Illuminator Chemiluminescent Detection System

INSTRUCTION MANUAL

Catalog #300360 (Illuminator Chemiluminescent Detection System), and
#300362 (Illuminator Chemiluminescent Detection System
with Prime-It Fluor Fluorescence Labeling Kit)
Revision A.01

For In Vitro Use Only
300360-12

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MATERIALS PROVIDED

Materials provided[a]	Quantity	Storage temperature
Antifluorescein-antibody-AP conjugate	30 rxns	+4°C
CSPD® chemiluminescent substrate	1.2 ml	+4°C
Diethanolamine	30 ml	Room temperature
Southern block reagent (20% BSA)	30 ml	–20°C
Tween® 20	12 ml	Room temperature
Northern block reagent	7.5 ml	Room temperature

[a] The Illuminator chemiluminescent detection system contains enough reagents for processing 30 150-cm^2 membranes.

LONG-TERM STORAGE CONDITIONS

Southern Block Reagent: –20°C
CSPD®: +4°C
Antifluorescein-antibody-AP conjugate: +4°C
Diethanolamine (DEA): Room Temperature
Tween® 20: Room Temperature
Northern Block Reagent: Room Temperature

PRECAUTIONS

Diethanolamine is corrosive to skin and to mucous membranes
Northern block reagent causes eye burns and may cause respiratory tract irritation
Southern block reagent contains 0.01% sodium azide as a bacteriostat

ADDITIONAL MATERIALS REQUIRED

10× SSC
20% SDS
1 M $MgCl_2$
1 M Tris-HCl (pH 8.0)
5 M NaCl
Water bath or hybridization oven
Plastic wrap or heat-sealable bag
Pyrex® or plastic dishes
Nylon membranes
Sonicated salmon sperm DNA
Hybridization solution

Revision A.01

INTRODUCTION

The Stratagene Illuminator chemiluminescent detection system, a chemiluminescence-based nucleic acid detection kit, permits fast, safe, and sensitive detection of DNA and RNA immobilized on nylon membranes. As little as 0.2 pg of target plasmid DNA can be detected in a 30-minute exposure of the processed blot to X-ray film or, in a similar exposure time, 1 pg of a single-copy gene can be detected in less than 5.0 µg of genomic DNA.[1] The versatile Illuminator chemiluminescent detection system can also be used for rapid northern blot analysis of RNA. The Illuminator system is designed to be used in conjunction with the Prime-It® Fluor fluorescence labeling system, which incorporates fluoresceinated nucleotides into the probe fragments. After hybridization to nucleic acid immobilized on membrane, the fluoresceinated probe is detected with the reagents of the Illuminator system, which include an antifluorescein antibody conjugated to alkaline phosphatase and a dioxetane substrate for the enzyme. Upon cleavage by alkaline phosphatase, the substrate generates a chemiluminescent signal which can be captured on X-ray film. This nonisotopic nucleic acid detection method offers increased safety, alleviates radioactive waste disposal problems and shortens overall experimental time.

PROTOCOL

Probe Generation

Probe generation should be performed according to the Protocol described in the instruction manual for the the Stratagene Prime-It Fluor Fluorescence Labeling Kit (Catalog #300380). Labeled probe DNA should be purified from unincorporated nucleotides by ethanol precipitation or by using a NucTrap probe purification push-column. Failure to remove unincorporated nucleotides leads to increased background.

For a protocol for monitoring incorporation of fluorescent nucleotide into probe, see *Appendix II.*

Probe Hybridization and Detection

Southern Blot Protocol

See *Appendix I: Target Nucleic Acid Transfer to Membrane* for suggested gel electrophoresis and treatment conditions.

Note *Nylon membranes must be used for the following procedure.*

The following volumes of buffers and solutions have been optimized for a membrane that is 150 cm². If necessary, adjust the volumes at each step according to the size of your blot. Use a minimum of 2 ml of QuikHyb® hybridization solution for smaller-size blots.

Once the protocol has been started, do not allow the membrane to dry out.

1. After transfer and crosslinking, briefly rinse the membrane in dH_2O.

2. Add 10 ml of QuikHyb hybridization solution and prehybridize for 15 minutes at 68°C. (Standard overnight hybridization solutions and protocols may also be used.)

