For non-isotopic thermal cycle labeling of DNA probes

KPL Detector™

PCR DNA Biotinylation Kit

Catalog Number
5910-0031 (60-01-01)

Size
30 reactions

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INTRODUCTION

Description

The KPL Detector™ PCR DNA Biotinylation Kit provides a rapid method for biotinylating DNA probes through incorporation of biotin-N_4-dCTP via a thermostable DNA polymerase in the polymerase chain reaction*.[1] Biotinylated probes generated using this kit are highly sensitive and allow for the identification of low copy target sequences. The process of direct labeling during PCR results in specific labeling and amplification of the sequence of interest even from crude DNA samples.

The ratio of biotin dCTP to unlabeled dCTP is optimized to produce probes with maximal biotin incorporation for detection of low copies or rare targets in mRNA and plasmid or Genomic DNA. Control primers and template DNA are included as positive controls to monitor the integrity of the assay and the components of the kit. The pre-biotinylated quantitation standard is used to quantitate the yield of biotin labeled probe. Quantitation of the probe is essential as the concentration of probe used in the hybridization buffer is critical for greatest sensitivity and minimal background.

This kit does not contain a thermostable polymerase, 10X PCR reaction buffer or $MgCl_2$. Therefore, users are required to provide their own thermostable DNA polymerase, which is typically supplied with the appropriate buffers. The type of thermostable polymerase, the quality of template, the choice of PCR primers and the size of fragment being generated must be taken into consideration for successful labeling and amplification. For optimal and efficient labeling during PCR it is recommended that the target amplimer be in the range of 100-1100bp.

Applications

Biotinylated probes can be detected with enzyme-labeled streptavidin and visualized using chromogenic or chemiluminescent substrates. Applications for detecting nucleic acids using biotinylated probes include Southern, Northern and dot/slot blotting; colony and plaque hybridizations and *in situ* hybridization. Amplification and direct labeling from small samples of genomic (1 - 100 ng) or plasmid template (10 pg - 1 ng) is most easily achieved by first optimizing the conditions for standard PCR before attempting to label the probe during the reaction.

Direct detection of a PCR biotin-labeled fragment is also possible. The biotinylated PCR product is electrophoresed, transferred to membrane and subsequently detected **without a probe** using enzyme-labeled streptavidin. A signal is then generated using the appropriate chromogenic or chemiluminescent substrate.

***Purchase of this kit does not constitute a license for PCR. A licensed polymerase and licensed thermal cycler must be used in conjunction with this product. PCR is covered by patents owned by Hoffman-La Roche, Inc. and F. Hoffman-La Roche, Inc.*

MATERIALS AND EQUIPMENT

Kit Components	Product Code	Volume
KPL 10X PCR Labeling Mix 1.5 mM biotin-N_4-dCTP/0.5 mM dCTP/ 2 mM dATP/2 mM dGTP/ 2 mM dTTP in 10 mM Tris-HCl (pH 7.5)/ 1 mM Na_2EDTA	5960-0030 **(**600-0011)	150 µL
KPL Control Primers 50 µM Control Primers in 10 mM Tris-HCl (pH 7.5)/1 mM Na_2EDTA	5910-0036 (600-0009)	30 µL
KPL Control Template DNA 20 ng/µL λ *Hind III* fragments in 10 mM Tris-HCl (pH 7.4)/ 5 mM NaCl/ 0.1 mM Na_2EDTA	5910-0034 (600-0004)	25 µL
KPL DEPC Treated Water	5960-0019 (50-86-03)	1.0 mL
KPL Quantitation Standard Biotinylated nucleic acid in 50 mM Tris, 50 mM Na_2EDTA (pH 8.0)	5960-0028 (600-0007)	100 µL

Control Primer 1 sequence: 5′-GATGAGTTCGTGTCCGTACAACTGG-3′
Control Primer 2 sequence: 5′-GGTTATCGAAATCAGCCACAGCGCC-3′

Sufficient reagents are provided to perform 30 labeling reactions following the protocol provided in this manual. Reagents must be stored at -20°C and used as directed. Do not store kits in a frost-free freezer. Kit components are stable for a minimum of 1 year from date of receipt when stored as instructed.

