invitrogen™

Qdot® Conjugates Protocol Handbook

Quantum Dot
invitrogen nanocrystal technologies

Molecular Probes™
invitrogen detection technologies

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Immunocytochemistry with Qdot® Conjugates in Cultured Cells

PLEASE READ ENTIRE PROTOCOL AND APPLICATION NOTES BEFORE STARTING.
Additional information can be obtained from the product page on our website at http://probes.invitrogen.com/products/qdot/

This protocol describes an optimized fixation, permeabilization, and labeling procedure for use of Qdot® secondary antibody and Qdot® streptavidin conjugates in cultured adherent mammalian cells. This protocol has been developed and optimized for targets in cell lines such as HeLa adenocarcinoma cells and NIH-3T3 fibroblast cells. It is important to note that cell labeling protocols appropriate for Qdot® conjugates can differ depending on the cell type and growth status, target antigen, primary antibody source, and fluorescence detection method. Traditional cell-labeling protocols developed for organic fluorescent dye conjugates may need modification to be successful with Qdot® secondary antibodies and streptavidin conjugates. Thus, this basic protocol is a starting point for the optimization of your system for use with Qdot® secondary antibodies and Qdot® streptavidin conjugates. These conjugates perform optimally when used within the range of 10 nM to 40 nM, but the exact concentration needed should be determined by titration for each system. Additionally, imaging Qdot® conjugates is optimal with filters designed specifically for these nanocrystals. These filters are available from Omega Optical or Chroma (see *application note 1*).

Antigen labeling with Qdot® conjugates:

Detecting cellular targets with Qdot® secondary antibody and streptavidin conjugates can be performed individually by using a single Qdot® secondary antibody or Qdot® streptavidin conjugate with one primary antibody, or multiplexed by using a combination of primary antibodies and various Qdot® nanocrystal colors. Golgi, tubulin, mitochondrial, peroxisome, nucleolin, nucleosome and ki-67 targets have been validated with this labeling protocol (for a complete list of primary antibodies validated, see *application note 2*). Other targets and cell lines, however, may require further optimization of this protocol. For example, reducing the fixation time may improve cell penetration and conjugate access for some targets as may increasing the concentration of permeabilization reagent or incubation time with the permeabilization buffer. Furthermore, if biotinylated antibodies are to be detected with streptavidin conjugates, it becomes imperative to block endogenous biotin-containing proteins expressed in mitochondria (the Endogenous Biotin Blocking Kit (Invitrogen Cat. no. E21390) noted in the protocol is used for this purpose).

Materials

Fixative: 4% formaldehyde in PBS
Recommendation: freshly prepare 40 mL of 4% formaldehyde (ultrapure, MeOH-free, 16% formaldehyde solution; Polysciences, Inc. Cat. no. 18814) in 1X PBS by combining 10 mL of formaldehyde, 4 mL of 10X PBS, pH 7.4 (Invitrogen Cat. no. 70011-044) and 26 mL of distilled water.
Option: Prepare 40 mL of 3.7% formaldehyde (from 37% stock) in 1X PBS

Permeabilization buffer: 0.25% Triton® X-100 in PBS
Prepare 40 mL of permeabilization buffer by adding 100 µL of Triton® X-100 (Sigma Cat. no. T9284) to 40 mL of 1X PBS. Rock or stir until the Triton® X-100 goes into solution.

Rinse buffer: 1X PBS
Prepare 2 L of 1X PBS by combining 200 mL of 10X PBS and 1.8 L of distilled water.

Endogenous Biotin Blocking Kit (for use if performing streptavidin-based labeling): Invitrogen Cat. no. E21390 contains two ready-to-use reagents.

Blocking buffer: 6% BSA/10% normal serum in PBS

Add to 3 g of BSA (RIA grade, Fraction V, minimum 96%; Sigma Cat. no. A-7888) 5 mL of normal serum from the host species of the secondary antibody (preferably heat inactivated at 56˚C for 1 hour), 5 mL of 10X PBS, and distilled water to a total of 40 mL. Rock until the BSA is completely dissolved. Add distilled water to a final volume of 50 mL and mix well with gentle rocking or stirring. If storing the blocking buffer, add sodium azide to a final concentration of 0.02% and keep at 4˚C.
See application note 3.

Secondary incubation buffer: 6% BSA in PBS

Add to 3 g of BSA (RIA grade, Fraction V, minimum 96%; Sigma Cat. no. A-7888) 5 mL of 10X PBS, and distilled water to 40 mL. Rock until BSA is completely dissolved. Add distilled water to a final volume of 50 mL and mix well with gentle rocking or stirring.