3. Place the fluoresceinated probe in a microfuge tube containing 100 µl of sonicated salmon sperm DNA (10 mg/ml) stock.

   Note *The probe should be generated with the Prime-It Fluor fluorescence labeling kit using ~10 ng starting template per ml of hybridization solution.*

   Heat probe in a boiling water bath for 5 minutes. Pulse-spin to collect condensation and store on ice until ready to add to hybridization.

4. Add probe to prehybridization solution and hybridize, with shaking, at 68°C for 2 hours.

5. Wash once for 15 minutes at room temperature in 0.1× SSC/0.1% SDS (150 to 200 ml).

   Wash twice for 15 minutes at 60°C in 0.1× SSC/0.1% SDS (150 to 200 ml) for each wash.

   Note *This is a high-stringency wash. Lower stringency can be achieved by decreasing the temperature of the wash solution and/or increasing the concentration of SSC.*

6. Prepare 1 liter of TBS[§] as follows:

   50 ml 1 M Tris-HCl (pH 8.0)
   30 ml 5 M NaCl
   920 ml dH_2O

7. Prepare the blocking solution: mix 1 ml of stock Southern block reagent with 9 ml of TBS.

8. Rinse membrane in a large volume of TBS (~ 150 ml) to remove SDS.

9. Block for 30 minutes at 60°C, while rocking gently. (Blocking can be performed at room temperature, but background may be slightly higher.)

[§] See *Preparation of Media and Reagents*.

10. Prepare 800 ml of TBST (0.05% Tween® 20 in TBS) by adding 400 µl of Tween 20 to 800 ml of TBS. Briefly rinse the membrane twice in a large volume of TBST (~150 ml per wash). Dilute the antifluorescein-antibody-AP conjugate according to the dilution on the tube label in 10 ml of TBST.

11. Decant the TBST and add the 10 ml of diluted antibody. Incubate for 1 hour at room temperature with gentle rocking.

12. Wash membrane in TBST three times for 10–15 minutes each, at room temperature, with gentle rocking (~150 ml/wash step). Prepare the assay buffer as follows:

    Dilute 960 µl of diethanolamine (DEA) in 95 ml of dH_2O
    Add 100 µl of 1 M $MgCl_2$
    Bring final volume to 100 ml with dH_2O and mix well

13. Decant TBST. Equilibrate membrane in the assay buffer, rocking for 5 minutes at room temperature.

    Prepare the substrate buffer: dilute 35 µl of CSPD® in 7 ml of assay buffer.

14. Decant the assay buffer and add the substrate buffer. Rock gently for 10 minutes at room temperature.

15. Seal membrane in plastic wrap or a heat-sealable bag. When using a heat-sealable bag, cut open two of the three closed edges, open the bag on a flat surface and place the blot inside. Close the bag and remove excess substrate buffer by rolling a pipet over the bag in order to squeeze the buffer out of the edges onto absorbent paper.

    When using plastic wrap, place the blot on top of a sheet of plastic wrap and fold the sheet over to cover the blot. Remove excess substrate buffer by rolling a pipet over the plastic wrap or pressing gently with paper towels in order to squeeze the buffer out of the edges onto absorbent paper.

    Do not allow the membrane to dry out completely. Seal the edges of the bag or fold over the edges of the plastic wrap and expose to X-ray film (e.g., Kodak® X-OMAT® AR) for ~30 minutes at room temperature.

16. The membrane may be re-exposed for longer or shorter periods of time as necessary. Exposures may also be obtained the following day; the signal will be more intense, but the background may be slightly higher.

Northern Blot Protocol

See *Appendix I: Target Nucleic Acid Transfer to Membrane* for suggested gel electrophoresis and treatment conditions.

Notes *Nylon membranes must be used for the following procedure.*

The following volumes of buffers and solutions have been optimized for a membrane that is 150 cm². If necessary, adjust the volumes at each step according to the size of your blot.

Once the protocol has been started, do not allow the membrane to dry out.

1. After transfer and crosslinking, briefly rinse the nylon membrane in dH_2O.

2. Add 10 ml of QuikHyb hybridization solution and prehybridize for 15 minutes at 68°C. (Standard overnight hybridization solutions and protocols may also be used.)

