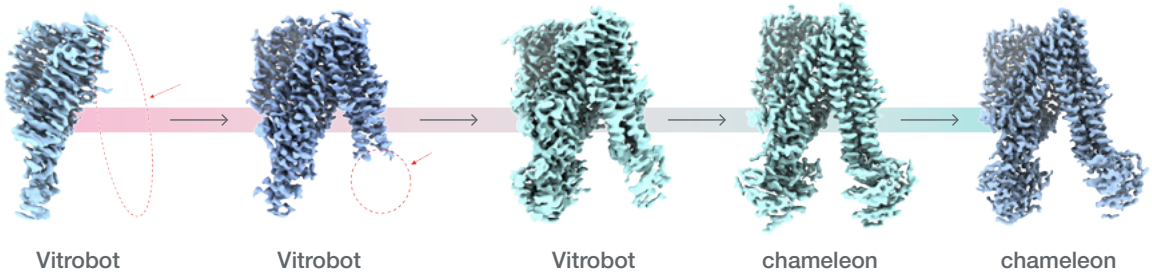
A Multi-Pronged Approach to Overcoming Challenges in CryoEM Sample Preparation

Summary

Cryo-electron microscopy (cryoEM) has become a core technology for resolving three-dimensional structures of biological macromolecules at near atomic resolution, particularly for membrane proteins. Sample preparation is a critical step that directly affects ice thickness, particle distribution, structural integrity, and final resolution. Suboptimal preparation may cause protein domain dissociation, particle aggregation, preferential orientation, or air-water interface (AWI)-induced denaturation[1].

In this case study, we demonstrate how we combined protein engineering and advanced cryoEM sample preparation using chameleon® to iteratively overcome challenges encountered in structural determination of an ABC Transporter ABCC2. The case study highlights the advantage of Biortus as a full-service gene-to-structure company, capable of offering solutions at various stages of the structural enablement process.



Background Information

ATP-Binding Cassette (ABC) transporters are a large superfamily of proteins found in all forms of life. They are molecular machines that use the energy from ATP hydrolysis to transport a wide variety of substrates across cellular membranes. ABCC2 (ATP-Binding Cassette Subfamily C Member 2) is a crucial transmembrane transporter widely distributed on cell membranes. It participates in key physiological processes such as drug metabolism, detoxification, and substance transport.

ABCC2 has the typical architecture of ABC transporters (Figure 1), consisting of two core functional domains:

- Transmembrane Domains (TMDs): These domains, typically composed of 6-12 transmembrane alpha-helices that span the lipid membrane, form the pathway for the substrate. They recognize and bind the specific molecule to be transported.
- Nucleotide-Binding Domains (NBDs): These domains bind and hydrolyze ATP. The energy released from this reaction drives conformational changes in the TMDs, powering the movement of the substrate across the membrane by alternating the transporter between inward-facing and outward-facing conformation.

The structural integrity of both NBDs is essential for maintaining protein function. Resolving the high-resolution structure of ABCC2 not only elucidates its mechanistic principles but also holds significant implications for drug development and disease research.

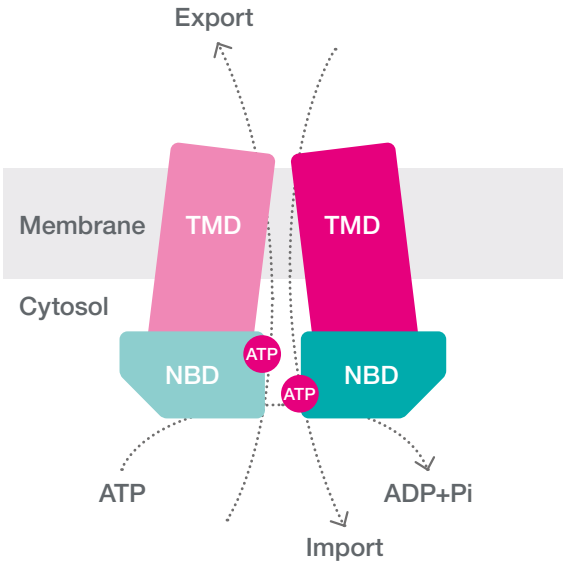


Figure 1. Typical architecture of ABC transporters.

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Wild Type ABCC2 is prone to denaturation during cryoEM sample preparation

We prepared cryoEM grids with the traditional blotting-based approach using the Vitrobot for the ABCC2 wild-type (WT) sample and a total of 5,712 movies were acquired. Following processing in cryoSPARC[2] and subsequent 2D classification, 317,798 particles were ultimately selected for further analysis. However, the 2D classification revealed that the vast majority of particles exhibited significant preferential orientation (Figure 2A). More critically, the subset of particles containing intact dual-NBD structures represented an exceptionally small proportion of the total.