Qdot® secondary antibody or Qdot® streptavidin conjugate:

DO NOT VORTEX PRODUCT VIAL. Centrifuge the Qdot® nanocrystal conjugate product vial for 3 minutes at 10,000 × g prior to use. If a pellet appears, only use the supernatant. Dilute the conjugate by adding 2 μL of the stock (1 μM) conjugate to 100 μL secondary incubation buffer immediately prior to use (in most cases this is optimal but it may need to be titrated between 10 nM and 40 nM final concentration). Use for current experiment, do not store diluted. See *application note 4*.

Dehydration solutions:

Prepare in containers suitable for a dehydration series EtOH/H_2O dilutions (v/v) of 30%, 50%, 70%, and 90%, as well as 100% EtOH and 100% toluene.

Mounting reagents:

The recommended mounting media is Cytoseal 60 (Richard-Allan Scientific Cat. no. 8310-4). This requires dehydration of the specimen in EtOH and toluene. Prepare in containers suitable for this process EtOH/H_2O dilutions (v/v) of 30%, 50%, 70%, and 90%, as well as 100% EtOH and 100% toluene.

Cell preparation:

Culture cells in the appropriate medium to the desired confluency and physiological state (typically 1–2 days for HeLa cells). It is recommended that cells be below 80% confluency at the time of fixation, depending on experimental requirements and imaging method.

A. Fixation and Permeabilization

All of the steps are performed at room temperature.

1.1 Rinse 2 times with 1X PBS. If cells were grown in a large dish (i.e., 100 mm petri dish), add a large enough volume of 1X PBS to completely cover the specimen (~25 mL for a 100 mm petri dish) swirl gently, pour off or aspirate the solution and repeat. If grown in a smaller container (i.e., 6-well plate), wash 3 times, 5 minutes each by adding enough 1X PBS to completely cover specimen. After 5 minutes, pour off or aspirate the rinse solution.

1.2 Incubate in 4% formaldehyde/PBS for 15 minutes to fix the specimen.

1.3 Repeat step 1.1.

1.4 Incubate in 0.25% Triton® X-100/PBS for 15 minutes to permeabilize the specimen (see *application note 5*).

1.5 Repeat step 1.1.

B. Target Labeling and Detection

All of the following incubations are performed in a humidity chamber at room temperature (see *application note 6*). Avoid specimen drying as this can cause high levels of nonspecific background and autofluorescence. At the end of each step, carefully remove or blot excess solution from the sample before moving to the next step.

If using Qdot® secondary antibody conjugates proceed to step 2.1.

If using Qdot® streptavidin conjugates, perform endogenous biotin blocking with the Endogenous Biotin Blocking Kit (Invitrogen Cat. no. E21390) or equivalent. If using Invitrogen product E21390:

a) Incubate in component A for 30 minutes.
b) Wash 3 times, 5 minutes each, in 1X PBS.
c) Incubate in component B for 30 minutes.
d) Wash 3 times, 5 minutes each in 1X PBS.

Proceed to step 2.1.

2.1 Incubate with blocking buffer for 1 hour.

2.2 Pour off blocking buffer and incubate for 1 hour with primary antibody diluted in blocking buffer (see *application note 7*).

2.3 Wash 3 times, 5 minutes each, in 1X PBS.
Note: If using a biotinylated secondary antibody, repeat steps 2.2 and 2.3 using the biotinylated antibody before continuing.

2.4 Incubate for 1 hour with Qdot® secondary antibody conjugate or Qdot® streptavidin conjugate, diluted to an optimal concentration (titrate between 10 nM and 40 nM for optimal results) in secondary incubation buffer.

2.5 Wash 3 times, 5 minutes each, in 1X PBS.
Note: If counterstaining is necessary, most counterstain procedures may be performed at this point, followed by any necessary wash steps and subsequent dehydration and mounting (see *application note 8*).

2.6 Dehydrate specimen. Submerge specimen for 5 seconds in 30%, 50%, 70%, and 90% EtOH/H_2O, twice in 100% EtOH, and twice in 100% toluene with the final dip ~10 seconds.

2.7 Mount in one drop Cytoseal 60 and let cure at least 4 hours before imaging.

Application Notes

Note 1: For optimal imaging of Qdot® conjugates, including reduced spectral bleed-through effects in multicolor applications, it is recommended that you use filter sets optimized for the excitation and emission of the Qdot® nanocrystal(s) in use. These filters are available from Omega Optical or Chroma. Additional information can be obtained from the Qdot® nanocrystals web page at http://probes.invitrogen.com/products/qdot/.

