

TranSignal™ TF-TF Interaction Arrays

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Product User Manual
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Contents

1. INTRODUCTION	3
2. MATERIALS PROVIDED	6
3. ADDITIONAL MATERIALS REQUIRED	7
4. PREPARING NUCLEAR EXTRACT FROM CELLS OR TISSUES	8
5. PREPARING TF-BOUND DNA	8
6. IMMUNOPRECIPITATION AND ELUTION	9
7. HYBRIDIZATION	11
8. DETECTION	12
9. RESULTS & ANALYSIS	13
10. TROUBLESHOOTING GUIDE	14
11. REFERENCES	15
APPENDIX A: Recipes & instructions for diluting stock solutions	15
APPENDIX B: Schematic diagram of the TranSignal TF-TF Interaction Array I	16
APPENDIX C: Schematic diagram of the TranSignal TF-TF Interaction Array II	17
APPENDIX D: Schematic diagram of the TranSignal TF-TF Interaction Array III	18
APPENDIX E: TranSignal TF-TF Interaction Array Grid	19
APPENDIX F: Stripping Procedure for TranSignal Arrays	20

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1. INTRODUCTION

The human genome encodes approximately 35,000 genes. While some of these, called housekeeping genes, are constitutively expressed, most are not. Non-housekeeping genes are expressed in a particular type of cell, under specific environmental conditions, or at a certain stage of development. The expression of such genes must be tightly regulated.

Gene expression is regulated by a group of proteins known as transcription factors (TFs). An estimated 2,000 TFs are at work in any given human cell. By binding to specific DNA *cis*-elements, each TF contributes a different functional role in gene expression.

Just as TFs regulate gene expression, the TFs themselves are regulated in a number of ways. Known as combinatorial regulation, these different levels of regulation allow gene expression to be tightly controlled. TFs may be regulated by protein modification, such as phosphorylation; protein translocation; and/or protein interaction. In the latter category, TFs may interact with upregulators or downregulators in the basal transcription machinery, or with other transcription factors.

A novel method for detecting TF-TF interactions

Traditionally, TF-TF interactions have been studied by co-immunoprecipitation and super-gel shift. But because these methods are notoriously time consuming and inefficient, they are not conducive to mapping the network of TF-TF interactions.

TranSignal™ TF-TF Interaction Arrays enable you to determine how a particular TF interacts with multiple other TFs—all in one experiment. This technology is based on the non-covalent binding of DNA binding sequences to TF proteins. After precipitation with specific antibodies against a target TF, the co-precipitated TFs are determined by array analysis of attached DNA sequences. The advantage of this technology is its capability of high-throughput analyzing interactions of native TF proteins in a given sample. Figure 1 illustrates how this simple procedure works. First, incubate your nuclear extract with the provided set of biotin-labeled double-stranded oligonucleotide probes, which represent a known library of *cis*-elements. During the incubation step, these probes bind to their corresponding TFs in your nuclear extract. The next step is immunoprecipitation: using a specific antibody against your TF of interest, pull out that TF and associated TFs, along with corresponding *cis*-elements. Next, wash away free *cis*-

elements and nonspecific binding proteins. Finally, elute the *cis*-elements and hybridize them to the TranSignal™ Protein/DNA Array membrane.

We currently offer three versions of the TranSignal™ TF-TF Interaction Array. Version I is spotted with 54 different consensus sequences; Version II is spotted with 96 different consensus sequences; and Version III is spotted with 94 different consensus sequences. (Maps of Versions I, II and III are provided in Appendices B, C and D, respectively.) By combining all of these versions, you can profile the interactions of 244 unique TFs. However, the TranSignal TF-TF Interaction Array system can be easily expanded to include up to 2,000 consensus sequences.

