Attachment 4.

Experimental Testing Procedure and Scoring Criteria for the Small Molecule Preliminary Round

I. Screening Phase (High-Throughput Preliminary Screening)

All candidate molecules will undergo preliminary screening using drug screening instruments such as microplate readers, through a cAMP detection assay based on the Glosensor protein probe. This assay characterizes the impact of small molecules on intracellular cAMP levels, **indirectly reflecting** their antagonistic activity against HCAR1. The evaluation procedure is as follows:

Each candidate molecule will be tested at two concentrations—30 μM (high) and 10 μM (low). Based on the screening results: ① Molecules that show no significant antagonistic activity at either concentration will be deemed inactive and excluded from efficacy ranking; ② Molecules that exhibit antagonistic activity at the high concentration but not at the low concentration will be classified as having low antagonistic activity; ③ Molecules that show antagonistic activity at the low concentration but not at the high concentration, or show antagonistic activity at both concentrations, will be considered to possess potential antagonistic activity. These molecules will be ranked **in descending order** based on their antagonistic efficacy at the low concentration.

Participating teams should be aware that during the selection of shortlisted molecules, this phase reduces the likelihood of false positives by comprehensively evaluating the dual-concentration assay results of submitted molecules. However, there remains a low probability that some false negatives may be **excluded**. To maximize the identification of high-quality molecules, the following selection rules

will apply:

(1) Based on the ranking of molecules classified under category ③ by descending antagonistic efficacy, the **top 100 molecules** will be shortlisted. Any additional molecules showing no statistically significant difference in antagonistic activity compared to the 100th-ranked molecule will also be included.

(2) If a participating team **fails to have any of its submitted molecules shortlisted**, the team may apply to the organizer within seven working days of the results announcement to have **one molecule** admitted by exception. This provision **does not apply to teams that already have at least one molecule shortlisted**. This phase will be independently validated through wet-lab experiments by relevant teams from East China Normal University and the Shanghai Institute of Materia Medica.

II. Primary Evaluation Phase (Confirmation of Antagonistic Activity and Initial Ranking)

All candidate molecules will undergo **multi-concentration antagonistic activity** testing using drug screening instruments such as microplate readers, based on the Glosensor cAMP assay. Dose-response curves of the small molecules against HCAR1 will be generated, and the half-maximal inhibitory concentration (IC_{50}) will serve as the primary metric for evaluation. Molecules **other than those classified as inactive or low-activity** will be ranked **in ascending order** based on their **IC_{50}** values. The top 10 participating teams, as determined by this ranking, will advance to the secondary evaluation round. The organizer will assign scores and rankings to the small molecules based on the wet-lab experimental results.

This phase will be independently validated through wet-lab experiments conducted by relevant teams from East China Normal University and the Shanghai Institute of Materia Medica.

III. Secondary Evaluation Phase 1 (Antagonistic Activity Assessment)

Shortlisted small molecules from the primary evaluation will undergo antagonistic activity assessment using the HTRF cAMP assay. This assay enables high-sensitivity quantification of intracellular cAMP levels, providing a more precise measurement of a small molecule ' s antagonistic effect on HCAR1 compared to the Glosensor cAMP assay. The HTRF method is based on the principle of fluorescence resonance energy transfer (FRET), offering not only accurate quantification of cAMP concentration but also a higher signal-to-noise ratio and lower nonspecific background interference, resulting in more stable and reliable outcomes. Therefore, the results from the HTRF cAMP assay will serve as a key basis for the ranking in the secondary evaluation. The evaluation procedure is as follows:

All shortlisted molecules from the primary evaluation will be subjected to **multi-concentration antagonistic activity** testing. Each molecule will be independently tested by teams from East China Normal University and the Shanghai Institute of Materia Medica. Final evaluation results will be determined based on IC_{50} values, and the molecules will be ranked accordingly.

All molecules advancing to the secondary evaluation phase will undergo multi-concentration testing to generate dose-response curves of the small molecules against HCAR1. Molecules will then be ranked **in ascending order** of

IC_{50} values, reflecting their relative antagonistic activity. Based on this ranking of antagonistic activity,

1. The top five molecules in the ranking will receive **antagonistic activity scores** according to the table below and will proceed to the subtype selectivity testing stage.