Note 2: If new to immunocytochemistry (ICC), it is recommended that you include a positive labeling control for protocol execution validation. For example, an optional control for Qdot® goat-anti-mouse secondary antibodies is anti-OxPhos Complex V Inhibitor Protein (Invitrogen Cat. no. A21355), which targets mitochondria. An optional control for Qdot® goat-anti-rabbit secondary antibodies is rabbit anti-giantin (Covance Cat. no. PRB-114C), which targets Golgi bodies. Other antibodies used in optimization of this protocol, and therefore suitable as positive controls include: rabbit anti-AIF (Cell Signaling Cat. no. 4642), mouse anti–ki-67 (Ventana Medical Systems Cat. no. 790-2910), mouse anti–α-tubulin (Sigma Cat. no. T6074), rabbit anti–α-tubulin (Affinity Bioreagents Cat. no. PA1-20988), mouse anti-nucleosome (BD Pharmingen Cat. no. 51-80591N), and mouse anti-nucleolin (Invitrogen Cat. no. 39-6400; Stressgen Cat. no. KAM-CP100).

Note 3: If background is observed with Qdot® secondary antibody conjugates, use a blocking buffer of 6% BSA in PBS with 10% heat-inactivated (56˚C for 1 hour) normal serum from the host species of the secondary (goat or rabbit). Please note that BSA can vary by lot and producer, so re-optimization of blocking conditions may be required for best results when substituting alternate sources for this component.

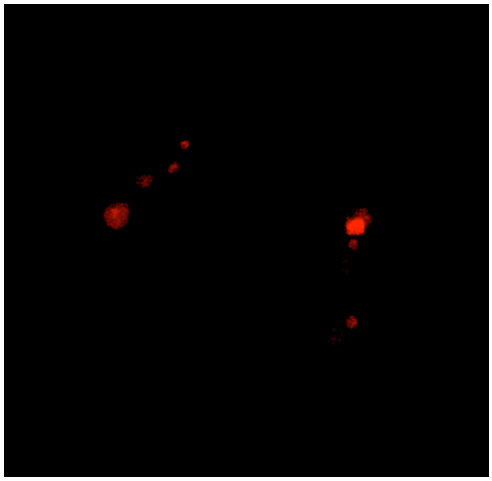
Note 4: Qdot® secondary antibody conjugates and Qdot® streptavidin conjugates should be stored at 4˚C in their concentrated (shipped) form. They should not be diluted to their working concentration until the day they will be used, as they may not be stable in dilute form. It is best to only make up as much as is needed for each experiment rather than to create and store a stock of diluted working solution.

Note 5: Changing fixation time or permeabilization time or concentration may be necessary for achieving labeling of certain targets.

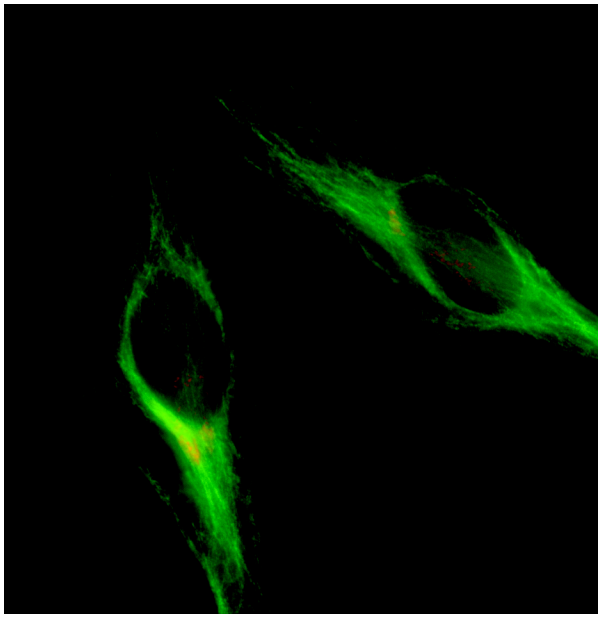
Note 6: A simple humidity chamber prevents labeling reagent concentration changes due to evaporation during incubations. To make a simple humidity chamber, place a piece of filter paper in a petri dish and saturate the filter paper with water. Next, place a piece of Parafilm® on the filter paper. During incubations, place a lid on the petri dish. Gentle agitation during incubations is optional.

Note 7: Briefly centrifuge the primary antibody prior to use. It may be necessary to titrate the concentration of the primary antibody for optimal target labeling.