After Local Refinement, the ABCC2 WT structure reached an overall nominal resolution of approximately 3.3 Å. The density map revealed that the transmembrane domains (TMDs) were resolved to a higher local resolution of about 3.0 Å, with clear structural details. However, the reconstructed map unequivocally lacked density for one of the NBDs (Figure 2B), preventing the determination of a complete, full-length ABCC2 structure. This significant limitation substantially constrained subsequent structural biological interpretation of ABCC2's functional mechanism.

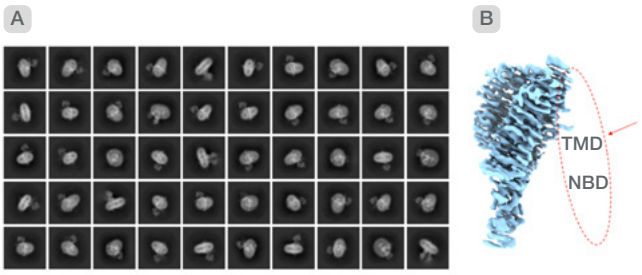

Figure 2. ABCC2 WT samples prepared using Vitrobot. (A) 2D classification and (B) the resulting density map of ABCC2 WT at 3.3 Å resolution. The red arrow indicates the position of the missing TMD and NBD domain, highlighting the structural incompleteness.

Structure Guided Mutagenesis Improves Sample Robustness

We reasoned that improving protein stability might improve sample robustness against structural damage commonly suffered at the air-water-interface. Based on information available in the literature[3], a specific amino acid substitution on ABCC2 was created and we prepared cryoEM samples of the mutant protein.

For the ABCC2 mutant sample, we again prepared cryoEM samples using the Vitrobot and data collection was scaled up to 7,986 movies. Following initial processing and two-dimensional classification, 965,624 particles were obtained. Despite this substantial increase in data volume, the 2D classification results demonstrated that particle integrity remained a significant challenge: only approximately 20% of the particles exhibited intact dual-NBD structures, while the remaining ~80% lacked one of the NBD domains (Figure 3). This widespread absence of structural domains suggests that during the conventional filter-paper blotting process of the Vitrobot, the protein structure likely suffered damage due to mechanical shear forces or air-water interface (AWI) effects.

