invitrogen™

BioPrime® Total FFPE Genomic Labeling System

For labeling DNA purified from formalin-fixed, paraffin-embedded tissue samples

Catalog nos. A10965-010 and A10965-011

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User Manual

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Kit Contents and Storage

Kit Sizes and Modules

This manual supports the following kit configurations.

Catalog no.	# of Reactions	Modules
A10965-010	10 (5 per dye)	Labeling and Purification
A10965-011	30 (15 per dye)	Labeling and Purification

Shipping and Storage

The BioPrime® Total FFPE Labeling Module is shipped on dry ice and should be stored at –20°C. The 10X Nucleotide Mixes may be stored at +4°C for up to 4 weeks (store at –20°C long-term).

The Purification Module is shipped and should be stored at room temperature.

Labeling Module

The following components in the BioPrime® Total FFPE Labeling Module should be stored at –20°C. The 10X Nucleotide Mixes may be stored at +4°C for up to 4 weeks (store at –20°C long-term).

Component	10-reaction kit	30-reaction kits
Alexa Fluor® 3 FFPE 10X Nucleotide Mix	25 µl	3 × 25 µl
Alexa Fluor® 5 FFPE 10X Nucleotide Mix	25 µl	3 × 25 µl
Exo– Klenow Fragment, 40 U/µl	30 µl	3 × 30 µl
Stop Buffer	0.5 ml	0.5 ml
2.5X Random Primers Solution	0.25 ml	3 × 0.25 ml
Control DNA (Salmon Sperm), (10 mg/ml)	10 µl	10 µl
DEPC-treated water	0.5 ml	2 × 0.5 ml

Important

The fluorescently labeled nucleotides in the Alexa Fluor® FFPE 10X Nucleotide Mixes are sensitive to photobleaching. Store the mixes protected from light.

Continued on next page

Kit Contents and Storage, continued

Purification Module

The following components in the BioPrime® Total FFPE Purification Module should be stored at room temperature.

Component	10-reaction kit	30-reaction kit
PureLink™ Spin Columns with Collection Tubes	11 columns/ tubes	31 columns/ tubes
Binding Buffer (B2) (combine with 100% isopropanol; see below)	9 ml	9 ml
Wash Buffer (W1) (combine with 100% ethanol; see below)	11 ml	11 ml
Elution Buffer (E1) (10 mM Tris-HCl, pH 8.5)	4 ml	4 ml
Amber Collection Tubes	12 tubes	36 tubes

Preparing Binding Buffer B2 with Isopropanol

Binding Buffer B2 supplied with the Purification Module must be mixed with 100% isopropanol prior to use.

Add the amount of isopropanol below directly to the bottle of Binding Buffer B2, and mark the checkbox on the bottle to indicate that you have added the isopropanol.

	Amount
Binding Buffer B2	9 ml (entire bottle)
100% Isopropanol	6 ml
Final Volume	15 ml

Store the buffer with isopropanol at room temperature.

Preparing Wash Buffer W1 with Ethanol

Wash Buffer W1 supplied with the Purification Module must be mixed with 100% ethanol prior to use.

Add the amount of ethanol below directly to the bottle of Wash Buffer W1, and mark the checkbox on the bottle to indicate that you have added the ethanol.

	Amount
Wash Buffer W1	11 ml (entire bottle)
100% Ethanol	40 ml
Final Volume	50 ml

Store the buffer with ethanol at room temperature.

Overview

Introduction

Array comparative genomic hybridization (aCGH) is a microarray-based method for analyzing genomic DNA to detect variations in gene copy number between samples (Pollack *et al.*, 1999; Pollack *et al.*, 2002). In aCGH, two genomic DNA samples are labeled with different fluorophores. The samples are hybridized to a microarray and the ratio of the fluorescent intensities of the fluorophores is measured for each feature on the array (Beheshti *et al.*, 2003; Cai *et al.*, 2002; Snijders *et al.*, 2001). This ratio provides a relative measure of the difference in gene copy number between the samples.

The BioPrime® Total FFPE Genomic Labeling System uses a mutant form of the Klenow fragment of DNA Polymerase I (Exo– Klenow) and nucleotides labeled with two novel, application-specific dyes (Alexa Fluor® 3 and 5) to differentially label genomic DNA samples for analysis by aCGH.

