Appendix 4. Small Molecule Track Experimental Testing Procedure and Scoring Rules

I. Initial Screening Step (High-Throughput Initial Screening)

Thermofluor ABI 7500 Fast equipment will be used to conduct thermal shift assay (TSA) screening. This step is intended to rapidly identify small-molecule compounds that bind the target protein, herpes simplex virus DNA polymerase (HSV DNA polymerase, HSV Pol), and significantly increase its thermal stability.

All candidate small molecules will be co-incubated with wild-type (WT) HSV Pol at a concentration of 100 μM. Each batch of experiments will include a blank control group containing protein only and no small molecule. A real-time PCR instrument or microplate reader will be used for gradient temperature detection. Fluorescence signal changes during protein unfolding will be monitored in real time. Melting curves will be generated to obtain the protein melting temperature (Tm). Based on the results, the change in melting temperature (ΔTm) of each candidate small molecule relative to the blank control group will be calculated. Candidate small molecules with ΔTm ≥ 2°C will be included in the Preliminary Round candidate molecule ranking. For reference, the positive drugs ACV-TP and Foscarnet have ΔTm values of > 4°C and > 7°C, respectively.

Participating Teams should note that TSA screening can reduce downstream experimental costs and false positives when selecting molecules for the Initial Screening step. However, a certain proportion of false-negative molecules may be excluded. To maximize the chance of identifying high-quality molecules, the Initial Screening rules will be implemented as follows:

(1) Candidate small molecules with ΔTm ≥ 2°C will be ranked by ΔTm from high to low. The top 100 candidate small molecules will enter the Initial Evaluation step. If fewer than 100 candidate small molecules meet the ΔTm ≥ 2°C criterion, all candidate small molecules that meet the criterion will enter the Initial Evaluation step.

(2) If none of the submitted molecules from a Participating Team enters the Initial Evaluation step, that team may apply to the Organizer within seven working days after the results are published to have one submitted molecule enter the Initial Evaluation step directly. This rule does not apply to Participating Teams that already have at least one molecule entering the Initial Evaluation step.

The above wet-lab validation will be conducted independently by relevant teams from Shanghai University and the Shanghai Institute of Major Infectious Diseases and Biosafety.

II. Initial Evaluation Step (WT Inhibitory Activity Confirmation and Preliminary Evaluation)

An in vitro DNA polymerase activity assay system will be used to conduct IC_{50}-based functional activity validation for the candidate small molecules obtained from Initial Screening. This step will quantitatively evaluate their ability to inhibit HSV Pol WT protein activity. Protein concentration will be optimized to ensure a stable signal-to-noise ratio. Multi-concentration gradient inhibition experiments will be conducted. Fluorescence signals will be monitored using a microplate reader to reflect enzyme activity. The resulting data will be fitted to dose-response curves to calculate the half-maximal inhibitory concentration (IC_{50}).

Candidate small molecules will be ranked by IC_{50} from low to high. The Participating Teams corresponding to the top 10 compounds will be selected to enter the Re-evaluation step. The Organizer will conduct comprehensive scoring based on wet-lab results. The above wet-lab

validation will be conducted independently by relevant teams from Shanghai University and the Shanghai Institute of Major Infectious Diseases and Biosafety.

Inhibition Activity Ranking (IC_{50} from low to high)	Inhibition Activity Score (WT)
1	100
2	95
3	90
4	85
5	80
6	75
7	70
8	65
9	60
10	55

If adjacent ranked molecules have no statistically significant difference in IC_{50}, the relevant molecules will share the highest applicable rank and receive the average of the original scores for those ranks. For example, if the molecules originally ranked 1st, 2nd, and 3rd have no statistically significant difference in IC_{50}, all three molecules will be assigned rank 1. Their score will be (100 + 95 + 90) / 3 = 95. The molecule originally ranked 4th will remain unaffected and receive 85 points.

III. Re-evaluation Step 1 (W781V Drug-Resistant Mutant Inhibitory Activity Evaluation)

Candidate small molecules that pass the Preliminary Round will be tested against the HSV Pol drug-resistant mutant W781V using multi-concentration gradient IC_{50} assays. This step will verify inhibitory activity against the mutant protein and evaluate the effect of the small molecules on W781V mutant HSV Pol. The results will support subsequent advancement and molecule optimization and will also serve as an important component of the Final Round score.