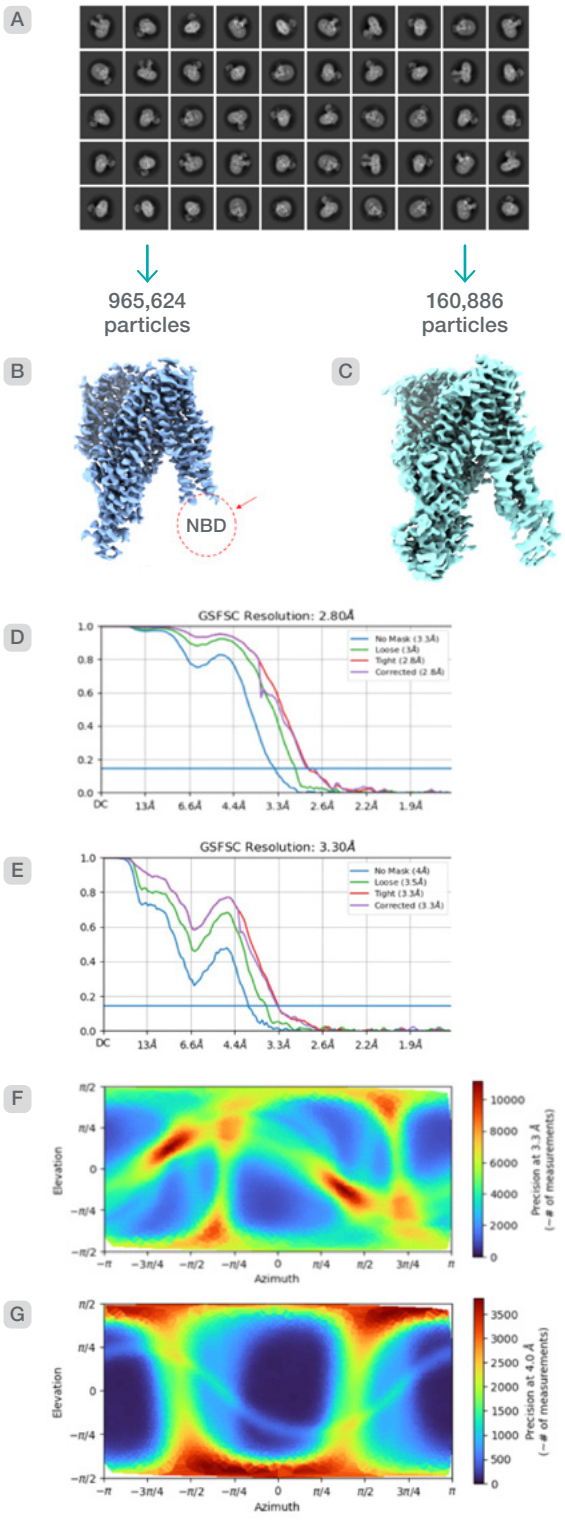

Figure 3. ABCC2 mutant sample prepared using Vitrobot. (A) 2D classification (B) 2.8 Å density map derived from the majority of the picked particles. One of the NBDs is missing (red arrow). (C) A minority of the protein particles with less AWI damage resulted in a 3.3 Å map with improved density of the NBD domain (D, E) Corresponding FSC curve and (F, G) angular distribution heatmap of particles indicates significant degree of preferential orientation in viewing angles.

chameleon blotless sample preparation preserves structural integrity of the nucleotide binding domain

To mitigate the challenges with traditional blotting-based approach to sample preparation, we used the chameleon system for sample preparation. chameleon employs an ultrafast vitrification and "blot-free" sample preparation approach. It utilizes self-wicking grids coated with nanowires on the grid bars to spontaneously adsorb excess sample deposited on grid, therefore completely avoids physical contact and disturbance from filter paper blotting. Furthermore, its nanolitre piezoelectric sample dispensing technology reduces sample consumption and shortens the entire process from sample deposition to plunge freezing to the millisecond scale (fastest 54 ms). This ultrafast freezing minimizes exposure of the protein to the air-water-interface and instantaneously trap the protein in vitreous ice before dissociation or denaturation can occur.

We initially prepared samples of ABCC2 mutant on chameleon using the same sample concentration as used on the Vitrobot (3.14 mg/mL). To reduce air-water-interface effects, the grid was frozen on chameleon with a plunge time of 400 ms. A total of 3,129 movies were acquired. Following the same processing in cryoSPARC2 and two-dimensional classification as before, 62,577 particles were selected from this relatively modest dataset. The 2D classification results demonstrated exceptional particle integrity, with nearly all particles clearly exhibiting intact dual-NBD structural features (Figure 4A). This indicates that the "blot-free" preparation method and millisecond freezing speed of the chameleon system effectively preserved the structural integrity of the ABCC2 protein.

Three-dimensional reconstruction and local refinement using the selected particles yielded an overall resolution of 2.94 Å (Figure 4C). More importantly, the density map exhibited uniform quality with continuous and well-defined density for both the TMD and dual-NBD domains, demonstrating high resolution consistency (Figure 4B). The angular distribution of particles also showed improved coverage compared to Vitrobot-prepared samples (Figure 4D).