Note 8: UV excitation of DAPI (Invitrogen Cat. no. D1306) resulting in emission around 600 nm has been observed and may not be appropriate for use with all Qdot® nanocrystals. Other nuclear counterstain options, many available in kit form with optimized protocols for their use, include SelectFX® Nuclear Labeling Kit (Invitrogen Cat. no. S33025), SYTOX® Green nucleic acid stain (Invitrogen Cat. no. S7020), 7-aminoactinomycin D (7-AAD) (Invitrogen Cat. no. A1310), propidium iodide (Invitrogen Cat. no. P3566), and TO-PRO®-3 iodide (Invitrogen Cat. no. T3605).



Multiplex labeling of HeLa cells using Qdot® conjugates. HeLa cells were prepared according to the protocol above. Tubulin was labeled with a mouse anti-tubulin primary antibody (Sigma, Cat. no. T6074) followed by a Qdot® 525 goat anti–mouse IgG secondary antibody (green, Cat. no. Q11041MP). Giantin was labeled with a rabbit anti-giantin primary antibody (Covance, Cat. no. PRB-114C) followed by a Qdot® 655 goat anti–rabbit IgG secondary antibody (red, Cat. no. Q11421MP). Images were captured using the optimal filter sets recommended in Appendix 1 of the Supplemental Information Guide (available online at http://probes.invitrogen.com/media/pis/mp19009.pdf).



Nuclear labeling in HeLa cells using Qdot® conjugates. HeLa cells were prepared according to the protocol above. Nucleoli were labeled with a mouse anti-nucleolin primary antibody (Novus, Cat. no NB 600-241A2) followed by a Qdot® 655 goat anti–mouse IgG secondary antibody (red, Cat. no. Q11021MP) and imaged using the optimal filter sets recommended in Appendix 1 of the Supplemental Information Guide (available online at http://probes.invitrogen.com/media/pis/mp19009.pdf).

Immunolabeling Formalin-fixed Paraffin Embedded Tissue Sections

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.
Additional information can be obtained from our website at www.invitrogen.com.

This protocol is intended to be a starting point for immunolabeling formalin-fixed tissues that were embedded in paraffin. It is expected that for a given antibody, certain steps in this procedure will change to improve immunolabeling and reduce background staining. Antigen retrieval conditions, primary antibody concentrations and incubation times, and secondary antibody concentrations and incubation times that successfully immunolabel tissue with other detection methods should be used as a starting point for this protocol. Steps are included for using Qdot® Streptavidin Conjugates as tertiary reagents in conjunction with biotinylated secondary antibodies or for using Qdot® Secondary Antibody Conjugates.

Materials

Primary Antibody: 1 – 10 µg / ml in TBS with 3 – 10% IgG free BSA
Control Tissue: positive and negative controls as appropriate
Antigen Retrieval:
- DAKO® Target Retrieval Solution 10X Concentrate (DakoCytomation, Cat. # S1699)

Blocking Reagents:
- TBS-BSA: 2% bovine serum albumin (IgG-free BSA, Jackson Immunoresearch, Cat # 001-000-162 or equivalent) in Tris buffered saline
- Normal serum(s) matching secondary antibody hosts (Jackson Immunoresearch)
- Borate-BSA: 2% BSA (see above) in 50 mM borate buffer (pH 8.3)

Secondary Detection Reagents Antibody: Dilutions are in TBS-BSA.
- Qdot Secondary Antibody Conjugate
- Biotin rabbit anti-mouse Fab (DakoCytomation, Cat # E0413 or equivalent)
- Biotin swine Anti-rabbit Fab (DakoCytomation, Cat # E0431 or equivalent)
- Biotin donkey anti-goat IgG (Jackson Immunoresearch, Cat # 705-065-147 or equivalent)

Tertiary reagents:
- Qdot Streptavidin Conjugate

Nuclear counter stain
- Hoechst 33342 , trihydrochloride, trihydrate *10 mg/mL solution in water* (Molecular Probes, Cat # H3570) diluted 1:10000 – 1:20000 in TBS

Buffers:
- TBS; Tris buffered saline, (DakoCytomation, Cat # S1968)
- TBST, TBS with 0.2% Tween-20 (DakoCytomation, Cat # S3306)
- PBS (Sigma, Cat # P3563)
- 50 mM Borate buffer (pH 803)

Mounting media:
- Cytoseal™ 60 (Richard-Allan Scientific, Cat # 8310)
- Polyvinyl alcohol with DABCO (Sigma, Cat # 10981)
- 90% Glycerol in 50 mM borate buffer

ImmEdge Hydrophobic Barrier Pen (Vector Laboratories, Cat # H-4000)

