Catalog #LNB0004

Transcription Factor
Buffer Reagent Kit

www.invitrogen.com
Invitrogen Corporation
542 Flynn Rd, Camarillo, CA 93012
Tel: 800-955-6288
E-mail: techsupport@invitrogen.com

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INTRODUCTION

Signal transduction events stimulate the association of transcription factors with specific DNA sequences, where they then can influence gene expression profiles. Invitrogen's Transcription Factor Assays provide a novel method for monitoring signaling events by assessing the DNA-binding activity of particular transcription factors within the nucleus. This Transcription Factor Buffer Reagent Kit is intended for use with Invitrogen's Multiplex Transcription Factor Bead Sets for the analysis of multiple transcription factors contained in nuclear extracts. A nuclear extraction kit for preparing nuclear proteins that retain their ability to bind to DNA is available from Invitrogen as a separate product (Invitrogen Cat. # FNN0031).

This kit has been configured for research use only and is not to be used in diagnostic procedures.

Read entire protocol before use.

PRINCIPLE OF THE METHOD

Invitrogen's Transcription Factor (TF) Assays are multiplexed bead assays designed for simultaneously quantifying the levels of multiple transcription factors within nuclear extracts.

To perform an assay, cells are first stimulated as desired, and nuclear extracts are prepared using Invitrogen's Nuclear Extraction Kit (Cat. # FNN0031). These extracts can be prepared in advance and stored at -80°C until the Transcription Factor Assay is performed.

In the first phase of a Transcription Factor Assay, biotin labeled DNA probes, provided in a separate Multiplex Transcription Factor Bead Set,

are pipetted into the wells of a 96 well PCR thermocycler-compatible microtiter plate. To these wells, controls or nuclear extract samples are added. Controls include the Positive Reagent Control, Negative Reagent Control, a protein control (Invitrogen Cat. # CP101), nuclear extract from unstimulated cells (Invitrogen Cat. # NE101U), and nuclear extract from stimulated cells (Invitrogen Cat. # NE101T). Samples are nuclear extracts from the cells under investigation. All controls and samples are incubated with the DNA probes for 20 minutes at 25°C in a PCR thermocycler. During this incubation, transcription factors contained in the nuclear extract bind to the DNA probes.

In the second phase of the assay, the Positive Reagent Control wells receive Digestion Buffer, while other wells receive Digestion Buffer plus Digestion Reagent (nuclease). The DNA digestion step is allowed to proceed for 20 minutes at 37°C in a PCR thermocycler. During this phase of the assay, transcription factors bound to their DNA probes protect the probes from nuclease. The amount of biotin that remains incorporated in the probe permits quantitation of the protection afforded by transcription factor in the samples.

In the third phase of the assay, fluorescently encoded microspheres are added to the digestion mixture and incubated for 45 minutes at room temperature. These beads are conjugated to capture DNA sequences designed to bind to the probes through sequence complementarity.

In the fourth phase of the assay, the individual mixtures are transferred to the wells of a filter plate. The beads are then washed, and incubated with streptavidin-RPE. The streptavidin-RPE binds to the biotin associated with beads. Excess streptavidin-RPE is removed in a final washing step, and the beads are analyzed in the Luminex® 100™ or 200™ instrument.

The efficiency of digestion of the DNA probes in the absence of transcription factors can be calculated from the Positive Reagent Control and the Negative Reagent Control. The amount of specific transcription factors bound to the DNA probes is then calculated by subtracting the MFI of the Negative Reagent Control from the MFI of the sample.

REAGENTS PROVIDED

Note: *Store all reagents at -20°C.*

Common Reagent Kit	***100 Test Kit***
Sample Diluent; 0.75 ml per vial.	1 vial
Binding Mix 1; 1.6 ml per vial.	2 vials
Digestion Reagent (Nuclease); 30 µl per vial.	1 vial
Digestion Buffer; 3.0 ml per vial.	1 vial
Hybridization Buffer; 1.8 ml per vial.	1 vial
Hybridization Accelerator; 120 µl per vial.	1 vial
Detection Reagent (SAV-RPE) in glycerol; 55 µl per vial.	1 vial
10x Assay Wash Buffer*. Contains 0.05% sodium azide; 6.5 ml per bottle.	1 bottle
*10x Assay Wash Buffer should be stored at 2 to 8°C following initial thawing.	

