

Chapter 10

Multiplex Immunoassay Profiling of Serum in Psychiatric Disorders

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10.1 Introduction

Schizophrenia patients have a decreased life expectancy due to increased incidence of comorbidities, including insulin resistance and type II diabetes [1]. Although these effects can be brought about by treatment with antipsychotics [2], there is emerging evidence that such metabolic disturbances can also occur in schizophrenia subjects prior to treatment at their first clinical presentation [3]. The bloodstream contains hundreds of bioactive and regulatory proteins including hormones such as insulin and growth hormone which are likely to be involved in these metabolic disturbances. However, most of the polypeptide hormones are present at very low concentrations in the circulation, which necessitates that the biomarker measurement systems used should be highly sensitive. One of the best means for achieving this is through the use of antibody-based approaches like the multiplex immunoassay technique [4]. We showed that this system can be used to simultaneously measure abundant serum proteins such as clotting factors, immunoglobulins and apolipoproteins as well as hormones such as the insulin-related peptides and growth factors, in first onset patients with schizophrenia prior to antipsychotic treatment [5, 6].

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Most immunoassays rely on antibodies to capture the protein of interest in a biological matrix such as serum or plasma. Traditional immunoassays are targeted at detecting a single protein and often rely on reactions associated with covalently linked enzymes. New technological developments over the past two decades have now led to the situation where multiple proteins can be detected in a single measurement vessel. The capability of measuring multiple proteins simultaneously maximizes the amount of information that can be obtained from single sample. At the same time, this reduces sample volume requirements and laboratory analysis time and decreases costs. However, multiplexed assays have potential problems that would not be encountered if single assays were used for each individual analyte, as described elsewhere [7]. Examples of these problems include the potential for cross-reactivity and false positives due to antibody interactions. These challenges, if not carefully addressed, can generate misleading results [8–13].

The chapter describes the development of a 5-plex immunoassay for the Luminex® system, although the same principles will apply to other similar platforms (Fig. 10.1) [14–17]. The samples used were serum samples from schizophrenia patients and controls in attempt to gain some insights into the circulating metabolism-related hormones which may be affected in this disorder.

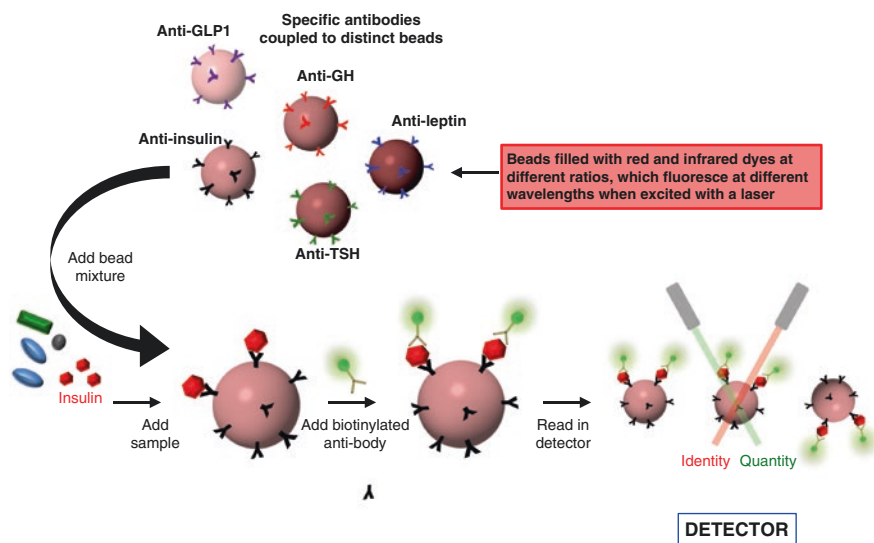


Fig. 10.1 Overview of the multiplex immunoassay protocol. Samples are added to dye-coded microbead-antibody conjugates that capture specific targets. Following incubation with a second antibody containing a biotinylated label to form a “sandwich” configuration, the mixtures are streamed through the Luminex instrument which uses lasers for identification of the antibody-microbead conjugates and quantitation of the bound hormones. The example shows a 5-plex assay capable of binding the targets glucagon-like peptide 1 (GLP1), growth hormone (GH), insulin, leptin and thyroid stimulating hormone (TSH) (although only the binding to the anti-insulin beads is shown in the flow chart)

Table 10.1 Demographic information associated with serum samples used in the study

	Schizophrenia patients	Controls
Male/female	161/75	152/78
Age (years)	31 ± 9	30 ± 9
Body mass index (kg/m ²)	24 ± 4	24 ± 4

