

The Development of an Indirect Competitive Immunomagnetic-Proximity Ligation Assay for Small-Molecule Detection

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Supporting Information

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1. Oligonucleotide sequences and modifications used in our studies

Icipla 1:

5'-Biotin-AAAACTCAAATCAACAGGCGAGCCGGACGCTACCAGCTTCTATACCGCAAG
CAGCTTGGCCTGAATCTGCTC-OH-3'

Icipla 2:

5'-P-TACGCCTCGACAGGACGCTGTGGCATTGCAGAGCGTGGCGCTTTACCTATGATAT
GATCGTGGTGATATCCGTC-Biotin-3'

Forward primer (Pla1): 5'-AAAACTCAAATCAACAGGCG-3'

Reverse primer (Pla2): 5'-GACGGATATCACCACGATCA-3'

Connector: 5'-TTTTTCGAGGCGTAGAGCAGATTCAA-3'

2 Buffers used in the assays

The buffers used for our assays included PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, PH 7.4), storage buffer (1 × PBS, 0.1% BSA), PBST wash buffer (1 × PBS, 0.05% TWEEN® 20), coating buffer (15 mM Na₂CO₃, 35 Mm NaHCO₃, PH 9.6), DNA buffer (4.6 × SSC buffer, 0.5% Blocking Reagent CA, 0.01 M EDTA, 0.1% TWEEN® 20), PLA buffer (1 × PBS, 0.1% BSA, 1 mM d-biotin, 0.05% TWEEN® 20, and 0.1 mg/ml salmon sperm DNA), ligation buffer (400 nM connector oligonucleotide, 40 mM Tris-HCl, 10 mM MgCl₂, 0.5 mM ATP, 5% polyethylene glycol 4000 solution, and 0.5 U T4 DNA ligase), and real-time PCR buffer (0.4 μM of each primer (Pla1 and Pla2) and 1 × SYBR Premix Ex Taq II)

3 Biotinylation of RAC-BSA conjugates and the RAC monoclonal antibody

EZ-Link Sulfo-NHS-Biotin was used according to the manufacturer's instructions for biotinylating proteins in solution. Once proper function and labeling of the protein was confirmed by ELISA, the labeled protein was purified for optimal performance and stability using dialysis in PBS overnight at 4°C with rotation. The concentrations of the purified biotinylated RAC-BSA conjugates (Bio-RAC-BSA) and the RAC monoclonal antibody (Bio-RAC-mAb) were measured in a micro-ultraviolet (UV) spectrophotometer.

4 Preparation of the tissue extracts

Muscle tissue (RAC-free) was finely chopped, and a 3-g sample was weighed and added to a

50-ml centrifuge tube. Then, 8 ml acetonitrile and 1 ml ethylacetate were added to the tube. After vigorously vortexing the sample for 3 min, the mixture was centrifuged at 10 000 rpm for 5 min. Then, 6 ml of the supernatant was transferred to a beaker and evaporated at RT until dry. The residue was dissolved in 1 ml n-hexane, and the n-hexane solution was vigorously shaken for 1 min with 2 ml PBS. The PBS layer was collected for further analysis after centrifugation at 10 000 rpm for 5 min.

5 Optimization of the microparticles and probes

To determine the optimal concentration for the RAC-BSA coated onto the microparticles, 6 different concentrations of Bio-RAC-BSA were used in an ICIPLA experiment while holding the concentration of the PLA probes constant (1000 pM). As shown in Figure S-1a, as the concentration of Bio-RAC-BSA increased, the corresponding Ct value—obtained via qPCR—gradually decreased from 30.45 (0 ng·ml⁻¹) to 16.11 (50 000 ng·ml⁻¹). The difference in the Ct value from 17.9 (5000 ng·ml⁻¹) to 16.11 (50 000 ng·ml⁻¹) was not obvious, though the latter sample contained ten times the concentration of Bio-RAC-BSA. Therefore, we chose 5000 ng·ml⁻¹ as the final Bio-RAC-BSA concentration for coating the microparticles.

To optimize the probe concentration for the ICIPLA method, 5 different probe concentrations were tested while maintaining a Bio-RAC-BSA concentration of 5000 ng·ml⁻¹. Each concentration contained a negative control in which no Bio-RAC-BSA was coated onto the microparticles. As shown in Figure S-1b, 1000 pM of probe provided the greatest difference in the Ct values between the negative control and the sample group, indicating that produced very strong proximity effect while the background signal was much lower. Thus, 1000 pM was ultimately chosen as the probe concentration for the following assays.