3. Place the fluoresceinated probe in a microfuge tube containing 100 µl of sonicated salmon sperm DNA (5 mg/ml).

   Note *The probe should be generated with the Prime-It Fluor fluorescence labeling kit using 5–10 ng starting template per ml of hybridization solution.*

   Heat probe in boiling water bath for 5 minutes. Pulse-spin to collect condensation and store the probe on ice until ready to add to the hybridization.

4. Add the probe to the prehybridization solution and hybridize at 68°C for 2 hours.

5. Wash once for 15 minutes at room temperature in 0.1× SSC/0.1% SDS (150 to 200 ml).

   Wash three times for 15 minutes at 60–68°C in 0.1× SSC/0.1% SDS (150 to 200 ml) per wash.

6. Prepare 1 liter of TBSN[§] in the following manner:

   50 ml of 1 M Tris-HCl (pH 8.0)
   30 ml of 5 M NaCl
   100 µl of northern block reagent
   920 ml of H_2O

   Rinse membrane in large volume of TBSN (~150 ml) to remove SDS.

[§] See *Preparation of Media and Reagents*.

7. Prepare the block solution:

   Add 40 µl of northern block reagent to 20 ml of TBSN.

8. Using 10 ml of prepared block solution, incubate for 30 minutes, with gentle rocking, at 60°C. Replace the blocking solution with the remaining 10 ml of fresh solution, and incubate again for 30 minutes.

9. Prepare 800 ml of TBST-N by adding 400 µl of Tween 20 to 800 ml of TBSN. Briefly rinse membrane twice in a large volume of TBST-N (~150 ml/rinse).

   Dilute antibody conjugate according to the dilution on the tube label in 10 ml of TBST-N.

10. Decant TBST-N wash and add 10 ml diluted antibody conjugate (prepared above) to membrane. Incubate 1 hour at room temperature, with rocking.

11. Wash membrane in TBST-N at room temperature three times for 15 minutes each time, with rocking (~150 ml/wash). Prepare the assay buffer as follows:

    Dilute 960 µl of DEA in 95 ml of dH_2O
    Add 100 µl of 1M $MgCl_2$ and bring to a final volume of 100 ml with dH_2O
    Mix well

12. Decant TBST-N. Equilibrate membrane in the assay buffer for 5 minutes at room temperature, with rocking.

    Prepare the substrate buffer: dilute 35 µl of CSPD in 7 ml of assay buffer.

13. Decant the assay buffer and add the substrate buffer. Rock for 10 minutes at room temperature.

14. Seal blot in plastic wrap or a heat-sealable bag. When using a heat-sealable bag, cut open two of the three closed edges, open the bag on a flat surface and place the blot inside. Close the bag and remove excess substrate buffer by rolling a pipet over the bag, allowing the buffer to be squeezed out of the edges onto absorbent paper.

    When using plastic wrap, place the blot on top of a sheet of plastic wrap and fold the sheet over to cover the blot. Remove excess substrate buffer by rolling a pipet over the plastic wrap or pressing gently with paper towels in order to squeeze buffer out of the edges onto absorbent paper.

    Do not allow the blot to dry out completely. Seal the edges of the bag or fold over the edges of plastic wrap and expose the blot to x-ray film (e.g., Kodak X-OMAT AR) for ~30 minutes at room temperature.

15. The blot may be re-exposed for longer or shorter periods of time as necessary. Exposures may also be obtained the following day; the signal will be more intense, but the background will be slightly higher.

Stripping Membranes

Southern Blots

1. Rinse the membrane briefly in dH_2O twice for 5 minutes each at room temperature.

2. Wash the membrane in 0.2N NaOH/0.1% SDS at 45°C for 30 minutes.

3. Rinse the membrane in dH_2O twice for 5 minutes each at room temperature.

   To check that the probe has been completely removed from the membrane, repeat the detection procedure described above, beginning at step 6 of *Probe Hybridization and Detection, Southern Blot Protocol* section.

