Product Manual

CYTAG® SuperCGH Labeling Kit

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

Instruction Manual

ENZ-GEN120-0010

2x10 reactions for 4x180K arrays

2x20 reactions for 8x60K arrays

ENZ-GEN120-0100

2x100 reactions for 4x180K arrays

2x200 reactions for 8x60K arrays

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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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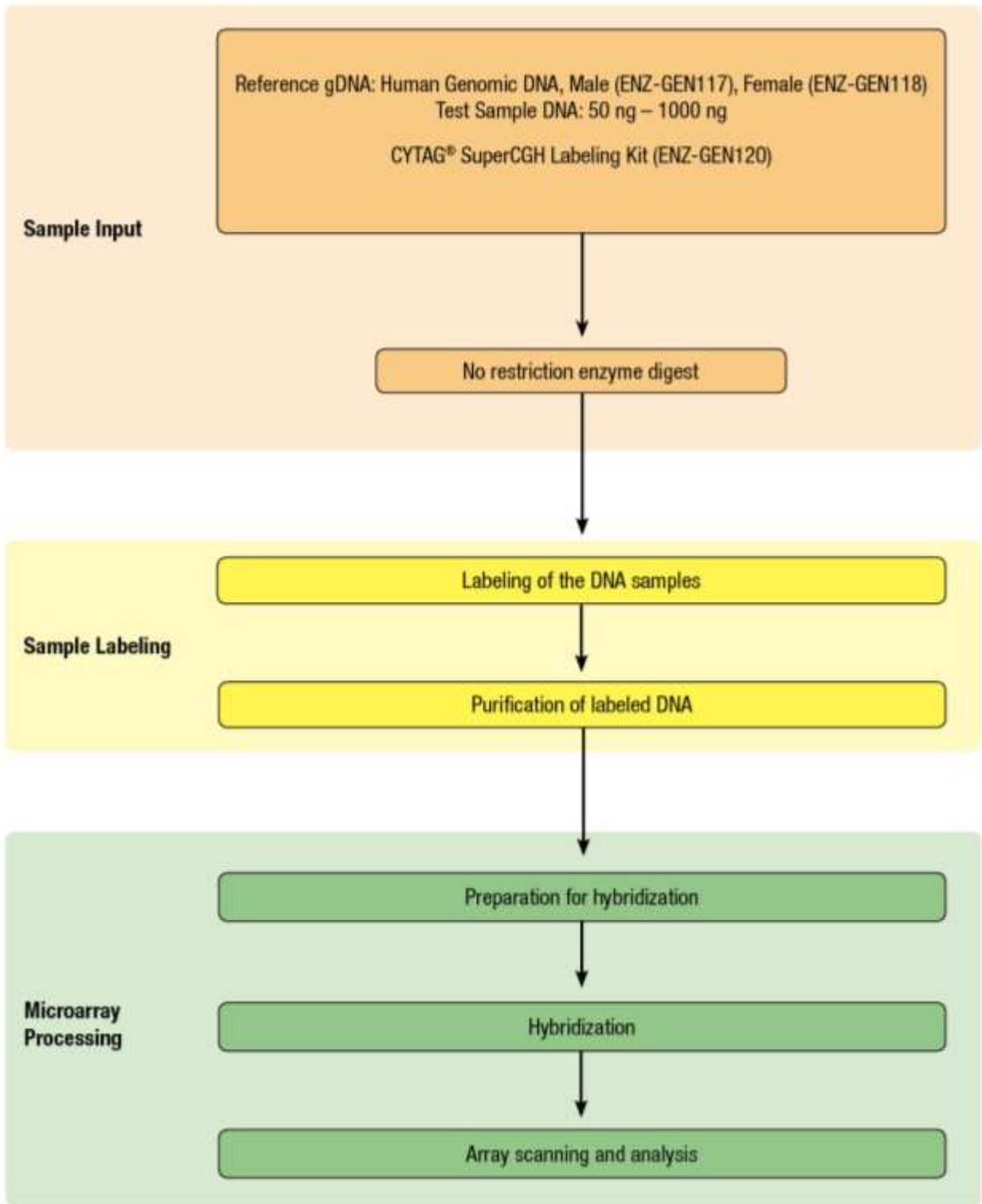
INTRODUCTION

Array-based comparative genomic hybridization (aCGH) is a powerful platform for detecting DNA copy number gains and losses associated with chromosomal abnormalities in genetic diseases and cancers.