REQUIRED SUPPLIES AND EQUIPMENT NOT INCLUDED

- Licensed DNA Polymerase with 10X PCR buffer
- $MgCl_2$ stock solution
- Primers
- 1X TE (See Solution Preparation)
- Licensed Thermal Cycler
- KPL 20X SSC
- Gloves
- Micropipetters and sterile tips
- Ice bath
- Microcentrifuge
- 37°C water bath
- UV Crosslinker or vacuum oven
- Nylon or nitrocellulose membrane (SeraCare recommends Pall Biodyne® B/ 5960-0025 (60-00-50), MSI MagnaGraph or MSI Nitropure)

TO QUANTITATE PROBE

In order to quantitate the concentration of labeled probe, the following detection reagents must be used:

For HRP Detection:
KPLDetector™ HRP Blotting Kit 5910-0027 (54-30-00)

For AP Detection:
KPL DNADetector™ AP Southern Blotting Kit 5910-0028 (54-30-01)

SOLUTION PREPARATION

KPL 20X SSC 5960-0021 (50-86-05)
3.0 M NaCl
300 mM Sodium Citrate
pH 7.5
sterile filter or autoclave solution

10X TE
100 mM Tris
10 mM EDTA
pH 8.0
sterile filter or autoclave solution

ASSAY OPTIMIZATION

Any sequence that can be successfully amplified by PCR may be used with this protocol. It is highly recommended that the optimal conditions for PCR be determined with unmodified nucleotides for each individual template and primers before using this kit to label probe. There are many resources available for optimization of PCR. (See References 2 - 5.)

Optimization of the reaction should include the following considerations:

- Quality, purity and concentration of template
- PCR primer design, including melting temperature, secondary structure
- Size of desired amplimer (100 - 1100 bp is optimal for efficient labeling)
- $MgCl_2$ concentration
- Denaturation time/temperature
- Annealing duration/temperature
- Extension duration/temperature
- Enzyme concentration
- Number of cycles

Notes:

- **Templates containing a high percentage of AT-rich sequence may label weakly as the biotin in this kit is coupled to dCTP.**
- **This kit has been used to successfully amplify and label DNA using templates that range in size from 100 bp to 1.1 Kb.**

Polymerase choice:

In general, any thermostable polymerase that routinely and specifically amplifies the desired product when unmodified nucleotides are used will work with this kit. For complete information on individual thermostable polymerases, please refer to the specific enzyme supplier.

SeraCare has qualified this kit with a number of thermostable polymerases including: Taq DNA Polymerase, Native or Recombinant (MBI Fermentas and Life Technologies), Taq DNA Polymerase (Promega), Taq Bead™ Hot Start Polymerase (Promega), Tth DNA Polymerase (Promega), Expand™ High Fidelity Polymerase (Roche Biochemical).

PROBE LABELING

Allow all reagents to thaw out completely, then vortex briefly and spin down in a microcentrifuge before pipetting. Keep all reagents on ice while in use except for the 10X Labeling Mix. This solution should be thawed on the lab bench and used at room temperature. Mix well before use. Failure to thaw completely may result in no amplification. Pipette reagents slowly and carefully to avoid errors.

Note: The following protocol was designed to be used specifically for PCR labeling of the control template included with this kit. PCR reaction conditions should be optimized for each new template/primer set with unmodified nucleotides before use of this kit. Use the following protocol as a guideline only.

By adjusting the final concentration of the KPL 10X Labeling Mix included in the kit probes of up to 1 kb in length may be generated. For probes less than 500 base pairs, a final concentration of 200 µM of each nucleotide is suggested (5 µL/50µL PCR reaction), and for probes greater than 500 base pairs, 350 µM of each nucleotide is suggested (8.75µL/50µL PCR reaction).

