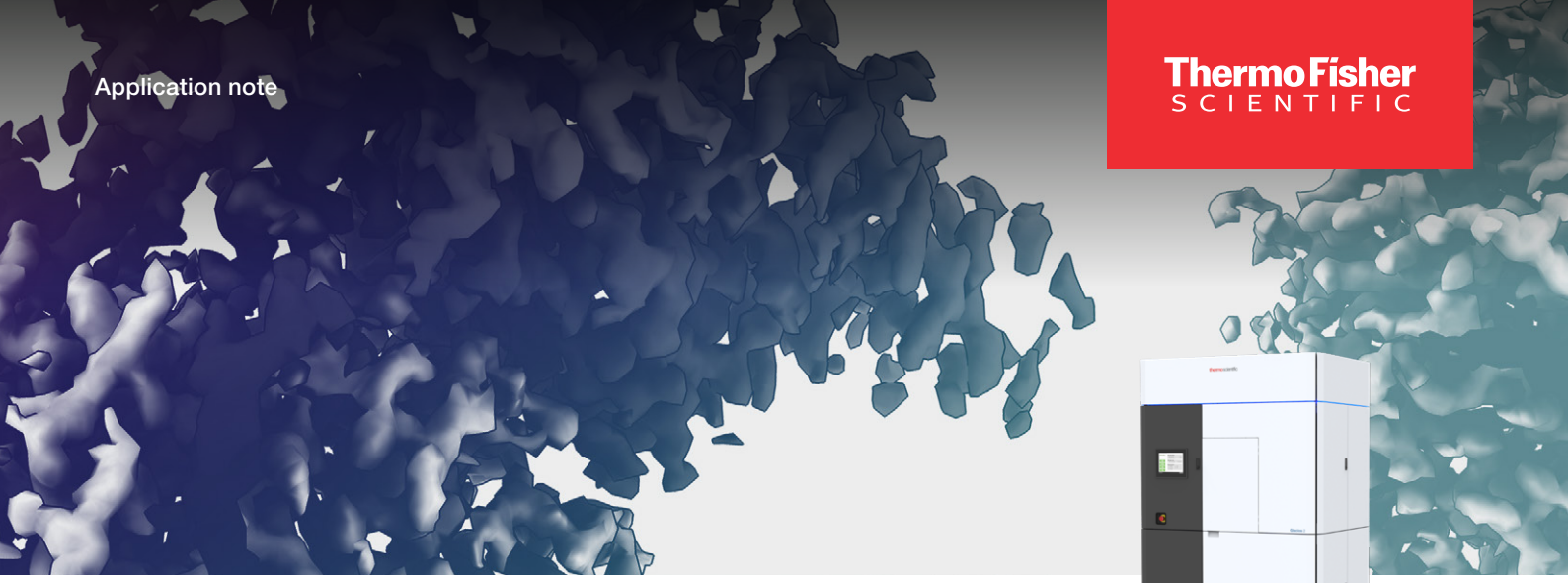
Application note



# Powering drug discovery with one structure per hour using cryo-EM

## Introduction

Cryo-electron microscopy (cryo-EM) techniques are an essential tool for the structural determination of proteins and macromolecules. Recent technological advances leading to improved throughput and resolution have revolutionized the potential applications of cryo-EM in drug discovery.

Rational design of next-generation therapeutics benefits greatly from high-resolution, direct observation of drug targets bound to their small-molecule inhibitors. This structure-based approach to drug design can result in increased specificity, leading to more effective drugs with reduced side effects.

With ongoing advancements in hardware and software, cryo-EM has continued to push the limits of structure-based analysis for macromolecules, particularly those that have been difficult to crystallize and characterize with traditional structural methods. Cryo-EM techniques such as single particle analysis (SPA) have been tailored to the structural analysis of proteins and small molecules, enabling the visualization of drug-target complexes at high resolution, as well as the interactions of small molecules or large macromolecular assemblies. Molecular structures determined with SPA offer near-atomic-level insights for drug development, guiding compound design from hit to lead and beyond. Single particle cryo-EM is particularly well suited for identifying biologics structures and providing critical information for multiple other drug discovery applications such as therapeutic antibody epitope mapping and the structural analysis of increasingly larger biopharmaceuticals.

Cryo-EM is meeting the rising pressure to reduce the time-to-market for new drugs by providing novel insights into structure-activity relationships. This can reduce the number of compounds that must be synthesized and assayed, thus enabling the identification of highly efficient drug candidates in fewer iterations.

One of the continued challenges for novel therapeutics is specificity. With structural characterization, inhibitor selectivity can be determined through high-resolution information from protein-small-molecule complexes. This requires high-throughput analysis of small (<100 kDa) and often asymmetric complexes, which have traditionally been challenging for cryo-EM. In this application note, a method for rapid, high-resolution analysis of protein-drug complexes is highlighted, which was developed by researchers at the Institute of Cancer Research (UK) in collaboration with scientists at Thermo Fisher Scientific. Through the use of the latest Thermo Scientific™ Glacios™ 2 Cryo-Transmission Electron Microscope (Cryo-TEM) they were able to determine over two dozen 3.5–4.5 Å kinase structures at ~1 structure per hour.

