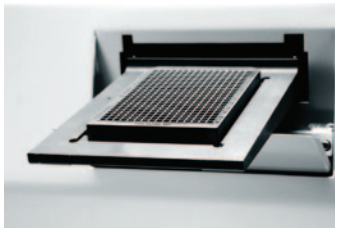


High Throughput Arrayed NF-κB Transcription Factor Profiling

Transcription factors are sequence-specific DNA-binding proteins intensively studied because of their critical role in gene regulation. The transcription factor nuclear factor-κB (NF-κB) is a key component for the inducible expression of a wide variety of target genes involved in the cellular response to infection and inflammation. NF-κB is composed of homodimeric and heterodimeric complexes of related proteins of the Rel superfamily. The prototypical NF-κB complex is composed of a heterodimer containing p65(RelA) and p50 proteins. The ability to accurately monitor the activation of NF-κB coupled with the identification of the proteins involved in the transcription factor complex is essential for many drug and therapeutic development efforts. Until recently, such research projects were time-consuming and difficult to implement in the context of high throughput screening environments.

This application note describes the use of a transcription factor profiling assay consisting of arrayed double-stranded oligonucleotides (ds-DNA) for the detection of sequence specific DNA binding proteins as measured with the **ImageXpress® Velos** System. A schematic depiction of the assay is shown in **Figure 1**. The **ImageXpress Velos** System is a high throughput multiparametric screening platform for fluorescence measurements from array, bead and cell-based assays. Fast scanning speeds allow throughputs of 120 seconds/plate, at a resolution of 10 microns regardless of the plate well density. As shown here this arrayed, highly-parallel assay format coupled with fast scanning by the **ImageXpress Velos** System meets the requirements for implementation in high throughput screening environments showing robust sensitivity, specificity, and reproducibility.

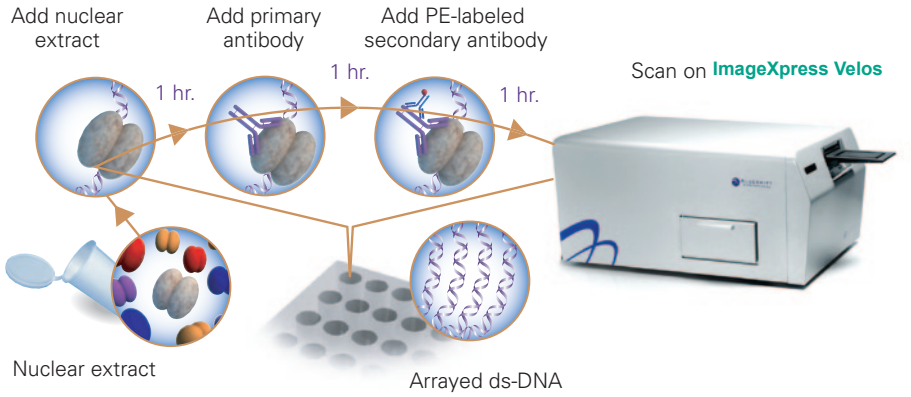


Figure 1. Arrayed Transcription Factor Profiling assay schematic.

Experimental

Oligonucleotides containing the wild type κB motif 5′-GGGACTTTCC-3′ or mutant 5′-G**CC**ACTTTCC-3′ (bold face type indicates the mutated sites) were obtained from Active Motif (Carlsbad, CA). One strand contained an amino modified 5′end. The amino-modified ds-DNA binding sequence for NF-κB was pin-spotted to epoxy coated glass slides (Slide E, Schott-Nexterion) by Advansta (Menlo Park, CA) in a 2x8 well format (96 multi-well plate spacing). The array in each well consisted of eight spots (~150μm diameter) of ds-DNA of wild-type and eight of the mutant κB motif. The slides were printed, blocked, sealed in moisture barrier resistant bags (Advansta), shipped to Active Motif and stored at 4°C until use. The protocol from Active Motif's NF-κB TransAM™ kit (cat. #40096) was adapted for these experiments.

Protocol

1. Add samples consisting of recombinant p65 (Active Motif, cat. # 31102) or stimulated (TPA + calcium ionophore) Jurkat nuclear extract (Active Motif, cat. #36013) diluted in the TransAM™ lysis buffer (20μl) to each well containing 30μl of binding buffer. Incubate on a shaker for 1 hour at RT.
2. Wash 3 times with wash buffer.
3. Add 100μl of binding buffer containing a 1:1000 dilution of rabbit anti-p65 antibody (Active Motif, NF-κB TransAM™ kit, cat. # 40096) to each well and incubate at RT for 1 hour with shaking.
4. Wash 3 times with wash buffer.
5. Add 100μl (1:1000 dilution) of phycoerythrin (PE)-conjugated anti-rabbit IgG (Molecular Probes, Eugene, OR) to each well and incubate at RT for 1 hour with slide covered by aluminum foil.
6. Wash 4 times with wash buffer.
7. Disassemble slide chamber gasket, hold slide at an angle and rinse once with wash buffer.
8. Place slide into a 50ml plastic conical tube, spin dry and scan on the **ImageXpress Velos** System configured with a 488nm laser. The fluorescent signal was filtered through a 540-610nm band pass filter.