Enzo Life Sciences' **CYTAG® SuperCGH Labeling Kit** has been optimized to label low-input DNA for dual sample comparative genomic hybridization (CGH) assays on oligonucleotide arrays. Our proprietary labeling technology allows you to achieve superior labeling efficiency, greater sensitivity, and fewer failed runs, all while using a simpler workflow that has been validated on Agilent systems. All required reagents for labeling are provided, including fully optimized nucleotide mixes that contain either Cyanine 3 or Cyanine 5-labeled dUTPs and purification components. Please note that our CGH kits are not compatible with Affymetrix and other arrays that do not detect Cyanine 3 and Cyanine 5. The CYTAG® SuperCGH Labeling Kit has been designed to provide higher labeled DNA yields, while still maintaining high specific activity, for precious low-input DNA samples as low as 50 ng.

CYTAG® ARRAY CGH WORKFLOW



REAGENTS PROVIDED AND STORAGE

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

Purification components are shipped at room temperature and recommended to be stored at room temperature upon receipt.

Avoid repeated freezing and thawing of the kit reagents. We recommend to aliquot ENZ-GEN120-0100 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.

Labeling Components (store at -20 °C):

Reagent	Vial ID	Minimum Volume Supplied	
		ENZ-GEN120-0010	ENZ-GEN120-0100
Primers/Reaction Buffer	1	400 µL	4 mL
Super Cyanine 3-dUTP Nucleotide Mix	2	100 µL	1 mL
Super Cyanine 5-dUTP Nucleotide Mix	3	100 µL	1 mL
Klenow DNA Polymerase	4	40 µL	400 µL
Stop Buffer	5	100 µL	1 mL
Nuclease-free Water	W	1 ml	10 mL

Purification Components (store at room temperature):

Component	Minimum Amount Supplied	
	ENZ-GEN120-0010	ENZ-GEN120-0100
PCR & Gel Clean-up Columns	20	200
Collection Tubes (2 mL)	20	200
Binding Buffer	20 mL	4 x 50 mL
5X Wash Buffer	8 mL	4 x 20 mL
Elution Buffer	4 mL	4 x 10 mL

PREPARATION OF WASH BUFFER WITH ETHANOL

The Wash Buffer supplied with purification components must be mixed with molecular biology grade ethanol (not supplied) prior to use.

Add the following amounts of ethanol below directly to each bottle of Wash Buffer, and indicate on the bottle that you have added the ethanol.

	ENZ-GEN120-0010	**ENZ-GEN120-0100**
5x Wash Buffer (entire bottle)	8 mL	20 mL (4 bottles included)
Ethanol, Molecular Biology Grade	32 mL	80 mL
Final Volume	40 mL	100 mL

***NOTE**: Store the buffer in ethanol at room temperature.*


Avoid repeated freeze/ thaw cycles.


Protect from light


Handle with care

PRECAUTIONS

- This product is for research use only. It is not intended for the diagnosis of diseases in humans or animals. DO NOT use internally or externally in humans or animals.
- 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 using Human Genomic DNA, male (Enzo Prod. No. ENZ-GEN117) or female, (Enzo Prod. No. ENZ-GEN118)
2. Water bath, heating block, or incubator set at 37 °C, 65 °C, and 99 °C or a PCR machine with heated-lid
3. Ice bath or cold block (0-4 °C)
4. Vortex mixer
5. Microcentrifuge
6. 1.5 mL microcentrifuge tubes (for collection of labeled DNA after purification
7. Ethanol, Molecular Biology Grade (for preparing purification Wash Buffer, see page 5)
8. Optional: TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)