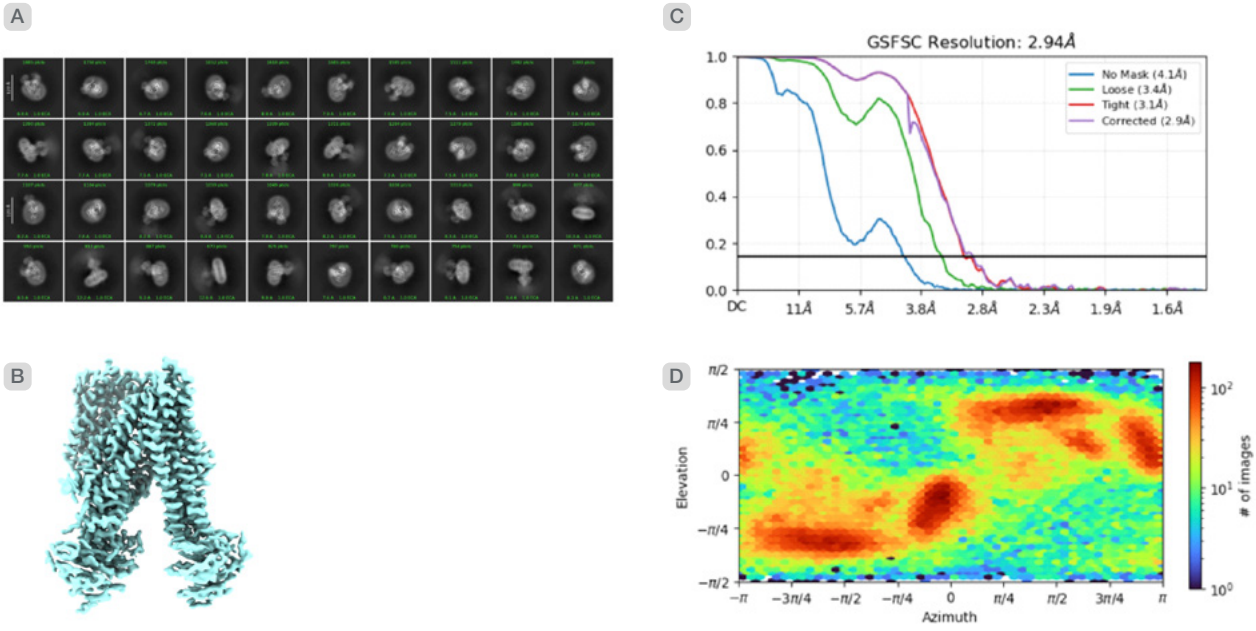

Figure 4. ABCC2 sample (3.14 mg/mL) plunged at 400 ms by chameleon. (A) All 2D classes clearly show intact dual-NBD structure. (B) 3D reconstruction density map at 2.94 Å resolution, showing the complete and well-defined dual-NBD structure. (C) Corresponding FSC curve. (D) Angular distribution heatmap of particles, indicating improved particle orientation distribution.

Increased sample concentration and shorter plunge time further improved grid quality

With the aim of getting high particle density on the micrographs to collect a larger dataset, we prepared cryoEM grids again using higher sample concentration on the chameleon (5.5 mg/mL). To further reduce air-water-interface effects, we further reduced the plunge time to 200 ms. A total of 8,152 movies were collected. Following processing and two-dimensional classification, a substantially increased number of 749,051 high-quality particles were ultimately obtained. The 2D classification results once again confirmed that the proportion of intact particles remained at 100% (Figure 5A). Furthermore, the particles exhibited more uniform distribution within the ice layer, and the projection angles displayed in the 2D class averages were more diverse (Figure 5A), suggesting a likely improvement in orientational distribution due to shortened plunge time. The homogeneity of particles was significantly superior to that achieved with Vitrobot-prepared samples.

Reconstruction utilizing the large number of high-quality particles obtained in this data collection resulted in significantly enhanced global resolution, reaching 2.51 Å as validated by GSFSC (Figure 5C). As a testimony to the quality of the particle set, fewer particles from the chameleon prepared grid are necessary to achieve a higher resolution reconstruction compared to the Vitrobot prepared grid (Figure 6). In this high-resolution density map, not only was the protein backbone trace clearly discernible, but many side-chain densities could also be distinguished (Figure 6). This result fully demonstrates the considerable potential of chameleon technology for high-resolution structural determination of complex membrane proteins such as ABCC2. The excellent particle orientation distribution (Figure 5D) provided a solid foundation for high-resolution reconstruction.