Procedure

1. Deparaffinize sections.
   1.1. 3 changes of xylene or xylene substitute, 5 min
   1.2. 3 changes 100% ethanol, 3 min each
   1.3. 1 change 95% ethanol, 3 min
   1.4. 1 change 90% ethanol, 3 min
   1.5. 1 change 70% ethanol, 3 min
   1.6. 1 changes deionized water > go to antigen retrieval step if needed, i.e. tissue fixed in formalin.
   1.7. 2 changes of TBS > go to step 3 blocking buffer if antigen retrieval is not needed (i.e. tissue not fixed in formalin).
2. Antigen retrieve formalin-fixed tissues. (p.3)
3. Place in dH_2O.
4. Optional: A PAP pen (or hydrophobic pen) can be used to circumscribe the tissue section in order to conserve on reagent usage. However, some PAP pens can quench the fluorescence of quantum dots. We recommend that the ImmEdge Pen be used for this purpose.

The following steps can be done on an autostainer if available

5. Block for 20 min in TBS-BSA.
6. Shake off the blocking reagent (do not rinse).
7. Incubate in primary antibody in TBS-BSA (1-10 μg/ml).
   Suggested times – these can be changed to suit the antibody and antigen conditions.
   7.1. 60 -120 min at room temperature RT in humid chamber
   7.2. Overnight at 4°C or room temperature
8. Wash in TBS 2 x 3min.
9. Block in TBS-BSA.
10. Qdot Secondary Antibody Conjugate: 1 – 20 nM in TBS-BSA @ room temperature or 37°C for 30 - 60 min. Skip to step 13.
    Or Biotinylated secondary antibody: 1 -2 μg/ml in TBS-BSA for 30 – 60 min at room temperature or 37°C.
11. Wash TBST (TBS-Tween) 2 changes @ 3 min each.
12. Qdot Streptavidin Conjugate in TBS-BSA (1-10 nM) @ RT for 30 min.
13. Wash TBST 2 x 3 min.
14. Counterstain nuclei with Hoechst 33342 in TBS for 15 min. (dilute 10mg/ml stock by 1:5000 in TBS) (see Technical note at end)
15. Wash in TBS 2 changes for 5 min.
16. Coverslip
    16.1. Polyvinyl alcohol mounting media with DABCO
    Or Cytoseal 60 mounting media
    16.2. After buffer, place in water bath 20-30 sec.
    16.3. Then 30% alcohol 30 sec.
    16.4. Then 70% alcohol 30 sec.
    16.5. Then 95% alcohol 30 sec.
    16.6. Then 100% alcohol 30 sec.
    16.7. Then xylene or toluene 30 sec.
    16.8. Place one drop of mounting media on section and apply coverslip.
17. Image sections on microscope with filter set optimized for quantum dots.

ANTIGEN RETRIEVAL

Restores antibody binding to proteins in formalin-fixed paraffin embedded tissues.

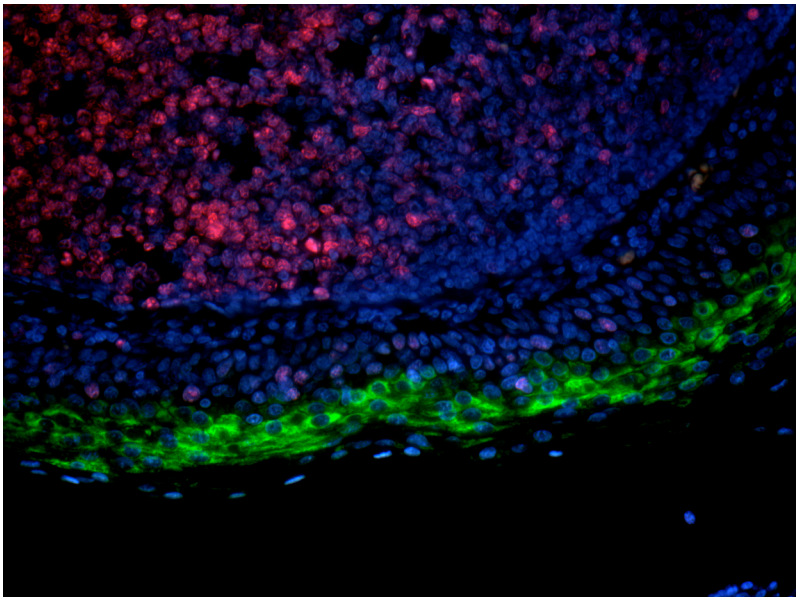
Materials

1 part - DAKO® Target Retrieval Solution 10X Concentrate
9 parts - dH_2O (Milli-Q® Ultrapure or distilled)

1) Preheat water bath or steamer to 95° C.
2) Heat working retrieval solution to 95° C in an appropriate container.
3) Place deparaffinized slides to the heated target Retrieval Solution. (Step #2)
4) Leave at 95° C for **10 – 40 min**.
5) Remove container with slides and cool on bench (to 50°C or less) in Retrieval solution.
6) Remove slides and rinse in deionized water for 3 minute.
7) Proceed with blocking step 9 above.

