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Obelin mutants as reporters in bioluminescent dual-analyte binding assay

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Two obelin mutants distinctly different in the color of bioluminescence were successfully applied to simultaneous detection of two analytes in a single well. The flash-type signals were triggered by single injection of Ca^{2+} and were discriminated by the spectral and time resolutions. The technique was developed in a high-throughput format, and applied to simultaneous immunoassay of two gonadotropic hormones – luteinizing (lutropin or hLH) and follicle stimulating (hFSH) – in clinical sera, and to simultaneous detection of two gene allelic variants at single nucleotide polymorphism genotyping of the human F5 gene encoding factor V Leiden polymorphism 1691 G→A (R506Q). A considerable number of samples were investigated and the results obtained were in good correlation with those obtained by using traditional techniques.

Introduction

Ca^{2+}-regulated photoprotein obelin, like other coelenterate photoproteins, is a stable complex of a single-chain apoprotein and peroxycoelenterazine. Upon binding of Ca^{2+}, obelin undergoes a conformation change that causes the decarboxylation of peroxycoelenterazine and the emission of visible light ($\lambda_{max} = 482$ nm).[1,2] The relationship between the light emission and the amount of obelin is linear in the presence of an excess of Ca^{2+}. A high quantum yield of the reaction, virtual absence of the background, and high sensitivity of modern photometers make it possible to detect the photoprotein down to the attomole level. The recently developed analytical techniques use Ca^{2+}-regulated photoproteins aequorin and obelin as effective reporters.[3] Binding assays involving these bioluminescent reporters provide high sensitivity, simplicity of measurement, reproducibility and safety. With the application of site-directed mutagenesis, obelin mutants with substantially altered bioluminescence spectral and kinetic characteristics were obtained in our laboratory: W92F,H22E emits a fast ($k_d = 0.6$ s^{-1}) violet signal ($\lambda_{max} = 387$ nm) and Y138F – a slow ($k_d = 6.1$ s^{-1}) greenish light ($\lambda_{max} = 498$ nm) with small spectral overlapping.[4] Using these as reporters, we developed a dual analyte single-well bioluminescence assay based on the spectral resolution of signals, in combination with time resolution. The technique allows enhancement of the assay capability for the sake of proper diagnostics, especially in cases requiring the detection of two analytes in one sample.

Recently, we successfully applied the approach to the simultaneous immunoassay of two forms of prolactin, the total and IgG-bound ones, in crude serum.[5] It was performed in a microplate format and allowed the detection of the two prolactin forms without any additional manipulation. The assay provided a high sensitivity in detecting total prolactin, close to that of RIA, and a capability to visualize both prolactin forms like in the case of gel-filtration chromatography, but in a much shorter time.

In this study, the application of our approach was broadened to: (a) simultaneous immunoassay of two gonadotropic hormones – luteinizing (lutropin or hLH) and follicle stimulating (hFSH) – in clinical sera, and (b) simultaneous detection of two gene allelic variants at SNP genotyping of the human F5 gene encoding factor V Leiden polymorphism 1691 G→A (R506Q). Many clinical samples were investigated and the obtained results were in good correlation with those obtained by traditional techniques.

Experimental

Materials

The highly purified photoproteins (violet mutant W92F,H22E and green mutant Y138F of obelin) were obtained according to ref. 4. Streptavidin, 2-iminothiolan, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) were from Sigma (USA).

Monoclonal mouse anti-hLH IgG (clone 5301) and anti-hFSH IgG (clone 6602) were from MedixBiochemica (Finland);

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monoclonal mouse anti-hLH/hFSH (α-chain) IgG (clone XF-1) – from Xema-Medica (Russia); goat polyclonal anti-FITC IgG – from Abcam (UK). The standard sera (calibrated according to hLH WHO 1st IRP 68/40 and hFSH WHO 2nd IRP 78/549) were from DIAS (Russia).