10.2 Materials

10.2.1 Samples

1. Serum samples collected from 236 schizophrenia patients and 230 controls (Table 10.1) [6] (*see Note 1*)

10.2.2 Bead Conjugation

1. Magnetic separation device
2. Copolymer tubes and labels
3. 1–4 mL of magnetic 12.5×10^6 beads/mL Luminex microspheres/beads
4. 125 µg/mL monoclonal capture antibodies against glucagon-like peptide 1, growth hormone, insulin, leptin, and thyroid-stimulating hormone (*see Note 2*)
5. Sulfo-NHS, N-hydroxysulfosuccinimide
6. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)
7. Activation buffer: 100 mM NaH₂PO₄, pH 6.0
8. Coupling buffer: 0.05 M 2-morpholino-ethane-sulfonic acid monohydrate (MES) pH 5.0
9. Blocking/storage buffer: 10 mM NaH₂PO₄, pH 7.4, 150 mM NaCl, 0.02% Tween 20, 0.1% bovine serum albumin (BSA) and 0.05% NaN₃ (PBS-TBN)

10.2.3 Biotinylation of Detection Antibody

1. Epitope-distinct antibodies (from above) to glucagon-like peptide 1, growth hormone, insulin, leptin and thyroid-stimulating hormone (*see Note 2*)
2. Sulfo-NHS-LC biotin (ThermoFisher Scientific; Waltham, MA, USA)
3. PBS

10.2.4 Multiplex Development

1. Assay buffer: PBS, 1%BSA
2. Washing buffer: PBS, 0.02% Tween-20

3. 100 µg/mL streptavidin, R-phycoerythrin (SAPE)
4. 96-well plate
5. Magnetic separator (*see Note 3*)
6. Recombinant protein standards for growth hormone, insulin, leptin and thyroid-stimulating hormone
7. Heterophilic blocking reagents (*see Note 4*)

10.3 Methods

10.3.1 Bead Conjugation

1. Place vials containing 1–4 mL of stock microspheres upright on a flat magnetic separator for 2 min so that the beads can settle (*see Note 5*).
2. Be careful not to disturb the beads and remove and discard 0.8 mL of buffer for every 1 mL bead stock.
3. Pool the remaining volume into a single copolymer tube.
4. Place tube in the separator for 30–60 s.
5. Remove and discard the supernatant leaving the tube in the separator.
6. Add 0.5 mL activation buffer and suspend the beads by vortexing and sonication.
7. Place in the magnetic separator for 30–60 s.
8. Remove and discard supernatant with the tube in the separator and resuspend in 0.4 mL of activation buffer as above.
9. Add activation buffer to Sulfo-NHS to give a final concentration of 50 mg/mL.
10. Add 50 µL of this solution to the tube and vortex.
11. Add activation buffer to EDC to give a final concentration of 10 mg/mL.
12. Add 50 µL of this solution to the tube and vortex.
13. Incubate 20 min in the dark while rotating at room temperature (*see Note 6*).
14. Place the tube in the magnetic separator for 30–60 s, remove the supernatant and add 0.5 mL of coupling buffer.
15. Repeat for a total of 2 washes and resuspend in 0.45 mL of coupling buffer.
16. Add 0.2 mL of each capture antibody to five sets of activated microspheres with immediate vortexing (*see Note 7*).
17. Incubate 2 h in the dark while rotating at room temperature.
18. Place tube in magnetic separator for 30–60 s, remove supernatant and add 1.0 mL of blocking/storage buffer.
19. Resuspend and incubate 30 min with rotation in the dark at room temperature.
20. Place the tube in magnetic separator for 30–60 s, remove the supernatant and wash twice as above with 0.25 mL blocking/storage buffer.
21. Resuspend in 0.25 mL blocking/storage buffer, count the beads with a haemocytometer, adjust to 50×10^6 beads/mL and store at 2–8 °C.

10.3.2 Biotinylation

1. Prepare a 10 mM solution of the biotin reagent immediately before use.
2. Add 10 mM biotin reagent to the 5 antibody solutions at a 20:1 biotin-antibody molar ratio (*see Note 8*).
3. Incubate on ice for 2 h (*see Note 9*).
4. Remove excess biotin by dialyzing the reaction mixture using a minimum of three exchanges into PBS.
5. Add BSA to a final concentration of 1% and a preservative for long-term stability.