METHODS AND PROCEDURES

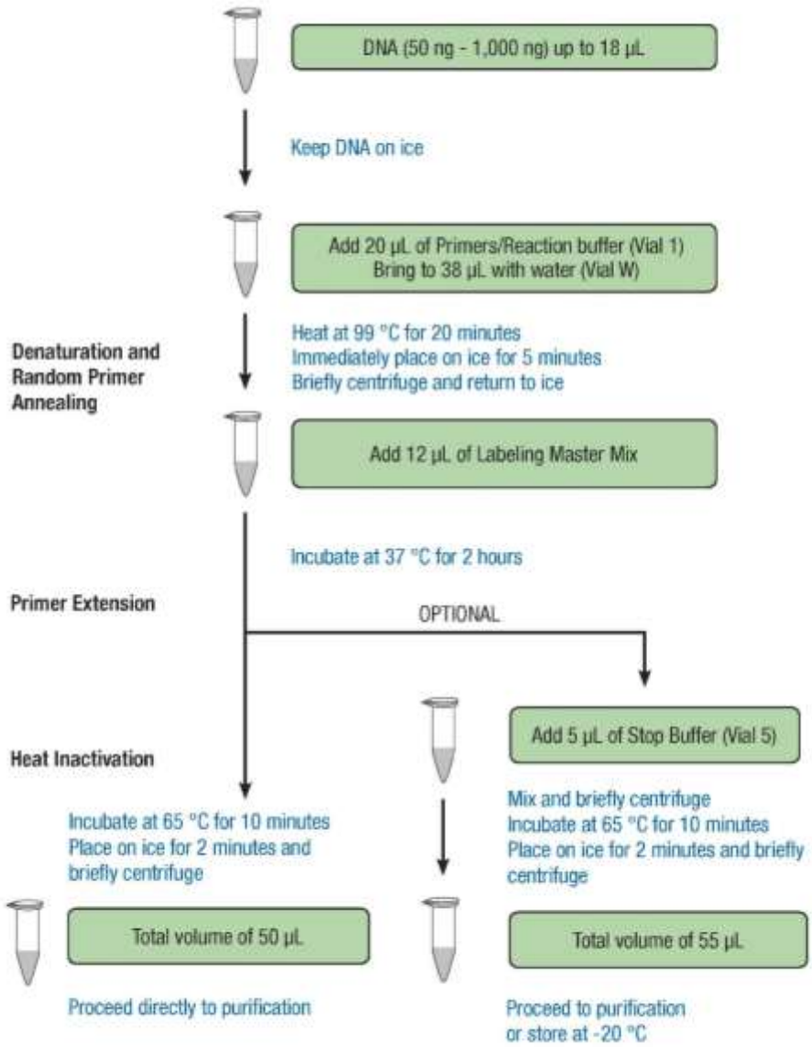
AMOUNT OF DNA REQUIRED

- Enzo's **CYTAG® SuperCGH Labeling Kit** is optimized for use with 50 ng - 1000 ng of genomic DNA as starting material for Agilent and other oligonucleotide arrays, without restriction enzyme digest.
- In general, 50 ng input of high quality genomic DNA is sufficient for labeling and hybridization to most Agilent array formats. In cases of lower quality DNA, an input of approximately 150 ng may be utilized to obtain acceptable array results.

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 260nm and 280nm. 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 and A_{260}/A_{230} ratio should be 2.0 or greater. Significant deviation from these ratios suggests the presence of contaminants and indicates that the sample should be re-purified.
- High quality, high molecular weight genomic DNA can be used directly for cyanine dyelabeling and array hybridization. Restriction enzyme digest is not recommended.

CGH DNA LABELING WORKFLOW: 4X180K ARRAYS



LABELING DNA FOR 4x180K CGH ARRAYS

Labeled DNA is prepared by the incorporation of cyanine dye labeled nucleotides according to the procedure summarized in Table 1 and described below. For each pair of genomic DNA to be compared, we recommend labeling the reference sample with Cyanine 3 and test sample with Cyanine 5. If further validation is desired, the labels can be swapped in a parallel or subsequent experiment.

When using Enzo's CYTAG® SuperCGH Labeling Kit, digestion with restriction enzymes is *NOT* recommended.

