# Characterisation of Mitochondrial Proteome Changes during SARS-CoV-2 ORF9b Expression by Rapid Immunopurification

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## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the pathogen that caused the **COVID-19 pandemic**[1] and has led to over 4.5 million deaths worldwide[2]. SARS-CoV-2 carries a single-stranded, positive-sense, and non-segmented RNA genome[3].


Fig. 1. SARS-CoV-2 genome (29.9 kbp).
Adapted from "Genetic and pathogenic characterization of SARS-CoV-2: a review," by A. Bahrami and G.A. Ferns, 2020, *Future Virology, 15*(8), 533-549.

**SARS-CoV-2 infection can trigger mitochondrial dysfunction through the expression of accessory viral protein ORF9b**, which is encoded by the second open reading frame of the nucleocapsid gene[4], thus impairing host antiviral innate immunity. Studies have also shown that SARS-CoV-2 ORF9b perturbs mitochondrial function through binding to and inhibiting TOM70[4]. However, the **complete molecular mechanism underlying SARS-CoV-2 ORF9b mediated mitochondrial dysfunction is yet to be deciphered**. Through unveiling the overall mitochondrial proteome changes during SARS-CoV-2 ORF9b expression, not only can we gain a better understanding of the intricate molecular virus-host interaction at mitochondria during SARS-CoV-2 infection, potential therapeutic drug targets for COVID-19 or related infectious diseases can also be identified.

## METHODOLOGY

### Optimisation of Rapid IP

**Cell Seeding**
Seed 10mL of HEK 293T cells on each 10-cm plate at 4×10^5/mL
**Transfection**
Transfect cells with DNA3.1+ ORF9b using GeneJuice for 24hr
**Rapid IP**
Refer to suggested protocol[5]
Amendment:
Homogenise cells with 10 strokes using a 25-gauge needle

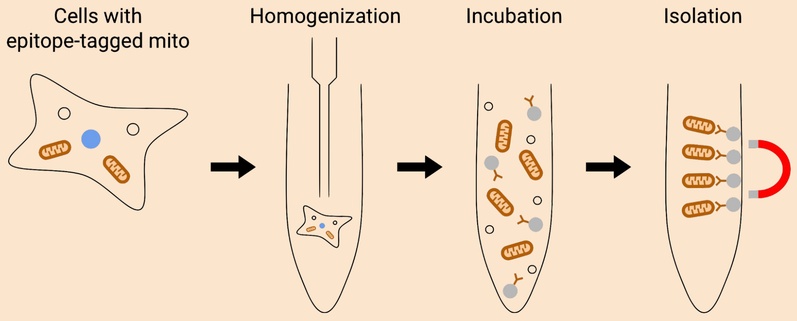


Fig. 2. Schematic diagram to illustrate the principle of rapid IP. Mitochondria can be isolated in 12 min with high organelle integrity and purity[6,7,8,9]. Isolated fractions are also suitable for LC-MS/MS analysis due to low salt concentration in isolation buffer[10,11,12].

### LC-MS/MS (Bruker timsTOF Pro)

**Screening Criteria**
1. At least 2 unique peptides
2. Final fold change >1.5 or <0.667
3. Target group (OMP25-HA) has a greater fold change than the control group (OMP25-myc)
4. Detected in both trials

### MitoProbe JC-1 Assay

**Cell Seeding**
Seed 1mL of HEK 293T cells on each 12-well plate at 1×10^5/mL
**MitoProbe JC-1 Assay**
Refer to suggested protocol[13]
Amendment:
Monomer Ex/Em: 485/535nm; Aggregate Ex/Em: 550/600nm

## RESULTS & DISCUSSION

### Optimisation of Rapid IP

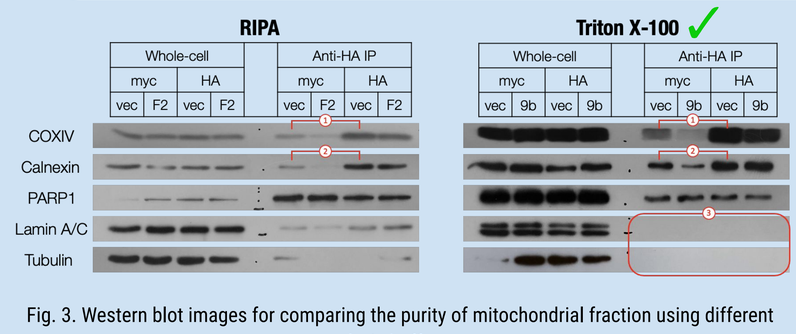


Fig. 3. Western blot images for comparing the purity of mitochondrial fraction using different lysis buffers.