4. Expose to film overnight. The autoradiograph should be completely blank. Repeat hybridization and detection procedures as described in steps 2–16 above in *Probe Hybridization and Detection, Southern Blot Protocol.*

Northern Blots

1. Rinse the membrane briefly in dH_2O twice for 5 minutes at room temperature.

2. Wash the membrane in 60% formamide/50 mM Tris-HCl (pH 8.0)/1% SDS at 80°C for 1 hour.

3. Rinse the blot twice in dH_2O for 5 minutes each time. To check that the probe has been completely removed from the membrane, repeat the detection procedure beginning at step 6 described above in *Probe Hybridization and Detection, Northern Blot Protocol* section.

4. Expose to film overnight. The autoradiograph should be completely blank. Rinse membrane in dH_2O twice for 5 minutes each time, and repeat hybridization and detection procedures as described above in steps 2–15 in *Probe Hybridization and Detection, Northern Blot Protocol*.

APPENDIX I: TARGET NUCLEIC ACID TRANSFER TO MEMBRANE

DNA Gels

1. Perform electrophoretic separation of target DNA; e.g., in a 0.8% agarose gel in TAE buffer at 60 mA for 2 hours. Stain the gel with ethidium bromide and photograph. The gel is now ready for Southern blotting treatment.

2. Transfer the gel to a Pyrex® baking dish and soak it in the solutions as outlined below:

   a. Soak the gel in 20 ml of 0.25 N HCl for 15 minutes at room temperature with constant rocking.

   b. Soak the gel in the following denaturation solution for 30 minutes at room temperature with rocking:

      87.66 g of NaCl [1.5 M NaCl]
      20 g of NaOH/liter of dH_2O [.5 N NaOH]

   c. Finally, soak the gel in the following neutralization solution for 30 minutes at room temperature with rocking:

      484 g Tris base [1 M Tris final concentration]
      350.4 g NaCl [1.5 M NaCl final concentration]
      Add dH_2O to 3 liters
      Adjust pH to 7.5 with concentrated HCl
      Bring to a final volume of 4 liters with dH_2O

3. Transfer the DNA onto a nylon membrane using a pressure blotter or by capillary action overnight using 10× SSC transfer buffer.

4. Fix the DNA to the membrane by using either the Stratalinker® UV crosslinker on the automatic setting, *autocrosslink,* or by baking for 2 hours at 80°C. After blotting, stain the gel with ethidium bromide to check that transfer of the DNA is complete.

5. Proceed to step 1 of the *Southern Blot Protocol* under *Probe Hybridization and Detection*.

RNA Gels

1. Separate total and/or messenger RNA in a 0.8% (w/v) agarose/0.75 M formaldehyde vertical or horizontal gel.

   Caution *Formaldehyde is a suspected carcinogen and must be used and disposed of in accordance with federal, state and local regulations. Always use formaldehyde in a fume hood.*

   Prepare the gel:

   Add 0.8 g of agarose to 84 ml of dH_2O
   Heat the suspension to dissolve the agarose; then cool to 60°C
   Under a hood, add 10 ml of 10× MOPS buffer and 6 ml of formaldehyde and mix well
   Cast the gel in a vertical or horizontal gel apparatus

2. Prepare the sample loading buffer:

   Note *The sample loading buffer should be prepared fresh each time it is used.*

   720 µl of formamide
   160 µl of 10× MOPS
   260 µl of formaldehyde
   100 µl of sterile dH_2O
   100 µl of ethidium bromide (10 mg/ml)
   80 µl of sterile glycerol
   80 µl of saturated bromophenol blue in sterile dH_2O

3. Dry the RNA sample. Add 5 µl of loading buffer, mixing well to resuspend the RNA pellet. Heat the samples in a boiling water bath for 5 minutes, load on the gel and electrophorese in 1× MOPS buffer at 100 V for ~1 hour or until the dye is about one-third of the way down the gel.

4. Wash the gel in dH_2O twice for 5 minutes each time to remove formaldehyde.

5. Transfer the RNA directly to a nylon membrane using a pressure blotter or by capillary action overnight without pretreatment of the gel. Stain the gel after blotting with ethidium bromide to check that RNA transfer is complete.

