**Cleland immunoblotting protocol**

**1.Buffers**

**Table 1. Buffer recipes**

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| **Buffer** | **Recipe** | **Storage** |
| 10X running buffer | Add 144.2 g glycine and 30.3 g Tris to 900 ml ddH2O.  Mix until they are in solution.  Add SDS 10 g and top up to 1L with 100 ml ddH2O | RT |
| 1X running buffer | 100 ml 10X transfer buffer in 900 ml ddH2O. | RT |
| 10X transfer buffer | Add 144.2 g glycine and 30.3 g Tris to 900 ml ddH2O.  Mix until they are in solution. | RT |
| 1X transfer buffer | 100 ml 10X running buffer in 800 ml ddH2O. Add 100 ml methanol. pH should be 8.3 | RT |
| 1X reducing transfer buffer | 0.5 g DTT in 10 ml transfer buffer | n/a |
| 10X TBS | 24 g Tris, 88 g NaCl in 900 ml ddH2O.  pH to 7.6 with 12 mole hydrochloric acid.  Top up to 1 L with ddH2O. | RT |
| 1X TBST | 100 ml 10X TBS in 900 ml ddH2O. Add 1 ml Tween20. | RT |
| Blocking buffer | 1 ml 10X fluorescent blocking buffer in 9 ml ddH2O. | n/a |
| Antibody buffer | 1 ml Superblock in 9 ml 1X TBST | RT |

**Useful links**

**Mass calculator** [**https://www.sigmaaldrich.com/GB/en/support/calculators-and-apps/mass-molarity-calculator**](https://www.sigmaaldrich.com/GB/en/support/calculators-and-apps/mass-molarity-calculator)

**Solution dilution calculator** [**https://www.sigmaaldrich.com/GB/en/support/calculators-and-apps/solution-dilution-calculator**](https://www.sigmaaldrich.com/GB/en/support/calculators-and-apps/solution-dilution-calculator)

**2.Reagents**

**Table 2. List of reagents.**

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| **Procedure** | **Material/reagent** | **Manufacturer (country)** | **Catalogue #** |
| 2PB synthesis | SPDP-dPEG®36-NHS ester | Sigma (UK) | QBD10867 |
| 2PB synthesis | NH2-PEG11-Biotin | ThermoFisher (UK) | 26136 |
| 2PB synthesis | DMSO | Sigma (UK) | 276855 |
| 2PB synthesis | PBS tablets | Fisher Scientific (UK) | 10388739 |
| Cysteine labelling | IP-lysis buffer | ThermoFisher (UK) | 87787 |
| Cysteine labelling | NEM | ThermoFisher (UK) | 23030 |
| Cysteine labelling | Neutral-TCEP | Sigma (UK) | 580561 |
| Cysteine labelling | Protease Inhibitor tablet | Sigma (UK) | 11697498001 |
| Cysteine labelling | 6 kDa spin column | Bio-Rad (UK) | 7326222 |
| Cleland immunoblotting | 10 ml, premixed 4x Laemmli protein sample buffer for SDS-PAGE | Bio-Rad (UK) | 1610747 |
| Cleland immunoblotting | 4-15% precast gels | Bio-Rad (UK) | 4561085 |
| Cleland immunoblotting | All blue protein standard | Bio-Rad (UK) | 1610373 |
| Cleland immunoblotting | DTT | Sigma (UK) | D9779 |
| Cleland immunoblotting | PVDF membranes | Bio-Rad (UK) | 1620261 |
| Cleland immunoblotting | Blocker™ FL Fluorescent Blocking Buffer (10X) | Thermo (UK) | 37565 |
| Cleland immunoblotting | Cdc20 Monoclonal Antibody (BA8) | Thermo (UK) | MA1-46040 |
| Cleland immunoblotting | Goat Anti-Mouse IgG H&L (Alkaline Phosphatase) | Abcam (UK) | ab97020 |
| Streptavidin immunoblotting | Recombinant Streptavidin protein (Alkaline Phosphatase) | Abcam (UK) | ab279314 |
| General | Tween20® | Sigma (UK) | P9416 |
| General | Superblock™ | ThermoFisher (UK) | 37580 |
| General | Vector[R] Red Substrate Kit, Alkaline Phosphatase (AP**)** | 2Bscientific (UK) | SK-5100 |
| General | Glycine | Sigma (UK) | G7126 |
| General | Sodium chloride | Sigma (UK) | S9888 |
| General | SDS | Sigma (UK) | L3771 |
| General | PBS tablets | Fisher Scientific (UK) | 10388739 |

**3.Preparing 2PB**

1. Dissolve SPDP-dPEG®36-NHS ester (SPN, Sigma, QBD10867) in DMSO or an anhydrous solvent to make the stock solutions. *\*Omit ddH2O-it will hydrolyse the NHS group.*
2. Dissolve NH2-PEG11-Biotin (NPB, ThermoFisher, 26136) in 1X PBS to make a stock solution.
3. Mix NPB and SPN in a 3:1 ratio (i.e., 3-parts NPB to 1-part SPN) to make 50 mM or working reagent (this can be stored at minus 20).
4. Dispense SPN into the reaction vial\* and then add NPB before topping the solution up to the desired volume with 1 X PBS.
5. Seal the reaction vial and rapidly mix (vortex) for 5-20 seconds.
6. Allow the reaction to proceed for 1 h. Mix often (vortex every 5 min, and every 1 min during the first 15 min of the reaction).
7. Store 2PB at -20oC.
8. **Optional**: Quench any unreacted NHS with 0.5 mM Tris (pH 7.2)

\*Seal the SPN stock so that no moisture which will hydrolyse the NHS group spoils the compound.