10.3.3 General Protocol

1. Create a capture bead mini-pool by adding 5 μL of each bead solution to a final volume of 1.4 mL assay buffer.
2. Make an 8 standard (S8) mini-pool by adding 0.2 μg of each recombinant protein to a final volume of 0.2 mL assay buffer, and create seven tenfold serial dilutions to generate a standard curve.
3. Make a mini-pool mix of detection antibodies by adding 5 μg of each biotinylated antibody to a final 5 mL assay buffer.
4. Produce 1:5 and 1:10 serial dilutions of serum and plasma samples in assay buffer (*see Note 10*).
5. Add 30 μL of each standard or sample to different wells of the 96-well plate.
6. Add 10 μL of blocking solution and then add 10 μL of the 5-plex capture beads.
7. Incubate the plate for 1 h on a shaker at room temperature.
8. Wash 3 times with 100 μL wash buffer, add 40 μL of the detection mini-pool to each well and incubate the plate for 1 h on a shaker at room temperature.
9. Add 20 μL SAPE to each well plate, and mix for 30 min on a shaker at room temperature (*see Note 11*).
10. Wash 3 times with 100 μL wash buffer.
11. Add 100 μL assay buffer.
12. Incubate the plate for 2–5 min on a plate shaker at room temperature.
13. Analyse on the Luminex 100 analyser.

10.3.4 Data Analysis

1. Carry out data analyses and determine the levels of each hormone in each sample (*see Note 12–14*).
2. Identify significant differences ($P < 0.05$) between schizophrenia patients and controls using Student's t-test for each hormone measurement (Table 10.2).

Table 10.2 Schizophrenia/control (SCZ/CONT) hormone ratios as determined using the 5-plex assay. The results showed significantly higher levels of insulin and significantly lower levels of growth hormone and thyroid-stimulating hormone in patients

Hormone	SCZ/CONT ratio	<i>P</i> -value
Glucagon-like peptide 1	2.03	0.1491
Growth hormone	0.54	0.0200
Insulin	1.65	0.0002
Leptin	1.16	0.2658
Thyroid-stimulating hormone	0.83	0.0213

10.3.5 Packaging and Use

1. Package all reagents individually in assay buffer using the volumes listed in the general protocol above.
2. Store beads, SAPE, and assay buffer at 4 °C and all other components at –80 °C.
3. Make 2–3 levels of assay quality controls (QC) by spiking recombinant protein into serum samples, and package these individually and store at –80 °C.
4. Screen assay performance and determine assay acceptance criteria by testing a minimum of 20 of each QC in duplicate prior to the next analysis.

10.4 Notes

1. The protocols of the study were approved by ethical committees from the Universities of Cologne, Muenster, Magdeburg in Germany, and from Erasmus University in the Netherlands [6].
2. For each hormone, one antibody is needed for capture and another for detection, and these should recognize distinct epitopes on the protein. For best results, a monoclonal antibody of high affinity is needed for capture and either a monoclonal or purified polyclonal antibody should be used for detection.
3. A plate washer can also be used.
4. We use Tru-Block from Meridian Life Sciences (Memphis, TN, USA).
5. Prior to coupling or biotinylation of the antibodies, they should be free of any amines and other protein. If using a Tris-based buffer, dialyse the antibody into 1× PBS using a minimum of three buffer exchanges. If the antibody preparation contains stabilizer proteins such as BSA or gelatin, purify with Protein A or Protein G columns, followed by dialysis.
6. The antibodies can be prepared during this incubation.
7. 1 mL of microspheres is sufficient for ~40 plates of assays.
8. The molecular weight of immunoglobulin G (IgG) is 150,000 kDa, so the amount of biotin required for a 1 mL of a 1 mg/mL antibody solution could be calculated as follows: 1 mL IgG × 1 mg/mL × 20 mmol biotin/1 mmol

$\text{IgG} \times 1 \text{ mmol IgG}/150,000 \text{ mg IgG} \times 1000 \text{ ul/mL} = 0.133 \text{ mmol biotin} = 13 \text{ uL of the } 10 \text{ mM biotin solution.}$

9. The incubation can also be carried out at room temperature for 30 min.
10. The assay will be multiplex based on the dilution requirement of the samples. In general, if the expected levels are in the pg/mL or low ng/mL range, they will require the same dilution of approximately 1:5 or 1:10.
11. The SAPE concentration will vary with the number of analytes in the multiplex.
12. Carry out curve optimization by varying sample dilutions if required as described previously [7].
13. Carry out blocker optimization using different reagents if required as described previously [7].
14. Assess cross reactivity by comparison of the multiplex results with corresponding single-plex assays if required as described previously [7].

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