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Luminex® 100™ or 200™ with XY platform. Please contact Invitrogen for instrument placement services.
2. Invitrogen recommends the use of StarStation™ software from Applied Cytometry Systems for data acquisition and analysis with the Luminex® 100™ or 200™.
3. Multiplex Transcription Factor Bead Kits. These kits contain the spectrally encoded fluorescent beads and binding mix (DNA probes in buffered solution).
4. Millipore Multiscreen™ Filter Plate (Millipore, Cat. # MSBVN1250).
5. Extra filter plate covers.
6. Microfuge.
7. 1.5 ml microfuge tubes.
8. Glass or plastic tubes.
9. Distilled or deionized water.
10. Beakers and graduated cylinders in various sizes.
11. Calibrated, adjustable precision pipettes, preferably with disposable plastic tips.
12. A manifold multi-channel pipette.
13. Filtration manifold for bead washing (e.g., Pall, Cat. # 5017 or Millipore, Cat. # MAVM 096 0R).

14. 96 well PCR plate.
15. 96 well PCR thermocycler.
16. 37°C water bath.
17. Sonicating water bath (e.g., Cole Parmer, Cat. # 08849-00).
18. Vortex mixer.
19. Orbital shaker.
20. Aluminum foil.
21. Absorbent paper towels.

SAFETY

All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. **Do not mix or interchange different reagent lots from various kit lots.**
2. Do not use reagents after the kit expiration date.
3. **The beads provided in the Multiplex Transcription Factor Bead Sets are light-sensitive.** Protect the beads from light to avoid photobleaching the embedded dye. Aluminum foil should be used to cover tubes containing beads during the assay. Filter plates containing beads should be shielded with an aluminum foil-wrapped plate cover.

4. Do not place filter plates on absorbent paper towels during loading, as liquid will be lost due to wicking. An extra plate cover serves as a good surface upon which to rest the filter plate. Following plate washing, excess liquid may be blotted from the bottom of the plate by pressing the plate on clean paper towels.
5. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
6. We recommend that with the initial use of this kit, samples and controls be analyzed in triplicate. Experienced users of this kit may consider decreasing the number of replicates with subsequent analyses.
7. Unstimulated Cell Nuclear Extracts (Invitrogen Cat. # NE101U), Stimulated Cell Nuclear Extracts (Invitrogen Cat. # NE101T), and Protein Controls (Invitrogen, Cat. # CP101) are optional in the assay. We recommend that these controls be included with the initial assay performed with this kit, however.

SAMPLE PREPARATION

1. Invitrogen's Transcription Factor Assays are intended for use with nuclear extracts generated with the Nuclear Extraction Kit. Please see the Nuclear Extraction Kit protocol for complete details.
2. Each assay requires 1-3 µl of nuclear extract at a protein concentration ranging from 1-10 µg/µl.
3. If assays with different volumes of cell extract will be compared, it is important to keep the total reaction volume constant by adding Sample Diluent to the samples with the lower volume.

DIRECTIONS FOR WASHING BEADS

This assay includes bead washing steps, which are performed in a filter bottom plate. The filter plate is designed to retain the beads while permitting the flow of liquids which are removed through the bottom of the plate by gentle aspiration using a vacuum manifold. During the assay, the plate must be kept in the upright position to prevent bead loss.

When preparing a vacuum manifold for use with these reagents, the vacuum setting should be adjusted so that 5 to 10 seconds are required to empty solution from the wells. Vacuum pressure should not exceed 5 inches Hg. Excessive vacuum can cause the membrane to tear, resulting in bead loss. Vacuum surge should be prevented by opening and adjusting the vacuum on the manifold before placing the plate on the surface.

