



Promega

Technical Bulletin

Gel Shift Assay System

INSTRUCTIONS FOR USE OF PRODUCTS E3050 AND E3300.



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Gel Shift Assay System

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 of this system. E-mail: techserv@promega.com.

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1. Description

The gel shift, or electrophoretic mobility shift, assay provides a simple and rapid method for detecting DNA-binding proteins (1). This method has been used widely in the study of sequence-specific DNA-binding proteins such as transcription factors. The assay is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments or double-stranded oligonucleotides. The gel shift assay is performed by incubating a purified protein, or a complex mixture of proteins (such as nuclear or cell extract preparations), with a ³²P end-labeled DNA fragment containing the putative protein binding site. The reaction products are then analyzed on a nondenaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

1. Description (continued)

Promega has developed Gel Shift Assay Systems that contain target oligonucleotides, a control extract containing DNA-binding proteins, binding buffer and reagents for phosphorylating oligonucleotides. The Gel Shift Assay Core System includes sufficient HeLa nuclear extract to perform 20 control reactions, Gel Shift Binding 5X Buffer, an SP1 Consensus Oligo and an AP2 Consensus Oligo. This is a reliable system for obtaining experience with gel shift assays because AP2 binding activity is stable and produces a strong gel shift. The complete system contains five additional double-stranded oligonucleotides that represent consensus sequences to characterized binding sites (Section 10.B, Table 3). These oligonucleotides can be end-labeled and used as protein-specific probes or as specific or nonspecific competitor DNA in competition assays.

2. Product Components and Storage Conditions

Product	Cat.#
Gel Shift Assay Core System	E3050

Each system contains sufficient reagents for 100 gel shift assays, including extract, to perform 20 control assays. Includes:

- 40µl HeLa Nuclear Extract
- 100u T4 Polynucleotide Kinase (PNK)
- 100µl T4 Polynucleotide Kinase 10X Buffer
- 200µl Gel Shift Binding 5X Buffer
- 35pmol AP2 Consensus Oligo (1.75pmol/µl)
- 35pmol SP1 Consensus Oligo (1.75pmol/µl)

Product	Cat.#
Gel Shift Assay System	E3300

Each system contains all of the components of the Gel Shift Assay Core System plus:

- 35pmol AP1 Consensus Oligo (1.75pmol/µl)
- 35pmol OCT1 Consensus Oligo (1.75pmol/µl)
- 35pmol CREB Consensus Oligo (1.75pmol/µl)
- 35pmol NF-κB Consensus Oligo (1.75pmol/µl)
- 35pmol TFIID Consensus Oligo (1.75pmol/µl)

Storage Conditions: Store HeLa Nuclear Extract at -70°C in small aliquots; avoid multiple freeze-thaw cycles. Store all other components at -70°C or -20°C.

3. Labeling of Consensus Oligonucleotides

Materials to Be Supplied by the User

(Solution compositions are provided in Section 10.A.)

- [γ - ^{32}P]ATP (3,000Ci/mmol at 10mCi/ml)
- 0.5M EDTA
- TE buffer
- 0.5M Na_2HPO_4 (pH 6.8)
- Whatman[®] DE81 2.3cm circular filters
- Nuclease-Free Water (Cat.# P1193)

3.A. Phosphorylation Reaction

1. Assemble the following reaction in a sterile microcentrifuge tube:

Consensus Oligonucleotide (1.75pmol/ μl)	2 μl
T4 Polynucleotide Kinase 10X Buffer	1 μl
[γ - ^{32}P]ATP (3,000Ci/mmol at 10mCi/ml)	1 μl
Nuclease-Free Water	5 μl
T4 Polynucleotide Kinase (5–10u/ μl)	<u>1μl</u>
Total volume	10μl

Note: If higher specific activity probes are required, the amount of ATP can be increased to 2.2 μl per 10 μl reaction to provide a 1:1 ratio of ATP to DNA ends.

2. Incubate at 37°C for 10 minutes.
3. Stop the reaction by adding 1 μl of 0.5M EDTA.
4. Add 89 μl of TE buffer. Remove an aliquot to determine percent incorporation, if desired, as described in the following section (Section 3.B). Alternatively, proceed directly to Section 4.

