Published in final edited form as:

Methods Mol Biol. 2014 ; 1187: 231–245. doi:10.1007/978-1-4939-1139-4_18.

Analyzing the nuclear complexes of Notch signaling by Electrophoretic Mobility Shift Assay

Kelly L. Arnett¹ and Stephen C. Blacklow^{1,2}

¹Harvard Medical School, Boston, MA 02115

²Dana Farber Cancer Institute, Boston, MA 02115

Summary

An Electrophoretic Mobility Shift Assay (EMSA) is a sensitive technique for detecting protein-DNA and protein-protein interactions in which complexes are separated by native (non-denaturing) gel electrophoresis. EMSAs can provide evidence for specific binding between components prepared from a wide range of sources, including not only highly purified proteins but also components of crude cellular extracts. EMSA experiments were critical in identifying the minimal protein requirements for assembly of transcriptionally active nuclear Notch complexes as well as the DNA sequence specificity of Notch transcription complexes. Here, we describe a radioactive EMSA protocol for detection of Notch transcription complexes.

1. Introduction

Upon activation of the Notch signaling pathway, the intracellular portion of Notch (NICD) is released from the membrane, translocates to the nucleus, and assembles into a transcriptional activation complex (1–4). In the nucleus, NICD interacts with the DNA-binding protein called CSL (gene name *RBPJ* in mammals) and a member of the Mastermind family, such as mammalian Mastermind-Like-1 (MAML1). The minimal regions of Notch required for assembly of the core Notch transcription complex are a highly conserved region immediately internal to the cell membrane called RAM, and an ankyrin-repeat domain (ANK; together, this part of NICD is referred to as RAMANK).

CSL has three structured domains, an N-terminal Rel-homology region (NTD), a β -trefoil domain (BTD), and a C-terminal Rel-homology region (CTD). The Notch RAM peptide binds to the isolated β -trefoil domain with high affinity (5, 6), and the ANK domain of Notch, which has only weak intrinsic affinity for CSL, engages the Rel-homology domains of CSL to create a composite binding surface for MAML1 (7, 8). Only a very short region of 1016 amino acid MAML1 (13–74) is required for cooperative binding to CSL and Notch-RAMANK (Figure 1)(9). Transcriptional activation of Notch-responsive genes is also dependent on the recruitment of additional coactivators linked to the general transcription machinery, such as p300 (E1A binding protein p300) or CREB-binding protein (CBP) (10, 11). A number of co-repressors have been shown to interact with CSL in order to repress

Notch target gene expression, including MINT/SHARP, SMRT, SKIP, CIR, and KyoT2 in mammals (12–17).

Initial findings about the composition of Notch nuclear complexes came from organismal and cellular systems. Many of the details of the interactions involved in assembly of the NTC have been elucidated by experiments involving highly purified components, using experimental approaches that include X-ray crystallography, isothermal titration calorimetry, circular dichroism, analytical ultracentrifugation, and hydrogen exchange coupled mass spectrometry. The electrophoretic mobility shift assay (EMSA) is a useful experimental tool for detecting protein-DNA and protein-protein interactions from a wide range of sources, from crude cellular extracts to highly purified proteins. A number of the key findings about the minimal requirements for nuclear Notch complex assembly were derived from EMSA experiments (5, 9, 10, 13, 18–23).

An electrophoretic mobility shift assay (EMSA), or gel shift assay, is a sensitive method for detecting molecular interactions. EMSAs are based on the observation that under native gel electrophoresis conditions, higher order complexes tend to migrate more slowly than the individual components. Rates of mobility in a non-denaturing native gel are determined by both molecular weight and charge, so the EMSA approach is particularly applicable in detecting protein-DNA interactions, as DNA carries a negative charge due to the phosphate backbone and its mobility is strongly correlated with molecular weight. Therefore, a protein-DNA complex will almost invariably migrate more slowly than free DNA. DNA is very easy to label specifically, allowing detection of DNA binding from a crude nuclear extract, without the need for extensive protein purification. Protein-protein EMSAs are also possible but can be complicated by the wide range of molecular charges that proteins adopt, which more significantly modulate electrophoretic behavior. Most EMSAs performed today are closely based on the original assays described over thirty years ago by Fried and Crothers (24) and Garner and Revzin (25). Extensive review, updated protocols, and troubleshooting of protein-DNA EMSAs are also available (26, 27).