**Triton X-100 is preferred over RIPA due to greater mitochondrial enrichment (see ①), less ER enrichment (see ②), and effective removal of nuclear and cytosolic fraction (see ③).**

Fig. 4. Western blot images for comparing the purity of mitochondrial fraction using different no. of times of beads washing by KPBS after the binding of mitochondria to beads.

**Washing beads more increases mitochondrial enrichment (see ①). Expression of TOM20 is enriched upon ORF9b expression (see ②). May be triggered to counterbalance the reduction in TOM70 during ORF9b expression to maintain mitochondrial import efficiency.**

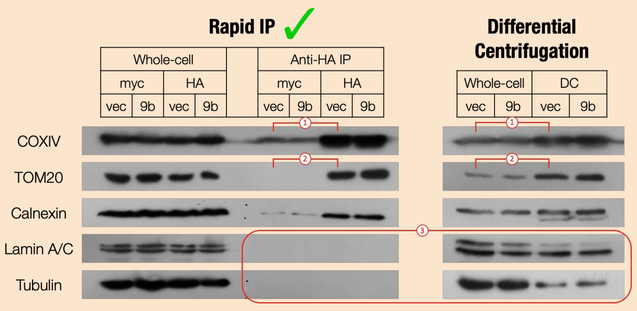


Fig. 5. Western blot images for comparing the purity of mitochondrial fraction obtained from rapid IP and differential centrifugation.

**Rapid IP is a more superior method due to greater mitochondrial enrichment (see ①&②) and effective removal of nuclear and cytosolic fraction (see ③).**

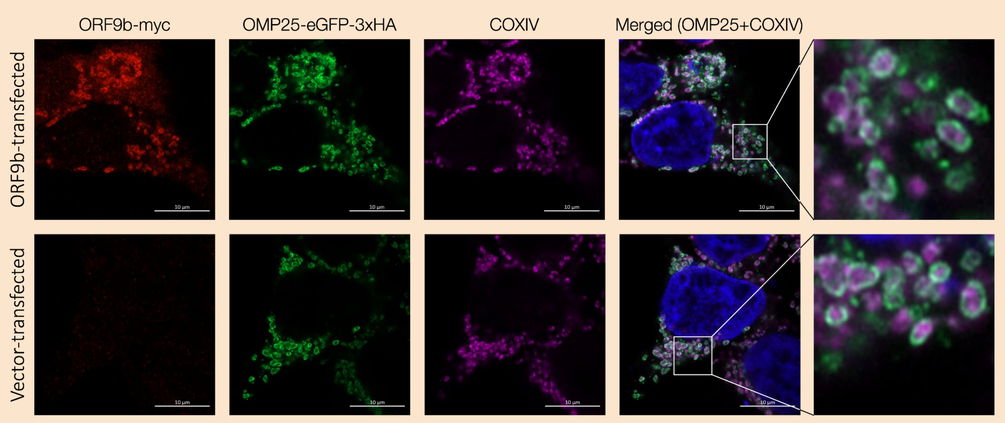


Fig. 6. Confocal microscopy (Airyscan) images of HEK 293T cells expressing OMP25-eGFP-3xHA transfected by ORF9b or vector for 24h. 1°Ab: mouse anti-myc (1:100); rabbit anti-COXIV (1:100). 2°Ab: 555nm anti-mouse (1:200); 633nm anti-rabbit (1:200); DAPI (1:1000).