## Methods

Overall, the method consists of rapid grid characterization and intermediate-resolution structural determination on a 200 kV cryo-TEM followed by high-end data collection on a higher resolution 300 kV Thermo Scientific™ Krios™ G4 Cryo-TEM. This allows initial structures to be obtained quickly while identifying promising candidates for higher quality data collection. Ideal samples should exhibit good ice thickness, suitable particle density and orientation, the presence of the inhibitor, and a promising initial structural resolution based on dynamic data processing performed during data collection.

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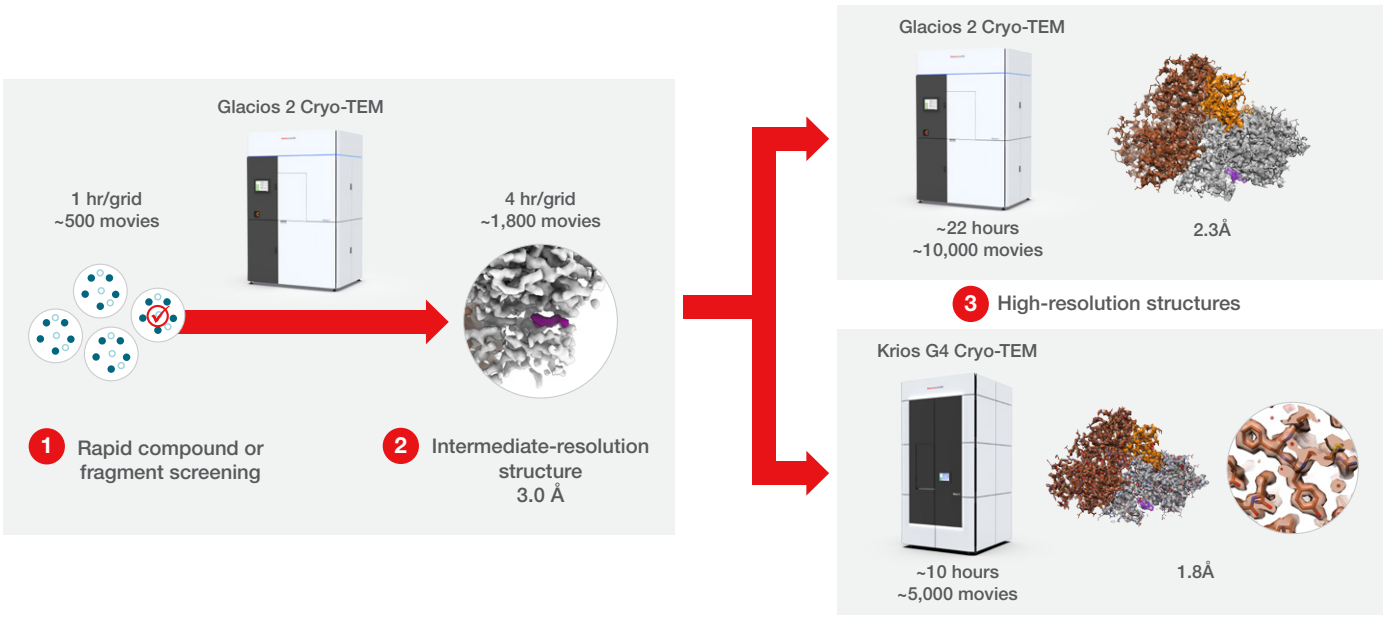


**Figure 1. Workflow utilized for evaluating CDK-targeting compounds. Rapid sample optimization with the Glacios 2 Cryo-TEM is followed by high-resolution data collection on a Glacios 2 and/or Krios G4 Cryo-TEM. For more information, see Reference 1.**

Sample optimization was performed on a 200 kV Glacios 2 Cryo-TEM, equipped with a Thermo Scientific™ Selectris™ X Energy Filter and a Thermo Scientific™ Falcon™ 4i Detector. Sample handling and data collection was controlled with Thermo Scientific EPU Multigrid Software. Data was collected using fringe free imaging (FFI) and aberration-free image shift (AFIS) at a throughput of ~500 images/hour.

High-resolution data was subsequently collected on a 300 kV Krios G4 Cryo-TEM, also equipped with a Selectris X Energy Filter and Falcon 4i Detector, along with a cold-field emission gun (cold-FEG). This configuration also offered a throughput of ~500 images/hour.

## Results and discussion

### High-resolution cryo-EM of human CAK

The human CDK-activating kinase (CAK) is a heterotrimeric protein complex consisting of cyclin-dependent kinase 7 (CDK7), cyclin H, and MAT1. CAK acts as a master regulator of cell growth and division by regulating initiation of transcription and the cell cycle. Due to its central role in cellular physiology, CAK is a promising target for cancer therapeutics and a possible target for antivirals.