Several labeling strategies are commonly used in protein-DNA EMSA protocols. The original labeling method, and still most commonly employed method due to its high sensitivity, is radioactive labeling with α - ^{32}P -dNTP. Several non-radioactive DNA detection methods are also routinely used in EMSA protocols, including chemiluminescent detection of biotinylated DNA, detection of fluorescently labeled DNA, and staining with intercalating fluorescent dyes (28–31). Unlike isotopic DNA labeling, which has little or no effect on protein binding affinity, use of non-isotopic labels including fluorophores or biotin can interfere with protein-DNA interactions. Higher protein and DNA concentrations are typically required for methods using fluorescent detection and DNA staining. The requirement for transfer to nitrocellulose is a complicating factor in chemiluminescence-based methods for detecting biotin-DNA. Equivalent transfer of disparately sized protein-DNA complexes is difficult, making this method less quantitative than direct detection methods.

Here we outline an EMSA protocol that has been optimized for detection of purified nuclear Notch complexes bound to ^{32}P -labeled DNA. The protocol takes 2–4 days to complete with

purified proteins in hand. We have added, as an appendix, protocols for purification of bacterially produced constructs encoding the minimal human NTC components, Notch1-RAMANK(1760–2126), CSL(9–435), and MAML1(13–74).

2. Materials

Ultrapure water should be used for all protein purification, DNA labeling and protein/DNA binding steps. Deionized water is sufficient in the gel electrophoresis buffers.

2.1. Reagents

1. Protein of interest at a concentration sufficient to bind DNA. For the method outlined here, 100–300 ng human CSL(9–435), 500–1000 ng human Notch-RAMANK(1760–2126), and 100–1000 ng human MAML1(13–74) per binding reaction are used. See Appendix (section 4) for expression and purification protocols for each of these proteins. If the protein concentration is near the estimated K_d , then a significant fraction of DNA will be shifted by bound protein.
2. DNA Oligonucleotides to be annealed. Purchase or synthesize single-stranded DNA oligonucleotides designed with at least 18 base-pairs of overlap and 3–5 nucleotide overhangs at each 5'-end. Include at least 3 nucleotides complementary to the ^{32}P -nucleotide being incorporated (see Note 1). If protein concentrations are significantly below the K_d , then more DNA or more ^{32}P incorporation into the labeled DNA duplex may be required for a detectable DNA shift.
3. α - ^{32}P -dCTP 250 mCi, 6000 Ci/mmol (Perkin Elmer).
4. 10× DNA labeling buffer: 500 mM NaCl, 100 mM Tris-HCl, 100 mM MgCl_2 , 10 mM DTT, pH 7.9. This can be made or purchased (NEB Buffer 2, New England Biolabs).
5. 20× dNTPs: 670 μM each of dATP, dTTP, dGTP. Stored at -20°C .
6. Klenow Enzyme, 5 U/l (New England Biolabs).
7. Siliconizing agent (Sigmacote, Sigma-Aldrich).
8. 10× Running Buffer: 250 mM Tris base, 2.5 M Glycine, (10 mM EDTA, optional) in 1 liter. Eliminate EDTA if metal-coordinating proteins will be used in the assay. For each experiment, prepare a sufficient volume of 1× Running buffer (see Note 2).
9. 10% (w/v) Ammonium Persulfate (APS). May be stored at 4°C for 1 month or frozen.
10. TEMED (N,N,N',N'-tetramethylethylenediamine).
11. 30 (w/v) Acrylamide solution (AcrylaGel, National Diagnostics).