SPN- <https://www.sigmaaldrich.com/GB/en/product/aldrich/qbd10867?gclid=EAIaIQobChMIkYS4n7-EggMVl-3tCh1h1gILEAAYASAAEgIKLvD_BwE>

NPB: <https://www.thermofisher.com/order/catalog/product/26136>

**4.Cysteine labelling**

1. Lyse the sample of interest in lysis buffer supplemented with 1-10 mM *N*-ethylmaleimide (NEM) or iodoacetamide (IDA). Ensure the pH of the lysis buffer is optimised for cysteine labelling. For example, pH 7-7.3 for NEM.
2. Centrifuge the sample (e.g., 14,000 *g* for 5 min at RT) to remove insoluble proteins.
3. Pass the soluble supernatant through a pre-equilibrated spin column, or equivalent, to remove unreacted NEM or IDA. *\*Do not exceed the volume capacity of the spin column.*
4. Determine the protein content of the eluent using an appropriate test, such as a Bradford assay.
5. Add 5 mM neutral-TCEP to the eluent (50 µg protein) and incubate for 30 min. For example, add 0.7 µl 500 mM TCEP to a 70 µl sample containing 50 µg protein.
6. Pass the soluble supernatant through a pre-equilibrated spin column, or equivalent, to remove unreacted TCEP. *\*TCEP must be removed or it will reduce 2PB.*
7. Add 5 mM 2PB and incubate for 90 min. For example, add 7 µl 50 mM 2PB to a 70 µl sample
8. Store the labelled samples at -20oC.

**Note:** Vortex the samples every 5-10 min during each incubation.

**5. Cleland immunoblotting**

1. Add an appropriate amount of 4X non-reducing loading buffer to each sample. \*Be sure to omit DTT or 2-metcarptoethanol as they will reduce 2PB.
2. Load the samples alongside a molecular weight marker onto a precast or hand-cast polyacrylamide gel.
3. Perform SDS-PAGE in 1X running buffer until the bromophenol blue dye front reaches the end of the gel.
4. Prepare the 1X transfer buffer.
5. Make 10 ml of reducing transfer buffer by adding 5% fresh DTT (Make this up just before the time of use). Add 0.5 g DTT. (It is easier to add the DTT powder to the falcon tube beforehand and then dispense the buffer in just before use).
6. Carefully excise the gel, rinse in ddH2O, and add the reducing transfer buffer.
7. Incubate for 15 min at RT with gentle agitation in a fume hood.
8. Activate the PVDF membrane in 100% methanol for 1-2 min before incubating it with transfer buffer for 15 min (do this just before step 5).
9. Assemble the transfer stack.
10. Electrically transfer the proteins from the gel to the PVDF membrane for 1 h at RT.
11. Carefully excise the PVDF membrane.
12. Add blocking buffer for at least 1 h at RT with gentle agitation.
13. Wash the blocked membrane once with 5% TBST for 5 min.
14. Incubate the blocked membrane with primary antibody solution overnight at RT or 4oC with gentle agitation.
15. Discard the antibody solution.
16. Wash the membrane (3 x 5 min in TBST).
17. Incubate the membrane with secondary antibody solution for 1 h at RT with gentle agitation.
18. Wash the membrane (3 x 5 min in TBST).
19. Detect target-specific oxiforms on a gel scanner using an appropriate technique, such as fluorescence or chemiluminescence.

**6. Data analysis**

1. Manually count the number of target-specific bands in each lane.
2. Assign a percentage cysteine redox state oxiform class specific each band (see note 1 &2).
3. Use appropriate software to obtain background subtracted densitometry values for each band.
4. Quantify the percentage abundance of each oxiform class using equation 1.
5. Quantify the cysteine redox state of the target using equation 2.
6. Statistically analyse the data using appropriate software.
7. Visualise the data using an appropriate software.

**Equation 1**: Abundance of each oxiform class in percentages = (class band value/ total)\*100

*Worked example = 50 AU of 20% oxiform class/ 100 AU of total = 50% of the protein is in the 20% oxidised form.*

**Equation 2**: %Reduced = sum of n %band (v%red). Where n is the number of bands containing a reduced form of the protein and v%red is the percentage amount of n in that band. (r = sum n%(V%).

Worked example = weighted mean of a protein with 2 cysteines whereby the percentage abundance the redox state classes is 100% reduced = 70%, 50% = 30%, and 0%reduced = 0%. To calculate it, we must divide 30% by 2= 15%. And then add 15% to 70% = 85%. The protein is 85% reduced. Or 15% oxidised.

**Note 1**: The percentage cysteine redox sate state class assigned depends on the number of cysteines the target possesses. For example, for a protein with 10 cysteines would produce 11 bands. In this case, band 2 would represent the 10% oxidised form.

**Note 2**: In some cases, there may not be 11 bands, and hence the cysteine redox state must be assigned on the basis of a predicted mobility shift based on ~3 kDa mass shift per oxidised cysteine. The molecular mass of the target and the percentage gel used with influence the degree of the observed mobility shift.