3.B. Determination of Percent Incorporation

1. Spot 1 μl of the labeled oligonucleotide (from Section 3.A) onto each of 4 Whatman[®] DE81 2.3cm circular filters.
2. Dry the filters briefly under a heat lamp. Place two filters aside for the direct determination of total cpm in the sample.
3. Wash the other two filters in 50ml of 0.5M Na_2HPO_4 (pH 6.8) twice for 5 minutes each wash to remove the unincorporated label.
4. Dry the washed filters under a heat lamp.
5. Place the filters into individual vials, add appropriate scintillation fluid and count in a scintillation counter.

3.B. Determination of Percent Incorporation (continued)

6. Calculate the average cpm for the total and incorporated filters.

$$\text{percent incorporation} = \frac{\text{cpm incorporated}}{\text{cpm total}} \times 100$$

Using the labeling conditions described above, typically 50% or more of the radioactivity is incorporated in the 5' end-labeling reaction. We recommend using the oligonucleotides if the percent incorporation is 30% or above. If the percent incorporation is under 30%, the gel shift assay may give a weak signal. For labeling conditions different from those described above, we recommend determining the specific activity to ensure successful labeling. Ideally, the specific activity of the labeled oligonucleotide will be 5,000–20,000cpm per 10–100fmol.

4. Removal of Unincorporated Label from Oligonucleotides

Materials to Be Supplied by the User

(Solution compositions are provided in Section 10.A.)

- G-25 spin columns
- TE buffer

The removal of unincorporated nucleotides from the DNA probe is an optional step that may improve the quality of gel shifts. Labeled oligonucleotides may be separated from unincorporated nucleotides by chromatography through a G-25 spin column equilibrated in TE buffer (1).

5. Gel Shift Assays

All parameters of this assay may need to be optimized if you are examining a novel DNA-binding activity. These parameters include extract preparation (proteases, nuclease and phosphatase contaminants may degrade target proteins or probe DNA), binding conditions (salt concentration, temperature, pH and special properties such as metal requirements for binding activity, including zinc, cadmium, etc.), addition of detergent, and the conditions under which the gel is run (gel electrophoresis conditions such as pH, temperature, polyacrylamide concentration and ionic strength). See reference 2 for a thorough experimental design example addressing these points.

Materials to Be Supplied by the User

(Solution compositions are provided in Section 10.A.)

- 4% nondenaturing acrylamide gel (see Table 1)
- Nuclease-Free Water (Cat.# P1193)
- gel loading 10X buffer
- TBE 0.5X buffer

5.A. Gel Preparation

The reactions from Sections 5.B may be analyzed using a Novex® 6% DNA retardation gel. Alternatively, these reactions may be analyzed on a 10 × 12cm, nondenaturing, 4% acrylamide gel, 0.75mm thick. Other gel sizes may be used for the analysis of various DNA-protein complexes. Prepare the gel as described in Table 1. Clean all glass plates using only distilled water. It is critical that the plates are completely free of ionic detergent (e.g., SDS).

Table 1. Formulation of 4% Acrylamide, 60:1 Acrylamide:Bisacrylamide Gel (20ml).

Component	Volume
TBE 10X buffer	1.0ml
37.5:1 acrylamide/bisacrylamide (40%, w/v)	1.25ml
40% acrylamide (w/v)	0.75ml
80% glycerol	625µl
distilled water	16.2ml
TEMED*	10µl
10% APS**	150µl

*N,N,N',N'-tetramethyl-ethylenediamine

**ammonium persulfate, freshly prepared (10% in distilled water)

Note: Gels with an acrylamide:bisacrylamide ratio of 60:1 are very soft, polymerize slowly and should be allowed to polymerize overnight for optimal results. A ratio of 40:1 may also be used (1) and has the advantage of producing a more rigid gel with much shorter polymerization times (1-2 hours).

5.B. DNA Binding Reactions

The following example uses the SP1 Consensus Oligo as a probe to detect SP1 binding activity in HeLa Nuclear Extract. Similar reactions can be set up using other Consensus Oligos. The positive control reaction can also be used to detect AP2 binding activity using the HeLa Extract and AP2 Consensus Oligo. We recommend setting up four reactions: a negative control, a positive control and two competition assays to demonstrate binding specificity.

