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# Differentiating Primary Human Cells in Rapid-Throughput Discovery Applications

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## Summary

Primary cultures of human cells provide an increasingly important alternative to using virally transformed or otherwise immortalized cell lines or to using cloned cell lines derived from human or animal tumors. Advances in primary cell culture techniques, media formulations, and other reagents have enabled routine culture of primary cells derived from human tissues for biomedical research and drug discovery approaches such as high content screening. That primary cells retain the phenotypic characteristics of the original tissue is one main advantage over immortalized cell lines. However, securing reliable supplies of primary cells on a large scale has been problematic. Here, two primary differentiating cell types, preadipocytes and preosteoclasts, are described to illustrate the utility of commercially produced cell systems in discovery research and rapid-throughput applications.

**Key Words:** Differentiating cell systems; gene discovery; in vitro toxicology; preadipocytes; preosteoclasts; primary cell cultures; target validation.

## 1. Introduction

Primary cultures of human cells provide an increasingly important alternative to using virally transformed or otherwise immortalized cell lines or to using cloned cell lines derived from human or animal tumors. Advances in primary cell culture techniques, media formulations, and other reagents have enabled routine culture of primary cells derived from human tissues.

The use of primary cell cultures isolated from human tissues has several distinct advantages over immortalized cell lines. Primary human cell cultures retain many of the phenotypic characteristics of the tissue of origin, including normal physiological functions, and, therefore, can be highly relevant models for gene discovery, target validation, drug testing, and in vitro toxicology. The biological relevance is a key advantage in functional and toxicological studies, because immortalized cell lines might have compromised mechanisms of apoptosis, cell cycle checkpoint controls, or altered proliferative responses *(1–4)*.

The availability of primary cells of consistent viability and performance allows the widespread use of cell systems in research. Key to this is the acquisition of human tissues under proper legal and ethical oversight. It is often difficult and time-consuming for researchers to build the relationships and infrastructure necessary to have tissues available routinely. Access to human tissues is typically sporadic, and the expense of shipping, processing and characterizing the tissues, and ensuing cells can be prohibitive. In addition, primary cells have limited life span, reaching senescence within a finite number of cell divisions *(5–7)*. Therefore, reliable supplies

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of primary cells serve an important research need. In addition, utilizing multiple donors for samples of tissue to generate independent isolates of primary cells allows researchers to demonstrate consistent responses among individuals *(8)*.

Among the most useful primary cell types are progenitors that are not terminally differentiated in function or morphology *(4,9)*. Such differentiating cell systems often allow for significant expansion of the numbers of cells under mitogenic conditions, followed by functional differentiation under controlled conditions. This process adds another dimension to discovery research using primary cell cultures, allowing researchers to study factors that promote or inhibit differentiation of particular cell types. Two particular primary differentiating human cell types, preadipocytes *(8–11)* and preosteoclasts *(12,13)*, will be described to illustrate the utility of such cell systems in discovery research and rapid-throughput applications.

## 2. Materials

1. Laminar flow hood.
2. Tissue culture incubator, 5% $CO_2$, w vacuum aspiration.
3. Cryopreserved primary human preadipocytes (*see* **Note 1**).
4. Cryopreserved primary human osteoclast precursors (*see* **Note 2**).
5. Sterile pipets.
6. Sterile 50-mL polypropylene conical tubes with caps.
7. Cell culture media (*see* **Notes 3–5**).
8. Fetal bovine serum (FBS) (*see* **Note 6**).
9. Insulin (E. Lilly, Indianapolis; IN).
10. Macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis; MN).
11. Soluble receptor activator of nuclear factor-κβ (RANK) ligand (Chemicon International; Temecula, CA).
12. Dexamethasone (Sigma; St. Louis, MO).
13. 3-Isobutyl-1-methylxanthine (IBMX [Sigma]).
14. Indomethacin (Sigma).
15. Trypan blue.
16. Glutamine.
17. Penicillin.
18. Streptomycin.
19. Adipored (Cambrex; Walkersville, MD; *see* **Note 7**).
20. Time-resolved fluorescence-capable plate reader with injector.
21. 96-well cell culture plates (black walls with clear bottoms).
22. OsteoLyse-collagen assay kit (Cambrex; *see* **Note 8**).
23. Phosphate-buffered saline (PBS).