Antagonistic Activity Ranking (Based on IC_{50}, from lowest to highest)	**Antagonistic Activity Score**
1	100
2	90
3	85
4	80
5	75

1. If any molecule exhibits no statistically significant difference in IC_{50} compared to the **fifth-ranked** molecule, it will share the fifth-place ranking and receive the same score (75 points).

2. If **adjacent ranked** molecules exhibit no statistically significant difference in their IC_{50} values, those molecules will share the highest ranking among them and receive **the average score of the combined original point values**. For example, if the originally ranked 1st, 2nd, and 3rd molecules have no significant differences in IC_{50}, they will all be assigned a rank of 1st, and their antagonistic activity scores will be calculated as (100 + 90 + 85) / 3 = 91.67 points. The 4th place ranking will remain unaffected and retain its original score of 80 points.

IV. Secondary Evaluation Phase 2 (Selectivity Assessment)

Molecules that have received antagonistic activity scores will undergo further

testing to assess their antagonistic activity against HCAR2 and other members of the GPCR family. The detailed procedure is as follows:

All molecules advancing to the selectivity assessment stage will be tested at multiple concentrations, and dose-response curves of the small molecules against HCAR1, HCAR2 and other GPCR family members will be generated. For each compound, the IC_{50} value against HCAR1 will be defined as A, and the IC_{50} values against HCAR2 and other GPCR members will be defined as B. A molecule with a B/A ratio < 10 will be classified as a low-selectivity HCAR1 antagonist, while a molecule with a B/A ratio > 1000 will be classified as a high-selectivity HCAR1 antagonist. Molecules will be ranked in descending order based on their B/A ratios. According to the selectivity ranking results, the top five molecules will receive **selectivity scores** as outlined in the table below.

Selectivity Ranking for HCAR1 (Based on B/A ratio, from highest to lowest)	**Selectivity Score**
1	100
2	90
3	80
4	70
5	60

V. Total Preliminary Round Score (Weighted Sum of Scores)

Scores generated from each evaluation phase will be weighted according to the following rules:

Item	**Weight (%)**

Antagonistic Activity Score	80
Selectivity Score	20

Total Score = (Antagonistic Activity Score) × 80% + (Selectivity Score) × 20%

VI. Notes

1. If a participating team submits multiple molecules, only the molecule with the highest antagonistic activity will be considered for award purposes. Awards are mutually exclusive—i.e., if a team wins a First Prize, the other molecules submitted by the same team will not be eligible for additional prizes such as Third Prize.

2. For the **possibility of false negatives** during the screening phase, participating teams should be aware that the occurrence of false negatives: ① **is not** the result of any subjective intent on the part of the organizer; ② **does not constitute** a testing error by the organizer; ③ **is** an unavoidable low-probability event inherent in high-throughput screening processes.

3. As stated in the "Screening Phase" of the *Experimental Testing Procedure and Scoring Criteria for the Small Molecule Preliminary Round*, "If a participating team fails to have any of its submitted molecules shortlisted, the team may apply to the organizer within seven working days of the results announcement to have one molecule admitted by exception". The organizer will **not accept any requests for molecule substitution** after shortlisting. Any actions that violate the spirit of the competition will be subject to **formal warnings**. Any expenses incurred by the participating teams during this phase shall be **borne solely by the teams themselves**. Once the procedures outlined in Attachment 3.4: Testing Procedure and Scoring have been implemented, the organizer shall **bear no**

responsibility for any false-negative-related incidents, and participating teams shall **have no right to hold the organizer liable**.

4. If a participating team raises a legitimate challenge to the results of any stage following the primary evaluation (including the primary evaluation itself), it must notify the organizer and submit the molecule for retesting by **a third-party institution designated** by the organizer. The test results must be submitted to the organizer within **seven working days** after the public announcement of the relevant stage’ s results. The organizer will review the third-party testing results and, if the outcome may affect the final award status of the molecule, will conduct a re-evaluation. If the re-evaluation confirms that the **molecule’ s award status is impacted**, the **testing cost incurred at the third-party institution will be covered by the organizer**. Otherwise, the cost will be borne by the participating team.