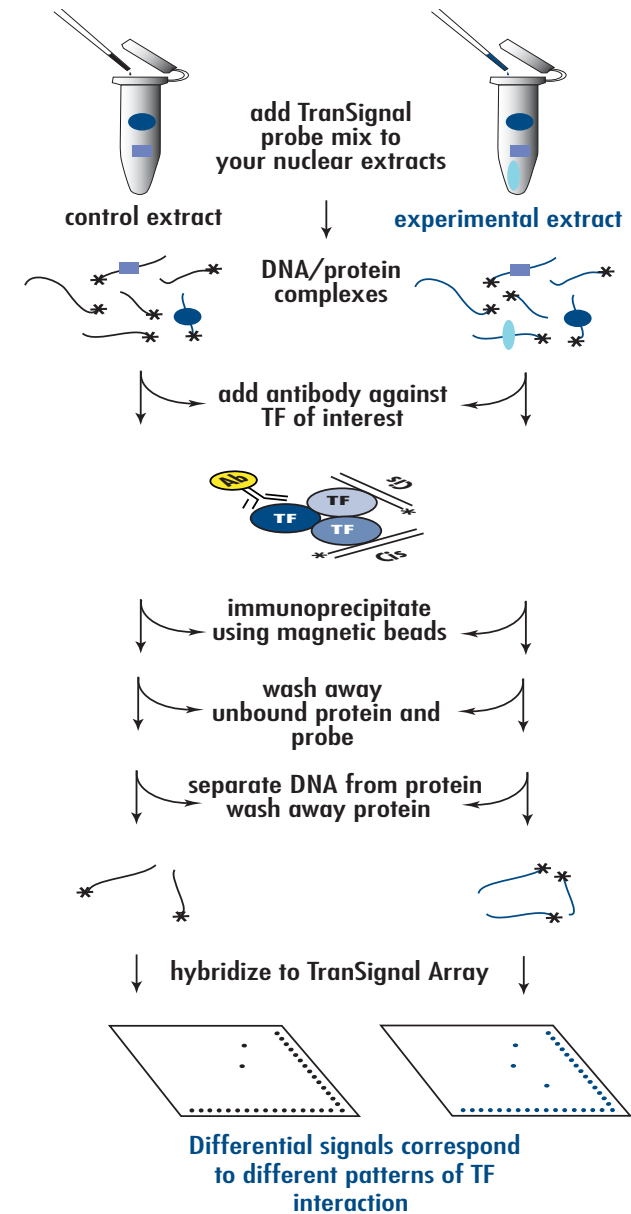


Figure 1: Schematic flow chart of the TranSignal™ TF-TF Interaction Array procedure.

2. MATERIALS PROVIDED

(See product label for appropriate storage conditions)

Box 1: Array Membranes & Hybridization Reagents

- TranSignal TF-TF Interaction Array (3 each)
- Hybridization Buffer (15 ml)
- p53 Goat Polyclonal, IgG (10 µl; 200 µg/ml)
- Substrate Solution I (3 ml)
- Substrate Solution II (3 ml)
- Substrate Solution III (5 ml)
- 1000x Streptavidin HRP Conjugate (60 µl)
- 20X SSC (32 ml)
- 20% SDS (15 ml)
- 4X Wash Buffer (45 ml)*
- 10X Detection Buffer (25 ml)*
- Distilled H₂O (RNase & DNase free; 300 µl)

Box 2: Reaction Kit

- TranSignal TF-TF Probe Mix (60 µl)
- Poly d(I-C) (50 µl)
- Control Nuclear Extract (from A431-PMA; 10 µl)
- 2X Blocking Buffer (30 ml)*
- 5X Binding Buffer (100 µl)
- 5X IP Wash Buffer (2 x 1.5 ml)*
- 1X IP Dilution Buffer (1.5 ml)
- 1X IP Elution Buffer (0.5 ml)

* These reagents are provided as stock solutions. See Appendix A for instructions on how to dilute these solutions/buffers.

3. ADDITIONAL MATERIALS REQUIRED

3.1 Reagents and Solutions

- Nuclear Extraction Kit (Panomics, Cat. # AY2002)
- Hybridization Wash I (2X SSC/0.5% SDS)*
- Hybridization Wash II (0.1X SSC/0.5% SDS)*
- Deionized H₂O
- Antibody directed against your TF of interest.
- [Optional] Normal IgG Antibody for your negative control during immunoprecipitation. (Choose a normal IgG antibody that is specific to your antibody of choice.)

* See Appendix A for recipes.