## 3. Methods

The methods outlined in this section describe:

1. The culture and differentiation of primary human preadipocytes and their use in an assay of intracellular triglyceride accumulation.
2. The culture and differentiation of primary human osteoclast precursors and their use in an assay of in vitro bone resorption.

### *3.1. Culture of Primary Human Preadipocytes*

1. Prepare Preadipocyte growth medium by adding FBS, L-glutamine, penicillin and streptomycin to Preadipocyte basal medium (The final concentrations of the supplements will be 10%, 2 m*M*, 100 U/mL, and 100 µg/mL, respectively). The medium should be warmed to 37°C before use.
2. Remove a cryovial of cells from liquid nitrogen storage and thaw rapidly in a 37°C water bath. Decontaminate the external surfaces of the cryovial of cells with 70% v/v ethanol or isopropanol.
3. Using a micropipet, gently add the thawed cell suspension to 50 mL of Preadipocyte growth medium.

4. Rinse the cryovial with medium and add the rinse to the cell suspension.
5. Centrifuge at 300*g* for 10 min at room temperature.
6. When washing the cells, do not attempt to remove too much of the wash. Leave a minimum of 1 mL of wash at the bottom of the tube. If the final cell count is low, some of the pellet might have been removed with the wash.
7. Add 2 or 3 mL of preadipocyte growth medium to the remaining 1 mL of wash and resuspend the pellet of cells. Dilute 20 µL of the cell suspension in 20 µL of 0.4% Trypan blue, do a cell count and determine percentage viability. Recovery should be greater than 90%.
8. Resuspend the preadipocytes at 100,000/mL in preadipocyte growth medium and plate the preadipocytes at 10,000 cells/well of a 96-well cell culture plate in 0.1 mL of preadipocyte growth medium (*see* **Note 9**).
9. Incubate at 37°C, 5% $CO_2$ and 90% humidity for 24 h. At this point, the preadipocytes should be confluent. Optimal differentiation of preadipocytes is obtained when the cells are confluent before to treatment with differentiation agents.
10. Prepare "2X" Adipocyte differentiation medium by the addition of insulin, dexamethasone, indomethacin, and IBMX to 100 mL of preadipocyte growth medium. After addition of the differentiation medium to the cells, the final concentrations of the differentiation agents, having been diluted 1/1, will be 10 µg/mL insulin, 1 µ*M* dexamethasone, 50 µ*M* indomethacin, and 200 µ*M* isobutyl-methylxanthine.
11. Induce the preadipocytes to begin differentiating into adipocytes with the addition of 0.1 mL of "2X" Adipocyte differentiation medium to each well.
12. If the cells are to be treated with a series of test samples, set up several 24-well dilution plates with the appropriate volume of adipocyte differentiation medium/well and make the required serial dilutions of the test samples. Add 0.1 mL of each different concentration of test sample to wells of the preseeded confluent preadipocytes. Each assay should be done in triplicate.
13. "Control" wells should be setup, which contain **(A)** no added test sample, **(B)** "solvent only" treatments if the test samples were dissolved in solvents such as dimethyl sulfoxide (DMSO), ethanol, and so on and **(C)** 100 µL of preadipocyte growth medium instead of differentiation medium.
14. Culture the cells in a tissue culture incubator in 5% $CO_2$ and 90% humidity at 37°C. No further additions or medium changes are required for a period of 10 d (*see* **Note 10**).