Technical note:

The introduction of Hoechst to the sections allows for visualizing nuclei and imaging with a standard RGB camera. However, Hoechst and DAPI have a long emission tail that can bleed into the single band-pass filters for 525, 565, and 585 nm quantum dots. Hence nuclear immunolabeling that requires the use of these DNA labels should use the 605 nm or 655 nm Qdot Conjugates to visualize nuclear antigens. Other DNA dyes are available that excite at different wavelengths and can be more compatible with the appropriate colors of quantum dots. Typically these dyes would be selected to absorb and emit at longer wavelengths than the quantum dots being used. One such dye is DRAQ 5™ (Biostatus Limited)



Multiplex labeling of human tonsil section for Ki67 and keratin with Qdot Conjugates. Ki67 in the nuclei of proliferating B cells (red) were labeled with Rabbit anti-Ki67 (Lab Vision, Cat # RB-1510) followed by Qdot 585 Anti-rabbit Secondary Conjugate (Cat # Q11411MP). Keratin in the peripheral stromal cells (green) was labeled with mouse monoclonal anti-pan keratin antibody (Lab Vision, Cat # MS-356) followed by biotin-goat anti-mouse IgG and Qdot 525 Streptavidin Conjugate (Cat #Q10143MP). Nuclei (blue) were counterstained with Hoechst 33342.

Immunolabeling Frozen Tissue Sections

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.
Additional information can be obtained from our website at www.invitrogen.com.

This protocol is intended to be a starting point for immunolabeling paraformaldehyde-fixed tissues that were frozen. It is expected that for a given antibody, certain steps in this procedure will change to improve immunolabeling and reduce background staining.

Materials

2% Paraformaldehyde
30% Sucrose
Superfrost™ Plus Slides (Erie Scientific Company, Cat # 4951 or equivalent)
Phosphate Buffered Saline (PBS; Sigma Chemical, Cat # P3563)
Primary Antibody
Wash Buffer: 0.5% bovine serum albumin, IgG-free BSA, (Jackson Immunoresearch, Cat # 001-000-162 or equivalent) in PBS
Blocking Buffer: 2% bovine serum albumin, IgG-free BSA, (Jackson Immunoresearch, Cat. # 001-000-162 or equivalent) in PBS
Qdot Secondary Antibody Conjugate
Nuclear counter stain
DAPI: 10 µg/ml DI water (Molecular Probes, Cat # D1306)
Mounting media:
Gelvatol: Prepared according to recipe at the following link:
http://www.feinberg.northwestern.edu/cif/gelvatol.htm
ImmEdge Hydrophobic Barrier Pen (Vector Laboratories, Cat. # H-4000)

Procedures

Preparing Tissue Sections:

Fix tissue in 2% Paraformaldehyde.
1. Cryo-protect in 30% sucrose overnight at 4° C.
2. Prepare 6 µm sections using a cryostat and place on Superfrost™ Plus slides.
3. Keep slides at -20° C until ready for use.

Immunolabeling:

Note: All wash steps are 4 minutes.

4. Rehydrate sections with 3 washes in PBS.
5. Wash 3 times with Wash Buffer.
6. Block for 45 minutes at room temperature in Blocking Buffer.
7. Wash 5 times with Wash Buffer.
8. Incubate with Primary Antibody for 60 minutes at room temperature (antibody diluted in Wash Buffer).
9. Wash 5 times with Wash Buffer.
10. Incubate with Qdot Secondary Antibody Conjugate for 60 minutes at room temperature (antibody diluted in Wash Buffer).

11. Wash 5 times with Wash Buffer.
12. Wash 5 times with PBS.
13. Stain with DAPI for 30 seconds at room temperature.
14. Wash 2 times with PBS.
15. Mount coverslip with Gelvatol and refrigerate.
16. Dry slides overnight at 4º C in the dark.

Note: Some PAP pens can quench the signal from the quantum dots. If your protocol requires the use of a PAP pen, we recommend the ImmEdge Hydrophobic Barrier Pen (H-4000) from Vector Labs.