3.2 Materials and Equipment

- Dynabeads with either protein A (DynaL Biotech, Cat# 100.01) or protein G (DynaL Biotech, Cat# 100.03) depending on antibody used for immunoprecipitation. *Please do not use agarose or sepharose beads as these bind non-specifically to the DNA probes, providing false positive results.*
 - Magnetic separation stand (Promega, Cat. # Z5332)
 - 0.5-ml and 1.5-ml tubes
 - Pipetman and tips
 - Microcentrifuge
 - Hybridization oven and bottles † (Stratagene, Cat.# 401030)
- † **Note:** skirted centrifuge tubes with screw caps may be used in place of the hybridization bottles (VWR, Cat. # 21008-480). Hybridization bottle dimensions: 150 mm x 35-mm diameter tubes.
- Plastic containers (~4.5" x 3.5"; equivalent to the size of a container for 200-ml pipet tips)
 - Heating block or water bath
 - Shaker
 - Plastic sheet protectors or overhead transparencies
 - Hyperfilm ECL (Amersham, Cat.# RPN3114K)
- OR
- Chemiluminescence imaging system (e.g., FluorChem from Alpha Innotech Corp.)

4. PREPARING NUCLEAR EXTRACT FROM CELLS OR TISSUES

Nuclear extracts can be prepared using the method described by Dignam *et al.* (1). Alternatively, you can use a commercially available kit, such as Panomics' Nuclear Extraction Kit (Cat. # AY2002).

5. PREPARING TF-BOUND DNA

In this step, you will incubate your nuclear extract with the provided set of biotin-labeled double-stranded oligonucleotide probes. During the incubation step, TF probes will associate with extract TFs to form labelled complexes. Be sure to dilute solutions and buffers, as described in the Appendix.

- 5.1 For each nuclear extract sample, combine the following components into a sterile 1.5-ml microcentrifuge tube. Prepare an additional sample for your negative control.

Nuclear Extract*	10 µl
Poly d(I-C)	10 µl
TranSignal TF-TF Probe Mix	10 µl
5X Binding Buffer	15 µl
dH ₂ O (RNase, DNase free)**	30 µl
Total Volume	75 µl

*Use Control Nuclear Extract (A431-PMA) for positive control. We recommend using 30-100 µg of nuclear extract per reaction.

**Adjust the volume of dH₂O based on the concentration of your nuclear extract.

- 5.2 Mix well by pipeting.
- 5.3 Incubate samples at 15°C for 30 min. Afterwards, incubate on ice for an additional 30 min.

6. IMMUNOPRECIPITATION & ELUTION

BEFORE YOU START: Chill all solutions to 4°C prior to use. Dilute solutions accordingly (see Appendix A). If you plan to proceed directly to overnight hybridization, we recommend that you first prehybridize your membranes (see Section 7.1, 7.2) before beginning with Section 6. **Estimated time: 2–3 hours.**

Using a specific antibody against your TF of interest, you will pull out that TF and any associated TFs, along with corresponding cis-elements. Wash away free cis-elements and nonspecific binding proteins; then, elute the cis-elements for use as probe.

NOTE: Antibodies bind with different affinities to protein A or protein G. We recommend that you use the chart below to choose the best affinity Dynabeads for your specific antibody. **Note:** Do not use agarose or sepharose beads as these bind non-specifically to DNA, providing false positive results.

Ig origin	Dynabeads
mouse IgG1	protein G
mouse Ig2a, Ig2b	protein A*
mouse IgG3	protein G
rabbit IgG1	protein A* or protein G
goat IgG	protein G

*Add 0.1% BSA to 1X IP Dilution Buffer and 1X IP Wash Buffer for protein stability.

- 6.1 To the tube prepared in step 5, add:

IP Dilution Buffer, 200 µl
Antibody for your TF* (~200 ug/ml), 10 µl

Note: The amount of antibody for immunoprecipitation is variable; adjust the amount accordingly.

*For your negative control, replace antibody with normal IgG. For your positive control, replace antibody with p53 Goat Polyclonal, IgG.