Biotinyl uridine triphosphate (B-dUTP), oligothymidine, carrying a 5′-hexamethylamino group, 5′-NH_2-$(CH_2)_6$-p-T_{30}, (NH_2-dT_{30}); primers for amplification of genomic DNA: 5′-CATCATGAGAGACATCGCCTC-3’ (LeiUp) and 5′-CATGTTC-TAGCCAGAAGAAATTC-3′ (LeiDn), primers for the PEXT reaction: 5′-FAM-AGCAGATCCCTGGACAGGCG-3′ (LeiN) and 5′-$(A)_{27}$AGCAGATCCCTGGACAGGCA-3′ (LeiM) were obtained from Biosan (Russia). Hot Start Taq DNA polymerase and dNTPs were from SibEnzyme (Russia). SNPdetect polymerase was from Evrogen (Russia).

Conjugates W92F,H22E-anti-hLH IgG (clone 5301), Y138F-anti-hFSH IgG (clone 6602), and Y138F-anti-FITC IgG were synthesized according to the method described in ref. 6.

To obtain the W92F,H22E-$(dT)_{30}$ conjugate, the protein was incubated with a 5-fold excess of 2-iminothiolan in 50 mM BICINE pH 8.5, 5 mM EDTA for 30 min at room temperature. Excess of reagent was removed by gel filtration on a D-salt Dextran Desalting column (Pierce, USA) equilibrated with 50 mM PIPES pH 7.5, 5 mM EDTA. The NH_2-dT_{30} was modified by a 100-fold excess of SMCC in 0.1 M $NaHCO_3$ for 2 h at room temperature followed by removal of excess reagent on a Sephadex G-25 spin-column equilibrated with water. The mixture of thionylated W92F,H22E and SMCC-activated oligonucleotide (molar ratio 2 : 1) was incubated overnight at 8 °C. The conjugate W92F,H22E-$(dT)_{30}$ was purified on a Mono Q column (GE Healthcare, Sweden) equilibrated with 20 mM Tris–HCl pH 7.0, 5 mM EDTA using gradient 1 M NaCl up to 70%.

All conjugates were stored at −18 °C in 20 mM Tris–HCl pH 7.0, 0.25 M NaCl, 5 mM EDTA, 0.1% BSA over 1 year without loss of bioluminescent activity.

Solid-phase immunoassay was carried out using white polystyrene stripwell microtiter plates (Corning, USA). In dual color assays, the photometer registered signals through band-pass optical filters VB6 (I) and YB16 (II) (Len-ZOS, Russia).

Sera samples were provided by the endocrinology diagnostic laboratory of Krasnoyarsk regional hospital no. 1. The hLH and hFSH in sera were previously quantified with separate RIA.

DNA samples were analyzed earlier by other methods and provided by Krasnoyarsk Branch of Hematological Scientific Center of the Russian Academy of Medical Sciences.

Simultaneous microtiter-based bioluminescent immunoassay of LH and FSH. The surface of wells was activated with 100 μL of mouse anti-hLH/hFSH (α-chain) (clone XF-1) immunoglobulin solution, 10 μg mL^{-1}, in PBS (0.15 M NaCl, 50 mM K–Na phosphate buffer pH 7.0) overnight at 4 °C. Then it was washed (three times, PBS, 0.1% Tween 20, 5 mM EDTA) and blocked by placing 1% BSA in PBS (140 μL per well) for 1 h, 37 °C. After washing, 100 μL standard (three replicates) and clinical (two replicates) sera were placed into the wells, incubated with shaking for 1 h at 37 °C and washed thereafter. Then the mixture of conjugates – W92F,H22E-anti-hLH IgG (clone 5301) and Y138F-anti-hFSH IgG (clone 6602) (100 μL, 1 μg mL^{-1} each, PBS, 0.1% BSA, 5 mM EDTA) – was placed into the wells, incubated with shaking for 1 h at 23 °C and washed thereafter. The bioluminescence was measured with a plate luminometer LB 940 Multimode Reader Mithras (Berthold, Germany) by rapid injection of $CaCl_2$ solution (100 μL, 0.1 M in 0.1 M Tris–HCl, pH 8.8) into the well. The measurements were carried out as follows: during the first second the photometer registered the violet signal transmitted through filter I; the replacement of filter II took the next 0.3 s; the green light was registered for the last 5 seconds.

