



## Cell and tissue sample preparation for 2D-PAGE

### Introduction

From our experience sample preparation is probably the most critical and variable step in 2D-PAGE. Even with a group of experienced labs, there was a significant improvement in cross-lab reproducibility when ready-to-go samples were used. That is the reason why steps are now being taken to provide a reference sample based on a HeLa cell lysate, which is accessible to many labs, but still comparable to 'real-life' samples in terms of complexity. What follows is a description of how these reference samples are going to be prepared, a procedure that can be used for any mammalian cell line and almost unmodified for many mammalian tissue samples as well.

This procedure is not necessarily the 'best' procedure, but it is one that has proven to be very robust across a wide range of samples that have been analyzed in our laboratory. We recommend anyone, especially newcomers to the 2D-PAGE field, to start by applying this exact procedure to a cell line sample and only start improving the method once a good result with the basic method has been achieved. There are so many parameters in 2D-PAGE that can be varied, that it is sometimes very difficult to determine why a specific method does not give the expected results. For the procedure described here, we know and guarantee it will work with the most common samples.

### DIGE vs. post-staining

In our lab, we typically solubilize all samples in the [DIGE sample buffer](#), as recommended by GE Healthcare, because samples in DIGE buffer will be compatible with any of the common staining methods, be it by pre-labeling with CyDyes (DIGE) or by post-staining with silver, Coomassie, or fluorescent dyes. However, DIGE buffer tends to be slightly more difficult to use than another popular lysis buffer, which we refer to as [Rabilloud buffer](#) (introduced by Thierry Rabilloud's lab: Rabilloud et al., Electrophoresis. 1997, 18:307), because certain cell lines, such as Jurkat, have a strong tendency to clump when they are lysed.



*Adding Rabilloud lysis buffer to a cell (Jurkat) pellet*

In Rabilloud buffer the presence of the Pharmalytes prevents that clumping. We sometimes use sonication to shear the DNA. Temperature control is essential when sonicating, so use 2 or 3 short (max. 10 s) bursts at low power and cool on ice for at least 1 min in-between sonications. If you are not using DIGE, it is more convenient to simply lyse the sample in Rabilloud buffer.



*Tip sonication on ice for completely dissolving DIGE samples. Note: the ultrasound should be off when moving the tip close to or through the air-liquid interface. Otherwise your sample will turn into foam. If this happens, centrifuge at high speed for a few minutes and start again*

### **Harvesting of cultured cells**

Harvesting of cells for 2D gel analysis can be done in different ways, which are all aimed at maintaining the proteome and reducing the contamination with proteins from the culture medium. For suspension cultures, cells should be collected by centrifugation from the culture medium and then washed at least twice in protein-free buffer (usually PBS). Be careful to remove the supernatant completely to avoid getting salt in the lysate.

#### **TIP**

We collect suspension cells from a T75 flask (20 ml of medium) first in a 50 ml Falcon tube, wash one or two times with 25 ml of PBS and finally transfer the pellet to an Eppendorf tube in 1 ml of PBS. A short spin in the Eppendorf centrifuge (3', 3000 rpm) and then buffer can be readily removed, much easier than from a Falcon tube. Perfectionists can use a gel loader tip to remove the very last microliter of buffer.

Adherent cells can be lysed either in the dish/flask or released from the flask/dish with Versene (EDTA) and then washed like suspension cells. Avoid trypsinization if possible.

For lysis in the dish/flask, wash the cell layer with PBS two to three times, remove the PBS completely after holding the dish/flask at an angle for 10 s and add lysis buffer directly on the surface. Here the protein concentration will inherently be lower. E.g. a well from a 6-well plate will require around 100  $\mu$ l lysis buffer for convenient handling resulting in a typical protein concentration of 2-3 mg/ml.