- 6.2 Mix well by gently tapping the tube. Place sample on rocking platform and rock gently for 90 min at 4°C.
- 6.3 Wash Dynabeads
 - 6.3.1 Resuspend the Dynabeads thoroughly in vial.
 - 6.3.2 Transfer 75 µl of the Dynabead solution to 1.5 ml microcentrifuge tube.
 - 6.3.3 Place tubes in magnetic stand for one minute (beads will precipitate out). Pipet off fluid and discard.
 - 6.3.4 Equilibrate the beads by resuspending them in 200 µl of 1X IP Dilution Buffer.
 - 6.3.5 Place tube in magnetic stand for one minute. Carefully, remove and discard the supernatant without disturbing the beads attached to the wall of the tube.
- 6.4 Transfer the DNA/protein/antibody complex (prepared in Step 6.2) to the tube containing the washed beads. Rock gently on the rocking platform at 4°C for 1 hr.
- 6.5 Collect beads:
 - 6.5.1 Briefly spin tube in a microcentrifuge at 2,000 rpm for 2 sec.
 - 6.5.2 Place tube in magnetic stand for 2 min to collect beads. Keeping tubes on magnetic stand, pipet off fluid and discard (do not disturb pellet).
- 6.6 Wash beads with 1X IP Wash Buffer:
 - 6.6.1 Add 400 µl of pre-chilled 1X IP Wash Buffer to tube containing beads. Completely resuspend the beads by gently tapping the tube. **IMPORTANT:** Gently **invert** the tube four times to clean the sides of the tube.
 - 6.6.2 Spin the tube in a microcentrifuge at 2,000 rpm for 2 sec.
 - 6.6.3 Place the tube in magnetic stand for 2 min to collect beads. Pipet off fluid and discard. **Important: Be sure to pipet off as much fluid as possible in this step.**
 - 6.6.4 Repeat Step 6.6.1–3 three times.
- 6.7 Elute probe from beads:
 - 6.7.1 Preheat heat block to 100°C. Heat the Elution Buffer to dissolve precipitates before use.

- 6.7.2 Add 60 µl of 1X IP Elution Buffer to tube containing beads. Gently mix.
- 6.7.3 Incubate at 100°C for 5 min.
- 6.7.4 Transfer to ice immediately. Keep on ice for 2 min.
- 6.7.5 Spin tube in a microcentrifuge at 2,000 rpm for 5 sec.
- 6.7.6 Place tube in magnetic stand for 2 min to collect beads on ice (i.e., place magnetic stand on ice with tubes).
- 6.7.7 **Important:** Transfer the fluid to a clean tube and place tube on ice. This is your eluted probe. The beads can be discarded. If you do not plan to use the probe immediately, store the probe at –20°C. Prior to use, thaw probe, and repeat steps 6.7.3 and 6.7.4, to ensure complete denaturation of double-stranded DNA.

7. HYBRIDIZATION

In this Section, you will hybridize the eluted probe (prepared in Section 6) to the array membrane.

- 7.1 Place each array membrane into a hybridization bottle. To wet the membrane, fill bottle with deionized H₂O. Then, carefully decant water.
- 7.2 To each hybridization bottle that contains an array membrane, add 3–5 ml of prewarmed Hybridization Buffer (provided). Place each bottle in the hybridization oven at 42°C for 2 hr.
- 7.3 Add the eluted probe from step 6.7.7 to each hybridization bottle and hybridize at 42°C overnight.
- 7.4 Decant the hybridization mixture from each hybridization bottle, and wash each membrane:
 - 7.4.1 Add 50 ml of prewarmed Hybridization Wash I*, incubate at 48°C for 20 min in a rotating hybridization oven. Decant liquid and repeat wash.
 - 7.4.2 Add 50 ml of prewarmed Hybridization Wash II*, incubate at 48°C for 20 min in a rotating hybridization oven. Decant liquid and repeat wash.

* See Appendix A for recipes.

8. DETECTION

IMPORTANT: Do not allow the membrane to dry during the detection. Dilute all solutions before use (see Appendix A). Pre-warm the 1X Blocking Solution and 1X Wash Buffer at 37°–50°C until all particulates are dissolved.