Amplification of genomic DNA. Amplification of genomic DNA was carried out in 25 μL buffer containing 67 mM Tris–HCl, pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 2 mM $MgCl_2$, dNTPs (0.2 mM each), the LeiUp and LeiDn primers (0.4 μM each), genomic DNA (1–10 ng), and Hot Start Taq DNA polymerase (2.5 U). PCR conditions: denaturation at 95 °C for 5 min, then 30 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s followed by heating of the reaction mixture at 72 °C for 10 min. PCR products were analyzed by electrophoresis in 1.7% agarose gel and stained with ethidium bromide. Concentration of PCR products was evaluated using the densitometry image of an electropherogram with Alpha EasyTM software (Alpha Innotech Corporation, USA). DNA Ladder 100 bp + 50 bp (SibEnzyme, Russia) was used as a calibration marker.

PEXT reaction. PEXT reaction was performed in 20 μL of a 1× SNP detect buffer containing 2.5 mM $MgCl_2$, dATP, dCTP, dGTP, B-dUTP (2.5 μM each), DNA template (0.2 pmol), LeiN and LeiM primers (1 pmol each), and SNP detect DNA polymerase (2 U). The reaction conditions were: denaturation at 95 °C for 5 min, then 3 cycles at 95 °C for 15 s, 60 °C for 10 s, and 72 °C for 15 s followed by heating of the reaction mixture at 95 °C for 5 min.

PEXT products microplate assay. The surface of the wells was activated with 50 μL of streptavidin solution, 10 μg mL^{-1}, in PBS for 1 h at 37 °C with shaking. After washing (three times, PBS, 0.1% Tween 20, 5 mM EDTA) the PEXT products (3 μL) in hybridization buffer (47 μL) containing 0.1 M maleic acid, 0.15 M NaCl, 5 mM EDTA, and 1% BSA were placed into the wells and incubated for 30 min at room temperature. The wells were washed again and the mixture of conjugates, Y138F-anti-FITC IgG and W92F,H22E-$(dT)_{30}$ (50 μL, each 0.1 μg mL^{-1}, in 20 mM Tris–HCl pH 7.0, 5 mM EDTA, 0.1 M NaCl, 0.1% BSA), was placed into the wells, incubated with shaking for 30 min at room temperature and washed thereafter. The bioluminescence intensity was measured with a plate luminometer LB 940 Multimode Reader Mithras by rapid injection of $CaCl_2$ solution into the well plate as was described above, excepting the green signal integrated for 3 seconds.

Results and discussion

Two kinds of the mutant of Ca^{2+}-regulated photoprotein obelin recently obtained in our laboratory[4] are characterized by their distinct difference in bioluminescence. The emission maxima of mutants W92F,H22E (violet) and Y138F (green) are separated by 113 nm with a small spectral overlap. It is possible to exploit this wide separation by the use of two wide band-pass optical

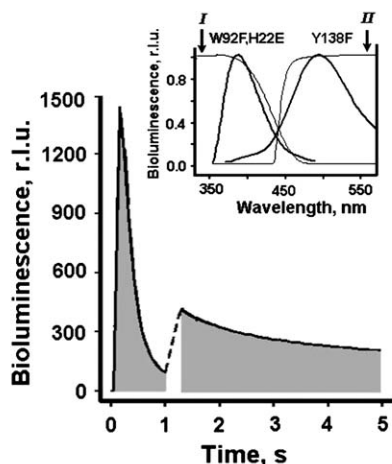

Fig. 1 Bioluminescence of the mixture of obelin mutants transmitted through filter I (fast violet signal) and filter II (slow green signal); dashed line, time for filter replacement. Upper inset: bioluminescence spectra of obelin mutants (thick lines) and optical filter transmission (thin lines). r.l.u. – relative light units.

filters (Fig. 1, inset). Because the bioluminescence kinetics of the green obelin is 10-fold slower than that of the violet obelin, the difference provides the possibility to resolve the signals in time. To implement the separation methods, we applied a plate luminometer Mithras LB 940. The construction of this device facilitates luminescence measurement through the optical filters placed on the wheel that allows fast filter replacement. Fig. 1 shows the bioluminescent reaction of the protein mixture. To divide signals effectively, a fast-kinetic violet signal was registered through optical filter I for 1 s, and a long-kinetic green signal – through optical filter II for 3–5 seconds. Filter replacement was done automatically for 0.3 seconds.