The BioPrime® Total FFPE Genomic Labeling System has been optimized for labeling DNA purified from formalin-fixed, paraffin-embedded (FFPE) tissue samples. It can be used to detect differences in gene copy number from as little as 500 ng of genomic DNA from these samples (depending on sample quality).

Advantages of the System

- Amplified products labeled with novel Alexa Fluor® 3 and 5 dyes have greater yields and higher signal intensities on the array.
- Exo– Klenow polymerase incorporates fluorescently modified nucleotides more effectively and provides higher yields than standard Klenow, for greater reproducibility of results.
- PureLink™ purification columns are designed to effectively remove all unincorporated nucleotides for the most accurate quantitation of labeled product and reduced background on the array.
- Dye-specific nucleotide mixes include labeled and unlabeled nucleotides for simplified reaction setup and workflow.
- Provides a complete solution for fluorescent labeling of genomic DNA.

Continued on next page

Overview, continued

Workflow Overview

Add the 2.5X Random Primers Solution and Alexa Fluor® 3 10X Nucleotide Mix or Alexa Fluor® 5 10X Nucleotide Mix to each reaction tube, then add the genomic DNA samples.

Heat each mixture briefly to denature the DNA, then cool to anneal the primers.

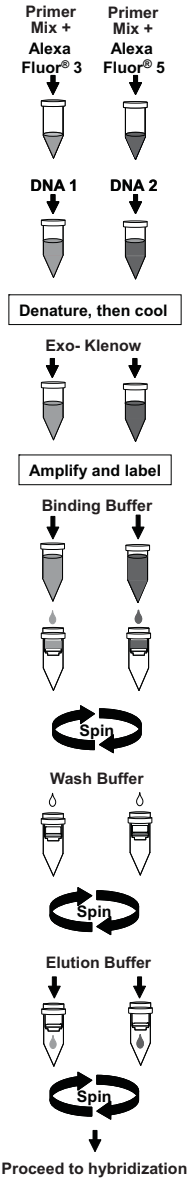
Add Exo– Klenow, then incubate at 37ºC for 2 hours to amplify and label the DNA.

Add Binding Buffer B1 to each labeled sample, transfer the mixture to PureLink™ spin columns in collection tubes, and centrifuge.

Add Wash Buffer W1 to each column and centrifuge.

Transfer the spin columns to Amber Recovery Tubes, add Elution Buffer E1 to each column, and centrifuge.

Proceed to hybridization.



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Overview, continued

Alexa Fluor® 3 and Alexa Fluor® 5

The novel, application-specific Alexa Fluor® 3 and Alexa Fluor® 5 dyes used in the kit are compatible with commonly used microarray scanners, and provide greater signal correlation (R^2) values than the spectrally similar Cy™3 and Cy™5 dyes, improving the resolution of two-color aCGH.

The table below shows the excitation/emission maxima and color of each dye:

Dye	Excitation/Emission	Color
Alexa Fluor® 3	555/565 nm	Pink
Alexa Fluor® 5	650/670 nm	Light blue

Control DNA

Control DNA (Salmon Sperm DNA) is included in the kit to help determine the efficiency of the labeling procedure. Note that the degree of labeling efficiency will depend on the quality of the FFPE sample. Equations for calculating labeling efficiency are provided on page 8.

Materials Supplied by the User

In addition to the kit components, you should have the following items on hand before using the BioPrime® Total Genomic Labeling System.

- Genomic DNA purified from FFPE sample (amount is array-dependent)
- Vortex mixer
- Microcentrifuge
- Heat block, air incubator, or thermocycler with heated lid
- Ice
- 1.7-ml DNase-free capped tubes or thin-walled PCR tubes
- Aerosol-resistant pipette tips
- 100% isopropanol and 100% ethanol (for preparing the purification buffers; see page vi)

Product Qualification

The Certificate of Analysis (CofA) provides detailed quality control information, and is available on our website. Go to www.invitrogen.com/support and search for the CofA by product lot number, which is printed on the box.