¹We design a 3–5 nucleotide overhang at the 5'-end of each oligo with a total of 3 dGTP (typically 2 on one 5'-end and 1 on the other 5'-end) for incorporation of 3 ^{32}P dCTP nucleotides for EMSAs performed with 0.2–1 pmol DNA per lane.

²For a large format gel apparatus, 3 liters of 1× Running buffer is sufficient.

12. 2% (w/v) Bis-acrylamide solution (Bis-AcrylaGel, National Diagnostics).
13. 2.5× Binding/Loading buffer: 25% glycerol, 50 mM HEPES pH 7.9, 150 mM KCl, 12.5 mM MgCl₂, 25 mM DTT, 25 µg/ml poly-dIdC, 0.5 mg/ml BSA. May be frozen for a month.
14. Marker dye containing bromophenol blue, such as 6× DNA loading buffer 30% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF.

2.2. Equipment

1. Microcentrifuge.
2. Microspin gel filtration columns (Illustra Microspin G25 or G50 column, GE Healthcare). Choice of resin will be guided by size of DNA being purified.
3. Waterbath, set to 30°C.
4. Gel electrophoresis apparatus (Protean II xi, Bio-Rad) (see Note 3).
5. Siliconized gel electrophoresis plates (see Note 4), 0.75 mm or 1 mm spacers and wide-tooth combs.
6. Cold room or gel cooling apparatus.
7. Gel dryer.
8. Film and developer or phosphorimager.

3. Methods

The protocol described here calls for the use of ³²P, which is a source of harmful β-radiation. Follow the safety protocols outlined by your institution for the safe handling and disposal of this isotope.

3.1. Anneal and Label DNA

1. Mix complementary oligonucleotides to a final concentration of 10⁻⁵ M of each in 25 mM Tris pH 8 (Mix 5 µl of each oligo at 10⁻⁴ M in 2 µl 0.5 M Tris pH 8 and 38 µl water).
2. Heat to 100°C for 10 minutes. Slowly cool to room temperature for 4–16 hours.
3. Radiolabel double-stranded DNA probes with a 3' fill-in reaction using Klenow fragment. Mix 2 µl annealed DNA duplex, 2 µl 10× DNA labeling buffer (NEB Buffer 2), 13.5 µl water, 1 µl 20× dNTP mix (670 µM), 1 µl α-³²P-dCTP, 0.5 µl Klenow enzyme (see Note 5).

³A large format is recommended in order to maximize resolution. This is especially true if proteins are dilute when loaded and therefore must be loaded in a large volume. 15–20 µl load volumes on a large format gel with a 15-well comb will generally result in well-resolved bands of a wide range of sizes.

⁴Siliconizing the inside of each plate before every use will facilitate transfer of the wet gel after electrophoresis.

⁵DNA can be labeled up to a week before the EMSA. As the ³²P-dNTP or DNA probe decays, exposure times will need to be increased.

4. Incubate at room temperature for 15 minutes. Stop reaction with 10 mM EDTA and dilute to 50 μ l for a final concentration of 0.4 μ M (Mix 1 μ L 0.5 M EDTA and 29 μ L water and add to 20 μ l reaction).
5. Remove excess α -³²P-dCTP on a microspin gel filtration column. For each labeling reaction, spin a new column at 3000 rpm for 1 minute in a microcentrifuge to remove the preservative fluid from the resin. Add reaction mix to the column, let sit 1 minute. Place the column in a clean microcentrifuge tube and spin at 3000 rpm for 2 minutes to recover the labeled DNA in the flowthrough. The excess nucleotide will remain on the resin.

3.2. Protein/DNA binding

1. Mix 6 μ l Binding/Loading buffer and proteins (100–300 ng CSL, 500–1000 ng Notch1-RAMANK, 100–200 ng MAML1). Dilute to 14 μ l.
2. Add 1 μ l ³²P-labeled DNA duplex (0.4 pmol) for a total reaction volume of 15 μ l.
3. Incubate for 30 minutes at 30–32°C in a waterbath.
4. Spin down briefly before loading onto the gel.