Human luteinizing hormone (lutropin or hLH) and follicle stimulating hormone (hFSH) belong to a subset of glycoprotein hormones, called gonadotropins that regulate gonadal function. Immunoassay is a useful tool for estimating the hormone levels in serum, and is important for discovering the disorders related to reproduction and puberty, such as hypogonadism, ovulation timing, and infertility. For proper diagnosis, it is strongly recommended to measure the levels of both hormones. The hLH and hFSH contain two different subunits, an α- and a β-chain, linked by noncovalent bonds. The primary structures of the α-subunits are almost identical, while their β-subunits are not identical. The β-subunits are responsible for the immunological and biological specificity of these hormones.

Fig. 2A presents the way we carried out the assay. The surface of wells was activated with immunoglobulin Ab1 (anti-hLH/hFSH, α-chain), then the samples of standard or clinical sera were placed into the wells, incubated and washed. The mixture of Y138F-Ab2 (anti-hFSH immunoglobulin) and W92F,H22E-Ab3 (anti-hLH immunoglobulin) conjugates was placed into the wells, incubated and washed thereafter. The violet label got bound with the hLH β-subunit, whereas the green one bound with the hFSH β-subunit. Bioluminescence of both labels was triggered by Ca^{2+} injection and measured sequentially: during the first second the violet signal was integrated through filter I, and after filter replacement the green signal was measured for 5

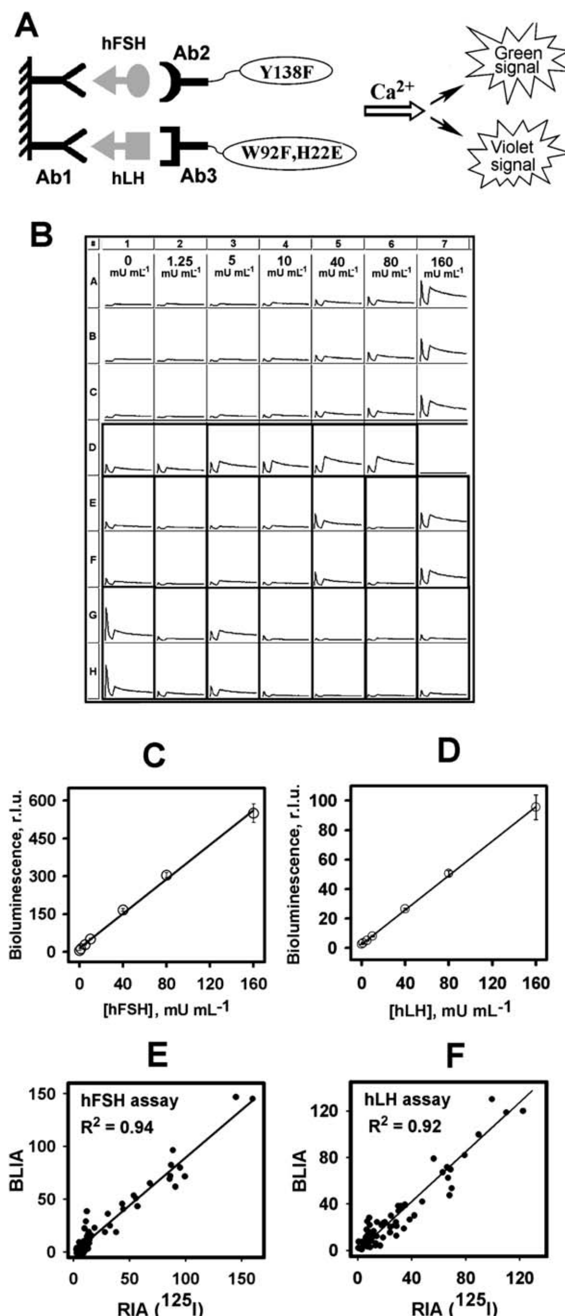

Fig. 2 (A). Schematic representation of the simultaneous solid-phase immunoassay of gonadotropic hormones. (B). Data output. Wells A1–C7, standard sera assay (in triplicates). Rows A1–A7, concentrations of hormones. Other cells present clinical sera assays (in two replicates each). (C and D) Bioluminescent standard sera microtiter-based immunoassay of hFSH (based on green signals) and hLH (based on violet signals), respectively. Each point is an average ± 1 standard deviation ($n = 3$). (E and F) Correlation of hFSH and hLH concentrations in 71 clinical samples obtained with simultaneous bioluminescent (BLIA) and separate isotopic (RIA) assays.