Table 1. Procedure Overview of DNA Labeling for 4x180K CGH Arrays

Step	Component/Condition	Amount
1. Add	DNA (50 ng – 1000 ng)	up to 18 µL
2. Add	Primers/Reaction Buffer (Vial 1)	20 µL
3. Add	Water (Vial W)	to 38 µL
4. Incubate	99 °C, 20 min	
5. Immediately Incubate	Ice, 5 min	
6. Centrifuge	Briefly centrifuge and put back on ice	
7. Add	Labeling Master Mix (as shown in Appendix A, Table 3)	12 µL
8. Incubate	37 °C, 2 hours	
Optional: Add	Stop Buffer (Vial 5)	5 µL
9. Incubate	65 °C, 10 min	
10. Purify	Purification using supplied columns	

***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. Equilibrate heat blocks or water baths to 37 °C, 65 °C, and 99 °C or use a thermal cycler.
2. Thaw all the reagents at room temperature except Klenow DNA Polymerase (Vial 4) which should be kept on ice while in use. Once thawed, flick the tubes to briefly mix, spin in a microcentrifuge and then keep the reagents on ice. Also, briefly spin the tube containing Klenow DNA Polymerase in a microcentrifuge tube and return to ice.
   ***NOTE**: Store all reagents on ice while in use and return promptly to -20 °C.*
3. Combine 50 ng – 1000 ng of genomic DNA (up to 18 µL) with 20 µL of Primers/Reaction Buffer (Vial 1), and add enough Nuclease-free Water (Vial W) to bring the reaction mixture to 38 µL. Mix well by gently pipetting up and down.
4. Heat at 99 °C for 20 min and immediately place on ice for 5 min. Briefly centrifuge, and return the reaction tubes to ice.

Primer Extension with Klenow DNA Polymerase

5. While on ice, add 10 µL of the appropriate cyanine dye-labeled nucleotide mix (Vial 2 or 3) and 2 µL of Klenow DNA Polymerase (Vial 4) to the primer annealed DNA sample.
   ***NOTE**: Making a Labeling Master Mix is recommended. See Appendix A, Table 3.*
6. Mix well by gently pipetting up and down.
7. Incubate each reaction tube at 37 °C for 2 hr.
8. Heat the reaction tubes at 65 °C for 10 min to heat-inactivate the enzyme.
9. Cool the reaction tubes on ice for 2 min and centrifuge briefly.
10. Proceed to purification step as described on page 15.
11. If you wish to stop the protocol and continue purification later:
    a. Add 5 µL of Stop Buffer (Vial 5) after Step 7.

    b. Mix and briefly centrifuge.

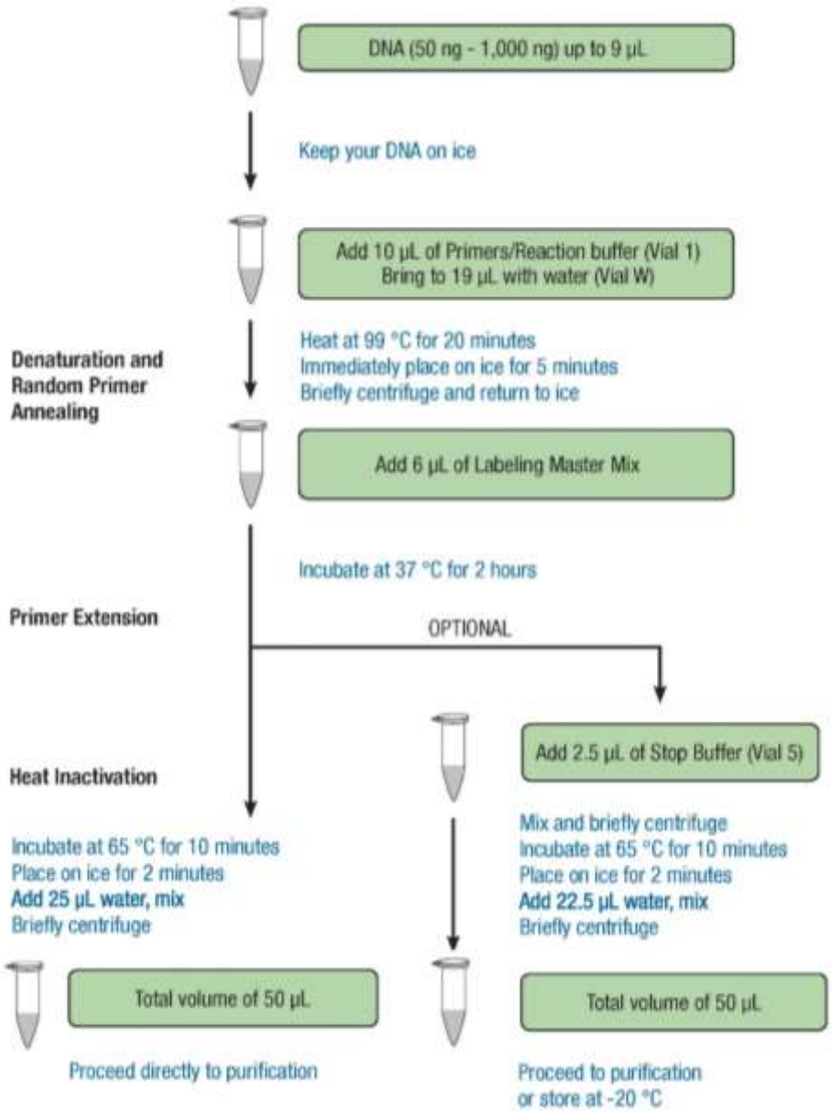
    c. Heat for 10 min at 65 °C to heat-inactivate the enzyme.