Prepare the reaction mix in a sterile PCR tube in the order as it appears below. Place the tube on ice while pipetting.

Component	Volume	Final
KPL DEPC Treated Water	variable	add to 50 uL
10X PCR Buffer	5 uL	1X
25mM $MgCl_2$	4 uL	2.0 mM
KPL 10X Labeling Mix	5 uL	200 uM each
KPL Control Primers	1 uL	0.5 uM each
Taq DNA Polymerase	variable	1.25 units/50 uL rxn
KPL Control Template	1 uL	20 ng/50 uL reaction
Total Mix	50 uL	

Mix the tube by tapping gently and centrifuging briefly. Cycling parameters will vary depending on the respective template, primers and the thermocycler used.

See page 8 for recommended cycling conditions for the control labeling reaction.

Recommended Temperature Cycling for Control Labeling Reaction

	Time	Temperature	Cycles
Initial Step	60 seconds	94°C	1 cycle
Denaturation	15 seconds	94°C	25 cycles
Anneal/Extend	60 seconds	68°C	25 cycles
Final Step	7 minutes	72°C	
Hold		4°C	

The control reaction generates an amplification product of 500 bp.

After PCR, transfer the reaction to a low retention microfuge tube (see required reagents) and store at 2 – 8°C. Run approximately 5 uL of sample on a 0.8-1% TBE or TAE agarose gel to determine the quality of the PCR product and to estimate the overall yield of the reaction. Transfer the remaining aliquot of sample to a silanized microfuge tube (see required reagents) and store at 2 - 8°C until quantitation (page 9).

Note: PCR biotinylated probes may be reused repeatedly. Store hybridization solution containing probe at 2 - 8°C (formamide-based solutions) or –20°C (aqueous solutions). Probe diluted in hybridization cocktail should be denatured either by heating to 68°C for 10 minutes (formamide-based hybridization solution) or by boiling the hybridization cocktail for 10 minutes (aqueous hybridization solution) before reuse.

Removal of non-specific amplimers, primers and unincorporated nucleotides:

Following biotinylation and analysis of the product on an agarose gel, separation of the newly labeled probe from unincorporated nucleotides, primers or non-specific amplimers may be desired if using the labeled product in hybridization assays. This is usually not necessary, as unincorporated nucleotides do not significantly increase background. If non-specific PCR products are evident in the final reaction, excise the correct band from an agarose gel and subsequently purify the product using well established methods.

In the unlikely event that oligonucleotides or unincorporated nucleotides need to be removed, SeraCare recommends using KPL Spin-Pure filters 5640-0001 (60-00-53) which will remove unincorporated nucleotides, primers or non-specific amplimers with minimal loss of labeled product. The use of 5’ $\rightarrow$ 3’ Select B columns, which have been developed specifically for purification of biotinylated probes, is also recommended. It is ***not*** recommended that G50 Microspin Purification Columns be used because biotin sticks to the resin resulting in loss of labeled PCR product.

PROBE QUANTITATION

Use this protocol to determine the amount of biotinylated DNA synthesized from the labeling reaction. The number of biotin-N_4-dCTPs incorporated on average per base remains constant. The amount of DNA synthesized will vary depending on the incubation time and the amount and purity of the template. See page 5 for a list of detection reagents.

NOTE: It is critical to the success of non-isotopic detection that the newly biotinylated probe be quantitated to determine the appropriate amount for the hybridization reaction. Biotinylated probes need only be quantitated once; they do not lose activity over time.

Probe Dilution and Dot Blotting

1. Prepare an initial 1:200 dilution of the newly labeled probe (0.5 µL DNA into 99.5 µL diluent). Make a series of two-fold dilutions from both the undiluted biotinylated DNA standard and the 1/200 dilution of probe, as follows: 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512. Use 2X SSC/0.1% SDS as the diluent or 1X TE if dilution tubes are silanized.

   Note: Pre-wet membrane in molecular biology grade water and soak in 5X SSC for at least 10 minutes. Dry the membrane for at least 10 minutes before use. 5X SSC equilibrated membrane may also be stored dry until use.