seconds. Fig. 2B gives the typically obtained results. The assay (in triplicate) of standard sera was performed in wells A1–C7. These samples are artificial sera, containing hLH and hFSH of a given equal concentration. The dependences of the violet bioluminescent signal upon hLH concentration and the green signal upon hFSH concentration (Fig. 2C and D) are linear for

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the whole concentration range, with $R^2 = 0.999$ and $R^2 = 0.997$, respectively. The hLH and hFSH bioluminescence assay sensitivities were 0.4 mU mL^{-1} and 0.34 mU mL^{-1} respectively (calculated from three replicates of standard zero sera assay, as mean + 2 standard deviation). These are close to sensitivities of separate radioisotope immunoassay – 0.34 mU mL^{-1} for hLH and 0.28 for hFSH (diagnostic kit from DIAS Ltd, Russia), and colorimetric immunoassay (0.3 mU mL^{-1}, diagnostic kit from XEMA, Russia). Using these dependence data as calibration curves, we determined the hLH and hFSH concentrations in 71 clinical sera. Some sera assays (in duplicate each) are presented in wells D1–H7. The data demonstrate good correlation between the proposed simultaneous bioluminescent assay and the traditional separate RIA (Fig. 2E and F): $R^2 = 0.92$, slope = 1.1 in the case of hLH and $R^2 = 0.94$, slope = 0.88 in the case of hFSH.

Another type of assay requiring detection of two targets (two gene allelic variants) is single nucleotide polymorphism (SNP) genotyping. We developed the method for SNP identification based on the primer extension reaction (PEXT)[7] followed by simultaneous alleles detection with bioluminescent solid-phase microassay. The study was performed as an example of SNP genotyping factor V Leiden polymorphism 1691 G→A (R506Q) in the gene encoding coagulation factor V. Identification of this polymorphism has prognostic value, which allows one to estimate the risk of cardiovascular diseases caused by blood clotting disorders and cardiovascular abnormalities in offspring. Coagulation factor V is the protein cofactor in the formation of thrombin from prothrombin. Mutation confers the resistance to the active form of factor V against proteolytic action of the regulating enzyme (activated protein C), which leads to hypercoagulation. Carriers of variant A have an increased propensity to vascular thrombosis, which represents a risk factor for venous and arterial thromboembolism, myocardial infarction, and stroke. This polymorphism is diagnostically valuable and widely analyzed in laboratory diagnostics.