To wash the beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum. Following each aspiration step, gently blot the bottom of the filter plate on absorbent paper towels to remove residual droplets of liquid. Remove the plate from the vacuum manifold and add 1x Assay Wash Solution to the wells, then empty the wells by aspiration with the vacuum manifold and blot. **Do not let the wells completely dry.** Repeat as directed under **ASSAY PROCEDURE**. Covering unused wells with an adhesive plate sealer improves the vacuum.

Unused wells may be left dry during the assay and used at a later time.

ASSAY PROCEDURE

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Preparation.

1. Initiate the Luminex® 100™ or 200™ warm up cycle.
2. Prepare reagents.

    - Allow all reagents to warm to room temperature for 30 minutes.
    - Place the Hybridization Buffer in a 37°C water bath to dissolve precipitate.
    - Briefly microfuge the Detection Reagent vial and the Digestion Reagent vial (contained in the Multiplex Transcription Factor Bead Set) to bring their contents to the bottom of the vials.

3. Allow the Protein Controls, Nuclear Extract Controls, and nuclear extract samples to thaw on ice.
4. Determine the number of wells required for the assay. Each assay requires a Bead Control well, Negative Reagent Control wells, Positive Reagent Control wells, plus wells for the samples under investigation. The number of replicates should be determined by the user. We recommend that with the initial use of this kit, the Negative Reagent Control wells, Positive Reagent Control wells, Protein Control, Unstimulated Cell Nuclear Extract Control, Stimulated Cell Nuclear Extract Control, and samples under investigation be analyzed in triplicate. With subsequent analyses,

the number of sample replicates may be decreased, and the Protein Control, the Unstimulated Cell Nuclear Extract Control, and the Stimulated Cell Nuclear Extract Control may be omitted. A suggested plate plan is presented in Figure 1. Please note that well A1 will be left dry until the assay is analyzed. Well A1 is designated for the beads to permit setting of the gates on the Luminex® 100™ or 200™ instrument.

Figure 1: Suggested Plate Plan.

Bead Control Well*	Protein Control	Sample 1	Sample 3		
Positive Reagent Control Well	Protein Control	Sample 1	Sample 4		
Positive Reagent Control Well	Unstimulated Cell Extract	Sample 1	Sample 4		
Positive Reagent Control Well	Unstimulated Cell Extract	Sample 2	Sample 4		
Negative Reagent Control Well	Unstimulated Cell Extract	Sample 2	Sample 5		
Negative Reagent Control Well	Stimulated Cell Extract	Sample 2	Sample 5		
Negative Reagent Control Well	Stimulated Cell Extract	Sample 3	Sample 5		
Protein Control	Stimulated Cell Extract	Sample 3			

*This well is left empty until the analysis on the Luminex® 100™ or 200™. This well is intended for setting the gates on the instrument and for estimating the bead background.

Phase 1: Protein : DNA Binding Step.

1. Mix the 20x Binding Mix vial(s) by inversion two times.
2. Prepare the 1x Binding Mix according to the table below:

1x Binding Mix	**Volume per 1 Reaction**	**Number of Reactions + 2**	**Volume x (Number of Reactions +2)**
20x Binding Mix	0.75 µl		
Binding Mix 1	14.25 µl		
Total Volume	15 µl		

Important: If you are multiplexing, add 0.75 µl of 20x Binding Mix for each transcription factor. Then add as much of Binding Mix 1 as needed to bring the total volume to 15 µl per 1 reaction.

3. Pipette 15 µl of the 1x Binding Mix into each well, except the Bead Control well, of a 96 well PCR plate.
4. Add 1 µl Protein Control, Unstimulated Control Cell Nuclear Extract, and Stimulated Control Cell Nuclear Extract to designated wells. Mix by gently pipetting up and down two times.
5. Add 1 – 3 µl of sample nuclear extract, diluted as indicated in **SAMPLE PREPARATION**, to designated wells. Mix by gently pipetting up and down two times.
6. Seal plate with an adhesive plate cover and incubate at 25°C for 20 minutes in a PCR thermocycler.