1. Assemble the following reactions in sterile microcentrifuge tubes in the order shown:

Reaction #1 (negative control)		Reaction #2 (positive control)	
Nuclease-Free Water	7µl	Nuclease-Free Water	5µl
Gel Shift Binding 5X Buffer	2µl	Gel Shift Binding 5X Buffer	2µl
HeLa Nuclear Extract	0µl	HeLa Nuclear Extract	2µl
Total volume	9µl	Total volume	9µl

5.B. DNA Binding Reactions (continued)

Reaction #3 (specific competitor)		Reaction #4 (nonspecific competitor)	
Nuclease-Free Water	4μl	Nuclease-Free Water	4μl
Gel Shift Binding 5X Buffer	2μl	Gel Shift Binding 5X Buffer	2μl
HeLa Nuclear Extract	2μl	HeLa Nuclear Extract	2μl
unlabeled competitor oligo (1.75pmol) (e.g., SP1 Consensus Oligo)	1μl	unlabeled noncompetitor oligo (1.75pmol) (e.g., AP2 Consensus Oligo)	1μl
Total volume	9μl	Total volume	9μl

2. Incubate the reactions at room temperature for 10 minutes, then add 1μl of ³²P-labeled SP1 Consensus Oligo to each reaction.

Note: We recommend that the incubation temperature be controlled for better experiment-to-experiment reproducibility.

3. Incubate the reactions at room temperature for 20 minutes.
4. Add 1μl of room-temperature gel loading 10X buffer per reaction, and analyze the reaction products as described in Section 5.C.

Note: The dyes in the gel loading buffer may interfere with the binding of some proteins to DNA. If this occurs, we recommend that the gel loading buffer be added only to the negative control.

5.C. Electrophoresis of DNA-Protein Complexes

1. If using 4% gels, pre-run the gel in 0.5X TBE buffer for 10 minutes at 350V before loading the samples. If using Novex® 6% DNA retardation gels, no pre-run is necessary. After loading the samples, run the gel at room temperature in 0.5X TBE buffer at 350V for 4% gels or in 0.5X TBE at 250–350V for 6% DNA retardation gels until the bromophenol blue dye is three fourths of the way down the gel. This should take less than 20 minutes. Maintain a gel temperature of <30°C.

Note: The electrophoresis conditions described here have been optimized for shifting the AP2 and SP1 Consensus Oligos provided with this system. Other factors and DNA probes may require substantially different conditions. We recommend optimizing electrophoresis conditions and exposure times whenever a new system is examined (2).

2. Open the gel plates and place the gel on a sheet of Whatman® 3MM filter paper. Cover with plastic wrap and dry on a gel dryer. Expose the gel to X-ray film 1 hour to overnight at -70°C with an intensifying screen. Alternatively, analyze the gel using phosphorimaging instrumentation.

6. Expected Results from Control Reactions

1. Unbound Consensus Oligos will run near the dye front.
2. If the complex is specific, the addition of unlabeled specific competitor should decrease the intensity of the band(s). In the presence of unlabeled nonspecific competitor, the specific band(s) should remain.
3. Nonspecific complexes may either remain in the presence of specific competitor or decrease with the addition of any type of DNA.
Table 2 describes the expected banding pattern after HeLa extract has been incubated with the different Consensus Oligos. Figure 1 shows the expected banding pattern after the HeLa Nuclear Extract has been incubated with the SP1 Oligo.

Table 2. Expected Banding Pattern after HeLa Nuclear Extracts have been Incubated with Different Consensus Oligos.

Consensus Oligo	Expected Results
AP1	One or two shifted bands.
AP2	One or several shifted bands.
CREB	Up to three shifted bands that may vary in relative intensity.
NF-κB	One or several bands that may vary in relative intensity.
OCT1	One or two shifted bands.
SP1	One to four shifted bands that may vary in relative intensity.
TFIID	One or several shifted bands that may vary in relative intensity.

