

## Cryo-EM data collection, refinement and validation statistics

|                                                     |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
|-----------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|
|                                                     | Cas9:sgRNA:DNA<br>( <i>S. pyogenes</i> ) with 0<br>RNA:DNA base<br>pairs, open-<br>protein/linear-DNA<br>conformation<br>(EMDB-24823)<br>(PDB 7S3H) | Cas9:sgRNA:DNA<br>( <i>S. pyogenes</i> ) with<br>0 RNA:DNA base<br>pairs, closed-<br>protein/bent-DNA<br>conformation,<br>DNA<br>conformation 1<br>(EMDB-24817)<br>(PDB 7S36) | Cas9:sgRNA:DNA<br>( <i>S. pyogenes</i> ) with<br>0 RNA:DNA base<br>pairs, closed-<br>protein/bent-DNA<br>conformation,<br>DNA<br>conformation 2<br>(EMDB-24820)<br>(PDB 7S39) | Cas9:sgRNA<br>( <i>S. pyogenes</i> )<br>in the open-<br>protein<br>conformation<br>(EMDB-<br>24818)<br>(PDB 7S37) | Cas9:sgRNA:DNA<br>( <i>S. pyogenes</i> )<br>forming a 3-base-<br>pair R-loop<br>(EMD-24819)<br>(PDB 7S38) |
| <b>Data collection and processing</b>               |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Magnification                                       | x81,000                                                                                                                                             | x81,000                                                                                                                                                                       | x81,000                                                                                                                                                                       | x36,000                                                                                                           | x81,000                                                                                                   |
| Voltage (kV)                                        | 300                                                                                                                                                 | 300                                                                                                                                                                           | 300                                                                                                                                                                           | 200                                                                                                               | 300                                                                                                       |
| Electron exposure (e <sup>-</sup> /Å <sup>2</sup> ) | 50                                                                                                                                                  | 50                                                                                                                                                                            | 50                                                                                                                                                                            | 50                                                                                                                | 50                                                                                                        |
| Defocus range (μm)                                  | -0.8 to -2                                                                                                                                          | -0.8 to -2                                                                                                                                                                    | -0.8 to -2                                                                                                                                                                    | -0.8 to -2                                                                                                        | -0.8 to -2                                                                                                |
| Pixel size (Å)                                      | 1.05 (nom.) / 0.525<br>(super-res.)                                                                                                                 | 1.05 (nom.) / 0.525<br>(super-res.)                                                                                                                                           | 1.05 (nom.) / 0.525<br>(super-res.)                                                                                                                                           | 1.115 (nom.) / 0.5575<br>(super-res.)                                                                             | 1.05 (nom.) / 0.525<br>(super-res.)                                                                       |
| Symmetry imposed                                    | C1                                                                                                                                                  | C1                                                                                                                                                                            | C1                                                                                                                                                                            | C1                                                                                                                | C1                                                                                                        |
| Initial particle images<br>(no.)                    | 8,386,564                                                                                                                                           | 8,386,564                                                                                                                                                                     | 8,386,564                                                                                                                                                                     | 1,594,370                                                                                                         | 5,006,233                                                                                                 |
| Final particle images<br>(no.)                      | 545,450                                                                                                                                             | 189,963                                                                                                                                                                       | 189,963                                                                                                                                                                       | 87,405                                                                                                            | 134,301                                                                                                   |
| Map resolution (Å)                                  | 2.5                                                                                                                                                 | 2.8                                                                                                                                                                           | 2.8                                                                                                                                                                           | 3.3                                                                                                               | 2.8                                                                                                       |
| FSC threshold                                       | 0.143                                                                                                                                               | 0.143                                                                                                                                                                         | 0.143                                                                                                                                                                         | 0.143                                                                                                             | 0.143                                                                                                     |
| Map resolution range<br>(Å)                         |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| <b>Refinement</b>                                   |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Initial model used (PDB<br>code)                    | 4ZT0, 5FQ5, 5F9R,<br>4CMP                                                                                                                           | 4ZT0, 5FQ5, 5F9R                                                                                                                                                              | 4ZT0, 5FQ5, 5F9R                                                                                                                                                              | 4ZT0, 5F9R,<br>4CMP                                                                                               | 4ZT0, 5FQ5, 5F9R                                                                                          |
| Model resolution (Å)                                | 2.9                                                                                                                                                 | 2.7                                                                                                                                                                           | 2.7                                                                                                                                                                           | 3.9                                                                                                               | 2.8                                                                                                       |
| FSC threshold                                       | 0.5                                                                                                                                                 | 0.5                                                                                                                                                                           | 0.5                                                                                                                                                                           | 0.5                                                                                                               | 0.5                                                                                                       |