The candidate small molecules entering the Re-evaluation step will undergo IC_{50}-based functional activity validation in the HSV Pol W781V drug-resistant mutant assay system. The same multi-concentration gradient used in the Initial Evaluation step will be applied to ensure data comparability. Fluorescence signals related to nucleic acid synthesis will be monitored using a microplate reader to evaluate the inhibitory effect on the W781V mutant. The experimental data will then be fitted to dose-response curves, and IC_{50} values will be calculated. Candidate small molecules will be ranked by IC_{50} from low to high.

Inhibition Activity Ranking (IC_{50} from low to high)	Inhibition Activity Score (W781V)
1	100
2	95
3	90
4	85
5	80

If a candidate small molecule has no statistically significant difference in IC_{50} from the molecule ranked 5th, it will share the 5th rank and receive the same score of 80 points.

If adjacent ranked molecules have no statistically significant difference in IC_{50}, the relevant molecules will share the highest applicable rank and receive the average of the original scores for those ranks. For example, if the molecules originally ranked 1st, 2nd, and 3rd have no statistically significant difference in IC_{50}, all three molecules will be assigned rank 1. Their score will be (100 + 95 + 90) / 3 = 95. The molecule originally ranked 4th will remain unaffected and receive 85 points.

IV. Re-evaluation Step 2 (N815S Drug-Resistant Mutant Inhibitory Activity Evaluation)

Candidate small molecules that pass the Preliminary Round will be tested against the HSV Pol drug-resistant mutant N815S using multi-concentration gradient IC_{50} assays. This step will verify inhibitory activity against the mutant protein and evaluate the effect of the small molecules on N815S mutant HSV Pol. The results will support subsequent advancement and molecule optimization and will also serve as an important component of the Final Round score.

The candidate small molecules entering the Re-evaluation step will undergo IC_{50}-based functional activity validation in the HSV Pol N815S drug-resistant mutant assay system. The same multi-concentration gradient used in the Initial Evaluation step will be applied to ensure data comparability. Fluorescence signals related to nucleic acid synthesis will be monitored using a microplate reader to evaluate the inhibitory effect on the N815S mutant. The experimental data will then be fitted to dose-response curves, and IC_{50} values will be calculated. Candidate small molecules will be ranked by IC_{50} from low to high.

Inhibition Activity Ranking (IC_{50} from low to high)	Inhibition Activity Score (N815S)
1	100
2	95
3	90
4	85
5	80

If a candidate small molecule has no statistically significant difference in IC_{50} from the molecule ranked 5th, it will share the 5th rank and receive the same score of 80 points.

If adjacent ranked molecules have no statistically significant difference in IC_{50}, the relevant molecules will share the highest applicable rank and receive the average of the original scores for those ranks. For example, if the molecules originally ranked 1st, 2nd, and 3rd have no statistically significant difference in IC_{50}, all three molecules will be assigned rank 1. Their score will be (100 + 95 + 90) / 3 = 95. The molecule originally ranked 4th will remain unaffected and receive 85 points.

V. Re-evaluation Step 3 (Y941H Drug-Resistant Mutant Inhibitory Activity Evaluation)

Candidate small molecules that pass the Preliminary Round will be tested against the HSV Pol drug-resistant mutant Y941H using multi-concentration gradient IC_{50} assays. This step will verify inhibitory activity against the mutant protein and evaluate the effect of the small molecules on Y941H mutant HSV Pol. The results will support subsequent advancement and molecule optimization and will also serve as an important component of the Final Round score.

The candidate small molecules entering the Re-evaluation step will undergo IC_{50}-based functional activity validation in the HSV Pol Y941H drug-resistant mutant assay system. The same multi-concentration gradient used in the Initial Evaluation step will be applied to ensure data comparability. Fluorescence signals related to nucleic acid synthesis will be monitored using a microplate reader to evaluate the inhibitory effect on the Y941H mutant. The experimental data will then be fitted to dose-response curves, and IC_{50} values will be calculated. Candidate small molecules will be ranked by IC_{50} from low to high.

