Technical Bulletin

Core Footprinting System

INSTRUCTIONS FOR USE OF PRODUCT E3730.

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I. Description

The Core Footprinting System provides the reagents necessary to identify transcription factor binding sites. The system includes a control transcription factor extract (AP2 Extract) and a purified SV40 Positive Control DNA in addition to the buffers and enzymes required to perform 5´-end labeling and digestion of the DNA.

Footprinting experiments require a double-stranded, single-end-labeled DNA probe. To obtain such a probe, an isolated DNA fragment is labeled with ^{32}P and digested with a restriction enzyme that releases one of the labeled ends. This procedure may be facilitated by subcloning the fragment into a polylinker. For example, if the fragment is cloned into the *Bam*H I site of the pUC polylinker, the *Eco*R I/*Hin*d III fragment can be isolated, labeled and digested with *Kpn* I or *Pst* I to generate a single-end-labeled fragment. Promega offers many plasmid vectors containing polylinkers that can be used for this purpose. (These include but are not limited to pGEM®-3Zf(+/–)(Cat.# P2261, P2271), pGEM®-5Zf(+/–)(Cat.# P2241, P2351) and pGEM®-7Zf(+/–)(Cat.# P2251, P2371) Vectors).

The SV40 Positive Control DNA provided with the Core Footprinting System consists of a gel-purified, 324bp DNA fragment of the SV40 early promoter/enhancer region. A bacterial extract containing the human recombinant transcription factor AP2 is supplied as a positive control footprinting extract. This extract produces approximately the same footprint on SV40 DNA as the purified transcription factor AP2. The extract is incubated with labeled SV40 Positive Control DNA, and the DNA is partially digested by incubating with RQ1 RNase-Free DNase. The region of AP2 binding, protected from DNase digestion, is visualized as a reduction of the intensity of at least one band in the region of the degradation pattern (region 74–81) and/or an enhancement of the intensity of the band above the 74–81 region (Figure 1). The sequence of the SV40 fragment and the location of the major AP2 binding site as well as the binding sites for other transcription factors are shown in Figure 2.

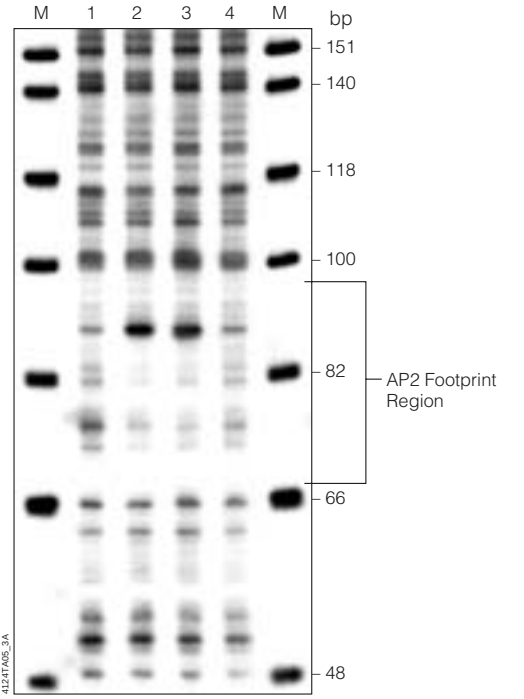

Figure 1. Autoradiogram of the footprint seen with the AP2 Extract on the SV40 Positive Control DNA. Lanes 1 and 4, no protein; lanes 2 and 3, 2µl AP2 extract; lanes M, ^{32}P end-labeled ϕx174/*Hin*f I markers. The film was exposed for 16 hours at –70°C with an intensifying screen.