### *3.1.1. Assay of Intracellular Triglyceride Accumulation*

1. For maximum throughput, the assay will require a fluorimeter capable of reading multiwell plates. The fluorimeter should ideally be equipped with an injector capable of delivering 1 or 5 µL of reagent to each well of 384- and 96-well plates, respectively. The fluorimeter should also have the ability to mix or shake the plate for 1 s immediately on reagent addition.
2. Following the instructions for the fluorimeter to be used, load the injector with Adipored reagent. Program the instrument to inject 5-µL of Adipored reagent/well (96-well plate) and shake/mix the plate immediately after each injection.
3. Remove the tissue culture plate containing differentiated adipocytes from the incubator and cool to room temperature.
4. The culture supernatant should be removed and each well carefully rinsed with 200 µL of PBS. Be extremely careful not to remove cells from the wells during this rinse step (*see* **Note 11**).
5. Fill each well with 200 µL of room temperature PBS.
6. Place the assay plate in the fluorimeter and initiate the Adipored reagent addition program. Make sure that the plate's lid/cover has been removed (*see* **Note 12**).
7. On completion of reagent addition, the assay plate should be incubated at room temperature for 10–15 min. The plate can be removed from the fluorimeter or remain in the instrument during the incubation period.
8. After 10 min, place the plate in the fluorimeter and measure the fluorescence with excitation at 485 nm and emission at 572 nm.
9. The actual readout in relative fluorescence units will vary with the fluorimeter used. However, after 10 d of differentiation, the ratio of relative fluorescence units (differentiated cells) to relative fluorescence units (undifferentiated cells) should exceed 20 (**Fig. 1**).

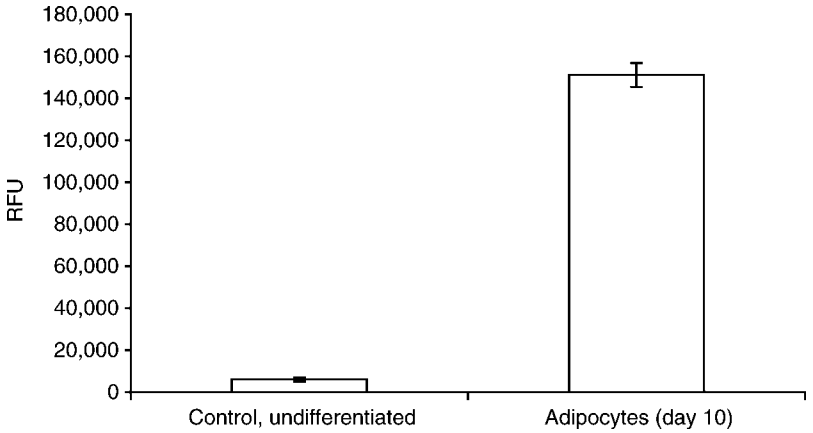


Fig. 1. An Adipored assay of intracellular triglyceride accumulation in differentiated primary human visceral adipocytes.

10. The differentiation of primary human adipocytes can also be assayed by measurement of the expression of a variety of "marker" genes such as those for leptin, AP2 or PPARγ (*see* **ref.** ***11***; **Note 13**).

### *3.2. Culture of Primary Human Osteoclast Precursors*

1. Prepare Osteoclast Precursor Culture Medium by adding FBS, L-glutamine, penicillin, and streptomycin to the basal medium (The final concentrations of the supplements will be 10%, 2 m*M*, 100 U/mL, and 100 µg/mL, respectively). Warm 100 mL of Osteoclast Precursor growth medium in a 37°C water bath.
2. Quickly but completely thaw the vial of frozen cells in a 37°C water bath. Wipe the outside of the vial with 70% ethanol and aseptically transfer the cell suspension to a 50 mL conical tube. Rinse the cryovial with 1 mL of Osteoclast precursor culture medium. Add the rinse dropwise to the cells while gently swirling the tube (approx 1 min). Slowly add additional medium dropwise to the cells until the total volume is 5 mL, while gently swirling after each addition of several drops of medium (approx 3 min). Slowly bring the volume up to 40 mL by adding 1–2 mL volumes of medium drop wise, while gently swirling after each addition of medium (approx 10 min).
3. Centrifuge the cell suspension at 200*g* at room temperature for 15 min.
4. Carefully remove by pipet all but 1 mL of the wash. Gently resuspend the cell pellet in the remaining medium and count. When washing the cells, do not attempt to remove too much of the wash.
5. Dilute 20 µL of the cell suspension in 20 µL of 0.4% Trypan blue, do a cell count and determine percentage viability. Recovery should be greater than 90%.
6. Prepare the osteoclast precursor differentiation medium by the addition of 60 ng/mL M-CSF and 125 ng/mL soluble RANK ligand (*see* **Note 14**). This medium will be "2X" relative to the two cytokine concentrations; the cytokines will be diluted 1/1 on addition of the test samples (0.1 mL) to the seeded cells. Resuspend the precursors in the differentiation medium at 100,000 cells/mL and seed the cells at 0.1 mL/well of a 96-well OsteoAssay plate.
7. If the cells are to be treated with a series of test samples, set up several 24-well dilution plates with the appropriate volumes of osteoclast precursor Culture medium (without M-CSF or RANK ligand)/well and make the required serial dilutions of the test samples. Add 0.1 mL of each different concentration of test sample to wells of the precursors. "Control" wells should be set up, which contain **(A)** no added test sample (i.e., osteoclast precursor culture medium, without cytokines, only), **(B)** "solvent only" treatments if the test samples were dissolved in solvents such as DMSO, ethanol, and so on, and **(C)** culture medium with M-CSF but no soluble RANK ligand instead of complete differentiation medium; these wells will serve as "undifferentiated" controls. Each assay should be done in triplicate.
8. Incubate the cells at 37°C, in a humidified atmosphere of 5% $CO_2$ for 7 d (*see* **Note 15**).