6. Proceed to step 1 of the *Northern Blot Protocol* under *Probe Hybridization and Detection*.

APPENDIX II: PROBE QUANTIFICATION USING THE ILLUMINATOR CHEMILUMINESCENT DETECTION SYSTEM

Label template DNA according to the protocol in the Prime-It Fluor Fluorescence Labeling Kit, and purify the labeled fragments from unincorporated nucleotides using a NucTrap probe purification push column or by ethanol precipitation. The efficiency of fluorescent nucleotide incorporation can be determined by performing serial dilutions on an aliquot of the labeled probe and determining the greatest dilution at which the probe can still be detected using the Illuminator chemiluminescent detection system. Add TE buffer[§] to make the final concentration of the DNA 0.25 ng of template/µl (e.g., if 50 ng of template were labeled, the final volume should be 200 µl).

Mix 8 µl of purified, diluted probe with 12 µl of TE buffer (= Dilution #1)
Mix 2 µl of Dilution #1 with 18 µl of TE buffer (= Dilution #2)
Mix 2 µl of Dilution #2 with 18 µl of TE buffer (= Dilution #3)
Mix 2 µl of Dilution #3 with 8 µl of TE buffer (= Dilution #4)
Final dilution 1:1250

Spot 1-µl aliquots of each dilution on a nylon membrane and crosslink using the Stratalinker UV Crosslinker on the *autocrosslink* setting (120,000 µJ/cm^2 of membrane). Detect the probe as described in the *Probe Hybridization and Detection* section of this manual (see step 6 in the *Southern Blot Protocol)*, or by using the abbreviated protocol described below.

The #2 dilution spot should be visible after a 1-minute exposure to X-ray film or the #4 dilution spot should be visible after a 30-minute exposure to X-ray film.

[§] See *Preparation of Media and Reagents*.

Abbreviated Detection Protocol for Probe Analysis

Note *All rinses are at room temperature.*

1. Briefly rinse the membrane, to which the spotted probe dilutions have been crosslinked, in TBS (50 mM Tris-HCl pH 8.0, 150 mM NaCl).

2. Block membrane for 10 minutes at room temperature in Southern block solution (see steps 6 and 7 of the *Southern Blot Protocol*).

3. Rinse membrane briefly in TBST (TBS, 0.05% Tween 20).

4. Incubate membrane for 30 minutes at room temperature with antifluorescein-antibody-AP conjugate (dilute according to tube label in TBST).

5. Rinse in a large volume of TBST (~150 ml/rinse) three times for 5 minutes each rinse.

6. Equilibrate the membrane in assay buffer for 5 minutes (see step 12 of the *Southern Blot Protocol*).

7. Incubate the membrane in substrate buffer for 10 minutes (see steps 13–14 of the *Southern Blot Protocol*).

8. Wrap in plastic wrap, remove excess buffer and expose to X-ray film for 1 minute—the #2 dilution spot should then be visible. The #4 dilution spot should be visible after a 30-minute exposure.

TROUBLESHOOTING

Observation	Suggestions
Weak signal	Incorrect membrane used as nucleic acid support. Only nylon membranes are effective in this chemiluminescent method.
	Inefficient transfer of nucleic acids from agarose gel. Restain gel with ethidium bromide following transfer. If transfer is incomplete, longer depurination and/or transfer time may help.
	Poor retention of transferred nucleic acids. Nucleic acid must be fixed to membrane. Use a Stratalinker UV Crosslinker set on the *autocrosslink* setting (120,000 μJ/cm$^{2\ of}$ membrane), or another UV crosslinker (consult the manufacturer's recommendations). Alternatively, bake at 80°C for 1–2 hours under vacuum.
	Insufficient nucleic acid sample loaded into the gel. Electrophoresis of a larger quantity of nucleic acids should result in more target transferred to the membrane.
	Poor incorporation of fluor-12-dUTP into probe. Incorporation of fluor-12-dUTP can be checked by dot blot analysis. Refer to *Appendix II*. If incorporation is inadequate, allow the labeling reaction to proceed for 30 minutes instead of 20 minutes.
	Low probe concentration due to insufficient template in the labeling reaction. Repeat the labeling reaction using twice as much template while keeping all the other reaction components constant. (Do not forget to boil the probe prior to addition to the hybridization solution.)
	Washing conditions are too stringent. Increase concentration of SSC and/or decrease temperature in the washing procedure, step 5.
	SDS in blocking, antibody–conjugate or wash buffer steps. SDS is a known enzyme inhibitor. Do not replace Tween® 20 with SDS in any of these buffers.
	Insufficient hybridization time. When using QuikHyb hybridization solution, hybridize for 2 hours. If using your own hybridization solution, hybridize for the maximum amount of time (or overnight)
Nonspecific background signal	Use deionized and filtered (0.2 μm) water to prepare all solutions. Bacterial contamination in the buffers can lead to general membrane background.
	Use carefully cleaned glassware in preparing solutions and buffers.
	Blocking and antibody buffers should be made fresh for each experiment.
	If the buffer will be stored, add 1 mM sodium azide to the assay buffer to prevent bacterial contamination.
	Do not let the membrane dry out at any point following the prehybridization step.
	Make sure that the antifluorescein-antibody-AP conjugate is diluted according to the description on the tube label.
	Reduce the amount of probe added to the hybridization buffer.
	Reduce exposure time.
	When working with multiple filters, make sure the buffer volumes are adequate, especially in the hybridization and blocking steps.
	Do not hybridize for longer than 2–2.5 hours when using QuikHyb hybridization solution.

