**Cell Permeabilization for PRO-seq**

**Purpose:** This protocol is used to permeabilize cells for nuclear run-on assays as part of PRO-seq.

**How it works:** Cells are harvested immediately into ice cold buffer to halt transcription. Subsequent treatment with mild detergents creates holes in the cell membranes, releasing intracellular NTP pools into the solution and further stalling transcription elongation.

**Application:** Although this protocol should be broadly effective for many cell types, the conditions were optimized for selected lines of mammalian cells in culture and additional optimization may be required for other cell types or tissues. Notably, initial cell harvest conditions will need to be altered for non-adherent cell types, and centrifugation speeds may need to be altered based on cell ‘delicacy’, to avoid cells bursting.

High quality permeabilized cells are the single most important factor for obtaining good PRO-seq data. Permeabilization is strongly influenced by cell type, cell handling, temperature, and amount of time that cells spend in Buffer P. These must be controlled as precisely and reproducibly as possible.

To help ensure good results:

* We **strongly** recommend that users new to the protocol or working with a new cell line:
  + Practice on control cells before preparing samples for PRO-seq.
  + Permeabilize at most 2-4 samples per batch and, even with practice, never more than 6 in one batch.
* Pairs of samples for comparison (e.g., A and B) should be permeabilized in parallel. Batch A1 with B1 not A1 with A2.
* We advise taking batches of samples through the protocol from start to finish before starting with the next batch. Though more time consuming, this approach offers the best control over the process.

The following situations will compromise the 3' end resolution of PRO-seq libraries.

* If cells are not kept ice cold prior to permeabilization, transcription elongation may continue during handling
* Failure to keep cells ice cold can also lead to RNA degradation.
* If cells are not thoroughly permeabilized, endogenous NTPs from intact cells may compete with biotinylated NTPs during nuclear run on.

We recommend you read the troubleshooting section at the end to get a feel for the protocol before starting. If you have any questions about the protocol or require assistance, please contact NTC staff.

**Expected Results:** With practice, it should be possible to recover at least 50% of the input cells with ≥95% permeabilization. Samples with <90% permeabilization, excessive clumping or cell debris are unsuitable for PRO-seq and cannot be processed by the core.

**Estimated Time Required:** ~1 hour per permeabilization batch. Allow additional time for cell counting and QC.

**Protocol Version Note:** This revision of NTC permeabilization protocol simplifies some instructions and makes minor tweaks to some steps. This version and all previous versions yield equally good cells so if you have already optimized permeabilization using a prior version there is no need to change your protocol.

**PROTOCOL:**

**Required Reagents:** *(buffer compositions are listed at end of protocol)*

DMEM + 10% FBS

Trypsin or Accutase for releasing adherent cells

1X PBS (without Mg/Ca; room temp and chilled)

Buffer P (permeabilization)

Buffer W (wash)  
Buffer F (freeze)

Liquid nitrogen

**Starting Material for Permeabilization:**

Cells, ideally 5-10 million (M) cells per sample

Note on cell numbers

In our hands, this protocol has a cell retention rate of ≥50% for most cell lines so, to be safe, plan to start with at least twice as many cells as you need for your downstream application. Ideally, you would start with 3-4X as many cells as you need – that way it’s not a disaster if your efficiency is lower! Currently, PRO-seq libraries are generated with 1M permeabilized cells (that is 1M cells counted after permeabilization, so this corresponds to ~2M starting cells for each library based on a 50% retention rate). It is difficult to permeabilize cells in batches of less than 1M without protocol optimization.

Please contact us about options if you plan to start with fewer than ~2M or greater than 10M cells per sample. If you anticipate difficulties obtaining the desired cell numbers, please let us know prior to submission. Our protocol can work with lower inputs, and we can discuss what is reasonable for your experiment. For very low input samples, we may ask for cells to be submitted at different concentrations than described below.