3.3. Gel Electrophoresis

1. Prepare 10 $\times$ Running buffer.
2. Assemble plates for gel electrophoresis with 0.75 or 1 mm spacers and partially insert combs of the same thickness.
3. Prepare an acrylamide solution with 40:1 ratio of acrylamide to bis-acrylamide in 1 $\times$ Gel Running buffer (see Table 1). Add the ammonium persulfate and TEMED last and mix quickly (see Note 6).
4. Pour the gel then insert the combs fully (see Note 7). Allow the gel to polymerize for at least 30 minutes.
5. Prepare 1 $\times$ Gel Running buffer. For a large format gel, 3 liters is sufficient.
6. After polymerization, remove comb from the gel while holding upside down. Rinse wells thoroughly to remove unpolymerized acrylamide (see Note 8).
7. Assemble gel apparatus and test for leaks (see Note 9). Fill with 1 $\times$ Gel Running buffer.

⁶We usually use a 10% acrylamide gel for EMSAs of Notch nuclear complexes. The high percentage gel results in the sharpest bands and a gel that is resistant to damage during transfer.

⁷It is critical to avoid forming air bubbles in the polymerizing gel. Make sure the plates are very clean and that the gel is poured along one spacer edge so that it fills from the bottom. We pour gels using a 30 ml syringe with a 16–18 gauge needle.

⁸One effective method of removing unpolymerized acrylamide is to rinse with water and then gently insert thin strips of Whatman paper into each well, allowing liquid to wick up the paper before removing carefully.

⁹Test for leaks very carefully. Even a very slow leak that doesn't empty the upper buffer chamber will decrease the rate of electrophoresis.

8. Load samples onto gel. The Binding/Loading buffer contains no dyes (as dyes such as bromophenol blue can interfere with some protein/DNA interactions). Load marker dye in an empty lane (see Note 10).
9. Run the gel at 4°C for 1–4 hours, at a voltage appropriate for the gel apparatus, being careful to avoid overheating the gel as this may denature protein/DNA complexes (see Note 11).

3.4. Detection of Bands on Gel

1. After electrophoresis is complete, remove gel and plate assembly. Carefully pry apart gel plates, allowing gel to remain attached to one of the plates.
2. Place Whatman paper on the gel to remove it from the glass plate. Cover with plastic wrap.
3. Dry the gel using a vacuum gel dryer, applying heat (70–75°C) and vacuum for up to 2 hours (see Note 12).
4. Autoradiography can be performed using film or phosphor screen. Expose for 30 minutes to overnight before development, depending on the amount and age of ³²P used and the concentrations of protein and DNA in the binding reaction.

Acknowledgements

This work was supported by NIH grants CA-092433 and CA-119070 (to S.C.B.).

Appendix: Expression and Purification of Nuclear Notch Proteins

4.1. Reagents for protein expression and purification

1. Competent bacterial cell lines suitable for IPTG-inducible expression: BL21(DE3), BL21(DE3)pLysS and Rosetta2(DE3)pLysS (EMD Millipore).
2. LB media and agar.
3. Antibiotics: ampicillin (100 mg/ml, sterile filtered), kanamycin (50 mg/ml, sterile filtered), chloramphenicol (34 mg/ml in ethanol).
4. Isopropyl-β-D-thiogalactopyranoside (IPTG).
5. Glutathione sepharose.
6. Ni-NTA agarose.
7. Ethylenediaminetetraacetic acid (EDTA).

¹⁰DNA duplexes that are 20–30 base-pairs in length will typically run near the bromophenol blue band. In order to see the free DNA on the gel, run until the bromophenol blue dye band is about 1 cm from the bottom of the gel.

¹¹Using a large format gel apparatus such as the Bio-Rad Protean II, run 1 hour at 180 V, then increase voltage to 190–200 V for up to 3 more hours.

¹²Removing the gel from the dryer before it has completely dried may lead to cracking of the gel.

