeling of limiting quantities of genomic DNA will often lead to better array results than if amplified DNA is used. As low as 100 ng of good genomic DNA can be used for labeling.
6. **DNA is degraded.** Ensure that you are storing your DNA correctly: at 4°C for general use, or -20°C for long term storing in aliquots to prevent freeze-thawing.
7. **Variability in labeling results.** Use optional stop buffer protocol to ensure all labeling reactions are labeled for the same duration.

APPENDIX A

PREPARING LABELING MASTER MIX

1. Make Labeling Master Mix just prior to use that includes 10% excess.
2. Mix the components in **Table 4** on ice in the order indicated to prepare Cyanine 3 (using material from Vial 2) and Cyanine 5 (using material from Vial 3) Labeling Master Mix. Make separate Labeling Master Mixes for Cyanine 3 and Cyanine 5.

Table 4. Preparation of Labeling Master Mix (example is shown for 10 reactions)

Component	Per reaction (µL)	x 11 reactions (µL) (including 10% excess)
Nucleotide Mix (Vial 2 or 3)	10	110
Klenow DNA Polymerase (Vial 4)	1	11
Final Volume of Labeling Master Mix	11	121

NOTE: *Keep the Labeling Master Mix on ice at all times and use within 30 min of preparation.*

APPENDIX B

PREPARING THE LABELED DNA FOR HYBRIDIZATION ON OLIGONUCLEOTIDE ARRAY

1. Combine the Cyanine 3- and Cyanine 5-labeled DNA eluates.
2. Bring the final volume of the combined eluates to the volume specified by the manufacturer of the particular array format with nuclease-free water.

   ***NOTE**: If the volume of the combined eluates are too high, the volume can be reduced by lyophilization in a centrifugal vacuum concentrator or by acetate/ethanol precipitation and resuspension in water. See Appendix C.*

3. Add Cot DNA, blocking agents and 2X hybridization buffer as specified by manufacturer for the particular array format.
   ***NOTE**: Use the recommended amount of Cot DNA as suggested by manufacturer.*
4. Perform hybridization, array washing and scanning as specified by manufacturer for the particular array format.

APPENDIX C

SODIUM ACETATE / ALCOHOL PRECIPITATION PROCEDURE OF LABELED DNA WITH COT DNA

1. Combine the labeled DNAs and the Cot DNA in a separate 1.5 mL microfuge tube.
2. Add 1/10th volume (25 µL if the total volume of the DNA is 250 µL) of 3M sodium acetate, pH 5.2, and 2.5 volumes (625 µL if the DNA volume was 250 µL) of ice-cold 100% ethanol. Mix well and store overnight at -20°C or 1 hr at -70°C.
3. Centrifuge for 20 min at 16,000 x g in a 4°C microcentrifuge.
4. Carefully remove the supernatant, add 500 µL of ice-cold 70% ethanol and spin for 5 min at 16,000 x g (room temperature).
5. Carefully remove supernatant, centrifuge for 1 min and remove residual fluid.
6. Air-dry pellet for 10 min or in a centrifugal vacuum concentrator for 2 min.
7. Resuspend the pellet in volume specified by the manufacturer of the particular array format with nuclease-free water.
8. Perform hybridization, array washing and scanning as specified by manufacturer for the particular array format.

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