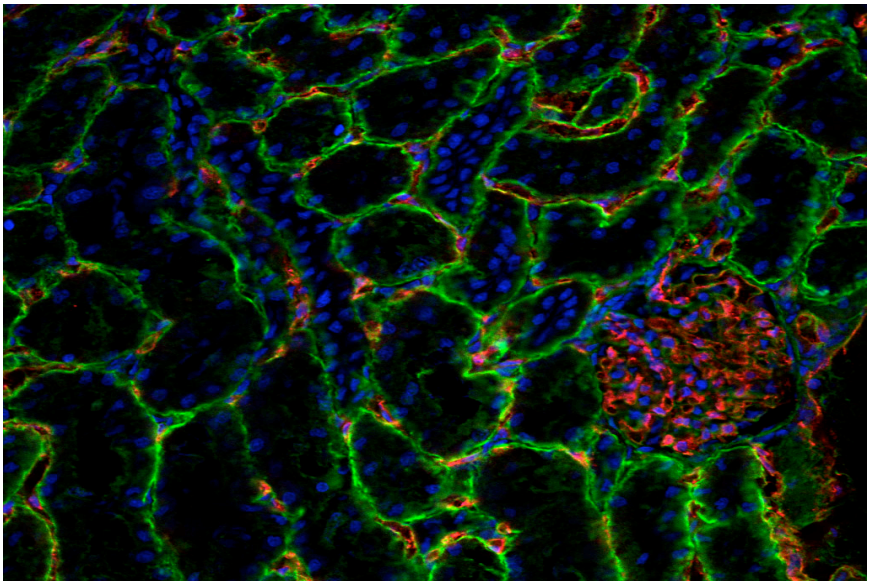


Figure 1. Laminin (Qdot 565 Goat anti-Rabbit IgG Conjugate; Green), PECAM (Qdot 655 Goat anti-Mouse IgG Conjugate; Red) and Nucleus (DRAQ5™; Blue) staining in a Rat Kidney Section.

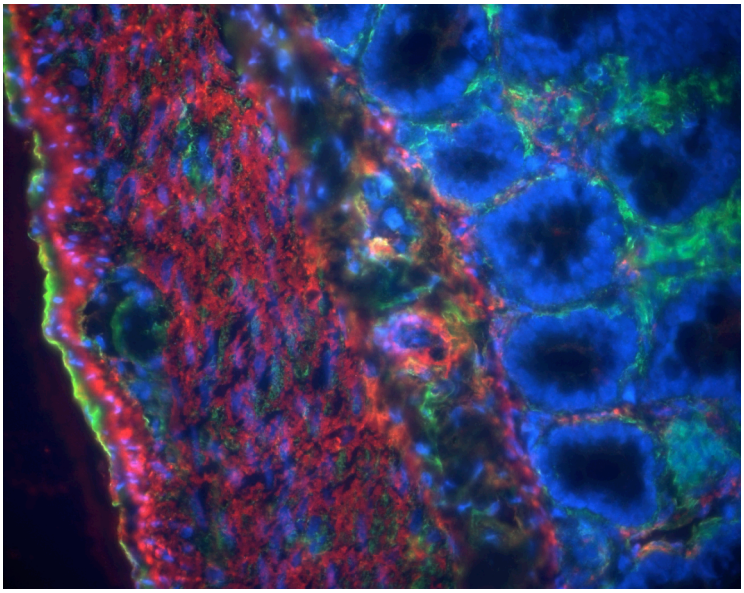


Figure 2. Laminin (Qdot 565 Goat anti-Rabbit IgG Conjugate; Green), Smooth Muscle Actin (Qdot 655 Goat anti-Mouse IgG Conjugate; Red) and Nucleus (DAPI; Blue) staining in a Rat Gut Section.

Western Blotting with Qdot Secondary Antibody Conjugates

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING
Additional information can be obtained from our website at www.invitrogen.com.

This protocol is based on standard polyacrylamide gel electrophoresis and Western blotting procedures. Concentrations of both the primary and secondary antibodies should be optimized for each user's particular system.