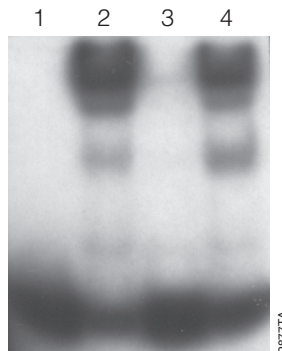


Figure 1. Gel shift assays. Gel shift assays were performed as described in Section 5. Lane 1, negative control (^{32}P -labeled SP1 Oligo without HeLa Nuclear Extract); lane 2, positive control (HeLa Nuclear Extract and ^{32}P -labeled SP1 Oligo); lane 3, HeLa Nuclear Extract with ^{32}P -labeled SP1 Oligo plus unlabeled SP1 Oligo (specific competitor); lane 4, HeLa Nuclear Extract with ^{32}P -labeled SP1 Oligo plus unlabeled AP2 Oligo (nonspecific competitor). DNA binding and electrophoresis conditions were as described in Section 5.

7. Gel Shift Assays Using Purified Transcription Factors

Modifications of the gel shift protocols described earlier may be necessary for assays using purified transcription factors. We have successfully shifted several Promega recombinant transcription factors with the corresponding consensus oligonucleotide using minor modifications to the protocols in Section 5. Suggested assay conditions for various factors are given below.

Note: These conditions are not recommended for use with nuclear extracts.

API (c-Jun): Use a final concentration of 0.01mg/ml poly(dI-dC)•poly(dI-dC) in the reaction (3). Other nonspecific competitor oligonucleotides (e.g., AP2) can also be used in place of poly(dI-dC)•poly(dI-dC). A final concentration of 5mM DTT in the binding buffer may enhance the in vitro DNA-binding ability of c-Jun. All other buffer component concentrations are identical to those in the Gel Shift Binding 1X Buffer. One to two fpu (footprinting units) of protein is generally sufficient for a strong gel shift.

NF-κB (p49 or p50): Assemble 20μl reactions containing 0.28pmol NF-κB Consensus Oligo in 10mM HEPES (pH 7.9), 50mM KCl, 0.1mM EDTA, 2.5mM DTT, 10% glycerol and 0.05% NP-40. Two hundred and fifty to three hundred nanograms of protein is sufficient for a strong gel shift. Incubate at 25°C for 30 minutes. Gel loading buffer should be added only to the negative control reaction. Protein-DNA complexes are resolved on Novex® 6% TBE gels. Run the gels for 15 minutes at 300V in 0.5X TBE.

8. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
No shift observed	<p>Insufficient amount of protein. Titrate the amount of protein used in the binding reaction.</p> <p>For nuclear extracts: The amount of extract that is necessary to produce an optimal shift will vary greatly depending on the extract preparation, DNA-binding affinity for the probe, extract quality and other factors. A general guideline is to use 1, 2, 5, 10 and 20μg of extract per reaction.</p> <p>For purified factors: Most purified protein preparations, particularly recombinant protein, will not be 100% active. Carefully titrate the amount of protein from an equimolar protein:DNA ratio up to a fivefold molar excess of protein. Try a positive control protein, if available, for your probe of interest.</p>

8. Troubleshooting (continued)

Symptoms	Causes and Comments
No shift observed (continued)	<p data-bbox="611 321 1102 513">Binding buffer is missing essential components. Optimize concentrations of salt and glycerol. Some proteins require additional factors, such as zinc, magnesium or heavy metal ions, to bind DNA. Other proteins may be required for binding to occur.</p> <hr/> <p data-bbox="611 534 1102 1046">Too much nonspecific competitor DNA or inappropriate type of competitor DNA. Some binding reactions require the use of nonspecific competitor DNA, such as poly(dI-dC)•poly(dI-dC). This may not be appropriate in all cases (e.g., when gel-shifting TFIID, poly(dG-dC)•poly(dG-dC) can be used). Avoid other types of competitor DNA, such as calf thymus DNA, because they may contain binding sites for the protein of interest. The dissociation rate of the probe:protein complex and the competitor:protein complex also need to be considered (4). For nuclear extracts: A general rule is to use 0.1µg of nonspecific competitor, such as poly(dI-dC)•poly(dI-dC), for every 2–3µg of extract. For purified factors: Many purified factors do not require a nonspecific competitor; if used, carefully titrate the amount. Final amounts of competitor generally should not exceed 50–100ng in a standard binding reaction.</p> <hr/> <p data-bbox="611 1232 1102 1357">Specific activity of the probe is low. Use only fresh ³²P label, label the probe properly and check the percent incorporation of the labeling reaction.</p> <hr/> <p data-bbox="611 1377 1102 1440">Probe denatured. Ensure target DNA is double-stranded.</p> <hr/> <p data-bbox="611 1460 1102 1684">DNA-protein complex labile. Perform the binding reaction and electrophoresis at 4°C, or use a different gel running buffer, such as TGE (1). Bromophenol blue in the gel loading buffer may interfere with the binding of some proteins to DNA. If this is the case, add loading buffer with dye only to the negative control reactions.</p>