PREPARATION OF MEDIA AND REAGENTS

TBS 50 mM Tris-HCl (pH 8.0) 150 mM NaCl	**10× SSC Transfer Buffer** Dissolve 87.65 g of NaCl and 44.1 g of sodium citrate in 400 ml of H_2O Adjust pH to 7.0 with a few drops of 10 N solution of NaOH Adjust volume to 1 liter with H_2O Sterilize by autoclaving
TBSN 50 mM Tris-HCl (pH 8.0) 150 mM NaCl 0.01% northern block reagent in dH_2O	**TE Buffer** 5 mM Tris-HCl (pH 7.5) 0.1 mM EDTA
1 M Tris-HCl (pH 8.0) 121.2 g Trizma base Add H_2O to 800 ml Add ~40 ml concentrated HCl Stir and adjust final pH to 8.0 with HCl Bring volume to 1 liter with H_2O. Sterilize by autoclaving.	**10× MOPS Buffer** 0.2 M MOPS (3-[*N*-morpholino]propane-sulfonic acid) 0.05 M sodium acetate 0.01 M EDTA Bring to a final pH of 5.5–7.0 with NaOH Do not autoclave

REFERENCE

1. Jerpseth, M., Ransom, S. L., Braman, J. and Moores, J. (1993) *Strategies* 6(2):47–49.

ENDNOTES

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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Illuminator Chemiluminescent Detection System

QUICK-REFERENCE PROTOCOLS

Southern Blot

- Transfer and crosslink DNA to membrane
- Rinse membrane in dH_2O
- Prehybridize in QuikHyb® hybridization solution for 15 minutes at 68°C
- Hybridize with Prime-It® Fluor probe in QuikHyb for 2 hours at 68°C
- Wash with 0.1× SSC/0.1% SDS for 15 minutes at room temperature
- Wash twice with 0.1× SSC/0.1% SDS for 15 minutes at 60°C
- Rinse membrane in TBS
- Block for 30 minutes at 60°C
- Rinse in TBST
- Incubate with antifluorescein-antibody-AP conjugate diluted in TBST for 1 hour at room temperature
- Wash 3 times in TBST for 10–15 minutes each time at room temperature
- Equilibrate in assay buffer for 5 minutes at room temperature
- Incubate with substrate buffer for 10 minutes at room temperature
- Expose to film at room temperature

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Illuminator Chemiluminescent Detection System

Northern Blot

- Transfer and crosslink RNA to membrane
- Rinse membrane in H_2O
- Prehybridize in QuikHyb® hybridization solution for 15 minutes at 68°C
- Hybridize with Prime-It® Fluor probe in QuikHyb for 2 hours at 68°C
- Wash once in 0.1x SSC/0.1% SDS for 15 minutes at room temperature
- Wash 3 times in 0.1× SSC/0.1% SDS for 15 minutes each at 60–68°C
- Rinse membrane in TBSN
- Block for 60 minutes at 60°C
- Rinse in TBST-N
- Incubate with antifluorescein-antibody-AP conjugate diluted in TBST-N for 1 hour at room temperature
- Wash 3 times in TBST-N for 15 minutes each time at room temperature
- Equilibrate in assay buffer for 5 minutes at room temperature
- Incubate in substrate buffer for 10 minutes at room temperature
- Expose to film