2. Prepare a dot blot by spotting 1 uL of each dilution onto a dry, pretreated membrane. Arrange samples so that corresponding dilutions of the standard and probe to be quantitated are side by side. It is not necessary to denature the DNA.

3. Allow the dots to dry for 10 minutes. UV crosslink the DNA to the damp membrane according to manufacturer's instructions or bake for 30 minutes at 80°C.

4. Detect the dots using one of the procedures on pages 10 - 11.

Detection using KPL HRP-Streptavidin and KPL LumiGLO®
(KPL Detector HRP Blotting Kit, 5910-0027 (54-30-00))
Note: Perform all incubations and washes with gentle agitation at room temperature (25°C ± 3°C).

1. Prepare 1X KPL Detector Block blocking/diluent solution according to the recommended procedure provided on the KPL Detector Block datasheet.

2. Incubate membrane for 30 minutes with 1X blocking/diluent solution (from step 1); use at least 0.1 mL diluted blocking solution per cm^2 membrane.

3. Incubate membrane in KPL HRP-SA diluted 1:500 in 1X blocking/diluent solution (from step 1) (i.e. 10 µL HRP-SA+ 5 mL diluted blocking solution) use at least 0.1 mL diluted KPL HRP-SA per cm^2 membrane. Incubate for 20 minutes.

4. Transfer membrane to a clean container and wash with KPL Biotin Wash Solution Concentrate diluted 1:10 in molecular biology grade water (i.e. 1 mL KPL Biotin Wash Solution Concentrate + 9 mL molecular biology grade water). Perform 3 washes for 5 minutes each using at least 0.4 mL diluted wash solution per cm^2 membrane.

5. Prepare enough KPL LumiGLO Chemiluminescent Substrate to completely immerse the membrane by mixing equal volumes of Solution A and Solution B. Incubate membrane in prepared KPL LumiGLO for 1 minute.

6. Remove excess KPL LumiGLO by blotting membrane on a piece of clean filter paper. Place membrane in a hybridization bag or between plastic sheets. Expose membrane to X-ray film for approximately 10 minutes. For more information on detection with KPL LumiGLO, consult the instructions accompanying the detection kit.

   Note: KPL TMB Membrane Substrate may also be used for chromogenic detection following the instructions in the product datasheet.

7. Determine relative specific activity of labeled probe following the instructions on page 12.

Detection using AP-Streptavidin and CDP-Star®
(KPL Detector AP Blotting Kit 5910-0028 (54-30-01))
Note: Perform all incubations and washes with gentle agitation at room temperature (25°C±3°C).

1. Prepare 1X KPL Detector Block blocking/diluent solution according to the recommended procedure provided on the KPL Detector Block datasheet.

2. Incubate membrane for 30 minutes with blocking/diluent solution (from step 1) use at least 0.1 mL diluted blocking solution per cm^2 membrane.

3. Incubate membrane in KPL AP-SA diluted 1:10,000 in blocking/diluent solution (from step 1), (i.e. 1µL KPL AP-SA+ 10 mL diluted blocking solution) use at least 0.1 mL diluted AP-SA per cm^2 membrane. Incubate for 30 minutes.

4. Prepare 1X KPL Phosphatase Wash Solution by diluting 5X KPL Phosphatase Wash Solution 1:5 in molecular biology grade water (i.e. 10 mL KPL Phosphatase Wash Solution + 40 mL water). Make sure the 5X Phosphatase Wash Solution is completely in solution before diluting. Warm to 37°C if necessary.

5. Place membrane in a clean container and wash 3 times for 5 minutes each using at least 1 mL of 1X Phosphatase Wash Solution per cm^2 membrane.

6. Dilute 10X Phosphatase Assay Buffer 1:10 in molecular biology grade water (i.e. 5 mL KPL Phosphatase Assay Buffer + 45 mL water). Rinse the membrane 2 times for 2 minutes each in 1X KPL Phosphatase Assay Buffer using at least 0.5 mL per cm^2 membrane.