7. During this incubation step, prepare the Complete Digestion Mix according to the table presented below. All wells require the Complete Digestion Mix except for the Bead Control well and Positive Reagent Control wells. It is suggested sufficient volume be made for at least two extra wells. Space is provided in the table below for performing calculations. Mix the Complete Digestion Mix by gently pipetting up and down ten times.

Complete Digestion Mix	**Volume per 1 Reaction**	**Number of Reactions + 2**	**Volume x (Number of Reactions +2)**
Digestion Buffer	24.75 µl		
Digestion Reagent (nuclease)	0.25 µl		
Total Volume	25 µl		

Phase 2: Digestion Step.

1. At the end of the 20 minute protein : DNA binding incubation step, remove the 96 well plate from the PCR thermocycler. Adjust the thermocycler temperature to 37°C.
2. Add 25 µl of Complete Digestion Mix to all wells except the Bead Control well and the Positive Reagent Control wells. Mix by gently pipetting up and down two times.
3. Add 25 µl of Digestion Buffer to the Positive Reagent Control wells. Mix by gently pipetting up and down two times.
4. Seal the plate with an adhesive plate cover.
5. Incubate wells for 20 minutes at 37°C in the PCR thermocycler.
6. During this incubation step, prepare for the next phase of the assay.

    - Mix Hybridization Buffer by inversion and check for precipitate. If precipitate is present, return the Hybridization Buffer to the 37°C water bath until the solution is clear.
    - Mix the Hybridization Buffer again by inversion. Mix the Hybridization Accelerator by brief, gentle vortexing and briefly microfuge the vial to bring the contents to the bottom.
    - Resuspend the 20x Bead Mix (provided in the Multiplex Transcription Factor Bead Set) by vortexing for 10 seconds and then incubating for 2 minutes in a sonicating water bath.

- Prepare the Complete Hybridization Mix according to the table presented below. Space is provided to perform calculations of volumes for each experiment. All wells require the Complete Hybridization Mix, including the Bead Control well. It is suggested that sufficient volume be made for at least two extra wells. Vortex the Complete Hybridization Mix for 10 seconds, then incubate the Complete Hybridization Mix for 2 minutes in a sonicating water bath. Protect this mixture from light by wrapping the tube in aluminum foil, as the beads are light-sensitive.

Complete Hybridization Mix	**Volume per 1 Reaction**	**Number of Reactions +2**	**Volume x (Number of Reactions +2)**
Hybridization Buffer	13.25 µl		
20x Bead Mix	0.75 µl		
Hybridization Accelerator	1 µl		
Total Volume	15 µl		

Important: If you are multiplexing, add 0.75 µl of 20x Bead Mix for each transcription factor. Then add as much of Hybridizitation Buffer as needed to bring the total volume to 15 µl per 1 reaction.

Phase 3: Hybridization Step.

1. At the end of the 20 minute digestion incubation step, remove the 96 well plate from the PCR thermocycler. All subsequent procedures will be performed at room temperature.
2. Add 15 µl of the Complete Hybridization Mix to each well except the Bead Control well. Mix by pipetting up and down two times. Save the extra Complete Hybridization Mix for use as the Bead Control. This Complete Hybridization Mix must be protected from light.
3. Seal the plate with an adhesive plate cover. An aluminum foil wrapped adhesive plate cover is recommended at this step to shield the beads from light.
4. Incubate wells for 45 minutes at room temperature on an orbital shaker (500-600 rpm). Protect the wells from light during this incubation.
5. During this incubation step, prepare the 1x Assay Wash Buffer and Complete Detection Mix.

    - Prepare the 1x Assay Wash Buffer according to the table presented below. Space is provided to allow calculations of volumes for each experiment. Mix the 1x Assay Wash Buffer by inverting several times. It is suggested sufficient volume of this reagent be made for at least two extra wells.