**Prior to Starting Protocol:**

* Warm Trypsin or Accutase to appropriate temperature
* Chill centrifuge(s) to 4ºC
  + You will need to spin down cells in 50mL conical tubes and 1.5mL Eppendorf tubes. All the spins are best done in a swinging-bucket rotor rather than fixed angle. Thus, you would ideally use adaptors that let you spin 1.5mL tubes in a swinging-bucket centrifuge*.*
* Chill appropriate volumes of buffers, medium, and 1X PBS on ice.
* Check that liquid nitrogen is available. It will be needed for freezing final cells.
* Immediately before use, supplement buffer aliquots as follows:
  + **Buffer W**: DTT to 0.5mM + 0.2μL/mL RNase inhibitor\*
  + **Buffer P**: DTT to 0.5mM + 0.2μL/mL RNase inhibitor\*
  + **Buffer F**: DTT to 0.5mM + RNase inhibitor\* to **1μL/mL** final

*\*we use SUPERaseIN from ThermoFisher (#AM2696)*

**Protocol**

***After harvest, cells must be kept ice cold at all times.***

1. Rinse cells on plate once with room temperature 1X PBS
2. Apply trypsin or Accutase to release adherent cells, using conditions typical for your cell type. After 1-2 minutes (or when cells detach), quench with **ice** **cold**DMEM+10%FBS (trypsin) or ice cold media (Accutase).
3. Collect cells in 50mL conical tube. Pool cells from multiple plates if appropriate. Place tubes on ice immediately.

*There is no need to precisely determine cell number at this stage. If you know the expected cell number based on plating, plate size, or just looking at the dish, that should be sufficient.*

*We achieve highest efficiency when 50 mL tube contains ~10M cells. Since the pellet after permeabilization can be ‘smeary’ and fairly translucent, it is difficult to keep track of and resuspend properly if cell numbers are very low. Conversely, if you have too many cells in a tube they tend to stick together after permeabilization and then shear during resuspension.*

1. Spin at 300xg for 4 min at 4ºC.
2. Remove supernatant. Gently resuspend cell pellet in 10mL cold 1X PBS.

*If you will be using the same cells for multiple purposes (e.g. PRO-seq and RNA-seq), take 10-20*μ*L of cells for counting so cells can be divided properly. If practicing the protocol, also count at this step as this "input" cell count is needed to calculate percent recovery.*

1. Spin again at 300xg for 4 min at 4ºC. Remove supernatant.
2. Resuspend cells in **250μL Buffer W** to get a single-cell suspension

*It is critical that cells are in single-cell suspension at this stage. If cells are still in clumps when Buffer P is added, they can become a gunky mass that gets sheared to pieces during resuspension and result in super-low final cell counts.*

1. Add **10mL Buffer P** and incubate sample(s) on ice for 5 min.

*Add Buffer P at medium speed on your automatic pipettor, dispensing along the wall of the tube (it may help to set the tube diagonally in an ice bucket and flow buffer P along the lower wall). This will enable mixing but avoid sloshing or foaming that will disrupt cells.*

*Keep amount of time in buffer P consistent across samples, especially when processing multiple samples at the same time.*

1. Spin at 400xg for 8 min at 4ºC.

***Check that the supernatants are clear and pellet is compact to avoid cell loss when removing supernatant. If the supernatant is cloudy and the pellet has a hazy/wispy appearance at the top, you should spin again before removing the supernatant.***

1. Remove supernatant from the first sample and immediately add **1 mL of buffer W** without resuspending. Do the same for the next tube and continue until all samples have been processed.

*The idea is to quickly dilute the residual Buffer P.*

1. Completely resuspend all pellets by pipetting.
2. Add an additional **9 mL Buffer W** to each tube.

*Add Buffer W at medium speed on your automatic pipettor, dispensing along the wall of the tube (it may help to set the tube diagonally in an ice bucket and flow buffer W along the lower wall). This will enable mixing but avoid sloshing that will disrupt cells.*

1. Spin at 400xg for 4 min at 4ºC.
2. Discard supernatant and carefully pipette off remaining buffer without disturbing the cell pellet.
3. Resuspend in **200μL Buffer F** and transfer to a 1.5mL low binding tube.
4. Rinse the conical tube with an additional **200μL Buffer F** and pool with the first resuspension to give 400uL total. Add more F buffer if necessary to get a good resuspension.
5. Pipet the pooled cells to fully mix and resuspend.