7. Drain off KPL Phosphatase Assay Buffer completely and incubate membrane 5 minutes in CDP-Star Chemiluminescent Substrate using at least 0.05 mL per cm^2 membrane.

8. Remove excess CDP-Star by blotting membrane on a piece of clean filter paper. Place membrane in a hybridization bag or between plastic sheets. Expose membrane to X-ray film for approximately 2 minutes. For more information on detection with CDP-Star, consult the instructions accompanying the detection kit.

9. Determine relative specific activity of labeled probe following the instructions on page 12.

PROBE RELATIVE SPECIFIC ACTIVITY (RSA)

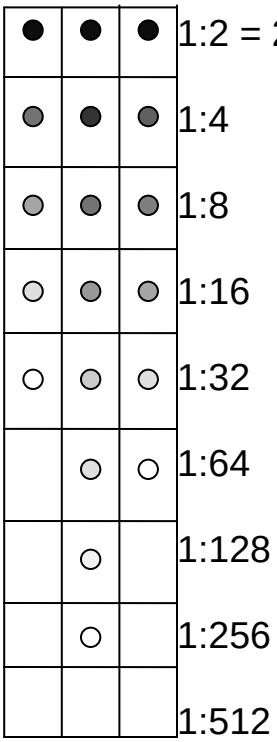
The relative specific activity (RSA) of the labeled probe is defined by the relative amount of biotin attached to the nucleic acid.

Calculate approximate probe RSA as follows:
Quantitation Standard RSA = 50 ng/µL
Reaction Volume = 50 µL

1. Calculate ratio of probe to standard (P/S):
P/S = probe endpoint dilution
standard endpoint dilution

2. Calculate probe RSA:
Probe RSA = standard RSA x P/S

3. Calculate total yield of biotinylated DNA probe:
Total yield = probe RSA x reaction volume



NOTE: When calculating the probe RSA it is not necessary to factor in the initial 1:200 dilution of probe. Following steps 1 - 3 above will give you the correct result.

Examples: (See Figure)
Probe 1 (P1) endpoint dilution = 1:32
Standard (S) endpoint dilution = 1:64

1. P:S = 32/64=1:2
2. Probe RSA = 50 ng/µL x 1/2 = 25 ng/µL
3. Total yield = 25 ng/µL x 50 uL = 1.25 µg biotinylated DNA

Probe 2 (P2) endpoint dilution = 1:256
Standard (S) endpoint dilution = 1:64

1. P/S = 256/64 = 4
2. Probe RSA = 50 ng/µL x 4 = 200 ng/µL
3. Total yield = 200 ng/µL x 50 µL = 10 µg biotinylated DNA

EXPECTED PROBE YIELD

The amount of biotinylated DNA synthesized will vary based on the template DNA, primers, experimental protocols, tubes and thermocyclers. For best results in hybridizations with low or single copy genes probes should be labeled to at least 50 ng/uL relative specific activity (RSA).

OPTIMIZATION AND TROUBLESHOOTING GUIDE

PROBLEM	POSSIBLE CAUSE	COMMENTS
No product is observed.	• A component may be missing.	• Always include a positive control.
	• The 10X KPL Labeling Mix was not warmed prior to use.	• The mix was not homogenous.
	• Too few cycles performed or extension time too short.	• 25 to 35 cycles is recommended. • Primer extension is usually carried out at 72°C for 20 - 40 seconds. Increasing the extension time may result in higher yield.
	• Annealing temperature too high.	• Primer annealing temperature must be optimized empirically. If temperature is too high, no annealing occurs. Decrease in 2 - 4°C increments.
	• Insufficient or poor template quality.	• Insufficient amount of template may result in primers unable to find their complementary sequence. Increase concentration of template or evaluate purity of template.
	• Insufficient denaturation.	• Denaturation should be done at 95°C. It may be necessary to optimize denaturation time (10 second increments).
	• Primers may not be optimally designed.	• Design primers that are 22-30 bp in length and a GC content of 45-60%.
	• Too little enzyme.	• 0.5 to 2.5 units is sufficient for most applications.

