Protocol for a Keratin-Free Environment

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1) Do I really need a keratin-free environment?

The first thing you must establish is whether you need to work in a keratin-free environment. Working in and maintaining a keratin free environment is most certainly more time consuming and tedious than working in a typical laboratory environment. However, under appropriate circumstances there is no substitute for a keratin-free environment. The choice of operating in a keratin-free environment over normal laboratory conditions largely depends on the protein samples you are working with. If the proteins of interest are in relatively high concentration or will not be analyzed by mass spectrometry, then a keratin-free environment is unnecessary. Knowing when the protein concentrations are such that a keratin-free environment is necessary comes with experience. However, we offer a few guidelines to get you started.

a) If the protein spot you are interested in stains lightly with SYPRO or silver stain, you will definitely need to work in a keratin-free environment.
b) If the protein spot/band stains dark with Coomassie blue then you probably don't need to work in a keratin-free environment.
c) Protein concentrations between these two extremes can only be evaluated through experience.

2) What are keratins?

Keratins represent a class of structural proteins found in all vertebrates. The word is derived from the indo-european word *ker* that means horn. The subclass α-keratins occur in mammals of which there are over 30 tissue-specific variants. α-keratins are the principal proteins found in skin, nails, hair, and hoofs. Variants are further classified by their acidic or basic amino acid content, acidic and basic keratins respectively. Or, keratin variants are classified by their cysteine content where hard keratins have high cysteine content and soft keratins have low cysteine content. The latter classification refers to the fact that the cysteines are usually involved in disufide bridges, the number of which defines their mechanical properties.

3) Why are keratins a problem?

Keratins represent a class of proteins that are identified by mass spectrometry in the majority of biological samples. More often than not they arrive in the sample through contamination from the environment rather than though natural abundance. If they are present in concentrations greater than that of the protein of interest, their abundance can overwhelm the analysis capacity of the LC-MS system and obscure the peptides of interest. This is particularly problematic when performing data dependent MS, as the MS/MS analysis will necessarily focus on the peptides from the more abundant keratins, providing less or no information about the proteins of interest. In low concentrations, compared to the proteins of interest, keratins are not a problem at all.

4) What is the source of contaminating keratins?

You may also be wondering what the source of the contaminating keratin might be. We and other mammals are constantly shedding bits of keratin-containing dead skin and hair that become bits of wind-blown dust themselves or adhere to other dust particles. Fingerprints provide a rich source of keratins as does wool clothing. We are swimming in a sea of keratin and keratin-carrying dust and fiber particles. In fact, we walk around each day with a veritable keratin fountain spewing from our heads. If these statements seem unlikely or unreasonable you are directed to read "The Secret Life of Dust" by Hannah Holmes (Wiley, John & Sons, Inc., August 2001). This keratin-polluted environment necessarily leads to contamination of experimental samples and is a significant problem for the analysis of low abundance proteins.

5) How do I establish a keratin-free environment?

Overview. There are two keys to establishing a keratin-free environment for your gel samples: Reduce the amount of exposure of the gel and gel equipment and supplies to primary sources of keratins, such as skin, hair, clothing, etc. Reduce the amount of exposure to dust and particulates, both of which can be a rich source of keratins. Best practices for a keratin-free environment therefore involve:

1) Perform as much work as possible in a biological safety cabinet (BSC) or laminar flow hood (**Figure 1**).
2) Assume that everything in the environment is a potential source of keratins. This includes microfuge tubes, pipette tips, reagent containers and reagents therein, exposed skin, etc.

Figure 1: Biological Safety Cabinet for Gel Processing.

For all intent and purpose, we define keratin-free to represent a state in which contamination by environmental keratins is minimized to a point below that of the low abundance proteins typically found in your biological samples. Removing keratin contamination in the BSC that is dedicated to the gel work should be the first step in the process. In addition to the decontamination of the BSC, there are additional steps that you should take to reduce the problem of keratin contamination.

Clothing. Get into the appropriate attire before preparing the BSC for de-keratination. It is recommended that you wear a disposable clean-room type lab coat with snaps on the sleeves to minimize the exposure of bare skin to the BSC environment. Cotton lab coats shed significant amounts of dust and particles that can be carriers of keratin and, at the very least, serve to reduce the life of the HEPA filter the BSC uses to purify the air. Always wear powder-free nitrile or vinyl gloves. We recommend nitrile. Never use latex gloves, as natural rubber contains significant amounts of keratin and other proteinaceous materials. For those of us with long arms, vinyl or Tyvek® sleeve protectors with elastic wrist and arm closures help minimize skin exposure at the wrist (**Figure 2**).