*Avoid introducing bubbles. The final suspension should look uniformly cloudy and free of clumps. If you draw the cells into a pipet tip and hold it up to the light, you should not see any "snowflakes" or particles. Permeabilized cells should be well resuspended for accurate counting.*

1. Count and QC the cells

*It’s critical to have an accurate cell count at this stage, as this cell count will be used as the basis of your PRO-seq input. Our suggested protocol for cell counting is below:*

1. Sample Dilution. Working one tube at a time:
   1. Pipet the sample to resuspend
   2. Remove 10μL and dilute into 90μL PBS to make a 1:10 dilution
   3. Pipet mix thoroughly
2. Total Cell Counting
   1. Use 10μL each of the 1:10 to count cells **without trypan blue**, either via cell counter or hemocytometer**.**
   2. If counts are outside the range of your counter, make additional dilutions as needed to obtain accurate counts.
3. Live/Dead Cell Counting with Trypan Blue
   1. Just before counting each tube, pipet an equal volume of trypan blue dye into the tube, tweak gently 2-3 times to mix and count 10μL. Trypan-positive cells are successfully permeabilized.
   2. If using an alternative live/dead dye such as AO/PI, adjust the sample:dye ratio accordingly

***A note on automated cell counters****:*

*Some cell counters will not read a sample if almost all cells are trypan-positive (i.e., assumed by the counter to be dead), and will give an error or tell you no cells can be detected. Since you already know the true cell number from the unstained count, you can interpret this error to mean that >98% of the cells are permeabilized. Alternatively, you can further corroborate by counting manually via hemocytometer. Not all cell counters have this problem, but many do.*

*If using an automated cell counter, assess each sample visually as well. Although correctly permeabilized cells will stain trypan-positive, so will dead cells. It’s hard to make any quantitative assessment of how many dead cells are in your sample (and, under normal circumstances, dead cells should be rare), but visual assessment should give you an idea of any major issues – permeabilized cells should look phenotypically intact, and may stain lighter than truly dead cells, since there are fewer holes in the membrane through which the dye can enter.*

*A sample that stains ~ 95% trypan-positive is successful. If the percentage staining positive is lower, let us know and we can work with you to optimize.*

1. Concentrate cells to **~25M permeabilized cells/mL** (i.e., ~1M cells per 40uL of buffer F) for submission to the NTC.
   * Spin at 400xg for 4 min at 4ºC.
   * Without disturbing the pellet, carefully pipet off the volume of supernatant necessary to increase the cell concentration to ~25M/mL.

*Cells are always recounted prior to run-on but the process is far more efficient if cells are submitted at roughly the requested concentration. Although ~25M/mL is preferable, concentrations between 10-25M cells/mL are acceptable for submission. Be careful not to disturb the pellet at this step: it is better for the sample to be a little more dilute than to lose cells while concentrating.*

1. Resuspend the cells gently but thoroughly in the remaining volume.
   * ***Tubes should be clearly labeled with the NTC ID#s provided in your sample manifest***
   * ***Report any protocol modifications to the core (note them in your manifest).*** *This is especially important for g-forces and spin times to ensure we handle, and recover, your cells properly during QC and run-on setup.*
2. Snap freeze in liquid nitrogen and store at -80°C.

**Permeabilized cells are stable at -80°C for at least 6 months.**

**Final Quality Control**

* If your final % permeabilized is <90%, do not submit these samples to the core. Excessive unpermeabilized cells can adversely affect the run-on reaction. Samples submitted to the core must have both **high % permeabilized** and **low % NON-permeabilized**.
* If your cells are heavily clumped, do not submit them to the core.Accurate interpretation of PRO-seq data relies on the assumption that all cells in the run on reaction have an equal chance of running on. Clumping may invalidate this assumption.

**Sample Submission for NTC**

The core uses 1M cells for each run-on reaction and requests samples be submitted at a concentration of ~25M cells/mL.

When possible, we recommend that users submit 3M or more cells. This allows sufficient excess in case of discrepancies between your counts and ours. Further, in case there is ever a need to rebuild a library, having more of the original stock available keeps potential batch effects to a minimum.

