

Biotin Chromogenic Detection Kit

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COMPONENTS OF THE KIT

Component	For 10 reactions (#K0661)	For 30 reactions (#K0662)
Streptavidin-AP Conjugate	40µl	120µl
50X BCIP/NBT Solution	4ml (2 x 2ml)	12ml (6 x 2ml)
Blocking Reagent (powder)	5g	15g
10X Blocking/Washing Buffer	200ml	600ml (3 x 200ml)
10X Detection Buffer	30ml	90ml

Materials and equipment to be supplied by the user

- Hybridization containers or bags for incubation/washing steps;
- Platform shaker for incubation/washing steps;
- High quality water (Milli-Q or double deionized);
- Labware for preparation and storage of solutions used in the experiments.

Related Products

- Biotin Decalabel™ DNA Labeling Kit #K0651, #K0652
- Biotin-11-dUTP #R0081
- NBT #R0841, #R0842
- BCIP-T #R0821, #R0822
- SensiBlot™ Plus Nylon Membrane #M1001, #M1002

STORAGE

All components of the kit are stable up to the kit expiry date if stored correctly.

Component	Storage conditions
Streptavidin-AP Conjugate	-20°C
50X BCIP/NBT Solution	-20°C (protect from light)
Blocking Reagent (powder)	-20°C or +4°C
10X Blocking/Washing Buffer	-20°C or +4°C
10X Detection Buffer	-20°C or +4°C

DESCRIPTION

The Biotin Chromogenic Detection Kit is a convenient tool for the chromogenic detection of biotinylated nucleic acid probes. The kit is optimized to reproducibly provide high detection sensitivity with low background in applications such as Southern, Northern, dot and slot blotting, as well as screening of viral plaques and bacterial colonies. The detection limit of a target sequence is 30-100fg.

PRINCIPLE

Biotin-labeled probe-target hybrids are detected with alkaline phosphatase-conjugated streptavidin (see Fig.1).

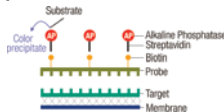


Fig.1. Chromogenic detection of a biotin-labeled probe-target hybrid.

Streptavidin is a biotin-binding tetrameric protein from *Streptomyces avidinii* with a molecular weight of approximately 60kDa. The streptavidin-biotin bond is the strongest known noncovalent biological interaction ($K_D \approx 10^{15} M$). Formation of such a complex is a very rapid process and, once formed, the complex is unaffected by external factors. Unlike avidin, streptavidin does not undergo any post-translational glycosylation. Its isoelectric point (pI 5-6) is ideal for use in the majority of applications. These properties result in high detection sensitivity and very low background.

Streptavidin is conjugated to alkaline phosphatase (AP), which facilitates chromogenic detection. Alkaline phosphatase cleaves the substrate, BCIP-T (5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt) which is supplemented with the enhancer chromogen NBT (nitro blue tetrazolium). This results in the formation of an insoluble blue precipitate, which appears as a well-defined spot or band at the reaction site on the membrane. No special equipment is required for chromogenic assays employing AP-conjugated streptavidin.

DETECTION PROTOCOL

The protocol is optimized for the detection of biotin-labeled probes hybridized to target nucleic acids on positively-charged membranes. It is not recommended for *in situ* hybridization techniques.

Preparation of Assay Solutions

All assay solutions should be prepared with high quality water (Milli-Q or double deionized).

Volumes of assay solutions required to develop a 100cm² membrane in an appropriately-sized container are given below.

Assay Solution	Required Volume
Blocking/Washing Buffer	200ml
Blocking Solution	50ml
Diluted Streptavidin-AP Conjugate	20ml
Detection Buffer	30ml
Substrate Solution	10ml

Scale the volumes of solutions according to the size of your membrane and container. Prepare sufficient solutions to allow the membrane to float freely in the tray.

Blocking/Washing Buffer. Dilute 1 volume of the concentrated 10X Blocking/Washing Buffer with 9 volumes of water. Diluted buffer can be stored at +4°C for 1 week. Add 0.1% sodium azide for a longer storage.

Blocking Solution. The Blocking Solution is prepared as a 1% (w/v) solution of Blocking Reagent in 1X Blocking/Washing Buffer. Weigh out the necessary amount of the Blocking Reagent, add the required volume of the Blocking/Washing Buffer, and stir the suspension on a magnetic stirrer until the Blocking Reagent completely dissolves.