Inhibition Activity Ranking (IC_{50} from low to high)	Inhibition Activity Score (Y941H)
1	100
2	95
3	90
4	85
5	80

If a candidate small molecule has no statistically significant difference in IC_{50} from the molecule ranked 5th, it will share the 5th rank and receive the same score of 80 points.

If adjacent ranked molecules have no statistically significant difference in IC_{50}, the relevant molecules will share the highest applicable rank and receive the average of the original scores for those ranks. For example, if the molecules originally ranked 1st, 2nd, and 3rd have no statistically significant difference in IC_{50}, all three molecules will be assigned rank 1. Their score will be (100 + 95 + 90) / 3 = 95. The molecule originally ranked 4th will remain unaffected and receive 85 points.

VI. Re-evaluation Total Score (Weighted Sum of Scores)

For the 10 candidate small molecules entering the Re-evaluation step, the interaction score based on protein thermal stability changes measured in the Initial Screening step will be assigned as follows:

Interaction Ranking Based on Protein Thermal Stability Change (ΔTm from high to low)	Interaction Score Based on Protein Thermal Stability Change (WT)
1	100
2	95
3	90
4	85
5	80
6	75
7	70
8	65
9	60
10	55

If adjacent ranked candidate small molecules have no statistically significant difference in ΔTm, the relevant molecules will share the highest applicable rank and receive the average of the original scores for those ranks. For example, if the candidate small molecules originally ranked 1st, 2nd, and 3rd have no statistically significant difference in ΔTm, all three candidate small molecules will be assigned rank 1. Their score will be (100 + 95 + 90) / 3 = 95. The molecule originally ranked 4th will remain unaffected and receive 85 points.

The scores generated in each step will be weighted as follows:

Item	Weight (%)
Inhibition Activity Score (WT)	20
Inhibition Activity Score (W781V)	20
Inhibition Activity Score (N815S)	20
Inhibition Activity Score (Y941H)	20
Interaction Score Based on Protein Thermal Stability Change (WT)	20

Total score = Inhibition Activity Score (WT) × 20% + Inhibition Activity Score (W781V) × 20% + Inhibition Activity Score (N815S) × 20% + Inhibition Activity Score (Y941H) × 20% + Interaction Score Based on Protein Thermal Stability Change (WT) × 20%.

VII. Notes

1. If the same Participating Team recommends multiple candidate small molecules, only the candidate small molecule with the highest total score will be considered for awards. Awards are mutually exclusive for the same participating team. For example, if a Participating Team receives

the First Prize, its other recommended candidate small molecules may not also receive the Third Prize.

2. Participating Teams should understand that false-negative events may occur during Initial Screening. Such false-negative events: (i) are not caused by the subjective intent of the Organizer; (ii) are not testing errors by the Organizer; and (iii) are unavoidable events in high-throughput screening.

3. The Organizer has stated in the Initial Screening section of these rules that if none of the submitted molecules from a Participating Team enters the Initial Evaluation step, that team may apply to the Organizer within seven working days after the results are published to have one candidate small molecule enter the Initial Evaluation step directly. The Organizer will not accept any request to replace candidate small molecules for Initial Evaluation. Any conduct inconsistent with the purpose of the competition will be subject to a warning. Any costs that may arise for the Participating Team in this step shall be borne by the Participating Team. After these rules take effect, the Organizer shall not assume responsibility beyond the scope agreed in the competition rules for any false-negative-related event that may occur in this step. Participating Teams shall not hold the Organizer liable for such events.

4. If a Participating Team has reasonable questions about the results of the Initial Evaluation step or any subsequent step, the team must report the matter to the Organizer and then entrust a third-party institution designated by the Organizer to conduct retesting. The testing results must be submitted to the Organizer within seven working days after the results of the relevant step are published. The Organizer will consider the test results issued by the third-party institution. If the result may affect the final award status of the candidate small molecule, the Organizer will retest the candidate small molecule. Based on the Organizer’s retest result, the Organizer will bear the testing cost of the third-party institution only if the result affects the final award status of that molecule. Otherwise, the cost shall be borne by the Participating Team.