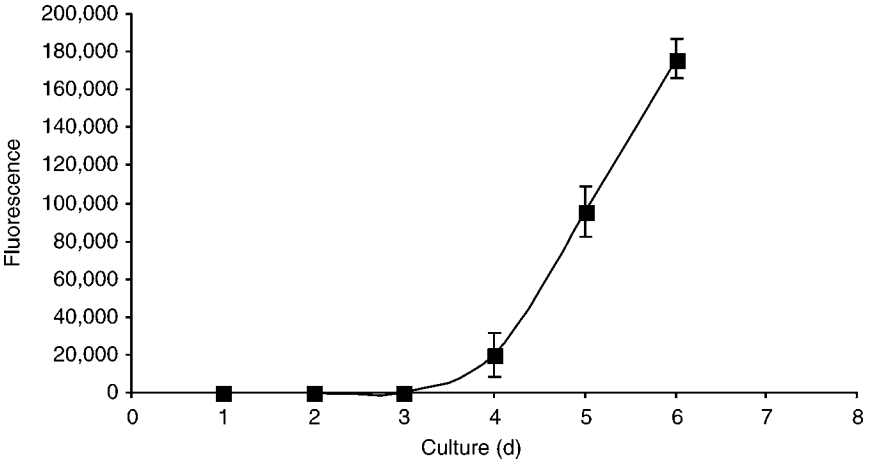


Fig. 2. Primary human osteoclast precursors were seeded onto an OsteoLyse-Collagen plate at 10,000 cells/well and differentiated with M-CSF and soluble RANK ligand for 6 d. At d 7–10 of culture, 10 µL of supernatant was removed and counted. The black bars represent counts obtained when the precursors were cultured with M-CSF only.

### *3.2.1. Resorption Assay*

1. Supplement the Osteoclast Resorption Medium with 10% FBS, glutamine, penicillin, and streptomycin and the same concentrations of M-CSF and soluble RANK ligand as in the original d 0 medium (*see* **Note 16**).
2. Replace the culture medium with fully supplemented resorption medium after 7 d and incubate for an additional 1–3 d at 37°C, 5% $CO_2$, and 90% humidity (*see* **Note 17**).
3. Before to sampling the cell culture supernatant, remove the Fluorophore releasing reagent from 4°C storage and let it warm to room temperature—do not warm this reagent in a water bath. Place 200 µL of Fluorophore releasing reagent in each well of a black-wall 96-well assay plate.
4. Transfer 10 µL of cell culture supernatant to the wells of the assay plate containing Fluorophore releasing reagent. Note that it is essential to change pipet tips each time a new cell culture supernatant is sampled (*see* **Note 18**).
5. Briefly mix the samples in the assay plate. Determine the fluorescence of each well of the assay plate in a time-resolved fluorescence fluorimeter (e.g., a Wallac Victor, with excitation at 340 nm and emission at 615 nm) over a 400 µs time period after an initial delay of 400 µs.
6. If the amount of collagen degraded, as a percentage of the total available collagen, is to be calculated, determine the total amount of intact collagen/well by placing 200 µL of Fluorophore releasing reagent in each of three unused wells of the OsteoLyse-Collagen plate. Mix the contents of the wells and then transfer 1 µL/well to corresponding wells in an assay plate containing 200 µL of Fluorophore releasing reagent/well. Determine the fluorescence of each well of the assay plate in a time-resolved fluorescence fluorimeter and multiply the result by 200 to calculate the total amount of intact collagen/well.
7. The fluorescence of the supernatant samples diluted in the wells of Fluorophore releasing reagent is directly proportional to the resorptive activity of the mature osteoclast. The fluorescent read-out of the OsteoLyse-Collagen assay is proportional to cell number, the degree of osteoclast differentiation and the duration of the assay (**Fig. 2**).

## 4. Notes

1. Primary human preadipocytes are available commercially from several sources including Cambrex Bio Science Walkersville, Walkersville, MD and ZenBio; Research Triangle Park, NC). Preadipocytes can also be isolated using published protocols (e.g., *see* **ref.** ***14***).

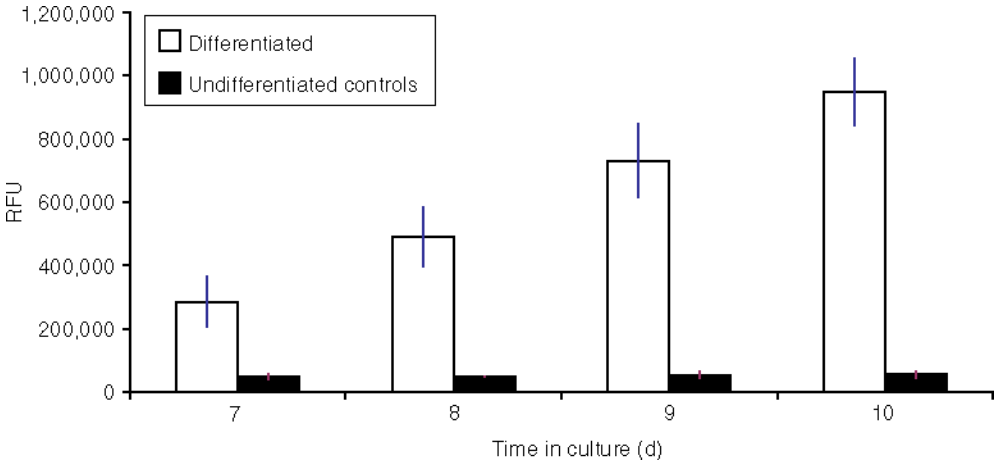


Fig. 3. The release of fluorescent collagen peptides by differentiating primary human osteoclasts requires 4 d of culture. Primary human osteoclast precursors were seeded onto an OsteoLyse-Collagen plate at 10,000 cells/well and cultured in medium containing M-CSF and soluble RANK ligand. Samples of culture supernatant (10 µL) were removed every 24 h and counted in 200 µL Fluorophore releasing reagent in a time-resolved fluorescence-capable plate reader.

2. Primary human osteoclast precursors are available commercially from Cambrex Bio Science Walkersville, Walkersville, MD). Alternatively, different types of precursors, of varying purity, may be obtained from either bone marrow or peripheral blood mononuclear cell fractions ***(15,16)***.
3. Commercial media for the proliferation and differentiation of preadipocytes are available from several sources including Cambrex Bio Science and ZenBio (Research Triangle Park, NC). Current literature also provides suggestions for basal media (e.g., Dulbecco's modified Eagle's medium containing 10% FBS) and the agents used to differentiate preadipocytes into adipocytes ***(17)***. Most protocols for the differentiation of preadipocytes use insulin, dexamthasone and IBMX. Glitazones are often used but, given that much research specifically addresses new types of glitazones, use of these agents may interfere with the assays. The Cambrex medium used in the current study, contains insulin (10 µg/mL), 1 µ*M* dexamthasone, 50 µ*M* indomethacin, and 200 µ*M* IBMX.
4. Commercial media for the proliferation and differentiation of osteoclast precursors is available from Cambrex Bio Science and was used in the current study. Current literature also provides suggestions for basal media (e.g., minimal essential medium α [αMEM] containing 10% FBS) and the agents used to differentiate osteoclast precursors into osteoclasts ***(18)***. The Cambrex medium used in the current study, contains both M-CSF (33 ng/mL) and soluble RANK ligand (66 ng/mL).
5. A Resorption Medium (Cambrex), formulated for maximal osteoclast resorptive activity through optimization of pH, calcium, and other parameters, is used for the actual bone resorption assay. Other media can be used (e.g., α-MEM containing 10% FBS) but the amount of resorptive activity may be substantially less because of excessive alkalinity ***(19)***.
6. The ability of FBS to support the optimal differentiation of both primary human preadipocytes and osteoclast precursors, varies widely from source-to-source and lot-to-lot. The FBS used in these studies was identified through extensive screening of multiple lots of serum from several commercial sources.
7. Adipored is an optimized formulation of the lipophilic dye Nile red (*see* **ref.** ***20***).
8. The OsteoLyse-Collagen plate is a sterile 96-well tissue culture plate to which human type I collagen has been covalently bound. The collagen is labeled with a Europium ion chelate; osteoclast-derived matrix metalloproteinases, secreted into the resorption bay of the cell, degrade the collagen and release europium chelate-labeled collagen peptides into the cell culture supernatant.
9. The primary human preadipocytes can also be cultured and assayed for intracellular lipid accumulation in 384-well plates. Seed wells of a black-wall, clear-bottom 384-well tissue culture plate with 3000 cells/40 µL preadipocyte growth medium. Note that as few as 1000 cells/well can be seeded but a longer period of culture will be required before the cells become confluent (i.e., before differentiation