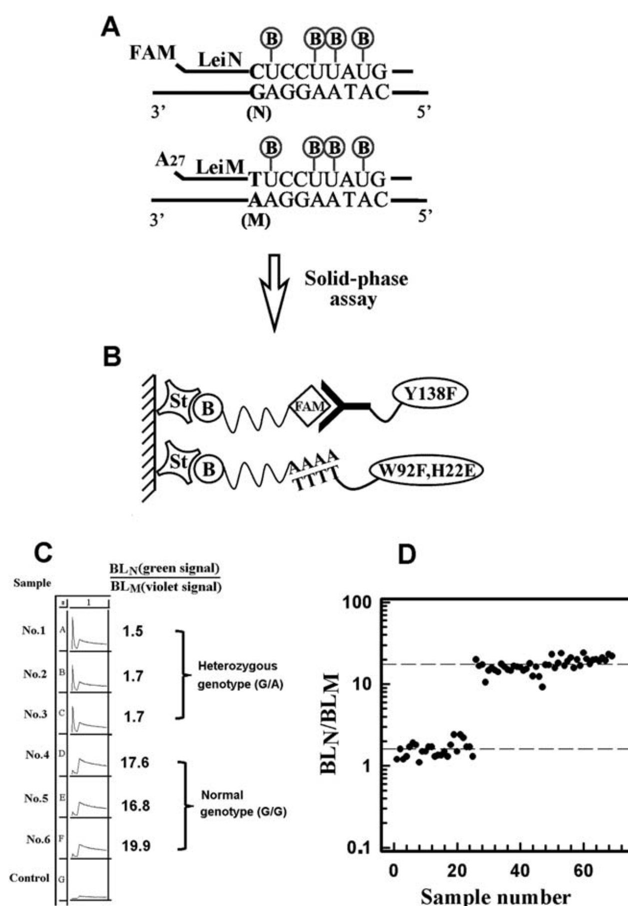

Fig. 3 (A) Schematic representation of PEXT reaction. (B) Simultaneous solid-phase assay of PEXT reaction products. (C) Data output on the assay of six clinical DNA samples. (D) Results of genotyping of 1691 G→A polymorphism in F5 gene (69 samples). St – streptavidin; B – biotinyl; FAM – fluorescein; BL_N/BL_M – green to violet bioluminescent signal ratio.

Specially selected DNA samples analyzed earlier by other methods were taken for the investigation. Genomic DNA was amplified by PCR using primers flanking a polymorphic site of 140 base pairs (dbSNP: rs6025). PCR products were used as templates for PEXT reaction with two primers containing 3′-terminal nucleotides, which were complementary to either normal or mutant alleles (Fig. 3A). The primer complementary to the normal allele was labeled with 6-carboxyfluorescein (FAM), the other with oligoadenylate $(dA)_{27}$. The reactions were carried out in the same tube. If the template and allele-specific primer were completely complementary, the latter was elongated with DNA polymerase. The resulting extension product contained a biotin residue due to the presence of biotinylated deoxyuridine triphosphate (B-dUTP) in the reaction mixture. The products were analyzed by solid-phase microassay in the same microtiter well according to the scheme presented in Fig. 3B. Biotin-containing oligonucleotides were immobilized on the streptavidin-activated well surface. Then the conjugate mixture (violet obelin–oligothymidylate and green obelin–anti-FAM IgG) was placed into the wells, incubated and washed. Obelins' bioluminescent reaction was initiated by Ca^{2+} and measured as was described above. The green signal is associated with the normal allele, while the violet one is associated with mutant allele availability. The genotype for each sample is defined by the ratio of the bioluminescent signals. Fig. 3C shows the assay of six clinical samples – three of them have normal genotype (with an average signals' ratio, BL_N/BL_M, of 18), whereas another three have a heterozygous genotype (with an average signals' ratio, BL_N/BL_M, about 1.6). The developed method was applied to investigate 69 clinical samples (Fig. 3D): 24 of them were of normal genotype, the others of heterozygous genotype. The samples with homozygous mutation have not been identified since this genotype is very rare. The results coincided completely with the real-time PCR data obtained by the staff of the Hematology Research Center (Krasnoyarsk Branch of the RAMS).

Reproducibility of the method was evaluated from the results of triplicate analysis (PCR of genomic DNA, PEXT reaction, and bioluminescent analysis of the reaction products) of two DNA samples of G/A and G/G genotypes. The variation coefficient of the discrimination factor (BL_N/BL_M) was 10.5% for the heterozygous G/A genotype and 14.4% for the normal G/G genotype.

There are no essential changes in the discrimination factor over a broad range of amplified DNA amount in PEXT reactions.

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So at assaying 10, 25, 50, 100 and 200 fmol of amplified DNA, the average discrimination factor values were 15.4 ± 2.85 and 1.58 ± 0.45 for G/A and G/G genotype samples, respectively.

Conclusion

In the present study, we have successfully applied two obelin mutants for simultaneous detection of two analytes in a single well, based on the spectral and time resolutions of bioluminescent signals. Both flash-type signals were triggered by a single injection of Ca^{2+} – no other substrates or co-factors were required. The total registration took only 4–6 s, despite the fact that the bioluminescence of labels was integrated sequentially. This distinguishes the proposed approach from the two-target simultaneous assays involving two different labels [*e.g.* see ref. 8–10]. We see obvious reasons for applying this technique in any dual assay regardless of its type – immune, hybridization or both. It is useful for large-scale research, and provides a fast and sensitive two-target assay that overcomes shortcomings of separate determination, in addition to the advantages in terms of cost and labor savings.

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