Note

- Shaking of the suspension at 50-60°C facilitates dissolution of the Blocking Reagent.
- Once prepared, the Blocking Solution should be store in aliquots at -20°C.

Dilution of the Streptavidin-AP Conjugate. Dilute the concentrated Streptavidin-AP conjugate 5000-fold in Blocking Solution. Prepare sufficient solution to allow the membrane to float freely in a tray. The diluted solution should be prepared **just prior to use**.

Detection Buffer. Dilute 1 volume of the concentrated 10X Detection Buffer with 9 volumes of water. The diluted buffer can be stored at +4°C for 1 week. Add 0.1% sodium azide for a longer storage.

Substrate Solution. Dilute 1 volume of 50X BCIP/NBT Solution with 49 volumes of 1X Detection Buffer. Do not add 50X BCIP/NBT directly to the concentrated 10X Detection Buffer. The Substrate Solution should be prepared fresh **just prior to use**.

Detection Procedure

Note

During all steps of the detection procedure, the membrane must float freely in the container and be evenly covered with solution. Solution volumes provided below have been calculated for a 100cm² membrane in an appropriately-sized container.

Step	Procedure
1	Wash the membrane (after hybridization/washing steps) in 30ml of Blocking/Washing Buffer for 5 minutes at room temperature on a platform shaker with moderate shaking.
2	Block the membrane in 30ml of the Blocking Solution for 30 minutes at room temperature with moderate shaking.
3	Prepare 20ml of diluted Streptavidin-AP conjugate.
4	Incubate the membrane in 20ml of diluted Streptavidin-AP conjugate for 30 minutes at room temperature with moderate shaking.
5	Wash the membrane at room temperature with moderate shaking as indicated below: <ol style="list-style-type: none"> Incubate with 60ml of Blocking/Washing Buffer for 15 minutes. Discard the solution and repeat once with fresh Blocking/Washing Buffer. Discard the solution Incubate with 20ml of Detection Buffer for 10 minutes and discard the solution.
6	Perform the enzymatic reaction. Incubate the membrane in 10ml of freshly prepared Substrate Solution at room temperature in the dark. The blue-purple precipitate becomes visible after 15-30 minutes of incubation. For the highest sensitivity, allow the color to develop overnight.
7	Stop the reaction. Discard the Substrate Solution and rinse the membrane with water (Milli-Q or double deionized) for few seconds.
8	Discard the water and air-dry the developed membrane to document the results. Note. The membrane should not be dried if stripping and re-hybridization are planned in subsequent experiments.

SUPPLEMENTARY PROTOCOLS

Hybridization Protocol

Solutions Required

100X Denhardt's solution	20X SSC (pH 7.0)
2%(w/v) BSA	3M NaCl
2%(w/v) Ficoll™	0.3M Na ₃ Citrate
2%(w/v) PVP (polyvinylpyrrolidone)	

Note. This hybridization procedure has been optimized for nylon membranes, and it is recommended for the Fermentas SensiBlot™ Plus Nylon Membrane (#M1001, #M1002).

Procedure

Step	Procedure
1	Prepare the following pre-hybridization solution (final concentration): 6X SSC 5X Denhardt's solution 0.5% SDS 50% (v/v) deionized formamide
2	Denature a 0.5 mg/ml aqueous solution of sonicated herring or salmon sperm DNA at 100°C for 5 minutes, and then chill on ice. Add the denatured DNA to the pre-hybridization solution to obtain a final DNA concentration of 50µg/ml.
3	Place the membrane in an appropriately-sized container (plastic bag or hybridization bottle), add the pre-hybridization solution with denatured DNA (0.2ml/cm ²) and pre-hybridize at 42°C for 2-4 hours with shaking.
4	Prepare the hybridization solution: <ol style="list-style-type: none"> Denature the biotin-labeled probe at 100°C for 5 minutes and chill on ice. Add the denatured probe to the pre-hybridization solution to obtain a final probe concentration of 25-100ng/ml.
5	Discard the pre-hybridization solution and add the hybridization solution to the membrane (60µl/cm ²).
6	Incubate overnight at 42°C with shaking.
7	Wash the membrane twice with 2X SSC, 0.1% SDS. Perform each wash for 10 minutes at room temperature.
8	Wash the membrane twice with 0.1X SSC, 0.1% SDS. Perform each wash for 20 minutes at 65°C.
9	Remove excess liquid from the membrane by briefly placing it on filter paper.
10	Detect the biotin-labeled DNA as described above (see Detection Protocol).