Methods

Before Starting

Amount of Starting Material

The amount of starting material depends on the recommendations of your array manufacturer. This kit can detect differences in gene copy number from as little as 500 ng of genomic DNA from FFPE samples (depending on sample quality).

Isolating Genomic DNA

Isolate genomic DNA using a high-quality purification system that is designed for use with FFPE samples. The PureLink™ Genomic DNA Mini Kit is a complete kit for the isolation of genomic DNA from various sample types, including FFPE tissues (see the **Important** note below). See page 13 for ordering information.

Important

If you are using the PureLink™ Genomic DNA Mini Kit, we strongly recommend performing an *overnight* digestion step of the FFPE sample lysate with Proteinase K.

The protocol in the PureLink™ manual suggests that the sample may be digested at 50°C for 3 hours to overnight. However, we have found that digesting overnight or even longer yields optimal results.

General Handling of DNA

When handling DNA, use sterile conditions to ensure that no DNases are introduced. All equipment that comes into contact with DNA should be sterile, including pipette tips, microcentrifuge tubes, snap-cap polypropylene tubes, and pipettes. Be sure pipettor barrels are clean and treated with ethanol.

Checking DNA Quantity and Quality

Genomic DNA may be run on an agarose gel to check for quantity and quality. Bufferless E-Gel® Pre-cast Agarose Gels are available from Invitrogen for fast and easy electrophoresis. See page 13 for ordering information.

Storing DNA

After isolating the DNA, we recommend that you proceed directly to **Labeling** on the next page. Otherwise, store the isolated genomic DNA at +4°C.

Labeling

Required Materials

In addition to the components of the Labeling Module, the following materials are supplied by the user:

- Genomic DNA (amount is array-dependent)
- Vortex mixer
- Microcentrifuge
- Heat block, incubator, or thermocycler with a heated lid
- Ice
- 1.7-ml capped tubes or thin-walled PCR tubes

Important

Fluorescently labeled nucleotides are sensitive to photobleaching. During all steps of the procedure, be careful to minimize exposure of the 10X Nucleotide Mixes and labeled DNA to light.

Preparing the Control DNA

The Control DNA is provided at a concentration of 10 mg/ml, and should be diluted in DEPC-treated water prior to use. To prepare 1 µg of Control DNA for labeling:

1. Dilute the Control DNA to a concentration of 1 µg/µl:

Control DNA (10 mg/ml)	1 µl
DEPC-treated water (provided in the kit)	9 µl
Final volume	10 µl

2. Add 1 µl of the diluted Control DNA to 21 µl of water, for a final volume of 22 µl.

Incubation Methods

The incubation steps may be performed in a heat block, air incubator, or thermocycler with a heated lid. Incubate the reaction protected from light.

Continued on next page

Labeling, continued

Labeling Procedure

1. If necessary, thaw the 2.5X Random Primers Solution and Alexa Fluor® 10X Nucleotide Mixes at room temperature. (Keep the labeled mixes protected from light.)
2. Briefly vortex each tube and centrifuge to collect the contents. Place the tubes on ice.
3. On ice, add the following to separate DNase-free 1.7-ml capped tubes or thin-walled PCR tubes:

Component	Tube 1	Tube 2
2.5X Random Primers Solution	20 µl	20 µl
Alexa Fluor® 3 10X Nucleotide Mix	5 µl	—
Alexa Fluor® 5 10X Nucleotide Mix	—	5 µl
Genomic DNA Sample 1	22 µl	—
Genomic DNA Sample 2	—	22 µl
Total volume	47 µl	47 µl

   Optional: You can also prepare a control reaction for each dye using 1 µg of Control DNA provided in the kit (diluted as described on the previous page).
4. Gently pipet up and down to mix and incubate at 95ºC, protected from light, for 5 minutes. Immediately cool on ice for 5 minutes.
5. On ice, add 3 µl of Exo– Klenow Fragment to each tube, for a final reaction volume of 50 µl.
6. Vortex tubes briefly and centrifuge to collect the contents.
7. Incubate at 37ºC for 2 hours in a heat block, air incubator, or thermocycler with a heated lid, protected from light.
8. After incubation, if you are storing the reaction for any length of time prior to purification, add 5 µl of Stop Buffer to each tube to quench the reaction. If you are proceeding directly to purification, you can skip this step.