    d. Cool reaction tubes on ice and centrifuge briefly.

    e. Labeled DNA can be stored at -20 °C overnight.

    f. Proceed to purification step as described on page 15.

CGH DNA LABELING WORKFLOW: 8X60K ARRAYS



LABELING DNA FOR 8x60K CGH ARRAYS

Labeled DNA for 8x60K CGH array is prepared according to the procedure summarized in Table 2 and described below. For each pair of genomic DNA to be compared, we recommend labeling the reference sample with Cyanine 3 and test sample with Cyanine 5. If further validation is desired, the labels can be swapped in a parallel or subsequent experiment.

When using Enzo's CYTAG® SuperCGH Labeling Kit, digestion with restriction enzymes is *NOT* recommended.

Table 2. Procedure Overview of DNA labeling for 8x60K CGH arrays

Step	Component/Condition	Amount
1. Add	DNA (50 ng – 1000 ng)	up to 9 µL
2. Add	Primers/Reaction Buffer (Vial 1)	10 µL
3. Add	Water (Vial W)	to 19 µL
4. Incubate	99 °C, 20 min	
5. Immediately Incubate	Ice, 5 min	
6. Centrifuge	Briefly centrifuge and put back on ice	
7. Add	Labeling Master Mix (as shown in Appendix A, Table 3)	6 µL
8. Incubate	37 °C, 2 hours	
Optional: Add	Stop Buffer (Vial 5)	2.5 µL
9. Incubate	65 °C, 10 min	
10. Add	Water (Vial W)	to 50 µL
11. Purify	Purification using supplied columns	

***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.*

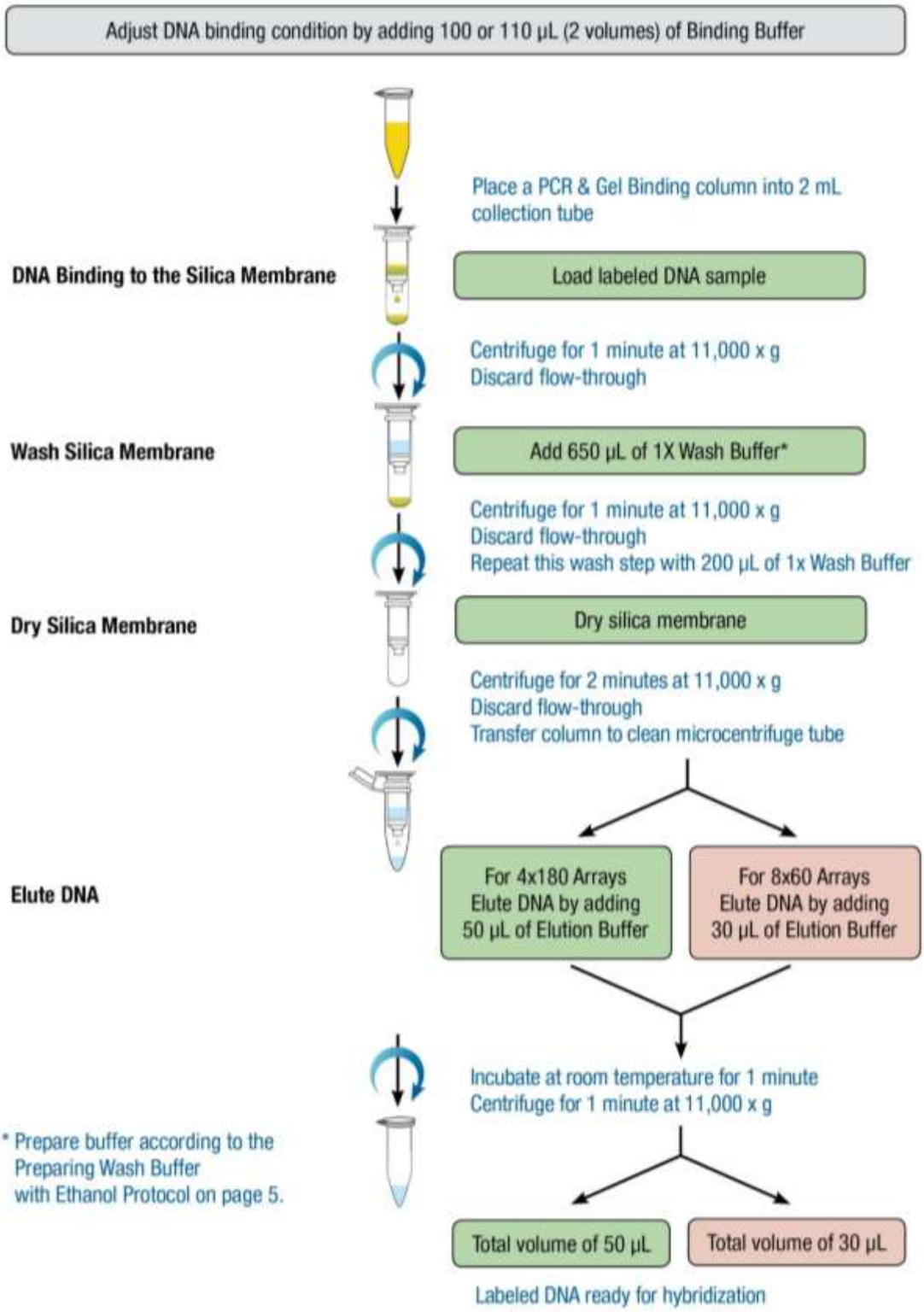
Denaturation / Heat Fragmentation of Genomic DNA and Random Primer Annealing

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 at room temperature except Klenow DNA Polymerase (Vial 4) which should be kept on ice while in use. Once thawed, flick the tubes to briefly mix, spin in a microcentrifuge and then keep the reagents on ice. Also, briefly spin the tube containing Klenow DNA Polymerase in a microcentrifuge tube and return to ice.
   ***NOTE**: Store all reagents on ice while in use and return promptly to -20 °C.*
3. Combine 50 ng - 1000 ng of genomic DNA (up to 9 µL) with 10 µL of Primers/Reaction Buffer (Vial 1), and add enough Nuclease-free Water (Vial W) to bring the reaction mixture to 19 µL. Mix well by gently pipetting up and down.
4. Heat at 99 °C for 20 min and immediately place on ice for 5 min. Briefly centrifuge, and return the reaction tubes to ice.

Primer Extension with Klenow DNA Polymerase

5. While on ice, add 5 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, Table 3.*
6. Mix well by gently pipetting up and down.
7. Incubate each reaction tube at 37 °C for 2 hr.
8. Heat the reaction tubes at 65 °C for 10 min to heat-inactivate the enzyme.
9. Cool the reaction tubes in ice for 2 min, add 25 uL Water (Vial W) to bring the volume to 50 µL and centrifuge briefly.
10. Proceed to purification step as described on page 15.
11. If you wish to stop the protocol and continue purification later:
    a. Add 2.5 µL of Stop Buffer (Vial 5) after Step 7.
    b. Mix and briefly centrifuge.
    c. Heat for 10 min at 65 °C to heat-inactivate the enzyme.
    d. Cool to room temperature, add enough Water (Vial W) to bring the volume to 50 µL and centrifuge briefly.
    e. Labeled DNA can be stored at -20 °C overnight.
    f. Immediately proceed to purification step as described on page 15.