Evaluation of Labeling Efficiency

The labeling efficiency is defined as the lowest detectable concentration of the labeled probe.

Step	Procedure
1	Prepare several dilutions of the biotin-labeled probe ranging from 1ng/ μ l to 10g/ μ l, and then spot 1 μ l of each dilution onto a nylon membrane strip.
2	Air-dry the spotted probe at room temperature for 30-45 minutes. Alternatively the spots can be dried at 80°C for 10 minutes.
3	Place the membrane on a UV trans-illuminator (spotted side down) and cross link the probe to the membrane for 1-5 minutes. Note. The spotted membrane can be stored indefinitely at +4°C or at room temperature in a plastic bag until needed.
4	Place the membrane strip into an appropriately-sized hybridization bag and perform the detection procedure as described above (see Detection Procedure). Adjust the volumes of the solutions to the size of the membrane strip and the bag.

Use the following guidelines for calculation of the necessary solution volumes:

- Blocking Solution 300 μ l/cm² of the membrane
- Diluted Streptavidin-AP Conjugate 200 μ l/cm² of the membrane
- Blocking/Washing Buffer 600 μ l/cm² of the membrane
- Detection Buffer 200 μ l/cm² of the membrane
- Substrate Solution 100 μ l/cm² of the membrane

The labeling efficiency is acceptable if the **30-100fg** spot of the labeled probe is easily detected after overnight incubation.

MEMBRANE RECOMMENDATIONS

The procedures and solutions described above have been optimized for the Fermentas SensiBlot™ Plus Nylon Membrane (#M1001, #M1002). The use of this membrane in combination with the Biotin Chromogenic Detection Kit is recommended to ensure the highest sensitivity of detection and the lowest background.

TROUBLESHOOTING

Low signal (sensitivity)

Possible cause	Recommendation
The probe concentration is too low	Increase the probe concentration
The hybridization time is too short	Increase the hybridization time
The labeling efficiency of the hybridization probe is too low	1. Check the labeling efficiency of hybridization probe (see the protocol above). 2. Prepare a new probe if the labeling efficiency is too low.
Post-hybridization washing conditions are too stringent	Decrease the temperature of post-hybridization washes.
The nucleic acid transfer procedure is inefficient	Increase the transfer time.

High/uneven background

Possible cause	Recommendation
Inefficient blocking	Prolong the blocking step
Inefficient washing	Prolong the washing step and/or increase the volume of the washing buffer
There are traces of agarose on membrane	Increase the membrane wash steps prior to the blocking step to ensure that no traces agarose gel are left on the membrane
The membrane does not float freely in solutions during detection procedure	Increase the volume of solutions in all steps
Impure water	Use only high-quality water
The working solution(s) have been contaminated by bacterial growth during storage	Add sodium azide to prevent bacterial contamination Prepare new working dilutions
Excess probe has been applied	Reduce the probe concentration
The pre-hybridization procedure is inefficient	Prepare a new pre-hybridization solution using only high quality non-specific DNA Increase the pre-hybridization time
Post-hybridization washes are not sufficient	Increase the temperature and/or the duration of washes

QUALITY CONTROL

The kit is tested in dot-blot hybridization on the SensiBlot™ Plus Nylon Membrane (#M1001). The biotin-labeled probe is generated by a random primer labeling reaction with the Fermentas Biotin Decalabel™ DNA Labeling Kit (#K0651). Hybridization and detection procedures are performed as described above (see sections the Hybridization procedure and the Detection Protocol). The labeled-probe spot corresponding to 30fg of the target DNA should be easily detected after overnight colour development.

Trademarks

Decalabel, **SensiBlot** are Fermentas trademarks.
Ficoll is a trademark of Amersham Pharmacia Biotech.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and in vitro use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

(2) Revised 02.03.2007

SAFETY INFORMATION



50X BCIP/NBT Solution

T Toxic

Hazard-determining component of labeling: **N,N-dimethylformamide**

Risk phrases

R61 May cause harm to the unborn child.
R20/21 Harmful by inhalation and in contact with skin.
R36 Irritating to eyes.

Safety phrases

S53 Avoid exposure - obtain special instructions before use.
S9 Keep container in a well-ventilated place.
S23 Do not breathe gas/fumes/vapour/spray.
S36/37/39 Wear suitable protective clothing, gloves and eye/face protection.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
S60 This material and its container must be disposed of as hazardous waste.