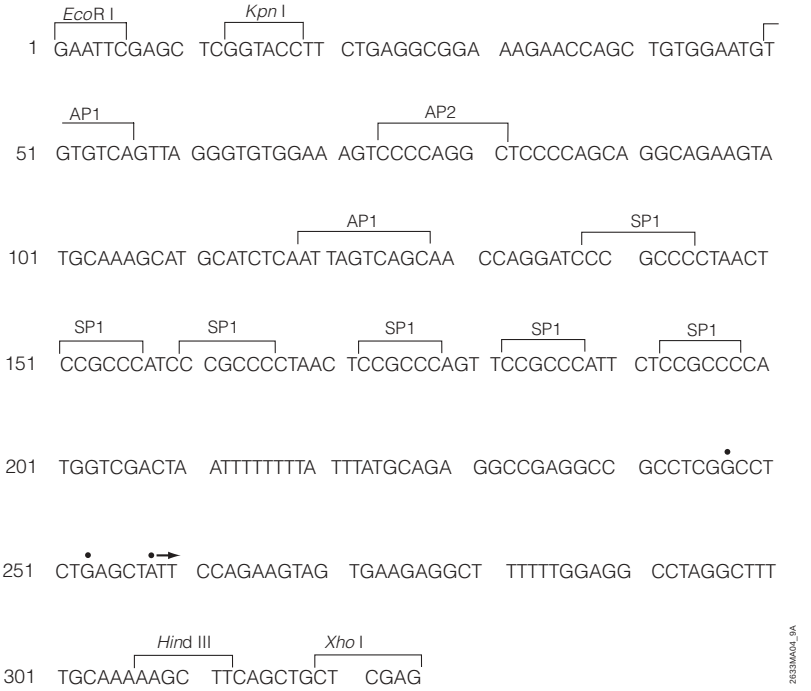

Figure 2. SV40 Positive Control DNA. The AP2 binding site (1–3) is located at positions 74–81. The (•) designates the early mRNA cap sites, and the arrow indicates the direction of mRNA synthesis. AP1 binding sites (4,5) are located at positions 50–56 and 119–128. SP1 binding sites (6) are located at positions 139–144, 151–156, 160–165, 172–177, 182–187 and 193–198.

II. Product Components and Storage Conditions

Product	Cat.#
Core Footprinting System	E3730

Each system contains sufficient reagents to perform 50 footprinting reactions and sufficient SV40 Positive Control DNA and extract to perform 10 positive control reactions. Includes:

- 20µl AP2 Extract
- 0.3µg SV40 Positive Control DNA (10µl)
- 5,000u *Hin*d III
- 1ml Restriction 10X Buffer E
- 1,000u Alkaline Phosphatase, Calf Intestinal (CIAP)
- 200u T4 Polynucleotide Kinase (PNK) (2 × 100u)
- 100µl Kinase 10X Buffer
- 1,000u RQ1 RNase-Free DNase (DNase I)
- 5ml Stop Solution
- 1.5ml 10mM Tris-HCl (pH 8.0)
- 3ml Ca^{2+}/Mg^{2+} Solution
- 1.5ml Binding Buffer
- 0.5ml Loading Solution
- 1 Protocol

Storage Conditions: Store the AP2 extract at –70°C and all other components at –20°C.

III. Preparation of Probe

The SV40 Positive Control DNA is a gel-purified, dephosphorylated 324bp fragment containing SV40 early promoter region sequences with binding sites for various transcription factors (Figure 2).

To use the SV40 Positive Control DNA in footprinting, it is first labeled at both ends using T4 Polynucleotide Kinase. A single-end-labeled probe (306bp) is then generated by digesting the label off one end with *Hin*d III (Figure 3). Alternatively, the opposing strand can be labeled by digesting the DNA with *Kpn* I (310bp probe) instead of *Hin*d III.

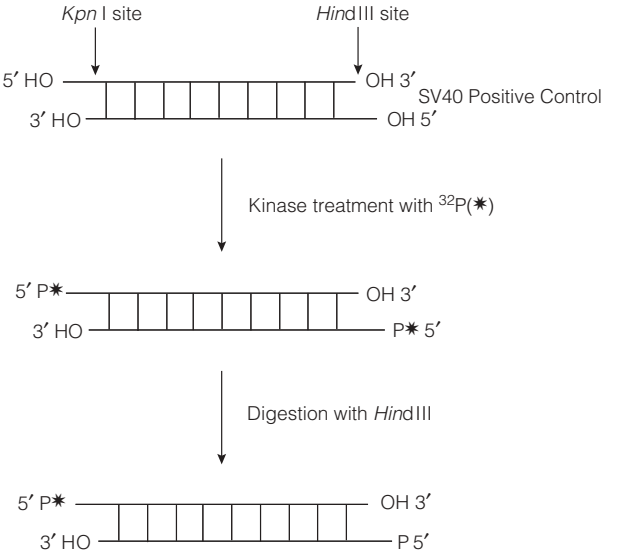

Figure 3. Schematic of preparation of the single-end-labeled SV40 Positive Control DNA.

Materials to Be Supplied by the User

(Solution compositions are provided in Section VI.)

- CIAP 10X Reaction Buffer
- 0.5M EDTA
- [γ-^{32}P]ATP (at 3,000Ci / mmol, 10mCi / ml)
- 100% ethanol
- TE buffer
- phenol:chloroform:isoamyl alcohol (25:24:1, equilibrated with TE buffer (pH 7.4) and 0.5M NaCl)
- 5M NaCl
- Nuclease-Free Water (Cat.# P1193)

III.A. Dephosphorylation of 5´-Ends

1. If DNA other than the SV40 Positive Control DNA is used, it must be treated with CIAP to remove the 5´-terminal phosphate groups prior to the labeling reaction. Add the following components directly to the DNA in a microcentrifuge tube:

CIAP 10X Reaction Buffer	10µl
CIAP (0.01u/pmol ends)	1–2µl
Nuclease-Free Water to a final volume of	100µl

Note: Each pmol of DNA ends will require 0.01u CIAP (1µg of 1,000bp DNA = 1.52pmol DNA = 3.03pmol ends). For assistance in calculating picomol ends for your DNA visit the Biomath Calculator at: **www.promega.com/biomath/**

2. Incubate using one of the following conditions, depending on the type of ends present:

   For 5´-protruding ends: Incubate for 30 minutes at 37°C. Add another 0.005u CIAP/pmol of ends and incubate an additional 30 minutes at 37°C.

   For 5´-recessed or blunt ends (or mixed ends): Incubate for 15 minutes at 37°C then for 15 minutes at 56°C. Add another 0.005u CIAP/pmol of ends and repeat incubations at both temperatures.

3. To stop the reaction, add 2.0µl of 0.5M EDTA and heat at 65°C for 20 minutes.

4. Purify the DNA. This can be done rapidly using the the Wizard® DNA Clean-Up System (Cat.# A7280) or by phenol extraction and ethanol precipitation.

III.B. Labeling and Digestion of the SV40 Positive Control DNA

1. The SV40 Positive Control DNA and any experimental DNA must first be labeled with T4 Polynucleotide Kinase. Assemble the following reaction in a microcentrifuge tube:

SV40 Positive Control DNA (0.3µg, 3pmol ends)	10µl
Kinase 10X Buffer	2µl
T4 Polynucleotide Kinase	1µl
[γ-^{32}P]ATP (at 3,000Ci/mmol, 10mCi/ml)	1µl
Nuclease-Free Water to a final volume of	20µl

Do not use DNA that has been suspended in, or precipitated from, buffers containing ammonium salts. Ammonium ions are strong inhibitors of bacteriophage T4 PNK.

2. Incubate at 37°C for 30 minutes. Add an additional 1µl of T4 Polynucleotide Kinase and incubate an additional 30 minutes at 37°C.

3. Remove the label from one end of the fragment by digesting with *Hin*d III. Assemble the following reaction in a microcentrifuge tube:

Phosphorylated DNA fragment (from above reaction)	21µl
Restriction 10X Buffer E	5µl
*Hin*d III	40u
Nuclease-Free Water to a final volume of	50µl

Note: When using DNA other than the SV40 Positive Control DNA, a different restriction enzyme may be required to remove the label from one end.

4. Incubate at 37°C for 1 hour.
5. Add 1µl of 5M NaCl and extract with 50µl of phenol:chloroform:isoamyl alcohol (25:24:1). Vortex and spin at 14,000 × *g* in a microcentrifuge for 5 minutes.
6. Transfer the upper, aqueous phase to a fresh tube and extract as above with an equal volume of chloroform:isoamyl alcohol (24:1).
7. Transfer the upper, aqueous phase to a fresh tube, add 2 volumes of 100% ethanol and precipitate for 20 minutes on ice.
8. Centrifuge at 14,000 × *g* in a microcentrifuge for 10 minutes and dry under vacuum. Suspend the probe in 100µl TE buffer. Store at 4°C.

IV. Use of Probe in Footprinting

Footprinting reactions using the SV40 Positive Control DNA are described below. These binding conditions can be used to footprint Promega purified AP1 (Cat.# E3061) and SP1 (Cat.# E6391), although these conditions may require further optimization when footprinting SP1. Several parameters must be optimized for footprinting of samples other than the provided positive control. Some of these parameters are briefly discussed in Table 1 (Section IV.A).

Note: Do not add competitor DNA such as poly(dl-dc)•poly(dl-dc) to the binding reaction when footprinting purified transcription factors.

Materials to Be Supplied by the User
(Solution compositions are provided in Section VI.)

- 100% ethanol
- 6% polyacrylamide 7M urea gel
- phenol:chloroform:isoamyl alcohol (25:24:1)
- 1M Urea (Cat.# V3171)

IV.A. Footprinting Protocol

1. Warm the Stop Solution to 37°C and hold until use. Bring the Ca^{2+}/Mg^{2+} solution to room temperature and hold until use. Immediately prior to use, dilute 5µl of RQ1 RNase-Free DNase (1u/µl) in 100µl of cold Tris-HCl (pH 8.0).

2. Assemble the following reactions in microcentrifuge tubes:

Control		**Sample**	
Binding Buffer	25µl	Binding Buffer	25µl
Nuclease-Free Water (at 4°C)	20µl	Nuclease-Free Water (at 4°C)	18µl
—		AP2 Extract	2µl
probe	5µl	probe	5µl
total volume	50µl	total volume	50µl

3. Mix and incubate on ice for 10 minutes.

   Note: It is important to treat each reaction identically in the following manipulations. If running more than two samples, process the samples two at a time to achieve similar results.

4. Add 50µl of room temperature Ca^{2+}/Mg^{2+} Solution and incubate at room temperature for one minute.

5. Add 3µl of diluted RQ1 RNase-Free DNase, mix gently, but thoroughly, and incubate at room temperature for one minute. The remaining diluted RQ1 RNase-Free DNase should be discarded.

   Note: There may be batch-to-batch variability in the RQ1 RNase-Free DNase activity, and the amount of RQ1 RNase-Free DNase added may need to be optimized for other probe samples. RQ1 RNase-Free DNase should be added at a concentration that allows approximately one random nick per molecule; the concentration must be determined empirically by titration of the enzyme.

6. Terminate the reaction by adding 90µl of Stop Solution. Mix well.

7. Extract each reaction with 200µl of phenol:chloroform:isoamyl alcohol (25:24:1) as in Section III.B, Step 5. Chloroform extraction is not necessary.

8. Transfer 170µl of the upper, aqueous phase to a fresh tube, add 500µl of 100% ethanol and precipitate on ice for 20 minutes.

9. Centrifuge at 14,000 × *g* for 5 minutes. Carefully remove the supernatant, wash with 70% ethanol and dry under vacuum.

10. Suspend the pellet in 8µl of Loading Solution and 2µl of 1M Urea by vortexing and flicking the tube. Heat at 95°C for 2 minutes and quick chill on ice for at least 2 minutes.

11. Load 5µl onto a 6% polyacrylamide, 7M Urea sequencing gel. Run the gel at 1500V, 60 watts in TBE 1X buffer until the bromophenol blue from the AP2 Extract Control reaction is at the bottom. Expose the gel on film at –70°C with an intensifying screen. Alternatively, process the gel using a phosphorimaging instrument.

Table 1. Critical Parameters to Optimize in DNase I Footprinting.

Parameter	Optimized Variable	Comments
DNA probe	High specific activity	Use approximately 15,000–20,000cpm per 1–20pmol DNA per assay.
	Purity	Purify the DNA by agarose or acrylamide gel electrophoresis, followed by electroelution and clean-up (phenol-extraction, reversed phase or ion exchange chromatography, or Wizard® PCR Preps DNA Purification System, Cat.# A7170).
	Size of DNA probe and location of DNA binding	The probe should be 100–600bp, with the protein - binding site no closer than 25bp from the labeled end. Protein binding sites as far as 400bp from the labeled end can be used but require longer electrophoresis and bands are not as sharp.
	Labeling	The probe must be labeled at only one end.
Protein	Purity	Only relative binding affinity can be measured in crude extracts. High purity is required for kinetic binding studies.
	Concentration	The number and intensity of bands corresponding to the protected region decrease as protein concentration increases. Footprinting can be used to obtain binding data (7) and to characterize the fully protected DNA region at saturating protein concentrations. In crude HeLa extracts, suggested titrations are 10, 20, 40 and 80µg protein.

(continued)

Table 1. Critical Parameters to Optimize in DNase I Footprinting (continued).

Parameter	Optimized Variable	Comments
DNA / protein binding conditions	Temperature	4–37°C.
	Time	10–60 minutes.
	pH	pH 5–9, with most reactions at pH 7–8.
	K^+ concentration	50–200mM. For most DNA binding proteins, specific binding affinity decreases with increasing K^+ concentration while specificity of binding increases.
	Mg^{2+} concentration	0–10mM. DNase I requires divalent metal ions Mg^{2+} (or Mn^{2+}) and Ca^{2+}. A Ca^{2+} / Mg^{2+} solution is supplied.
	Ca^{2+} concentration	0–1mM. DNase I requires the divalent metal ions Mg^{2+} (or Mn^{2+}) and Ca^{2+}. A Ca^{2+} / Mg^{2+} solution is supplied.
	PEG	0–5%. Use only high-quality PEG (8,000 MW). PEG may change the rate at which DNase I cuts.
	Nonionic detergents	0–0.1% Triton® X-100.
DNase I	Concentration	Adjust DNase I concentration such that 1 nick occurs per molecule of DNA probe.
	Reaction time	30–120 seconds. Optimize the time using the DNA probe in absence of binding proteins.

V. 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-protein control uneven, with more intense bands at the top of the gel and low band intensity at the bottom of the gel	Underdigestion by DNase I. Optimize reaction using a range of DNase I concentrations.
	DNase I not mixed adequately. Mix DNase I thoroughly with sample by gently pipetting, stirring or vortexing at lowest setting for 3 seconds.
	Conditions used in binding assay do not favor DNase I activity. Include the storage buffer of the DNA-binding protein in the no-protein control.
No-protein control uneven, with more intense bands at the bottom of the gel and less intense bands at the top of the gel	Overdigestion by DNase I. Optimize reaction using a range of DNase I concentrations.
	Specific activity of DNA probe too low. DNA probe concentration should be low relative to protein concentration, and labeling efficiency should be 40–50%.
	DNase I not inhibited by Stop Solution. Stop Solution should be heated and mixed well. It should be kept at 37°C until immediately prior to use, and samples should be vortexed after Stop Solution addition.
Faint signal in some lanes even when the same probe worked well in other lanes under the same conditions	Sample not completely dissolved in Loading Buffer. Vortex sample well and spin down prior to heating and loading on gel. We recommend using silanized microcentrifuge tubes.
	Pellet lost during ethanol precipitation. Place tubes in centrifuge with cap hinge at top and note position of pellet. Draw ethanol off gently so as not to disturb the pellet.
Some lanes overdigest while others underdigest under the same DNase I concentrations	Variation in handling reactions. Time the exposure of sample to DNase I, and handle all samples in identical fashion while mixing DNase I. Treat samples in groups of 1 or 2 to facilitate handling and ensure consistent treatment.
No-protein control overdigests while protein-containing reactions work well	DNase I digestion conditions altered by addition of protein. Optimize no-protein control with different, usually lower, DNase I dilutions than the sample containing DNA binding proteins.

V. Troubleshooting (continued)

Symptoms	Causes and Comments
Doublet bands in undigested probe and digestion pattern	Probe stored too long and beginning to degrade. Store labeled probes for no more than one to two weeks.
No DNase I ladder on gel, but probe signal is strong	Inactive or underactive DNase I. Optimize using a range of DNase I concentrations.
	Insufficient DNase I used. Optimize using a range of DNase I concentrations.
	DNase I inactivated during handling. Vortex DNase I for 3 seconds only at lowest setting or mix manually by stirring or pipetting.
	High concentrations of $(NH_4)SO_4$ or NaCl in DNA-binding protein(s) fractions inhibit DNase I. Dialyze DNA binding protein fractions against storage buffer at 4°C.
DNase I ladder narrows as samples run to bottom of gel	Excess salt or EDTA in sample. Wash samples 2 times in 70% ethanol before drying. Reduce amount of EDTA in Stop Solution.
DNA bands fuzzy	Intensifying screen and film not in direct contact with gel. Repeat exposure with tighter cassette or add extra layers of paper or exposed film for tighter contact.
	Impurities in acrylamide or gel buffer. Use ultrapure-grade reagents.
	Inadequate buffering during gel run. Check TBE concentration. Do not run gel at excessive voltage to avoid buffer breakdown.
	Excess nucleic acid in sample. Reduce amount or eliminate carrier tRNA and competitor DNA.
Weak signal	DNA probe will not kinase well with T4 PNK following CIAP treatment. Ammonium ions are strong inhibitors of T4 PNK. DNA should not be dissolved in, or precipitated from, buffers containing ammonium salts prior to treatment with kinase.
	High TCA-precipitable label incorporation but low amount of labeled probe at gel purification step. Sample may be contaminated with low MW RNA or bacterial genomic DNA, which can compete for kinase labeling. Prepare plasmid DNA by CsCl gradient centrifugation.

VI. Composition of Buffers and Solutions

Binding Buffer

50mM	Tris-HCl (pH 8.0)
100mM	KCl
12.5mM	$MgCl_2$
1mM	EDTA
20%	glycerol
1mM	DTT

Ca^{2+}/Mg^{2+} Solution

5mM	$CaCl_2$
10mM	$MgCl_2$

CIAP 10X reaction buffer

500mM	Tris-HCl (pH 9.3)
10mM	$MgCl_2$
1mM	$ZnCl_2$
10mM	spermidine

Kinase 10X Buffer

700mM	Tris-HCl (pH 7.6)
100mM	$MgCl_2$
50mM	DTT

Loading Solution

1:2	0.1M NaOH:formamide (v/v)
0.1%	xylene cyanol
0.1%	bromophenol blue

Restriction 10X Buffer E

60mM	Tris-HCl (pH 7.5)
1M	NaCl
60mM	$MgCl_2$
10mM	DTT

The pH of the 1X Buffer at 37°C is pH 7.5.

Stop Solution

200mM	NaCl
30mM	EDTA
1%	SDS
100µg/ml	yeast RNA

TBE 1X buffer

89mM	Tris-base
110mM	boric acid
2mM	EDTA

TE buffer

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

Tris-HCl (pH 8.0)

10mM	Tris-HCl (pH 8.0)

VII. Related Products

Product	Size	Cat.#
rhAP1 (c-jun)	50fpu	E3061
rhSP1	15µg	E6391
rhNF-κB (p50)	50gsu	E3770
rhTFIIB	50gsu	E3790

fpu = the amount of protein required to yield a complete footprint on SV40 Early Promoter DNA.
gsu = the amount of protein required to gel shift the NF-κB oligonucleotide under defined conditions.

VIII. References

1. Mitchell, P.J., Wang, C. and Tjian, R. (1987) Positive and negative regulation of transcription in vitro: Enhancer-binding protein AP-2 is inhibited by SV40 T antigen. *Cell* **50**, 847–61.
2. Williams, T. *et al.* (1988) Cloning and expression of AP-2, a cell-type-specific transcription factor that activates inducible enhancer elements. *Genes Dev.* **2**, 1557–69.
3. Mercurio, F. and Karin, M. (1989) Transcription factors AP-3 and AP-2 interact with the SV40 enhancer in a mutually exclusive manner. *EMBO J.* **8**, 1455–60.
4. Lee, W., Mitchell, P. and Tjian, R. (1987) Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell* **49**, 741–52.
5. Mermod, N., Williams, T.J. and Tjian, R. (1988) Enhancer binding factors AP-4 and AP-1 act in concert to activate SV40 late transcription in vitro. *Nature* **332**, 557–61.
6. Gidoni, D. *et al.* (1985) Bidirectional SV40 transcription mediated by tandem Sp1 binding interactions. *Science* **230**, 511–7.
7. Letovsky, J. and Dynan, W.S. (1989) Measurement of the binding of transcription factor Sp1 to a single GC box recognition sequence. *Nucl. Acids Res.* **17**, 2639–53.

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