**Before submitting samples:**

* Have you reviewed and approved an estimate for your project?
* Did you send an electronic copy of your completed sample manifest?
* Have you let us know when you plan to ship or drop off your samples?

**Buffer Compositions**

**Use RNase-free water and reagents to make all solutions.**

**Filter sterilize all solutions with 0.2 μm filters into RNase free plastic bottles.**

Large stocks of buffers W, P, and F can be prepared in advance (without DTT, and RNase inhibitors) and stored at 4°C. Aliquots of buffers containing DTT and inhibitors should be discarded after use. Buffers P and W are identical except for the addition of detergents Igepal and Tween-20 to Buffer P so it can be convenient to make a large stock of Buffer W, make 2 aliquots and add detergents to one of them to make Buffer P.

**Detergent Stock Solutions**

100% Tween-20 and especially 100% Igepal CA-630 stocks are viscous and slow to dissolve in aqueous solutions. It is convenient to first prepare 10% stock solutions in water. A positive displacement pipet such as an Eppendorf repeater with combi-tips can help dispense accurately and completely. Roll or invert the stock solution until all the detergent is fully dissolved then sterile filter through a 0.2um membrane.

**Buffer P (permeabilization)**

10 mM Tris-Cl, pH 8.0

10 mM KCl

250 mM Sucrose

5 mM MgCl2

1 mM EGTA

0.1 % (v/v) Igepal CA-630

0.5 mM DTT *(add only to working aliquots, not main stock)*

0.05 % (v/v) Tween-20

10 % (v/v) Glycerol

Before Use:

Add DTT to 0.5mM + 0.2μL/mL SUPERase-IN RNase inhibitor

**Buffer W (wash; identical to P without detergent)**

10 mM Tris-Cl, pH 8.0

10 mM KCl

250 mM Sucrose

5 mM MgCl2

1 mM EGTA

0.5 mM DTT *(add only to working aliquots, not main stock)*

10 % (v/v) Glycerol

Before Use:

Add DTT to 0.5mM + 0.2μL/mL SUPERase-IN RNase inhibitor

**Buffer F (freeze):**

50 mM Tris-Cl, pH 8.0

40 % (v/v) glycerol

5 mM MgCl2

1.1 mM EDTA

0.5 mM DTT *(add only to working aliquots, not main stock)*

Before Use:

Add DTT to 0.5mM + SUPERase-IN RNase inhibitor to **1μL/mL** final

**Troubleshooting**

The protocol above works well for a variety of cell types (e.g., HEK293T, mouse ES cells) but is not universal. Some customizations may be required for your cells. Below are some tips if optimization is needed.

***Low Yield***

*Recovery of 30-50% is typical but if you are recovering less than ~20%, optimization can help. Keep in mind there is no minimum yield you must obtain for samples to be useable for PRO-seq so don't drive yourself crazy trying to get perfect recovery.*

*Yield is mainly an issue if your starting cells are precious, or yields are so low that you cannot practically generate enough to submit. Since yield and tendency to clump are affected by some of the same parameters, keep in mind that it is better to have a lower yield of high-quality cells than a higher yield of low-quality cells.*

*To identify where you're losing cells:*

* Take aliquots of the supernatant from each step and count them for cells. You should see few or no cells.
* Take aliquots after each suspension step and count to calculate step yields.

*Possible Causes for Low Yield:*

* Incomplete pelleting during centrifugation.

*The g-forces in the protocol above are general and you may need to modify for your cell type. Buffer F is denser than the other buffers and slightly higher g-force may be needed to get good pellets. Recovery must be balanced against the tendency of some cells to clump at higher g-forces.* Increase g-force or spin time