| Model resolution range<br>(Å)                       |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Map sharpening <i>B</i> factor<br>(Å <sup>2</sup> ) |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Model composition                                   |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Non-hydrogen atoms                                  | 9,985                                                                                                                                               | 13,885                                                                                                                                                                        | 13,885                                                                                                                                                                        | 9,035                                                                                                             | 13,940                                                                                                    |
| Protein residues                                    | 1,034                                                                                                                                               | 1,358                                                                                                                                                                         | 1,358                                                                                                                                                                         | 1,026                                                                                                             | 1,360                                                                                                     |
| Nucleotides                                         | 76                                                                                                                                                  | 133                                                                                                                                                                           | 133                                                                                                                                                                           | 32                                                                                                                | 135                                                                                                       |
| <i>B</i> factors (Å <sup>2</sup> )                  |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Protein                                             | 54.44                                                                                                                                               | 71.86                                                                                                                                                                         | 71.08                                                                                                                                                                         | 100.33                                                                                                            | 86.34                                                                                                     |
| Nucleotide                                          | 70.84                                                                                                                                               | 73.37                                                                                                                                                                         | 77.80                                                                                                                                                                         | 101.75                                                                                                            | 73.92                                                                                                     |
| R.m.s. deviations                                   |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Bond lengths (Å)                                    | 0.003                                                                                                                                               | 0.004                                                                                                                                                                         | 0.004                                                                                                                                                                         | 0.004                                                                                                             | 0.004                                                                                                     |
| Bond angles (°)                                     | 0.772                                                                                                                                               | 0.788                                                                                                                                                                         | 0.793                                                                                                                                                                         | 0.851                                                                                                             | 0.790                                                                                                     |
| Validation                                          |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| MolProbity score                                    | 1.60                                                                                                                                                | 1.62                                                                                                                                                                          | 1.63                                                                                                                                                                          | 2.12                                                                                                              | 1.44                                                                                                      |
| Clashscore                                          | 8.03                                                                                                                                                | 7.03                                                                                                                                                                          | 6.84                                                                                                                                                                          | 12.39                                                                                                             | 7.41                                                                                                      |
| Poor rotamers (%)                                   | 0.98                                                                                                                                                | 0.57                                                                                                                                                                          | 0.57                                                                                                                                                                          | 1.86                                                                                                              | 0.41                                                                                                      |
| Ramachandran plot                                   |                                                                                                                                                     |                                                                                                                                                                               |                                                                                                                                                                               |                                                                                                                   |                                                                                                           |
| Favored (%)                                         | 97.08                                                                                                                                               | 96.45                                                                                                                                                                         | 96.23                                                                                                                                                                         | 95.58                                                                                                             | 97.86                                                                                                     |
| Allowed (%)                                         | 2.92                                                                                                                                                | 3.55                                                                                                                                                                          | 3.77                                                                                                                                                                          | 4.42                                                                                                              | 2.14                                                                                                      |
| Disallowed (%)                                      | 0                                                                                                                                                   | 0                                                                                                                                                                             | 0                                                                                                                                                                             | 0                                                                                                                 | 0                                                                                                         |