PURIFICATION WORKFLOW



PURIFICATION OF LABELED DNA

NOTE: *Purify each labeling reaction in* ***separate*** *columns (do not combine the labeled sample and labeled reference DNA prior to purification). Enzo's PCR & Gel Clean-up columns are provided and 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 molecular biology grade ethanol to 5X Wash Buffer. For example, for the 5X Wash Buffer included with ENZ-GEN120-0010, 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 during use of this solution.

   a. For each sample, place a PCR & Gel Binding Column into a 2 mL Collection Tube (supplied).
   b. Mix labeled DNA 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 the mixture to a PCR & Gel Binding Column.
   d. Follow same steps for the remainder of samples.
   e. Centrifuge for 1 min 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 of 1X Wash Buffer.
   b. Centrifuge for 1 min at 11,000 x g. Discard flow-through and place the PCR & Gel Binding Column back into the Collection Tube.
   c. Add an additional 200 µL of 1X Wash Buffer and repeat step b.
4. **Dry silica membrane**

   Centrifuge for 2 min at 11,000 x g to remove any remaining 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 labeled DNA**
   a. *For 4x180K array*: place the PCR & Gel Binding Column into a clean 1.5 mL microcentrifuge tube (not provided). Add 50 µL Elution Buffer to each column and incubate at room temperature for 1 min to increase the yield. Centrifuge for 1 min at 11,000 x g to isolate labeled DNA.
   b. *For 8x60K array*: place the PCR & Gel Binding Column into a clean 1.5 mL microcentrifuge tube (not provided). Add 30 µL Elution Buffer to each

column and incubate at room temperature for 1 min to increase the yield. Centrifuge for 1 min at 11,000 x g to isolate labeled DNA.

***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 and dye incorporation, we recommend using a NanoDrop ND-1000 (or equivalent) UV-VIS Spectrophotometer in the Microarray Measurement Mode, using the dsDNA setting. 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 50 ng of high quality genomic DNA, the expected yield should be ≥ 5.0 µg. The DNA should contain either at least 300 pmoles of incorporated Cyanine 3, or at least 200 pmoles of incorporated Cyanine 5 using the 4x180 protocol.

EXPECTED DERIVATIVE LOG RATIO SCORE

After hybridization to a 4x180K or 8x60K CGH array, typical Derivative Log Ratio (DLR) Score for 50 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 and/or the presence of degraded or cross-linked 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 and 12 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 50 ng of good genomic DNA may 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

PREPARATION OF LABELING MASTER MIX

1. Make labeling Master Mix just prior to use that included 10% excess.
2. Mix the components in **Table 3**, in the order indicated to prepare Cyanine 3 (using material from Vial 2) and Cyanine 5 (using material from Vial 3) Labeling Master Mix. Mix components by gently pipetting up and down, while samples are kept on ice. Make separate Labeling Master Mixes for Cyanine 3 and Cyanine 5.

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

	For 4x180K Arrays		For 8x60K Arrays	
Component	**Per reaction (µL)**	**x 11 reactions (µL) (including 10% excess)**	**Per reaction (µL)**	**x 11 reactions (µL) (including 10% excess)**
Nucleotide Mix (Vial 2 or 3)	10	110	5	55
Klenow DNA Polymerase (Vial 4)	2	22	1	11
Final Volume of Labeling Master Mix	12	132	6	66

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

APPENDIX B

PREPARATION OF 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 WITH COT DNA

1. Combine the labeled DNAs and the Cot DNA in a 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 20 µL nuclease-free water and determine the concentration of DNA. Calculate DNA concentration using the following equation:

$$\text{DNA concentration} = 50\ \mu\text{g/mL} \times \text{OD}_{260} \times \text{Dilution Factor}$$

Where 1 OD_{260} measured at 1 cm path length is equal to 50 µg/mL concentration of dsDNA.

NOTES

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
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Catalog Number: ENZ-GEN120 Rev. 01/25/18