8. Troubleshooting (continued)

<u>Symptoms</u>	<u>Causes and Comments</u>
Addition of protein causes probe to stick in wells	Too much protein used or insufficient amounts of nonspecific competitor added. Titrate the amount of protein and nonspecific competitor.
	Salt concentration not optimal. Titrate the salt concentration in the binding buffer. Excessive salt or impurities that copurify with the DNA probe can cause this problem.
Negative control reaction gives a shifted band	Probe artifact. This may be seen when larger DNA fragments are used as probes. Use a smaller DNA fragment, if possible. If the probe is generated by restriction digestion of a plasmid DNA, ensure that the DNA is not nicked.
	Partial denaturation of the probe. Very short probes may undergo partial denaturation during electrophoresis, particularly in AT-rich regions. Run the gel at 4°C to minimize overheating, or use a longer probe.
Multiple shifted bands produced with addition of protein	The protein oligomerizes. Try titrating the protein.
	Protein degradation. Add protease inhibitors to extract preparations and binding buffer. Avoid multiple freeze-thaw cycles of extracts or purified factors. Check for protein degradation by Western analysis if an antibody to the protein is available.
	Several proteins in extract recognize DNA sequence or interact with the protein of interest. Use antibody supershift reactions (1) to identify the protein of interest. Perform competition analysis with DNA fragments, poly(dI-dC)• poly(dI-dC) or oligonucleotides that contain point mutations in the protein-binding site.
Free DNA probe band not seen	Gel ran too long. Run the gel for a shorter time. Use tracking dyes to estimate the mobility of the free probe.
	Too much protein. Titrate the amount of protein added.

8. Troubleshooting (continued)

<u>Symptoms</u>	<u>Causes and Comments</u>
Free DNA probe band seen in negative control but no bands seen in other reactions	Nuclear extract or protein preparation may contain phosphatase or DNase activity. Test protein preparations or extracts for these activities and include appropriate inhibitors.
Bands appear smeared	<p>Incomplete gel polymerization. Allow the gel to polymerize longer. Use fresh ammonium persulfate solution (<1 week old).</p> <p>Electrophoresis buffer incorrectly prepared. Use fresh electrophoresis buffer. Do not use TBE buffer preparations that contain a precipitate.</p> <p>Incorrect voltage gradient. We recommend a voltage gradient of 10–15V/cm for most proteins. However, proteins that dissociate rapidly from DNA (e.g., TBP) will require a shorter run time and higher voltage gradient (30–35V/cm).</p> <p>Plates have detergent residues. Ensure that the gel plates are cleaned thoroughly and rinsed well to remove any traces of detergents. (For example, NF-κB is very sensitive to detergents.)</p> <p>Too much protein used. Titrate the amount of protein.</p> <p>Gel too warm during electrophoresis. Run gel in cold room to improve sieving properties of gel.</p> <p>Insufficient amount of nonspecific competitor. Titrate the amount of nonspecific competitor.</p> <p>Salt or glycerol concentration is not optimal. Optimize the salt and glycerol concentrations in the binding buffer.</p> <p>Protein may require purification. Purification of the protein of interest may be necessary.</p> <p>DNA-protein complex is labile. See comments under “No shift observed”.</p>



9. References

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10. Appendix

10.A. Composition of Buffers and Solutions

gel loading 10X buffer

250mM	Tris-HCl (pH 7.5)
0.2%	bromophenol blue
40%	glycerol

Gel Shift Binding 5X Buffer

20%	glycerol
5mM	MgCl ₂
2.5mM	EDTA
2.5mM	DTT
250mM	NaCl
50mM	Tris-HCl (pH 7.5)
0.25mg/ml	poly(dI-dC) • poly(dI-dC)

TE buffer

10mM	Tris-HCl (pH 8.0)
1mM	EDTA

T4 Polynucleotide Kinase 10X Buffer

700mM	Tris-HCl (pH 7.6)
100mM	MgCl ₂
50mM	DTT

TBE 10X buffer (1L)

107.80g	Tris base
~55g	boric acid
7.44g	disodium EDTA • 2H ₂ O

Add components in the order listed above to ~800ml of distilled water. Add slightly less than the total amount of boric acid. Mix until completely dissolved, check pH and adjust to 8.3 with boric acid. Bring final volume to 1L with distilled water.