Proceed to **Purification**, next page. The reaction can be stored at –20ºC overnight if necessary (following the addition of Stop Buffer).

Purification

Purification Module

In this step, you use the Purification Module provided with the system to purify the labeled DNA.

Purification Procedure

1. Add 200 µl of Binding Buffer B2 (prepared with isopropanol as described on page vi) to each tube from Step 8 on the previous page, and vortex to mix.
2. Load each sample onto a PureLink™ Spin Column, preinserted in a collection tube.
3. Centrifuge at 10,000 × *g* for 1 minute. Discard the flow-through and place the column back in the collection tube.
4. Add 650 µl of Wash Buffer W1 (prepared with ethanol as described on page vi) to the column.
5. Centrifuge at 10,000 × *g* for 1 minute. Discard the flow-through and place the column back in the collection tube.
6. Spin at maximum speed for an additional 2–3 minutes to remove any residual wash buffer. Discard the flow through.
7. Place the Spin Column in a new, sterile Amber Recovery Tube (supplied in the kit).
8. Add 55 µl of Elution Buffer E1 to the center of the column and incubate at room temperature for 1 minute.
9. Centrifuge at maximum speed (~20,000 × *g*) for 2 minutes. The flow-through contains the purified labeled DNA probes. (Discard the column after use.)

To determine the efficiency of the labeling reaction, proceed to the next page.

For a list of array hybridization reagents available from Invitrogen, see page 13.

Assessing the Efficiency of the Labeling Procedure

Calculating the Results

Note: Labeling efficiency will depend on the quality of the FFPE sample.

To calculate the amount of labeled DNA using a UV/visible spectrophotometer:

1. Transfer an appropriate volume of purified, labeled DNA from step 9, page 7, to a clean cuvette. Use an appropriate volume for your spectrophotometer. Blank the spectrophotometer using 10 mM Tris-HCl, pH 8.5.

   Important: The labeled DNA must be purified as described on page 7 before scanning, as any unincorporated labeled nucleotides will interfere with the detection of labeled DNA.

2. Measure the absorbance of the sample at A_{260}, A_{320}, A_{555}, A_{650}, and A_{750}. Wash each cuvette thoroughly between samples.

Yield:[1]

DNA (µg) = $(A_{260}–A_{320}) \times 50$ µg/ml × volume in ml

Dye Incorporation:[2]

Alexa Fluor® 3 (pmole) = $(A_{555}–A_{650})/0.15 \times$ volume in µl

Alexa Fluor® 5 (pmole) = $(A_{650}–A_{750})/0.24 \times$ volume in µl

Degree of Labeling:[3]

Alexa Fluor® 3 base/dye ratio =
$((A_{260} – A_{320}) – ((A_{555} – A_{650}) \times 0.04)) \times 150{,}000/(A_{555} – A_{650}) \times 6{,}600$

Alexa Fluor® 5 base/dye ratio =
$((A_{260} – A_{320}) – ((A_{650} – A_{750}) \times 0)) \times 239{,}000/(A_{650} – A_{750}) \times 6{,}600$

Notes:

[1]Subtracting A_{320} from A_{260} corrects for any silica particles that may leak from the purification columns and artificially increase the yield calculations.

[2]Subtracting A_{650} from A_{555} and A_{750} from A_{650} corrects for any fluorescent background that might artificially increase the measure of dye incorporation.

[3]Absorbance at A_{555} has a very slight effect on the A_{260} reading, and the formula $((A_{555} – A_{650}) \times 0.04))$ corrects for this. Conversely, there is no effect of A_{650} on the A_{260} reading; the multiplication by zero was added to the second formula to keep the formulas consistent.

