

parallel sample handling

improves performance of ligand binding assays

with solubilised receptor protein

ABgene®'s Dye Terminator Removal Kit is designed for the removal of unincorporated dye terminators from gene sequencing reactions in a 96-well format. This application note describes a novel screening application of this kit.

Handling solubilised G-protein coupled receptors is often quite difficult, especially in relation with ligand binding assays. Receptor activity is commonly assessed by rapid gel filtration allowing the separation of small, unbound ligand molecules from ligands bound to the receptor. This method, which is described for several receptors in detail in the literature¹⁻⁴, is performed with single columns, preventing high sample throughput.

In this application note we describe high throughput ligand binding assays with solubilised receptors employing rapid gel filtration in a 96-well format, allowing screening with the following advantages:

- dissociation of low affinity ligands from the receptor (of ligands binding with low affinity) is reduced by rapid application of the sample to the gel bed;
- the parallel processing of a high number of samples which allows high throughput screening;
- the use of purified membrane proteins, such as solubilised GPCRs, as target molecules minimises unspecific background signals;
- the small sample volume reduces costs significantly.

The company m-phasys also used the screening technology for ligand binding studies with refolded GPCRs, produced from *E. coli* inclusion bodies with its proprietary M-FOLD™ refolding technology.

Materials

- β2AR Sf9 membranes (APB, Biosignal, 6110106, X-21)
Specific activity: 1.8pmol/ mg membrane protein
Concentration: 3.4mg/ml in
50mM Tris/HCl pH 7.4, 10% glycerol, 1% BSA
- Dye Terminator Removal Kit (ABgene®, AB-0943)
- 96-well Standard Microplate, polypropylene (ABgene®, AB-0796)
- 96-well sample plate, Isoplate (Wallac, 1450-514)
- 1M Tris/HCl pH 7.4
- 1M NaCl
- 0.5M EDTA
- Glycerol
- PMSF (Sigma)
- Aprotinin (Sigma)

- Detergent
- ³H-dihydroalprenolol (³H-DHA, APB)
- (-)-Propranolol (Tocris)
- Centrifuge capable of processing 96-well plates
- Ultima gold MV (Packard)
- Microbeta 1450 (Wallac)

Membrane solubilisation

170µg of Sf9 membranes (50µl) were diluted in 500µl of ice-cold incubation buffer (20mM Tris pH 8.0, 100mM NaCl, 2mM EDTA, 10% glycerol). The membranes were pelleted by ultracentrifugation (5 min, 200,000 x g at 4°C). The pellet was solubilised carefully in 500µl of solubilisation buffer (20mM Tris pH 8.0, 100mM NaCl, 2mM EDTA, 10% glycerol, 10µM PMSF, 50µM Aprotinin and 10mg/ml detergent). The membranes were incubated for 1h at 4°C by gentle shaking. Unsolubilised material was removed by ultracentrifugation (5 min, 200,000 x g at 4°C).

Binding assay

For ligand competition assays, seven different ligand concentrations were used (in triple determination). Into each well of a standard polypropylene 96-well microplate the following was added in order:

- 8µl of the ligand solution (2.5nM ³H-DHA, 20mM Tris pH 7.4, 100mM NaCl, 2mM EDTA, 10% glycerol, 25mM MgCl₂),
- 2µl of the 10-fold concentrated cold ligand solution (1nM – 1µM (-)-Propranolol in 20mM Tris pH 7.4) and
- 10µl of the solubilised receptor.

After mixing, the sample was incubated at 25°C for 30 min by gentle agitation. At the end of the incubation time the plate was cooled down on ice.

Ligand separation

The storage buffer of the separation plate (Dye Terminator Removal Kit, ABgene®) was removed by centrifugation (Eppendorf centrifuge 5804 R with rotor A-2-DWP, 3 min, 200 rpm, 4°C) and the eluate was collected in a 96-well sample plate. The gel matrix was equilibrated by washing three times with 200µl of cold washing buffer (20mM Tris pH 7.4, 100mM NaCl, 2mM EDTA, 12.5mM MgCl₂, 0.4mg/ml detergent) with an additional centrifugation step for removal of any excess liquid. Then 20µl of the cooled samples were

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applied carefully onto the gel beds using a multichannel pipette. The plate was centrifuged immediately (3 min, 1,500 rpm, 4°C). The eluates were collected in a 96-well sample plate and mixed with 180µl of scintillation cocktail (Ultima gold MV, Packard). The activity was measured in a Microbeta 1450 (Wallac) scintillation counter.

Conclusion

One application of gel filtration, or size exclusion chromatography, is the separation of unbound ligands from receptor bound ligands in binding assays. Gel filtration separates mixtures of biomolecules according to size. Therefore, the performance is independent of the buffer conditions (detergent, salt, pH) and the state of the protein (charge, conformation, oligomerisation). Because the separation is driven by centrifugation the same force acts on each molecule at the same time and the macromolecules will be eluted equally and fast. In contrast to other filtration techniques, additional washing steps, which always result in additional dissociation of the bound ligands, are not necessary. Using the ABgene® Dye Terminator Removal Kit separation plates, parallel sample handling enables high throughput screening and the small volumes processed reduce costs significantly.

References

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For further details and ordering information for ABgene®'s Dye Terminator Removal Kit, please see page 6.