One of the major challenges in the development of CDK-targeting compounds is specificity; 20 different isoforms are classified as CDKs, and they often exhibit high sequence overlap near their catalytic sites. Reducing off-target effects and increasing potency for novel therapeutics will necessitate structure-based drug-design approaches.

Fourteen lead compounds were analyzed in this study, but the method can be scaled to larger sets of small molecule ligands. EPU Multigrid Software was used for both initial screening as well as data collection. Designed for autoloader-equipped cryo-TEMs, EPU Multigrid Software allows users to queue multiple grids for unattended screening and data collection. Up to 12 grids can be imaged in one microscope session.

Only 1 hour of data collection per dataset was needed to determine 26 structures on the Glacios 2 Cryo-TEM, confirming the presence of 12 different bound inhibitors from 13 samples. Most of these structures were determined at ~3.5–4.5 Å resolution.

Intermediate-resolution structural determination was also completed using the 200 kV Glacios 2 Cryo-TEM. Data collection time was extended to 4 hours per sample, resulting in resolutions of ~3 Å for 12 datasets. At this resolution, major conformational differences between the samples were visible and inhibitors could be visualized in the cryo-EM density.

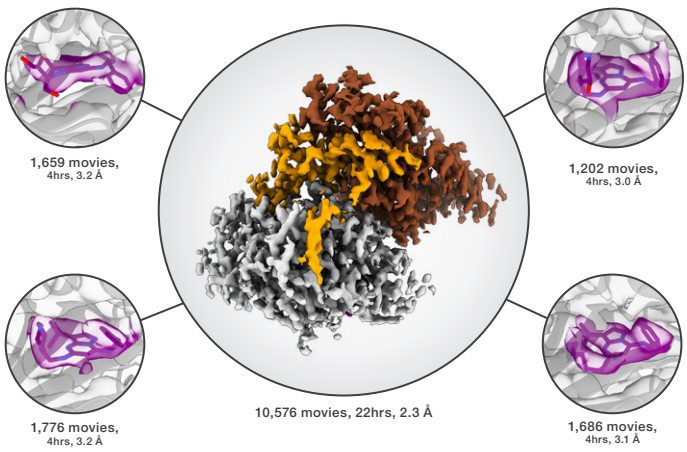


**Figure 2. Structures of CAK solved with the Glacios 2 Cryo-TEM. The middle structure was determined at 2.3 Å resolution in less than a day.**

**Glacios 2 Cryo-TEM**

The Glacios 2 Cryo-TEM showcases many of the advances that have made high-quality analysis possible on 200 kV instruments. Hardware optimization has focused on improved specificity and sensitivity in electron detection. For instance, the Thermo Scientific™ Falcon™ 4i Direct Electron Detector enables high detective quantum efficiency (DQE) access the full spectral range, capturing both small/flexible proteins as well as larger structures using fewer images. When coupled with the novel Thermo Fisher Selectris™ or Selectris X Energy Filters, signal is limited to the zero-loss elastically scattered electrons, producing high-contrast TEM data for higher throughput and higher resolution structures.

The high-resolution data collected on the 300 kV Krios G4 Cryo-TEM sought to accurately model the bound inhibitors and identify any water molecules that may be contributing to inhibitor binding and specificity.

In this study, structures of CAK were determined at up to 1.8 Å resolution for its free and nucleotide-bound states, as well as in complex with 14 inhibitors, with the best resolution at 1.7 Å. These structures provide detailed insight into inhibitor interactions as well as the network of water molecules found in the active site of CDK7.

## Conclusion

This application note highlights a three-step cryo-EM screening and early elimination workflow for structure-based drug discovery. This approach can help researchers understand how structurally diverse inhibitors interact with the active site of CDK7, providing a potential basis for the design of next-generation cancer therapeutics.1

This method is particularly well-suited for drug discovery, as the highest resolution structures are not always necessary to identify promising lead compounds. By providing structural information in only a few hours, the Glacios 2 Cryo-TEM showcases the value of this approach for early drug discovery. Once compounds are sufficiently advanced in the chemistry optimization pipeline, higher resolution data becomes increasingly more valuable, providing deeper insights that can improve drug design and reduce side effects.

As shown in this study, cryo-EM can now routinely gather data for ~2 Å structural determination of small protein complexes in less than a day. This enables high-throughput, high-resolution cryo-EM, even for difficult targets. Additionally, more than a dozen structures can be screened at ~3.5–4.5 Å in a day.

In summary, the Glacios 2 Cryo-TEM serves as a powerful screening system that enhances subsequent 300 kV analysis. This approach greatly improves throughput and productivity, optimizing the time spent on the higher-resolution Krios Cryo-TEM and ensuring that the highest quality data is collected for only the most ideal samples. This methodology facilitates the application of cryo-EM to iterative structure-based drug design, which is becoming increasingly necessary to address a range of global health challenges.

### References


1. Cushing, VI, et al. High-resolution cryo-electron microscopy of the human CDK-activating kinase for structure-based drug design. bioRxiv 2023.04.07.536029. doi: 10.1101/2023.04.07.536029


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