SI21: ORF9b

Table 1: General Information

1	**Protein Name**
	ORF9b
2	**Region/Name/Further Specification**
3	**Sequence of fl protein**
	MDPKISEMHP ALRLVDPQIQ LAVTRMENAV GRDQNNVGPK VYPIILRLGS PLSLNMARKT LNSLEDKAFQ LTPIAVQMTK LATTEELPDE FVVVTVK
4	**Protein boundaries of expressed construct**
	aa 1-97 (fl ORF9b)
5	**Ratio for construct design**
	fl protein
6	**Sequence homology (to SCoV)**
	Identity : 72.4%; similarity: 95.0%
7	**Published structures (SCoV2 or homologue variants)**
	SCoV2: PDB 6Z4U
8	**(Published) assignment (SCoV2 or homologue variants)**
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Table 2: Cell-free Protein Synthesis

1	**Expression vector**
	pEU-E01-MCS (Cell-Free Sciences)
2	**Purification-/Solubility-Tag**
	C-terminal Strep tag II (WSHPQFEK)
3	**Cleavage Site**
	-
4	**Molecular weight / Extinction coefficient / pI - of protein**
	11.99 kDa / 6,990 M^{-1} cm^{-1} / 6.73
5	**Comments on sequence of expressed construct**
	C-terminal “SAWSHPQFEK” ten artificial residues due to construct design.
6	**Feeding buffer**

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	30 mM HEPES-KOH (pH 7.6), 100 mM potassium acetate, 2.7 mM magnesium acetate, 16 mM creatine phosphate, 0.4 mM spermidine, 1.2 mM ATP, 0.25 mM GTP, 4 mM DTT and 6 mM (average concentration) amino acid mix
7	**Translation mix**
	50% (v/v) mRNA, 50% (v/v) home-made WGE, 40 μg/mL creatine kinase, and 6 mM (average concentration) amino acid mix
8	**Protein synthesis temperature and time**
	22°C for 16 h without agitation (bilayer method).

Table 3: Protein Purification

1	**Buffer List**
A	100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA.
B	100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin.
2	**Purification steps (with corresponding buffer(s) and incubation times)**
A	Harvest total CFS.
B	Incubate with benzonase for 30 min on a wheel, at rt.
C	Centrifuge for 30 min at 20,000 g, 4°C.
D	Harvest the soluble fraction (SN).
E	Equilibrate the Strep-Tactin column (IBA Lifesciences) with 2 CV of **1A** (all steps performed on the bench by gravity).
F	Load SN onto the column.
G	Wash the column with 5 CV of **1A**.
H	Elute the protein of interest with **1B**.

Table 4: Final sample

1	**Yield**
	0.64 mg/ml WGE and total production of 1338 μg for NMR samples.
1b	**A260/280 ratio**
	0.76
2	**Stability**
	Stable at 4ºC for a week.
3	**Comment on applicability**
	Protein studied at pH 6, 7.5 and pH 8. Methionine gets oxidized without DTT in the buffer.

Additional information

	Constructs	Conditions	Comments
A	Fl ORF9b; Strep tag II (pEU-E01-MCS (Cell-Free Sciences)); no cleavage site; C-terminal "WSHPQFEK" eight artificial residues.	As above with 0,1% (w/v) DDM	NMR shows severely broadened resonances due to oligomerization or protein micelles.
B		As above without DTT	Methionines get oxidated.

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