- 8.1 Using forceps, carefully remove each membrane from its hybridization bottle and transfer to a new container containing 20 ml of 1X Blocking Buffer; each membrane needs its own container. (We use a container that is equivalent to the size of a 200-ml pipet-tip container, ~4.5" x 3.5".)
- 8.2 Incubate at room temperature for 30 min with gentle shaking.
- 8.3 Transfer 1 ml of Blocking Buffer from each membrane container to a fresh 1.5-ml tube. To each, add 20 µl of 1000x Streptavidin-HRP Conjugate and mix well. Return each mixture to the appropriate container and incubate at room temperature for 20 min.
- 8.4 Decant the Blocking Solution containing the antibody. Wash each membrane three times at room temperature with 20 ml of 1X Wash Buffer, each 5 min.
- 8.5 Add 20 ml of 1X Detection Buffer to each membrane and incubate for 5 min at room temperature.
- 8.6 To prepare the substrate solution, mix 1 ml Substrate Solution I and 1 ml Substrate Solution II. Add 1 ml Substrate Solution III. Mix well.
- 8.7 Using a plastic sheet protector, overhead transparency film, or plastic wrap (whichever is readily available), place each membrane on a plastic sheet. Then, pipet 3 ml of substrate solution onto each membrane and overlay each with a second plastic sheet. Ensure that substrate is evenly distributed over the membrane with no air bubbles. Incubate at room temperature for 5 min.
- 8.8 Remove excess substrate by gently applying pressure over the top sheet. Using a paper towel, remove excess substrate that might be remaining on the surface of the sheets. Expose the membranes using either Hyperfilm ECL or a chemiluminescence imaging system, such as the FluorChem imager from Alpha Innotech Corp. In either case, we recommend that you try several different exposures of varying lengths of time (e.g., 2–5 min). Overexposure of your blot may result in excessive background.

9. RESULTS & ANALYSIS

The main advantage of the TranSignal TF-TF Interaction Array is that you can detect the interaction of a protein with multiple transcription factors simultaneously. Follow these guidelines to analyze your results:

- 9.1 Acquire the images using either x-ray film or a chemiluminescent imaging system.
- 9.2 Adjust the exposure time such that the biotin spots (along the right and bottom sides of the membrane) have equal signal intensity.
- 9.3 Compare the images between negative control (normal IgG) and experimental. All the spots that appear in your experimental should be positive; in contrast, the negative control should not have any spots. If the image of the negative control has some weak spots, then you'll need to check whether the same spots also appear in the experimental. If so, the spots in the experimental may represent false positives. To confirm your results, perform co-immunoprecipitation or super-gel shift assays.

10. TROUBLESHOOTING GUIDE

Problem	Cause	Recommendation
Uneven background	Substrate is not evenly distributed on the membrane.	Do not use thin cling wrap materials during the overlay procedure Re-detect with substrate that is evenly covering the membrane surface.
High Background	Incubation with substrate is too long.	Expose longer—10 to 15 min Incubation should not exceed 5 min.
Signal is too weak.	The yield of recovered DNA probe is low.	Confirm using control nuclear extract (provided). Check the concentration of nuclear extract.
No Signal	The antibody may be not suitable for IP	Test the antibody before applying to the array.

11. REFERENCES

- Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Research* 11:1475–1489.

APPENDIX A:

Recipes & instructions for diluting stock solutions

SECTION 6

- **15 ml of 1X IP Wash Buffer**
To 12 ml of deionized H₂O, add 3 ml of 5X IP Wash Buffer (provided).

SECTION 7

- **300 ml of 2X SSC/0.5% SDS (Hybridization Wash I)**
To 262.5 ml of deionized H₂O, add 30 ml of 20X SSC (provided) and 7.5 ml of 20% SDS (provided). Mix well.
- **300 ml of 0.1X SSC/0.5% SDS (Hybridization Wash II)**
To 291 ml of deionized H₂O, add 1.5 ml of 20X SSC (provided) and 7.5 ml of 20% SDS (provided). Mix well.

SECTION 8

- **60 ml of 1X Blocking Buffer**
To 30 ml of deionized H₂O, add 30 ml of 2X Blocking Buffer (provided). Mix well and store at 4°C.
- **200 ml of 1X Wash Buffer**
To 150 ml of deionized H₂O, add 50 ml of 4X Wash Buffer (provided). Mix well.
- **100 ml of 1X Detection Buffer**
To 90 ml of deionized H₂O, add 10 ml of 10X Detection Buffer (provided). Mix well.

APPENDIX F: Stripping Procedure for TranSignal Arrays

Note: We do not encourage stripping the TranSignal™ array membranes more than two times.

Procedure

1. Wash membranes in 0.4M NaOH at 45°C for 30 min .
2. Wash membranes in 0.2M Tris-HCL, pH 7.6; 0.1X SSC, and 0.1% SDS at 45°C for 15 min .
3. To ensure that stripping was successful, run it through the standard chemiluminescence detection procedure as described in this user manual.
4. After detection, wash the membrane in 1X Washing Buffer at 42°C for 30 min.
5. Membranes are ready for hybridization or dry the membrane in an 80°C incubator for later use.

Notes: