

Transcription Factor Microplate Assays

FREQUENTLY ASKED QUESTIONS

Q: Based on your experience, what are the optimal times after the addition of drug or compound to cells that samples should be assayed for changes in transcription factor activation? From the examples in the Marligen brochure, the observed changes are fairly rapid, at 15 and 30 min. Are there situations when one should look at shorter or longer treatment times?

A: We usually begin a time course study with the following timepoints: 15 minutes, 30 minutes, 2 hours, 4 hours and 24 hours. These timepoints allow for detection of transcription factors that are rapidly activated and translocated to the nucleus immediately after stimulation as well as transcription factors that must be transcribed and translated prior to activation and that are typically detected much later.

Q: How much sample do you need to add to each assay to give optimal results?

A: For most cell lines and tissues that we have tested, 2 μg of nuclear extract per replicate is sufficient. However, certain cell lines, tissues, and treatments may require extract amounts as low as 0.25 μg or as high as 10 μg per replicate. When using previously untested cell types or tissues, we recommend running a small pilot experiment in which the nuclear extract amount is titrated from 0.5-10 μg per replicate (0.5, 1, 2.5, 5, 10 μg is a good range).

Q. How many cells do you recommend using for an extraction?

A: For human cells, 1 million cells typically yield 8-15 μg of nuclear extract depending upon the cell type. Initially, it is best to use at least 10,000,000 cells per extraction to ensure there is plenty of material to work with. Preparing extracts from this number of cells provides cell and nuclear pellets of a size that are easier to work with if you haven't taken nuclear extracts before. Once one becomes proficient at preparing nuclear extracts, smaller numbers of cells can be processed.

Q: What is the minimal amount of nuclear extract that can be used in the assay?

A: The minimum amount of nuclear extract that can be used in the assay will vary depending on the cell or tissue type being assayed. We recommend that you perform a pilot titration of extract amount to determine the optimal sample amount for your cell, tissue, or sample type.

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Listed below is a table with the linear assay range we have for each assay. Note this is for specific conditions and can vary depending on treatment, cell or tissue type and sample.

Transcription Factor Assay	Sample	Treatment	Linear Assay Range*
AP-1	U937 cells	100nM PMA for 24 hours	0.4-24 µg of nuclear extract
	c-jun protein	na	3.0-50 ng of protein
CRE-ATF	K562 cells	10 µM Hemin for 45 minutes	0.5-15 µg of nuclear extract
CREB	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	0.1-15 µg of nuclear extract
EGR	THP-1 cells	100nM PMA for 24 hours	1.0-10 µg of nuclear extract
	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	0.25-8 µg of nuclear extract
GATA	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	0.5-15 µg of nuclear extract
MEF-2	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	1.0-15 µg of nuclear extract
Myc-Max	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	0.1-15 µg of nuclear extract
NF-1	Jurkat cells	100nM PMA for 4 hours	0.7-21 µg of nuclear extract
	NF-1 protein	na	0.005-2 ng of protein
NFκB	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	0.12-30 µg of nuclear extract
	NFκB p50 fragment recombinant protein	na	0.0003-0.13 gel shift units
Octamer	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	0.25-15 µg of nuclear extract
PPAR	THP-1 cells	100nM PMA for 24 hours	0.2-10 µg of nuclear extract
	HL-60 cells	100nM PMA plus 500nM ionomycin for 24 hours	1.0-15 µg of nuclear extract

* Maximum value is the highest amount of sample tested

Q: I have nuclear extracts that I made previously using a different protocol than you recommend. Will the assay still work?

A: Maybe. We highly recommend that customers use Marligen's Nuclear Extraction Kit (Cat. No. 11906-100) to generate nuclear extracts since all of the reagents and buffers have been developed for optimal assay performance. However, if you have previously generated extracts we recommend that you perform a small pilot study to determine if the extract is compatible with the assay.

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Q: Are there any chemicals that will interfere with the assay's performance?

A: High levels of metal chelators (e.g. > 1mM EDTA or EGTA) will interfere with the assay performance. In addition, the assay performance is dependent on the concentration of Dithiothreitol (DTT), greater than 0.2 mM DTT will adversely affect assay performance.

Q: How much does the Nuclear Extraction Kit (Cat. No. 11906-100) cost? And how many samples can you prepare from one kit?

A: The nuclear extraction kit lists for \$130 and you can run 100 extractions of about 10,000,000 cells each. Along with the kit you will also need to have the following reagents: Protease Inhibitor Cocktail (Sigma Cat. No. P8340, Phosphatase Inhibitor Cocktail (Sigma Cat. No. P5726), PMSF (Sigma Cat. No. P7626) and Dithiothreitol (Sigma Cat. No. D9779.)

Q: I wish to do a quick screen to determine if a transcription factor DNA binding activity is present in my cells. Is there an alternative protocol that doesn't require making nuclear extracts?

A: Yes. We have developed a Whole Cell Extraction Reagent (Cat. No. 11941-100) specifically for screening purposes and the whole protocol can be completed within 45 minutes. It is important to note that results obtained with samples prepared using the Nuclear Extraction Kit and the Whole Cell Extraction Reagent may differ significantly.

Q: I just want to do a small pilot study and don't have enough samples to fill a whole plate. Can I reuse the plate?

A: The plate comes in a strip-well format that allows 8 wells to be removed from the plate at a time. If you have fewer than 96 samples, simply remove the number of strips you need to carry out your experiment and store the plate until you are ready to run the rest. All buffers and solutions can be stored for further use.

Q: Can I use your Nuclear Extraction Kit protocol (Cat. No. 11906-100) to prepare nuclear extracts from animal tissues?

A: Yes. We have taken extracts from a wide range of different mouse and rat tissues with good success. When preparing extracts from tissues, please keep in mind the following:

Some tissues (muscle for example) are tougher to homogenize than others. For these tissues, it is important to make sure that during homogenization the samples do not get too warm. Perform all homogenization on ice. Also avoid excessive bubbling of the sample as this will denature proteins in the sample.

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Some tissues are “dirtier” than others (e.g. liver has a lot of blood whereas brain is relatively clean). When preparing nuclear extracts from “dirty” tissues, make sure the nuclear pellet is washed until the supernatant is clear. The protocol recommends two washes for most samples, but it may be necessary to perform as many as 4 or 5 washes. Transcription factors will remain associated with the nuclear DNA throughout these washes.

Q: What species cross-reactivity information do you have?

A: The probe sequences used are highly conserved among species and the probes were designed to have the maximal cross-reactivity with multiple species. We have demonstrated that the assay works with samples prepared from human, mouse, rat, mink and monkey cell lines and/or tissues.

Q: We understand that AP-1 can have several different proteins in the complex, such as c-jun/c-fos heterodimer, c-jun homodimer, etc. The specific family members that the assay detects is not mentioned. Why?

A: This transcription factor assay is designed to give the most comprehensive measurement of the activity of known and unknown transcription factors. The technology used in the assay is based on the use of specific DNA binding sites to detect all transcription factor complexes that bind to a specific DNA sequence, as opposed to antibodies that bind specific epitopes on individual proteins. For example, the AP-1 binding site used in our assay has a core sequence comprising TGAGTCA. This sequence binds the c-jun/c-fos complex with high affinity and also will detect c-jun homodimers. The broad screening capability of the assay ensures that changes in transcription factor activities are detected even if the protein isoforms in the complex are not previously known. Marligen's assays are therefore uniquely suited to measure gene regulation events mediated by known or previously unidentified protein complexes.

Q: What component contains the specific biotinylated probes?

A: Binding Mix 2 contains the biotinylated probes.

Q: According to the protocol, Digestion Mix (Digestion Buffer containing Digestion Reagent) removes unbound biotinylated probes. Does this affect the probes that have the transcription factor bound to them?

A: No. The binding of the transcription factors protects the probes from being digested. The protected probes are then detected through the biotin label. The signal generated is directly proportional to the amount of the activated transcription factor that was in the nuclear extract.

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Q: Is the transcription factor assay quantitative?

A: Yes, the assay is quantitative when comparing extracts. This means that if one sees a two-fold signal increase between a control and treated sample, it is reasonable to conclude that there is twice as much active transcription factor in the one sample compared to the other. However, since the transcription factors often exist as complexes of two or more different proteins it is not possible to quantitate specific mass amounts of individual proteins.

Q: When I calculate the percent digestion, it is < 95%. What can cause this?

A: Typically the mishandling of the Digestion Reagent or the Complete Digestion Mix causes the digestion step to be incomplete. The digestion reagents and resulting Complete Digestion Mix contains proteins that require careful handling. Some common mistakes include harsh mixing or vortexing of the Complete Digestion Mix, introducing bubbles into the solution when pipeting either the Digestion Reagent or the Complete Digestion Mix, leaving the Complete Digestion Mix on the bench for too long (>15 minutes) prior to use, or using polystyrene tubes or reservoirs when preparing the mix. In order to obtain optimal results, mix gently by pipeting up and down, do not mix the Complete Digestion Mix more than 15 minutes prior to use and use polypropylene or polycarbonate tubes in the assay.

Q: Can multiple transcription factors be measured on the same plate?

A: Yes. The first step of each assay is specific for the individual transcription factor being measured. All of these reactions are carried out in solution. However the same capture plate and detection method is used for all of the assays. Therefore when samples are ready to be captured, all the samples can be assayed using the same plate. This allows different transcription factors from the same sample to be measured side by side.

Q: The negative controls show higher signals than my experimental samples. Is this a problem? What causes this result?

A: Negative controls do not contain nuclear extract. If high levels of endogenous nuclease are present in extract samples, the biotinylated probes used in the assay will be degraded to cause this problem. Endogenous nuclease has been noted in extracts from certain samples from cells undergoing apoptosis or in cases where nuclear pellets are not washed well prior to extraction.

To determine if endogenous nuclease may be present in a sample, assays can be run without the addition of Digestion Reagent to Digestion Buffer. If samples contain endogenous nuclease DNA probes will be degraded and assay signals will be greatly reduced as compared to the positive control.

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To minimize the amount of nuclease in the samples it is necessary to thoroughly wash the nuclear pellets with the Wash Buffer in the Nuclear Extraction Kit. It will also help to homogenize the tissues as gently as possible to avoid disrupting compartmentalized nucleases.

A second cause for low sample signals compared to the negative control is that the correct negative control has not been used. The appropriate negative control is 3 μ L Sample Diluent Buffer.

For further technical assistance please contact our Technical Service Department at technical.support@marligen.com or (301)-874-4990.