10.B. Characteristics of Transcription Factors

Table 3. Characteristics of Transcription Factors for the Provided Oligonucleotides.

Transcription

Factor

(Reference)

Oligonucleotide Sequence and General Characteristics

SP1 (5)	5'-ATT CGA TCG GGG CGG GGC GAG C-3' 3'-TAA GCT AGC CCC GCC CCG CTC G-5'	O-glycosylated transcription factor with sequence specificity conferred through three zinc fingers in the DNA-binding domain.
AP1 (c-Jun) (6)	5'-CGC TTG ATG AGT CAG CCG GAA-3' 3'-GCG AAC TAC TCA GTC GGC CTT-5'	Forms DNA-binding dimers with other members of the AP1 family and with <i>c-fos</i> through leucine zipper formation.
AP2 (7)	5'-GAT CGA ACT GAC CGC CCG CGG CCC GT-3' 3'-CTA GCT TGA CTG GCG GGC GCC GGG CA-5'	May act independently as both a TPA- and cAMP-inducible element; AP2 is especially responsive to retinoic acid and may function in morphogenesis.
NF-κB (8)	5'-AGT TGA GGG GAC TTT CCC AGG C-3' 3'-TCA ACT CCC CTG AAA GGG TCC G-5'	Binds to κ light chain enhancer in B cells and is present in a covert cytoplasmic form in non-B cells.
OCT1 (9)	5'-TGT CGA ATG CAA ATC ACT AGA A-3' 3'-ACA GCT TAC GTT TAG TGA TCT T-5'	A member of the OCT family that is apparently ubiquitous in mammalian cells. The bipartite POU domain includes the POU-box and the homeodomain.
CREB (10)	5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3' 3'-TCT CTA ACG GAC TGC AGT CTC TCG ATC-5'	Confers responsiveness to cAMP; it contains a leucine zipper motif for dimerization, and the associated basic domain is homologous to <i>c-jun</i> DNA-binding domains.
TFIID (11)	5'-GCA GAG CAT ATA AGG TGA GGT AGG A-3' 3'-CGT CTC GTA TAT TCC ACT CCA TCC T-5'	A general transcription factor complex; it exhibits specific DNA binding to the TATA box. For many genes, TFIID is necessary, and in conjunction with RNA polymerase II, is sufficient to initiate basal transcription.

10.C. Related Products

Product	Size	Cat.#
Gel Shift Binding 5X Buffer	5 × 200µl	E3581
Nuclease-Free Water*	50ml (2 × 25ml)	P1193

*For Laboratory Use.

Transcription Factors

Product	Size	Cat.#
rhAP1 (c-Jun)	50fpu	E3061
rhNF-κB (p50)	50gsu	E3770

One fpu = one footprint unit; one gsu = one gel shift unit.

Nuclear Extract

Product	Size	Cat.#
HeLaScribe® Nuclear Extract, Gel Shift Assay Grade	3 × 40µl	E3521

Transcription Factor Consensus Oligonucleotides

Product	Size	Cat.#
AP1 Consensus Oligonucleotide	35pmol	E3202
	175pmol	E3201
AP2 Consensus Oligonucleotide	35pmol	E3212
	175pmol	E3211
TFIID Consensus Oligonucleotide	35pmol	E3222
	175pmol	E3221
SP1 Consensus Oligonucleotide	35pmol	E3232
	175pmol	E3231
OCT1 Consensus Oligonucleotide	35pmol	E3242
	175pmol	E3241
CREB Consensus Oligonucleotide	35pmol	E3282
	175pmol	E3281
NF-κB Consensus Oligonucleotide	35pmol	E3292
	175pmol	E3291



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