**(1) Mitochondria remain intact during expression of OMP25-eGFP-3xHA**
**(2) ORF9b did not distort OMP25 localization on OMM**
**⇒ Rapid IP can be applied on HEK 293T cells expressing both OMP25-eGFP-3xHA and ORF9b.**

### LC-MS/MS

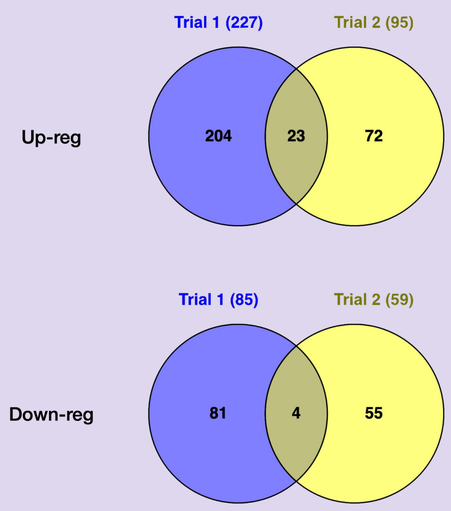


Fig. 7. Venn diagrams of number of differentially expressed mitochondrial proteins in HEK 293T cells after 24-hr transfection with ORF9b.

| Up-reg | | | Down-reg |
|---|---|---|---|
| VCP | **IDH2** | ERP44 | MRPL9 |
| MTERF3 | RRBP1 | ALDH5A1 | TMX3 |
| VWA8 | ELAC2 | PARK7 | LMNA |
| RAB21 | MICU1 | **NUBPL** | NONO |
| **COA1** | **PDPR** | **OGDH** | |
| MTO1 | NT5DC3 | PPOX | |
| FAR1 | SARM1 | MUT | |
| ACOT9 | ALDH9A1 | | |

Table 1. List of differentially expressed mitochondrial proteins detected in both LC-MS/MS trials. Proteins that regulate the TCA cycle and ETC are shown in red colour.

**Five proteins that involve in the citric acid cycle and respiratory electron transport were enriched during ORF9b expression.**
**Proposed mechanism: ORF9b dysregulates mitochondria by promoting the production of mitochondrial reactive oxygen species (mtROS)**

### MitoProbe JC-1 Assay

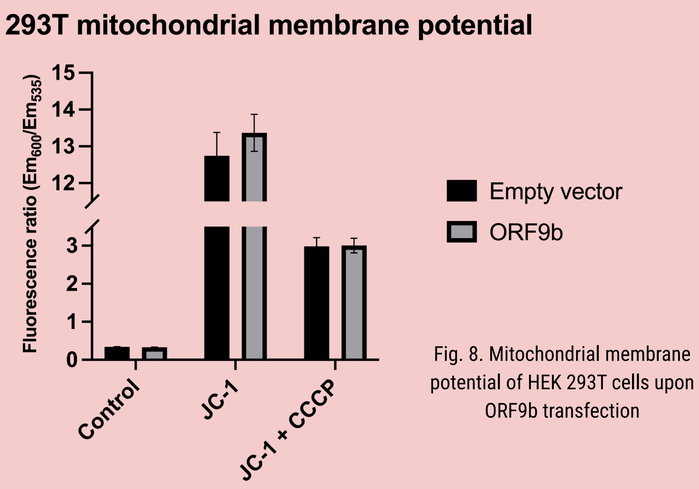


Fig. 8. Mitochondrial membrane potential of HEK 293T cells upon ORF9b transfection

**ORF9b increased mitochondrial membrane potential by 4.9%**
**⇒ ORF9b increased mtROS production[14].**

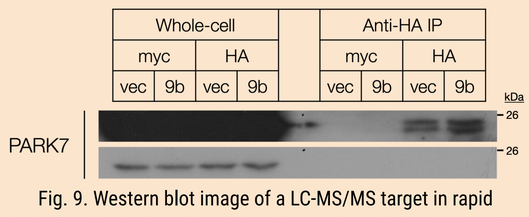


Fig. 9. Western blot image of a LC-MS/MS target in rapid IP-isolated mitochondria of ORF9b-transfected HEK 293T cells.

**Known that ROS simulates mitochondrial translocation of Parkin proteins[15]. Matches with WB results.**

## CONCLUSION

ORF9b may induce mitochondrial dysfunction by the production of mtROS. This provides insights into the treatment of COVID-19 and related diseases using therapeutic drugs that target mtROS.

### Acknowledgement

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