TROUBLESHOOTING GUIDE (continued)

PROBLEM	POSSIBLE CAUSE	COMMENTS
No product is observed.	• $MgCl_2$ concentration too low	• The optimal Mg^{2+} concentration varies from 0.5 mM to 5 mM. Increase in 0.1 mM increments.
	• Target template is difficult to amplify.	• In most cases this is due to unusually high GC content and or secondary structure. Addition of 2-Betaine and DMSO may help amplification.
Multiple products and/or products are smeared.	• Too many cycles performed.	• Reducing number of cycles may eliminate non-specific bands.
	• Annealing temperature too low.	• Increase annealing and or extension temperature in 2-3°C increments.
	• The primers may not be optimally designed.	• Design primers that are 22-30 bp in length and a GC content of 45-60%.
	• Too much enzyme in the mixture.	• 0.5 to 2.5 units is suitable for most applications.
	• $MgCl_2$ concentration may be too high.	• The optimal Mg^{2+} concentration varies from 0.5 mM to 5 mM. Decrease in 0.1 mM increments.
	• Template concentration too high.	• Too much template may lead to an increase in mispriming events. Reduce concentration of template in reaction.
Quantitation Standard appears weak	• Adherence of the biotin to the tube	• Heat quantitation standard at 95°C for 5 minutes and repeat protocol.

RELATED PRODUCTS

Product	Size	Catalog No.
KPL Detector™ HRP Blotting Kit	2400 cm^2	5910-0027 (54-30-00)
KPL Detector™ AP Blotting Kit	2000 cm^2	5910-0028 (54-30-01)
KPL Biodyne® B Nylon Membrane	20 cm x 1 m	5960-0025 (60-00-50)
KPL 20X SSC	1 Liter	5960-0021 (50-86-05)
KPL Hybridization Bags	50 bags	5960-0026 (60-00-51)
KPL Herring Sperm DNA	40 mg	5920-0003 (60-00-14)
KPL Spin-Pure Filters	5 filters	5640-0001 (60-00-53)
KPL GeneRuler Biotinylated DNA Ladder	100 µL	5960-0029 (600-0008)

DETECTION REAGENTS

Product	Size	Catalog No.
KPL HRP Labeled Streptavidin	1 mL	5950-0004 (474-3000)
KPL AP Labeled Streptavidin	1 mL	5950-0005 (475-3000)
KPL 5X Detector Block	1200 mL	5920-0004 (71-83-00)
KPL 10X Biotin Wash Solution	200 mL	5960-0015 (50-63-06)
KPL Phosphatase Wash Solution (5X)	600 mL	5960-0018 (50-63-15)
KPL Phosphatase Assay Buffer (10X)	200 mL	5960-0017 (50-63-14)
KPL LumiGLO Chemiluminescent Substrate	240 mL	5430-0040 (54-61-00)
KPL TMB Membrane HRP Substrate	200 mL	5420-0027 (50-77-03)

REFERENCES

1. PCR is covered by patents owned by Hoffmann-La Roche, Inc. and F. Hoffmann La Roche, Inc.
2. Innis, M.A., Gelfand, D.H., Sninsky, J.J., Eds. (1995). PCR Strategies. Academic Press, San Diego, CA.
3. White, B.A. Ed. (1993). PCR Protocols: Current Methods and Applications; *Methods in Molecular Biology*, Vol. 15. The Humana Press, Totowa, N.J.
4. Dieffenbach, C.W. and Dveksler, G.S. (1995). PCR Primer – A Laboratory Manual. CSHL Press, Long Island, N.Y.
5. Syrjanen, S., et. al. (1991). The Use of Polymerase Chain Reaction in Generation of Biotinylated Human Papillomavirus DNA Probes for *in situ* Hybridization. *J. of Virological Methods*. 31:147-160.

The product listed herein is for research use only and is not intended for use in human or clinical diagnosis.

Notes:

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