#### TIP

Remaining salt will often be visible on the 2D as 'holes' in the pattern, between pH 5.5 and 6.5. To eliminate that problem, use phosphate-buffered isotonic sucrose solution for the (last) wash step. Prepare 10 mM phosphate buffer like PBS but add 0.25 M sucrose instead of 0.15 M NaCl

#### Lysis protocol for cultured cells or tissue

We always aim to achieve a protein concentration between 5 and 10 mg/ml. For a cell line like HeLa that translates into 2.5 - 3 x 10<sup>7</sup> cells per ml of lysis buffer. For smaller suspension cells like Jurkat, we use around 8 - 10 x 10<sup>7</sup> cells/ml. For a tissue sample, a rule of thumb is to take a buffer/tissue ratio of 8, in other words 1 g of tissue is lysed in 8 ml of lysis buffer.

Cell pellets are thawed briefly - if necessary - by holding the tube/vial for 30 - 60 s and then resuspended quickly by careful pipetting up and down with the appropriate amount of lysis buffer. With big pellets, it sometimes takes a while since the inside is still frozen, but gradually the pellet will 'melt' and be resuspended. Make sure the buffer flow is quite rapid but do not capture air into the tip, because this will lead to strong foaming of the buffer.

Tissue samples can be homogenized directly in the appropriate amount of lysis buffer. We homogenize soft tissues using a Potter, with 8 strokes at 800 rpm. Move slowly to avoid foaming. Hard tissue can be pulverized under liquid nitrogen and resuspended in lysis buffer. Any other suitable homogenization protocol can be used, provided the sample is not heated above 25 °C and excessive foaming is avoided. It is not recommended to use any additional salts, detergents etc. because they will almost always cause problems in 2D-PAGE.

#### TIP

The pellet will add to the total volume, so if you keep the pipette fixed at the buffer volume, there will be little risk of introducing air.

After resuspension leave the lysate at room temperature for 10 min and then remove insoluble material centrifugation for 15 minutes at 15,000g (or more, for tissue samples

100'000g is recommended). The supernatant (yellowish but clear) is aliquoted and stored frozen at -80 °C. Sometimes some white material is floating on top of the solution, especially with lipid-rich tissues. This is mostly lipid and poses no problems for further processing. If desired, most of it can be removed by pipetting out the supernatant from below.

#### TIP

Often seemingly insoluble material will be present at the bottom of the tube, whenever a sample is thawed. This usually redissolves quickly after bringing the sample to room temperature and vortexing. In any case, it is always advisable to centrifuge samples again after a freeze/thaw cycle, not only for 2D-PAGE applications. In almost any buffer, freezing and thawing of concentrated cell or tissue lysates leads to precipitation.

### **Congratulations!**

By now you have created a reference sample that will generate reliable and reproducible results.

A general protocol for 2D-PAGE (24 cm IPG 4-7, 12% SDS-PAGE) is available from [www.fixingproteomics.org](http://www.fixingproteomics.org). The procedure described here has been proven and we guarantee it will work with most common samples prepared using the sample preparation protocol above.

## Lysis buffers:

The composition of the **DIGE lysis buffer** is,

Name	Conc.	Amount for 100 ml	Unit
Urea	7 M	42.04	g
Thiourea	2 M	15.22	g
CHAPS	4%	4	g
Tris (Merck 1.08382.1000)	30 mM	0.36	g

pH is set at 8.5 using HCl on ice. For this purpose we use 'precision' pH paper (such as Sigma P4536), since a pH electrode will not survive regular interactions with these buffers. Proper pH setting is critical for labeling efficiency. We always test a buffer batch with a known sample before using it for anything else.

The composition of the **Rabilloud lysis buffer** is,

Name	Supplier	Conc.	Amount for 100 ml	Unit
Urea	Fluka, 51456	7 M	42.04	g
Thiourea	Fluka, 88810	2 M	15.22	g
CHAPS	Fluka, 26680	4%	4	g
DTT	Sigma, D9779	1%	1	g
Pharmalyte 3 - 10	GE, 17-0456-01	2%	2	ml

Store aliquoted at -80°C for 3 - 6 months. Discard after thawing. Note: Many labs add nuclease (DNase, RNase or benzonase) to the lysisbuffer. While the activity of such enzymes in these buffers is questionable, they should be added freshly. We have used 3 µg/ml DNase I (Sigma D4527) and 1.5 µg/ml RNase A (Sigma R5500). Many also add protease inhibitor tablets (such as Roche Complete, 1697498) to the buffer (1 tablet per 50 ml).