Materials

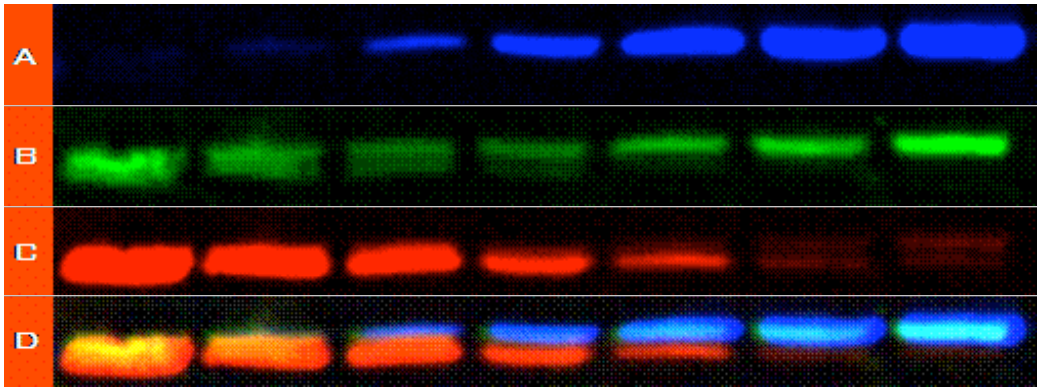
Qdot Secondary Antibody Conjugates
PVDF (recommended: Millipore Immobilon™ FL PVDF) or Nitrocellulose membrane
Blocking buffer (recommended: SEA BLOCK; Pierce Chemical, Cat. #37527)
1X TBS-T (0.1M Tris, 150 mM NaCl, 0.1% Tween-20)
1X TBS (0.1M Tris, 150 mM NaCl)
Electroblotting apparatus, power supply, and buffers
Rocking or oscillating platform
Methanol
100 X 15 mm square petri dish (such as Falcon, Cat. #351112) or plastic bags for incubation steps
Primary antibody: mouse and/or rabbit IgG
Gel Imaging instrument with filters appropriate for Qdot conjugates

Procedure

Polyacrylamide Gel Electrophoresis (PAGE) and electroblotting to PVDF should be performed as per laboratory routine or instructions provided with electroblotting apparatus.

Note: Always wear gloves and use forceps when manipulating gels and membranes. Blotting membranes should be handled with care to prevent scratching or folding.

1. All membrane incubations are designed to be performed with **8-10 ml volumes** of reagent in an appropriately sized container (such as a square petri dish or sandwich bag---Plastic food storage containers are not recommended). These incubations should be performed on a rocking table under continuous motion.
2. Wash membrane in 8 ml TBS-T 2 X 5 minutes with gentle shaking.
3. Incubate membrane in 8 ml SEABLOCK for 1 hour at room temperature or overnight at 4°C with continuous rocking.
4. Add primary antibody to petri dish containing membrane and SEABLOCK to a final concentration of 1-2 μg/ml (The amount of primary antibody may need to be optimized for specific experiments).
5. Incubate the membrane at room temperature for 1 hour with constant rocking.
6. Wash membrane in 8 ml TBS-T 3 X 5 minutes with gentle shaking.
7. Incubate membrane for 1 hr at room temperature in Qdot® Secondary Antibody Conjugates diluted 1:1000 in 8-10 mL SEABLOCK (1 nM final concentration).
8. Wash membrane in 8 ml TBS-T 3 X 5 minutes each with gentle shaking.
9. Transfer the membrane to TBS and wash 2 X 3 minutes each. Store in TBS at 4°C until imaged.



Western blots of serial dilutions of a mixture of lysates from *E. coli* which expressed individual epitope tags. In addition to the two secondary antibodies included in the 605/705 Western Blotting Kit (Q24021MP), the Qdot 565 Goat anti-GST Conjugate (Q14631MP) was also used. A: cMyc (705, pseudo-colored blue), B: GST (565, green), C: HA (605, red), D: combination of all three antibodies.

Blot Image Capture

Western blot data can be collected on a wide variety of instruments. Qdot Conjugate labeled blots can be imaged in gel imaging systems equipped with a digital camera, UV epi-illumination, and band-pass filters matched to the quantum dot emission wavelengths. Trans-illumination may also be used but the autofluorescent light from the membrane is significantly higher, resulting in an approximately 10-fold decrease in the overall sensitivity of detection.

For the highest sensitivity and multiplexed detection with the quantum dots, be sure to obtain custom filters for your instrument that are designed to discriminate the signal from the distinct Qdot Conjugates. These filters can be obtained from Omega Optical (www.omegafilters.com) or Chroma Technology (www.chroma.com), and have to be ordered in sizes appropriate to the particular system. Consult your instrument specifications to determine the appropriate size before ordering custom filters

An alternate imaging strategy is to use a color digital camera with a UV-transilluminator. In these cases, a high quality color camera with a standard Haze2A (UV Blocking) filter at the front of the lens can be used as a quick, inexpensive, semi-quantitative method for imaging single or multicolor blots with visibly distinguishable colors such as 565 and 655. If a Haze2A filter is unavailable, the image can be taken through the UV blocking plate or mask available in most laboratories.

NOTE: When using UV illuminators, one must wear appropriate eyewear to prevent UV exposure and serious health risks.

For additional information on detection of Qdot Conjugates or for recommendation on instruments, please refer to our web page: www.invitrogen.com.

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