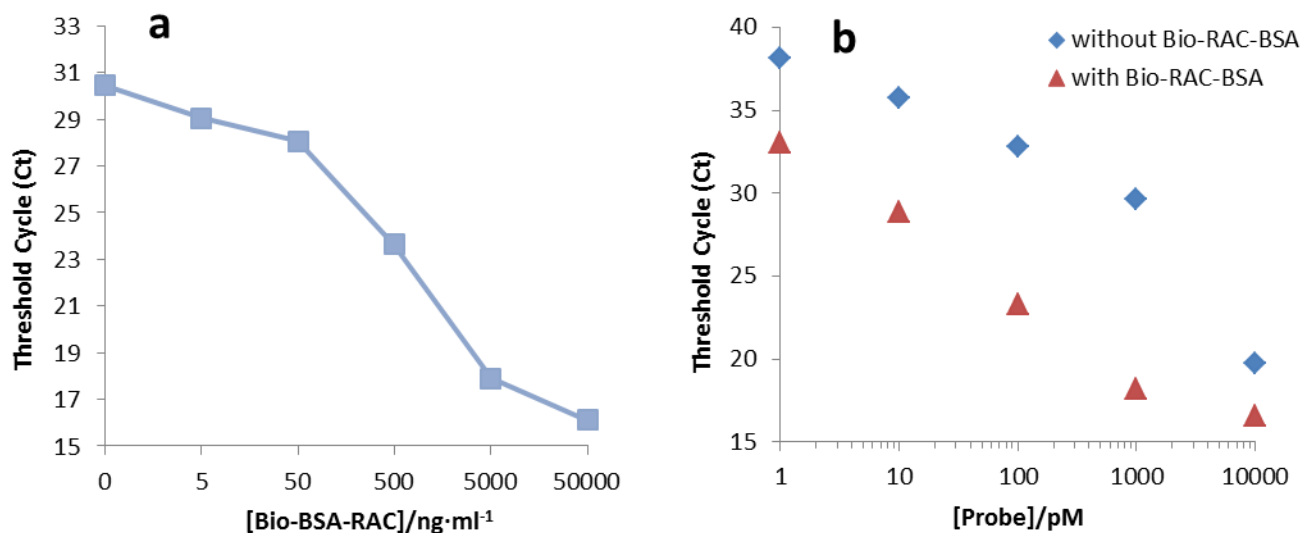


Figure S-1. Optimization of reagent concentrations for the ICIPLA technique. (a) Determination of the optimal Bio-RAC-BSA concentration for coating the microparticles. (b) Optimization of the PLA probe concentration. The Bio-BSA-RAC and probe concentrations are plotted on the x axes, and the Ct values are plotted on the y axes.

6 Indirect competitive ELISA

Microplates were coated with $100 \mu\text{l}\cdot\text{well}^{-1}$ of $0.2 \mu\text{g}\cdot\text{ml}^{-1}$ RAC-BSA conjugate in coating buffer overnight at 4°C . After washing three times with PBST wash buffer, nonspecific binding was blocked by incubation with $200 \mu\text{l}\cdot\text{well}^{-1}$ of 1% BSA at 37°C for 1 h. The solution was discarded, and the micropaltes were washed three times with PBST wash buffer. Then, $50 \mu\text{l}\cdot\text{well}^{-1}$ of RAC serially diluted in PBS was incubated with $50 \mu\text{l}\cdot\text{well}^{-1}$ of $0.5 \mu\text{g}\cdot\text{ml}^{-1}$ Bio-RAC-mAb at RT for 1 h. After another washing procedure, $50 \mu\text{l}\cdot\text{well}^{-1}$ of Streptavidin-horseradish peroxidase (STV-HRP) was added, followed by incubation at RT for 30 min. The plates were washed five times with PBST wash buffer, and $100 \mu\text{l}\cdot\text{well}^{-1}$ of freshly prepared TMB substrate solution was added. After incubating at RT for 10 min, the reaction was stopped with 2 M H_2SO_4 . The absorbance was measured at 450 nm using a Sunrise microplate reader.

A checkerboard method was used to determine the optimal amounts of RAC-BSA and Bio-RAC-mAb. The inhibition curve of optimized indirect competitive ELISA (icELISA) for RAC was shown in Figure S-2. The limit of detection (LOD) of the assay, which is represented by IC_{15} value, was $0.2 \text{ ng}\cdot\text{ml}^{-1}$.

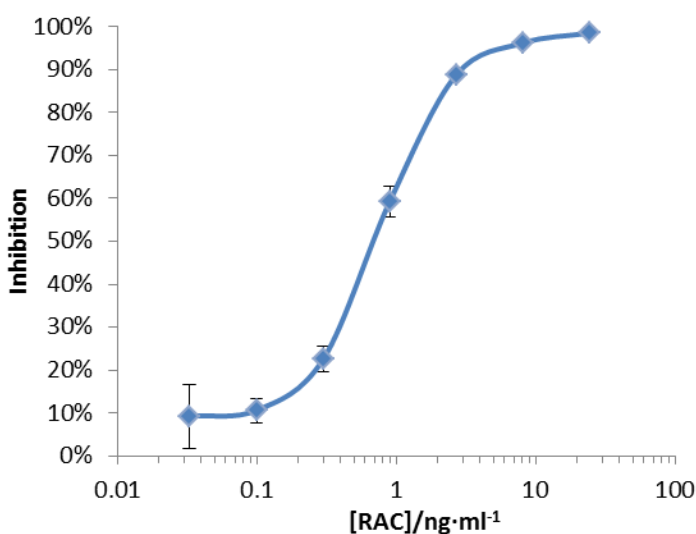
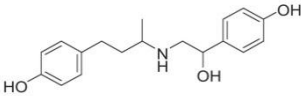
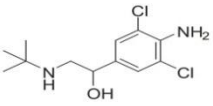
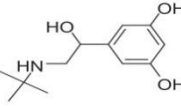
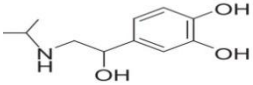


Figure S-2. The optimized icELISA inhibition curve for RAC.

7 Assessment of the assay specificity

The specificity of ICIPLA was evaluated using the RAC monoclonal antibody (8DA2) with clenbuterol, terbutanline and isoprenaline prepared in PBS. The cross-reactivity values (CR%) were calculated using the following formula: $\text{CR}\% = \text{IC}_{50}(\text{RAC})/\text{IC}_{50}(\text{cross-reactive compound})$. The IC_{50} was determined from the midpoint of the assay standard curve.

Table S-1. Cross-reactivity of the RAC monoclonal antibody with three RAC analogs for the ICIPLA technique.

Competitor	Chemical structure	Cross-reactivity (%)
Ractopamine		100
Clenbuterol		<0.01
Terbutanline		<0.01
Isoprenaline		<0.01