1x Assay Wash Buffer	**Volume per 1 Reaction**	**Number of Reactions +2**	**Volume x (Number of Reactions +2)**
10x Assay Wash Buffer	60 µl		
Deionized Water	540 µl		
Total Volume	600 µl		

- Prepare the Complete Detection Mix, according to the table presented below. Space is provided to perform calculations of volumes for each experiment. Mix the Complete Detection Mix by inverting several times. It is suggested sufficient volume of this reagent be made for at least two extra wells.

Complete Detection Mix	**Volume per 1 Reaction**	**Number of Reactions + 2**	**Volume x (Number of Reactions +2)**
1x Assay Wash Buffer	49.5 µl		
Detection Reagent	0.50 µl		
Total Volume	50 µl		

Phase 4: Detection Step.

1. Pre-wet the 96 well filter plate by adding 50 µl 1x Assay Wash Buffer to each well designated for the assay, including the Bead Control well.
2. Remove the 1x Assay Wash Buffer from each well by gentle aspiration with the vacuum manifold. Do not allow the filter plate to dry. Blot residual liquid from the bottom of the plate with absorbent paper towels.
3. Carefully transfer the contents of the wells of the PCR plate to the corresponding wells of the pre-wetted filter plate. A multi-channel pipette is desirable for this transfer. Care must be taken not to puncture the membranes of the filter plate or cross-contaminate the samples.

4. To the Bead Control well (well A1 in our example), add 50 µl 1x Assay Wash Buffer followed by 10 µl Complete Hybridization Mix.
5. Remove the liquid from the wells of the filter plate by gentle aspiration with the vacuum manifold. Do not allow the membrane to dry. Blot residual liquid from the bottom of the plate with absorbent paper towels.
6. Wash each well by adding 100 µl 1x Assay Wash Buffer. Remove the 1x Assay Wash Buffer by gentle aspiration with the vacuum manifold. Repeat this washing step two more times for a total of three washings. Blot residual liquid from the bottom of the plate with absorbent paper towels.
7. Add 50 µl of Complete Detection Mix to each well.
8. Incubate for 5 minutes at room temperature. Shield the plate from light during this incubation with an aluminum foil-wrapped plate cover.
9. Remove the liquid from the wells of the filter plate by gentle aspiration with the vacuum manifold.
10. Wash each well by adding 100 µl 1x Assay Wash Buffer. Remove the 1x Assay Wash Buffer by gentle aspiration with the vacuum manifold. Blot residual liquid from the bottom of the plate with absorbent paper towels.
11. Add 100 µl of 1x Assay Wash Buffer to each well. Shake the plate on an orbital shaker (500-600 rpm) for 2 to 3 minutes to resuspend the beads.

12. Analyze the assay on the Luminex® 100™ or 200™ instrument.
    - Set bead events to 75.
    - Set minimum number of events to 20.
    - Enter the number of samples.
    - Set Sample Size to 65 µl.
    - Set Flow Rate to Fast. Under the Settings Tab, click Bead.
    - Enter the bead region numbers as indicated on the Multiplex Transcription Factor Bead Set **PRODUCT INFORMATION SHEET**.
    - Check the probe height to accommodate Multiscreen™ MSBVN1250 filter plates.
    - Perform one alcohol flush and one sheath fluid wash.
    - Insert the plate into the instrument and initiate the analysis.
    - Set the gate for the analysis using the Bead Control well.
13. Representative data for each specific Multiplex Transcription Factor Bead Set and data analysis method are provided on the lot-specific **PRODUCT INFORMATION SHEET**.
14. Upon initial thawing and use in the assay, the Assay Wash Buffer should be stored at 2 to 8°C. All other reagents included in this kit (Digestion Reagent, Hybridization Buffer, Hybridization Accelerator, and Detection Reagent) should be returned to storage at -20°C after use.

LIMITATIONS OF THE PROCEDURE

This kit is for research use only.
Not for human therapeutic or diagnostic use.

REFERENCES

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Multiplex Assay Summary