medium can be added). Initiate differentiation of the cells by the addition of 40 µL of "2X" differentiation medium/well.

10. The extent of adipocyte differentiation may be noted by microscopic observation of lipid vacuoles in the induced cells. The intracellular lipid vacuoles will begin to appear 4–5 d after induction and will continue to increase in number and size for the duration of the culture. Noninduced cells will have few, if any, lipid vacuoles.
11. Differentiated adipocytes are delicate and care should be used to avoid disrupting the cells. In some cases, the cells might "peel" from the well surface when subjected to excessively vigorous aspiration. It is possible to forego the PBS rinse step and add the Adipored directly to the cell culture supernatant. Background readings will be slightly higher because of a very low level of fluorescence induced by the binding of the Adipored reagent to albumin *(20)*.
12. If the fluorimeter is not equipped with an injector, the Adipored reagent can be added with a multichannel pipet if the following protocol is used: Add the Adipored reagent with a multichannel pipet that can accurately deliver 5 µL of reagent/well. Mix the plate by rapping the edge of the plate against the lab bench several times immediately on addition of reagent to each row of wells—do not wait until the reagent has been added to all wells of the plate before mixing the plate's contents.
13. The gene expression profiles for over 47,000 transcripts, in both undifferentiated and differentiated adipocytes and osteoclasts, as determined with Affymetrix Human Genome U133 Plus 2.0 arrays, can be accessed at the following website: www.cambrex.com/content/bioscience/article.class.19.id.1706.
14. Primary human osteoclast precursors cannot be "passaged." They can be differentiated, but in the absence of specific differentiation signals, the cells will senesce.
15. Differentiated osteoclasts can be identified by phase microscopy as unusually large multinucleate cells. These cells will begin to form after approx 4–5 d of culture; the cells can be used in assays of resorption after 5 d of culture (**Fig. 3**). To document osteoclast differentiation, cultures may be stained for the $\alpha_v\beta_3$ integrin complex or for tartrate-resistant acid phosphatase *(21,22)* (*see* **Note 13**).
16. If the OsteoLyse-Collagen assay is to be used to screen or otherwise assay test samples, there are two different protocols that might be used. If the assay is used to measure differentiation of the osteoclast precursor, the test sample should be added at d 0 and removed at d 7. However, if the assay is to measure mature osteoclast function, the test sample should be added only at d 7, with the new Resorption medium addition.
17. The cell culture supernatant can be sampled at any time after the medium change. Because a very small volume (5–10 µL) of supernatant is sampled, it is very easy to do time-course studies by sampling the supernatant on sequential days. Note that supernatant volumes greater than 10 µL are unnecessary and may lead to inefficient counting of the fluorophore as the ratio of Fluorophore releasing reagent to sample decreases.
18. Note that the europium fluorophore is extremely sensitive. All pipet tips and other materials that come into contact with the probe must be discarded in appropriate waste containers. Probe that is spilled on a counter top and allowed to dry will give rise to dust and high background levels in the laboratory. Fluorimeters that are contaminated with the probe must be cleaned.

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