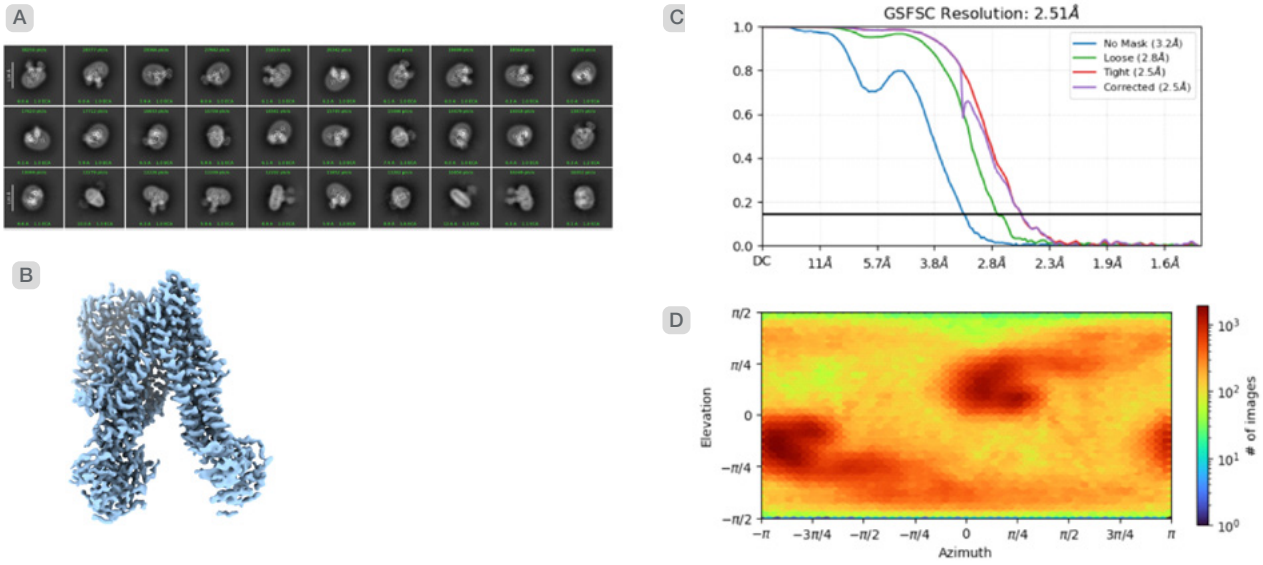

Figure 5. ABCC2 sample (5.5 mg/mL) plunged at 200 ms by chameleon. (A) 2D classification and (B) 3D reconstruction density map at 2.51 Å resolution. (C) Corresponding FSC curve. (D) Angular distribution heatmap of particles, demonstrating optimal particle orientation distribution.

Freezing Instrument	Vitrobot	Vitrobot	chameleon	chameleon
Plunge Time (ms)	n/a	n/a	400	200
Particles in Final Reconstruction	965,624	160,886	62,577	749,051
GSFSC Resolution (Å)	2.80	3.30	2.94	2.51

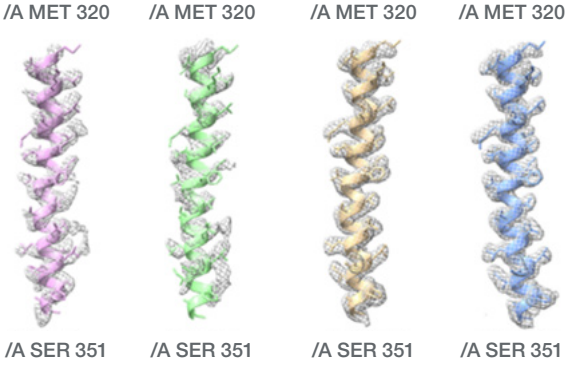

Figure 6. Improvement in density map quality of ABCC2 mutant through iterative improvements in cryoEM sample preparations.

Conclusion

Through a combination of expertise in protein engineering and advanced sample preparation technique using chameleon, we undertook iterative steps to improve the protein sample and the resulting 3D reconstructions of ABCC2, ultimately delivering the complete structure at 2.51 Å resolution. This showcases the strength of Biortus as a full-service gene-to-structure company, capable of providing solutions to tackle challenges encountered at different stages of the structural enablement process.

About Biortus

Biortus is a leading world-class contract research organization that provides a one-stop gene-to-structure service. Since 2009, Biortus has partnered worldwide with numerous research institutions, pharmaceutical and biotechnology companies to consistently provide high-quality custom proteins for all stages of biomedical research.

Biortus's core capabilities include target protein production, biochemical / biophysical / cellular assay development, compound screening and characterization, X-ray crystallography, cryo-EM for protein structure determination (SPA), and MicroED for small molecule crystal form and structure characterization. Our service is highly recognized by clients with co-authorship in more than 10 publications in prestigious journals including Science, Cell, Cell Research, Nature Communications, and JACS. Our exceptional technical capabilities and reliable service quality have garnered trust and established long-term partnerships with numerous multinational pharmaceutical companies (MNCs).

References

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"At Biortus, we have leveraged the advantages of SPT Labtech's chameleon to address cryoEM's notorious bottlenecks, such as preferred orientation and air–water interface-induced denaturation in single particle analysis (SPA), for challenging drug targets. In this case study, chameleon's blot-free and ultrafast vitrification enabled successful determination of the complete structure of ABCC2, while Vitrobot had failed, albeit with extensive efforts. As a CRO specializing in structural biology, we look forward to strengthening our strategic collaboration with SPT Labtech to further accelerate our clients' drug discovery."

Hui Shi, Head (Senior director) of cryoEM department, Biortus.

"SPT Labtech is very proud to be part of a collaboration with a progressive and increasingly globally recognised company such as Biortus. To see this already helping to overcome some of the bottlenecks in their workflow and provide valuable data is very encouraging for the future of us both in helping to speed up drug discovery workflows."

Paul Thaw, Head of Structural Sciences, SPT Labtech.

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