All instrument parameters for data acquisition were defined by a single "Method" file. BlueImage™ analysis software was used to quantify the degree of p65 binding to wild type and mutant ds-DNA by measuring the mean fluorescence intensity from eight spots. The appearance of the fluorescent signal due to p65 binding to the arrayed ds-DNA is shown in **Figure 2**.

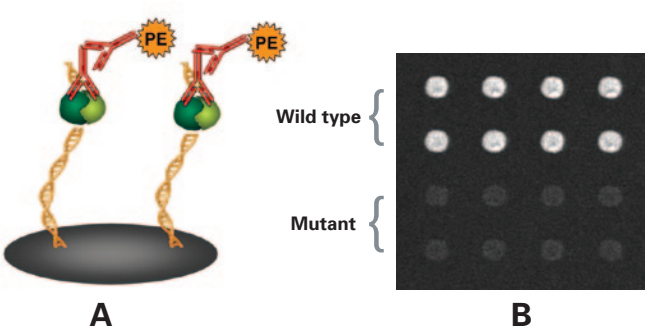


Figure 2. A. Assay schematic. **B. ImageXpress Velos** System image showing the binding of recombinant p65 (1 ng/well) to wild type ds-DNA (upper eight spots) with low binding to mutant ds-DNA (lower eight spots).

Assay Sensitivity, Specificity, & Reproducibility

The model assay format consisted of the same array of ds-DNAs printed in 16-wells on each slide for the detection of the p65(RelA) NF-κB family member. A standard curve demonstrating the binding sensitivity of recombinant p65 to the ds-DNA array was generated by performing a 1:2 dilution series of the protein, as shown in **Figure 3A**. Note, the data is not background subtracted in order to show the sensitivity, specificity, and background of the assay. The detection limit of the assay, measured as signal - background equivalent to three times the standard deviation of the background, was ~0.1 ng/well.

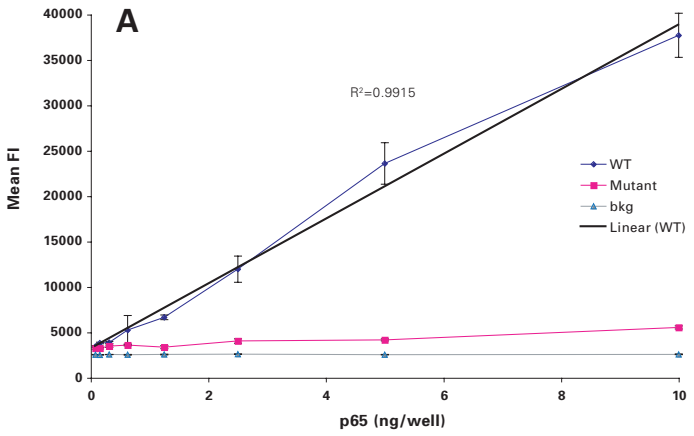
Figure 3B demonstrates the detection of p65 in increasing amounts of nuclear extracts prepared from TPA and calcium ionophore-stimulated Jurkat T cells. The data demonstrates that the assay has a linear response to the amount of p65 in cell nuclear extracts and shows sequence specificity with a high signal-to-noise ratio with a low background. To evaluate assay reproducibility 14 wells were incubated with 5 ng/well of p65. The assay was very reproducible with a mean ± standard deviation (SD) of 13,132 ± 537 fluorescence intensity (FI) units with a CV of 4%.

Conclusions

Compared with previously available methods the use of the **ImageXpress Velos** System enables fast data acquisition and analysis of multiple transcription factor binding sites per well, across multiple wells on each slide, and for higher throughput in 96 or 384-well epoxy-coated glass plates (Schott-Nexterion). Although the data presented here relate specifically to the p65(RelA) NF-κB family member, screening for a number of different transcription factors separately or in each well is enabled by simply printing ds-DNAs with the transcription factor binding site of interest. In summary, the **ImageXpress Velos** System is well suited for rapidly:

- measuring transcription factors in nuclear extracts
- profiling the consensus DNA-binding site for novel engineered transcription factors
- screening for small molecule inhibitors of sequence-specific DNA-binding proteins

Custom assays designed with the content needed to study any combination of transcription factors are now possible for high throughput screening environments. For more information please contact us (info@moldev.com).



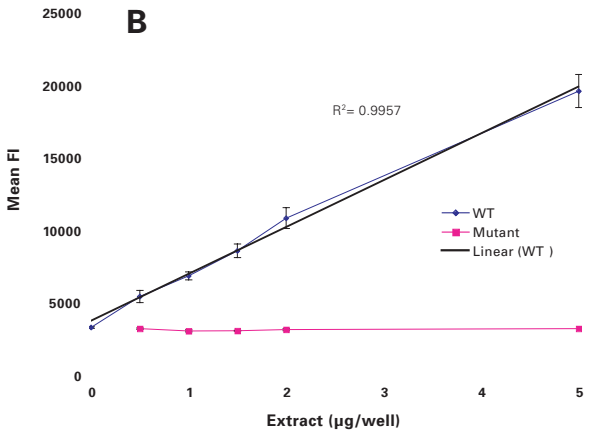


Figure 3. A. Standard curve for recombinant p65 binding to wild type or mutant ds-DNA. **B.** Quantitation of p65 in increasing amounts of stimulated nuclear extracts (TPA and ionophore-treated Jurkat cells).

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