* + Spin parameters
    - Decrease g-force & increase spin duration. Some cells may clump at higher g-force so longer, slower spins may help with delicate or sticky cells.
    - Some loss is anticipated during the buffer P step but it is a good idea to check that the supernatant after spinning out of buffer P is clear before removing it. If not, spin the tube again before proceeding.
* Labware
  + We typically use 50mL conical tubes for the high-volume steps (PBS, buffers W and P) and move to 1.5mL tubes for the low volume steps in buffer F. Some users have reported improved yields with 15mL conical tubes and 5mL low binding tubes such as Eppendorf #0030108310 or similar. For 5mL tubes, reduce the volume of the buffer W and P steps by half. Depending on your cells, this may require scaling down the number of input cells per tube.
* Over-permeabilization

*The standard amount of detergent may be too much for some delicate cells, though this is rare. We suggest trying the other optimization steps in this section before altering the buffers*

* + Reduce time in buffer P
  + Reduce detergent concentration by half. This may also require optimization of exposure time to maintain >90% permeabilization.

***Clumping***

*Clumps and cell debris are common problems and must be resolved. Accurate interpretation of PRO-seq data relies on the assumptions that:*

1. *Equal numbers of cells are used for all samples*
2. *All cells are equally permeabilized*
3. *All cells have an equal chance of running on*

*Clumps may contain incompletely permeabilized cells and the cells in them may be unequally exposed to run-on reaction buffers. Clumps and debris may make accurate cell counting and spiking difficult or impossible.*

*Possible Causes:*

* Excessive g-force.
  + Reduce g-force. To maintain yield, you may need to spin longer at the lower g-force. A longer, slower spin may also improve recovery of some delicate cells and reduce clumping.
* Inherently stick or clumpy cells
  + Try a cell strainer. After the initial resuspension of cells in Buffer W, pass them through a strainer. We've tested Corning Falcon Test Tube with Cell Strainer Snap Cap (#352235; 35um mesh). Place the end of a wide bore tip in contact with the strainer mesh and dispense the cells briskly through or, if your cells are very fragile, pass them through by gravity flow. Proceed with the rest of the protocol as usual.
  + Buffer composition may not be optimal for your cells. Modifying the buffers is not usually necessary but, if other optimizations fail, let us know and we can make suggestions.

***Low Percent Permeabilized (Below 90% Permeabilized)***

*This is an infrequent problem but very important to address if it does occur.*

*Possible Causes:*

* Too many cells for the volume of Buffer P
  + Decrease cells per tube
* Cells are resistant to permeabilization
  + Increase time in Buffer P
  + **Very** gentle nutation in buffer P. Lie the tubes flat on nutator rotate 2-5 minutes at 4°C at 15-25 RPM. Cells in buffer P are very fragile and any sloshing or foaming can greatly reduce yield. Use care when placing tubes onto and removing from the device to avoid knocking or dropping. Tubes may need to be secured so they can't roll off. Do not mix by hand as this is error prone and irreproducible.
* Debris and vesicles may not stain with Trypan but may end up counted. Make sure samples are free of debris.
* Poor automatic cell counter performance. See the note above on automatic counters. On some models with manual focus, different focal planes can give wildly different % live counts due to the low contrast of permeabilized cells. If your counter gives crazy numbers but inspection by eye indicates that ~100% of cells are permeabilized then the cells are probably fine. Please note this for the core on your sample manifest.

***Variable Permeabilization Between Sample Batches***

*For example: A1 is 95% permeabilized, B1 is 90% permeabilized, and C1 is 80% permeabilized.*

*Possible Causes:*

* Delays in sample processing. The steps between buffer P addition to Buffer F addition are very **time sensitive**. It is imperative that you are as quick and gentle as possible during steps 8-9. Any variability in processing and time spent in each buffer will affect the final cell counts/percent permeabilized and may affect PRO-seq results.
* All samples should be prepared by the same person using the same stocks of solutions as close in time as practical (i.e. batches prepped on the same day or consecutive days depending on experimental design). The more parameters you can keep uniform between batches, the less chance of variable results.

**A Note at the End**

The core collects information on the performance of this protocol with various cell types and would be grateful to know what works best for your cells. Over time, this will let us more easily advise all users the best way to permeabilize their cells. Finally, this protocol looks deceptively straightforward but has a lot of subtlety to the timing and handling of cells. Don't get discouraged if it doesn't work well the first few times. It always improves with practice, and we have yet to find a cell type that cannot be permeabilized.