Expected Results

Control DNA

Typically, if starting with 1 µg of Control DNA as specified on page 5, you should expect the following:

Yield: ≥7 µg of amplified DNA

Dye incorporation: ≥175 pmol Alexa Fluor® 3
≥300 pmol Alexa Fluor® 5

Degree of labeling: ≥0.7 for Alexa Fluor® 3
≥1.2 for Alexa Fluor® 5

Note on Signal Intensity

The Alexa Fluor® dye-labeled nucleotides and reaction conditions of the BioPrime® Total FFPE Genomic Labeling System have been optimized for use on microarrays. Signal intensity and signal/background on microarrays does not correlate directly with dye incorporation or degree of labeling when comparing different fluorescent dyes.

Labeling with Alexa Fluor® 3 and Alexa Fluor® 5 dye-labeled nucleotides typically yields microarray signal intensities and signal/background ratios greater than or equal to labeling with other labeled nucleotides, even with lower dye incorporation and/or degree of labeling.

Saturated Spots

Due to the higher signal intensities associated with Alexa Fluor® dyes, you may see more saturated spots with your standard scanner settings.

The percentage of saturated spots should be ≤10%. If it is higher, we recommend lowering the PMT (photomultiplier tube) setting on your scanner by 10% and rescanning the array. If it is still too high, lower the setting by another 10% and rescan. Repeat as necessary.

Troubleshooting

Problem	Cause	Solution
Yield of labeled DNA from both the control reaction and the experimental sample is low	DNA has been lost in the purification step after labeling	Make sure that isopropanol has been added to the Binding Buffer and ethanol has been added to the Wash Buffer, as specified on page vi. Measure the amount of labeled DNA in the control reaction before and after purification. Repeat the labeling and purification procedures, following all steps without modifications.
Yield of labeled DNA from the experimental sample is low, but the control reaction is fine	FFPE sample is degraded	Carefully evaluate the quality of your FFPE sample; some samples may be too degraded for effective labeling
	Starting amount of DNA sample is too low	Increase the amount of starting DNA.
	FFPE sample is not completely digested by Proteinase K	If you are using the PureLink™ Genomic DNA Mini Kit, we strongly recommend performing an *overnight* digestion step with PureLink™ Genomic Digestion Buffer and Proteinase K (see **Important** note on page 4).
Cannot detect labeled probes	DNA has been lost in the purification step after labeling	Make sure that isopropanol has been added to the Binding Buffer and ethanol has been added to the Wash Buffer, as specified on page vi. Measure the amount of labeled DNA in the control reaction before and after purification. Repeat the labeling and purification procedures, following all steps without modifications.
Amount of incorporated labeled nucleotides is low or fluorescence is low	Starting amount of DNA is too low	Increase the amount of starting DNA
	Reaction tubes have been exposed to light	Avoid direct exposure of the reaction tubes to light. Repeat the labeling procedure.
	Fluorescent nucleotides have been exposed to light	Repeat the labeling reaction, being careful to avoid direct exposure to light.
	Inefficient labeling due to improper purification	Follow all the purification steps as described in the procedures.
High background with Agilent scanners	High yields of DNA, a high level of dye incorporation and/or degree of labeling, and/or extended hybridization times	Adjust the PMT settings on the scanner to reduce background as described on page 9

Appendix

Technical Support

World Wide Web

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, MSDSs, FAQs, formulations, citations, handbooks, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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MSDS Requests

MSDSs are available on our website at www.invitrogen.com. On the home page, click on **Technical Resources** and follow instructions on the page to download the MSDS for your product.

Certificate of Analysis

The Certificate of Analysis provides detailed quality control information for each product. Certificates of Analysis are available on our website. Go to www.invitrogen.com/support and search for the Certificate of Analysis by product lot number, which is printed on the box.

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Technical Support, continued

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives. Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation's liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order. Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives. **Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.**

Additional Products

Additional Products

Invitrogen has additional reagents that may be used to prepare labeled probes for hybridization. Ordering information is provided below.

Product	Quantity	Catalog no.
PureLink™ Genomic DNA Mini Kit	50 preps 250 preps	K1820-01 K1820-02
E-Gel® 1.2% Starter Pak	6 gels and base	G6000-01
Human Cot-1 DNA®-Fluorometric QC	1 mg	15279-101
Human Cot-1 DNA®	500 µg	15279-011
Mouse Cot-1 DNA®	500 µg	18440-016
Yeast tRNA	25 mg 50 mg	15401-011 15401-029

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Notes:

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