Troubleshooting Forum

Molecular Biology Techniques Q&A

Cell Adhesion

This month's question from the Molecular Biology Forums (online at molecularbiologyforums.biotechniques.com) comes from the "Cell Culture" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

Will treating cells with trypsin interfere with a downstream cell adhesion assay?
(Thread 31046)

Q I am trying to develop an adhesion blocking assay for endothelial cells using antibodies against specific integrins. To begin, I incubate trypsinized cells with 10 μ M Calcein AM for 10-15 min, wash the cells, add the blocking antibody at various concentrations for 30 min, and then seed my treated cells onto 96 well plates at 3,000 cells per well. Prior to fluorescence detection, I change the media once to get rid of any floating cells.

My results have been inconsistent. The fluorescent signals increase or decrease when I don't expect them to and there is only a 4-fold difference between control cells not treated with antibody compared to those treated with the highest concentrations of antibody. I expected a stronger signal from Calcein.

I suspect that the integrins on the cell surfaces may be cut by trypsin. Perhaps the cells do not have enough time to reattach strongly, so I could be losing cells when changing the medium. I let the cells attach for 1.5 hrs, which I think should be enough. Can anyone recommend another way to detach the cells without affecting their surfaces?

To check for attachment, I plan on timing after trypsinization to see how long the cells take to attach. However, I would appreciate recommendations for better methods from anyone with experience performing blocking assays.

A Perhaps you could label the cells with Calcein prior to trypsinisation. I get a great signal from calcein after labelling with 4-8 μ M for 30 - 60 min in the dark. This signal can persist for a very long time, greater than 2 hours for certain, which should be long enough for your assay. Cells normally adhere within about 1 hour when washing and changing media, although that may vary with cell type to some degree.

There are alternatives to trypsinisation such as using a cell scraper, although you may have difficulty with the cells clumping.

I assume that you are trying to quantify the number of attached cells based upon fluorescence. You can get a more accurate assessment using a device that measures cell attachment such as a real-time cell electronic sensing (RT-CES) system. This is a label-free cell monitoring system that detects changes in electrical conductivity.

A You could try using TrypLE from Invitrogen. It is collagenase-based and will be gentle to integrins.

A I talked with a colleague who studied integrins during her PhD. She recommends cell dissociation buffer, enzyme free, PBS based, Invitrogen cat. 1-13151-014. Another trypsin-free method is Acutase, but I do not know if it is enzyme free.

Q: Another possible source of the problem may be the serum in the medium. Could serum proteins affect my assay?

A: I would not think so. When using trypsin, you generally need to add FBS to your cell suspension anyway to inhibit it; usually adding media with 10% FBS is sufficient. There are some proteases in serum such as plasminogen and fibrinogen, but nothing that should cut the integrins.

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Before adding any integrin blockers, you might want to do a simple pilot experiment first to ensure that you can get reproducible and detectable results. Try seeding labeled cells at varying concentrations in a 96-well plate for 0, 15, 30, and 60 min, wash 1-3 times with PBS, add 100 μ l PBS to each well, and read the signal with a plate reader. You will need to find the minimum time it takes to get a good adherent cell signal and determine the sensitivity of detection for different numbers of adherent cells with each dilution. You should probably repeat this a few times to look at the reproducibility.

You should also pre-label the cells prior to harvest. Remember that the plate reader gives a relative fluorescent signal, so don't be too worried if your control gives a signal of 30,000 one time and 8000 the next. As long as the other conditions change relative to this, it's okay.

Q: You may be right about the trypsin, but I found a study where a group incubated with the blocking antibody in the presence of BSA without serum. They did not mention why they did it that way, but I have heard other researchers say that trypsin can cut integrins at the cell surface. I suppose opinions vary.

I will let you all know what happens with my experiment. For now, I am re-seeding my cells because our incubator broke and I lost all of my cultures.

Q: I ran a cell concentration assay with Calcein AM and found that the numbers of cells I have been using fall within the linear range of the curve. My cell binding assay should be run at the moment the cells are specifically binding to the substrate, so I plan to run a time course assay now. My time points are planned in 5 min increments, so I need to do this outside the incubator; it is quite difficult to seed cells every 5 min into a 96-well plate while it is in the incubator. At this point, there is no need to be sterile, since the assay will run for just 30 min to 1 hour, so I can do this on the bench. But I am worried about the cells not having enough CO₂ to behave normally. I could use equilibrated medium, but I suspect that the phenol red in the medium is affecting my readings, so I prefer to use a buffer instead. Does anyone know of a good buffer for cell binding that I can use in this situation? I know calcium should be present, but I am not sure what else should be included.

A: Unfortunately, your options are limited. Since you are using a plate reader, you could use sealed flasks. Or you might try phenol-free media to remove the background. I suggest that you equilibrate the plate in the CO₂ incubator, quickly add the cells, and then seal the plate with parafilm to try to keep the remaining CO₂ near the cells for as long as

possible. This will require that you use a plate reader that can read plates with the lid on. Without putting the entire plate reader in an environment hood or purchasing a RT-CES, this is probably your only option.

A: You could stagger the time points. If the cells get an initial 5 minutes in the atmosphere of your lab during reading, they can then go back into the hood for 15 min to re-equilibrate before another reading at the 20 min mark. Another plate can carry the cells for the 10 minute and 25 minute marks, etc.

Or try working with duplicate plates. Read one in the regular lab air and read another identical plate at the time of the first plate's last reading. If the two readings match within 10% or so, then you can be confident that the hour outside the incubator has not affected cell binding. You won't need to use this control again in later experiments.

Q: I tried changing a few things and ran some experiments. First, I optimized my instrument settings such as bandwidth, dynamic range, top vs. bottom reading, etc. and since I have been reading from the top of the plates, I removed the plate cover. Then I increased the length of incubation times for my assay to 10 min increments, which worked well for creating a nice linear curve of increasing fluorescence. I incubated my cells with DPBS containing

Ca⁺⁺ and Mg⁺⁺ and equilibrated serum free medium in parallel with room air conditions. The cell phenotype changed in DPBS, so using buffer to maintain pH is not a good idea in this case. I still need to compare normoxic and 5% CO₂ conditions as previously suggested, but now I am just trying to determine the appropriate time points to use for future assays. Once I finish, I will do the comparison. I also decreased the volumes in each well to considerably diminish light diffraction and trypsinized for 3 min instead of 5.

These simple changes made a huge difference in my assay results. Previously, I saw a 10X difference between my unknown samples and negative control samples, but now I see 60- to 150-fold differences, depending on the number of cells attached. I am confident now that my assay is more robust. Most importantly, my curves are linear, and I no longer see unexpected or unexplainable changes in fluorescence. There may still be some cutting of the integrins by the trypsin. I attempted to detach cells with EDTA only, but after 10 min, only 50% were detached. I will investigate further.

Selected and edited by Kristie Nybo, Ph.D.

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