Working surfaces. Prepare the BSC by rinsing all internal surfaces with both water and absolute ethanol, and wipe dry with clean-room wipes. You have to wear the appropriate attire as described in the previous paragraph during this decontamination step for it to be effective. You must perform this operation with the BSC in full operation and while maintaining the front shield opening no larger than 10 inches. This may require a bit of gymnastics on your part. It is recommended that wash bottles of both distilled water and ethanol be kept within the hood for periodic BSC cleaning (**Figure 2**). Don't forget to wipe down the bottles before placing them in the BSC. Once prepared in this way, all materials placed in the BSC must be wiped free of keratins before setting them down in the BSC.

Equipment and supplies. Establish a list of equipment, supplies, and chemicals that will be used in the BSC and store adequate supplies of each in the BSC. Store as many of the required materials in the BSC as possible. Constant and continual movement of materials in and out of the BSC increases the chance that keratins will be transferred to the BSC. Periodic replenishment of supplies is not a problem. Make sure that the outsides of all equipment and supply containers (e.g. bags, boxes, etc.) are wiped down with ethanol and water-soaked clean room wipers outside of the BSC before setting them down in the BSC. Never place paper or cardboard containers in the BSC, as they are rich sources of dust. It is highly recommended that a set of pipettors be dedicated to the keratin-free BSC and not removed for other purposes. Never place previously opened bags or boxes of supplies in the BSC, as the risk of keratin contamination is significant. Open all supply containers (pipette tip boxes or bags, bags of microfuge tubes, etc.) in the BSC while it is in full operation and keep them in the BSC until the supply is used up.

Figure 2: Clothing and Equipment used to establish and maintain a Keratin free environment.

Clearly, not all equipment and materials can be located within the BSC at all times. If items are to be shuttled in and out of the BSC, it is good practice to locate them on a biohazard mat or clean room wipe when working with them outside and on another while inside the BSC. At the end of the day or experiment the mats and wipes can be discarded, reducing the possibility that keratins are transferred to the floor of the BSC. Lastly, keep the front shield of the BSC closed whenever not in use.

6) How do I know that my workbench environment is keratin-free?

It is always prudent to qualify the environment as keratin-free before evaluation of valuable samples. For this purpose we recommend that you run a gel with protein standards using all of the reagents, equipment and procedures you would use with your experimental samples. Remove plugs from both stained and unstained regions, process the plugs, and evaluate the resultant peptides by mass spectrometry. The absence of keratins signifies that the area is keratin free. Extremely low levels of keratins (peptides have low or marginal Sequest Xcorr values) can be ignored.

Summary of key points:

- Thoroughly wipe down the inside of the BSC (or laminar flow hood) with water and ethanol before use.
- Wipe down all containers and apparatus before placing them in the BSC.
- Store all reagents and as much of the required apparatus and supplies and as possible in the BSC.
- Never place previously opened bags or boxes of supplies in the BSC.
- Perform as much reagent preparation as possible in the BSC and limit exposure of reagents to open laboratory environment as much as possible.
- Handle all materials with nitrile or vinyl gloves and a wear clean-room lab coat, and sleeve protectors if necessary.
- Never use latex gloves or tubing.

7) How do I maintain a keratin-free environment?

Overview. There are three keys to maintaining a keratin-free environment for your gel samples:

1) Perform as much work as possible in a biological safety cabinet (BSC) or laminar flow hood.
2) Assume that everything in the environment is a potential source of keratins.
3) Minimize the number of transfers of materials to and from the BSC.

Reagent preparation is always an issue, as weighing and pH adjustments are typically not performed in the BSC. To minimize keratin contamination the following steps should be taken:

- Minimize the amount of time chemical bottles are open while outside of the BSC.
- Dispense reagents only with spatulas, funnels and the like that have been wiped free of keratin and into containers treated in the same precautionary manner.
- Wear a bouffant cap if the chemical bottle or reagent container will be open to the laboratory for extended periods (e.g. while adjusting pH). Bearded or mustached individuals should also wear a facemask.
- Maintain keratin-free acid and base solutions for pH adjustment and dispense with keratin-free droppers or pipette tips.
- Always wear appropriate gloves and lab coats.
- Thoroughly wipe down the work surfaces of the BSC (or laminar flow hood) with water and ethanol at the end of the day.
- Keep the front shield of the BSC closed whenever not in use.

8) What about my sample?

Protein samples can be contaminated with keratins before loading on the gel, while in the gel, as well as during and after post-gel processing. Preventing keratin contamination in the first- and last-listed periods may be facilitated by taking the precautions outlined above. If samples are placed in microplates for automated loading into a mass spectrometer, it is recommended that the plate be covered with a suitable piercible cover while in the auto sampler. More often than not, keratin contamination occurs while the protein is still in the gel. The large exposed surface area of the gel is an invitation to keratin collection. In addition to applying the aforementioned precautions to the preparation and running of the gels, the following should also be adhered to:

- Open the gel sandwiches only while they are in the BSC.
- If the gels are to be scanned, it is recommended that they be placed into pre-cleaned clear plastic and kept there at all times while they are not in the BSC. We recommend placing them in between the leaves of a clear plastic sheet protector. Plastic wrap works as well.
- Spot picking should be performed in the BSC if possible. If this is not possible, wear the appropriate attire as noted above
- Handle gels as though they were keratin magnets.

9) How do I re-establish a keratin-free environment once it is compromised?

Once the keratin-free environment is compromised, it should be reestablished before any work continues. We consider an area compromised if several plugs from the same gel are found to contain significant amounts and different types of keratins **(Figure 3).** For example, if the top 5 or 6 peptide IDs that Sequest gives are keratins, the gel spot is contaminated. An excellent confirming test is to process a gel plug or two from regions in the gel that are not stained. If the result shows the presence of keratins, the gel is definitively contaminated.

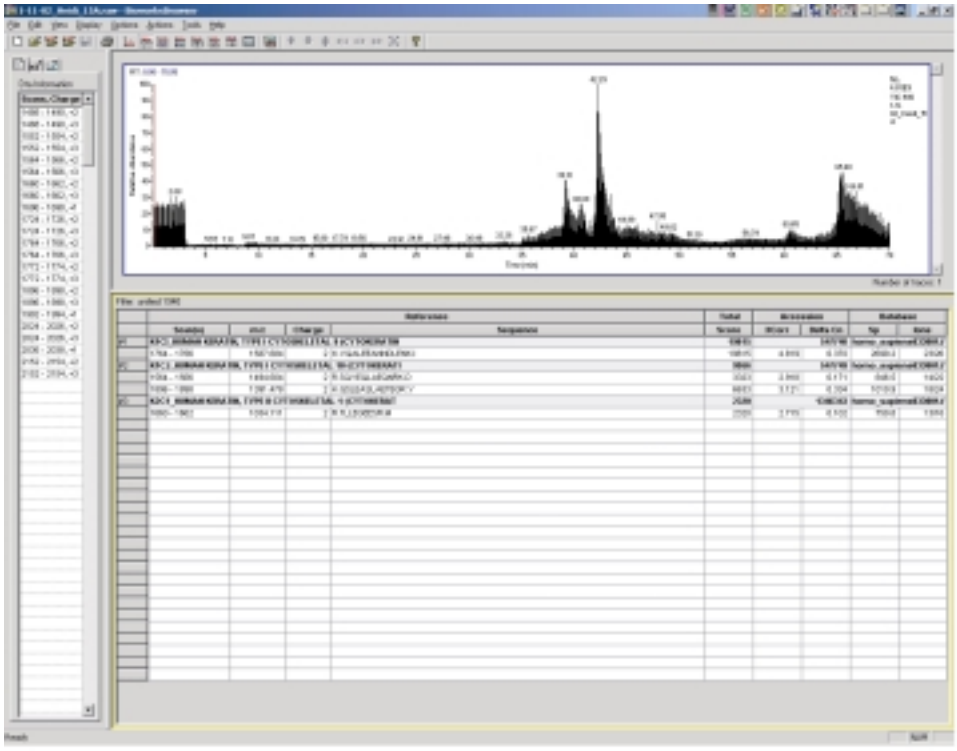

Figure 3: Bioworks Search Result indicating Keratin contamination.

Although you might be tempted to test each solution and each container of supplies one at a time to find the source of the contamination, we have found that it is less time consuming and less expensive to reestablish the keratin-free environment from scratch. Reagents and supplies stored in the BSC should be discarded or used only for experiments where keratin contamination is not a problem. The BSC and any equipment stored in it should be treated as noted above. The BSC should be restocked with new supplies. Finally the environment should be qualified as keratin-free as described above.

Good Luck!