8. Protease inhibitors: either one Roche Complete tablet with EDTA per cell lysis or 1:1000 each of 150mM PMSF, 0.7mg/ml Pepstatin A, 2mg/ml Aprotinin, 0.5mg/ml Leupeptin.
9. Reducing agents: β -mercaptoethanol (β ME) and dithithreitol (DTT).
10. Buffers and solution components: Tris, sodium phosphate monobasic, sodium phosphate dibasic, imidazole, Sodium Chloride, Guanidinium•HCl.
11. Acetic Acid.
12. Specific protease for cleavage of tags (*e.g.* Tobacco etch virus protease; TEV).

4.2. Equipment for protein expression and purification

1. Incubator 37°C, 18°C
2. Protein gel electrophoresis apparatus.
3. Centrifuge (4,000 *g*) and rotors to hold 1L bottles and 50 ml tubes.
4. Centrifuge (15,000 *g*) and rotor and appropriate 40–50 ml tubes.
5. Rockers at 4°C and room temperature.
6. FPLC with anion exchange, cation exchange and S200 gel filtration columns.
7. Spin concentrators, 30 KDa and 3.5 KDa molecular weight cutoff.
8. Lyophilizer.

4.3. Protocol for bacterial expression and purification of GST-TEV-RAMANK

1. Plasmid: pGEX-4T or pDEST15, ampicillin resistant, with an insert encoding a TEV cleavage site and human Notch1(1760–2126).
2. Transform competent BL21(DE3) cells with Notch1-RAMANK plasmid and grow on LBA plates (LB agar with 100 mg/L ampicillin) (see Note 13).
3. Grow 20–50 ml overnight culture from a single colony in LBA media (LB with 100 mg/L ampicillin).
4. Inoculate 2 L with 20 ml overnight culture, grow 37°C until culture reaches OD₆₀₀ of 0.5–0.8 AU.
5. Take aliquots before and after induction and at each step during purification (see Note 14).
6. Induce protein expression with 1 mM IPTG. Grow for 4 hours post induction.
7. Harvest cells by centrifugation in 1 L bottles at 4000 *g* for 25 minutes.

¹³Notch1-RAMANK and -ANK proteins can also be expressed in BL21(DE3)pLysS cells.

¹⁴Dilute cell pellets in a volume of SDS-loading buffer proportional to cell density at the time the aliquot was taken and load equal amounts.

8. Resuspend cell pellet in 40 ml lysis buffer (50 mM Tris pH 8.0, 300 mM NaCl) and freeze.
9. While thawing cells, add 5 mM β -mercaptoethanol and protease inhibitors (see Note 15).
10. Sonicate thoroughly to lyse cells and shear genomic DNA. Take care to cool between sonication steps to prevent overheating and possibly denaturing protein. (see Note 16)
11. Centrifuge 14,000–18,000 *g* for 30 minutes and collect supernatant.
12. Apply supernatant to 4 ml pre-equilibrated Glutathione sepharose beads, rock 4°C for 1 hour.
13. Wash beads in a column with 50–100 volumes or in batch at least 3 times with 10 column volumes Wash buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM DTT)
14. Remove GST-tag with TEV protease (100–200 μ g) overnight at 4°C (see Note 17).
15. Collect supernatant from cleavage reaction by spinning down beads and rinsing once with Wash buffer.
16. (Optional) Remove TEV protease by applying to 0.5 ml Ni-NTA agarose (TEV is His-tagged) for one hour and collecting supernatant (see Note 18).
17. (Optional) Purify further on MonoQ anion exchange column: Buffer A: 20 mM Tris pH 8.0, 5 mM DTT, 1mM EDTA, 100 mM NaCl; Buffer B: 20 mM Tris pH 8.0, 5 mM DTT, 1mM EDTA, 1M NaCl.
18. (Optional) Purify further on S200 size-exclusion column: Buffer: 20 mM Tris pH 8.5, 150 mM NaCl, 5 mM DTT.
19. Store at –80°C at concentrations of 1 mg/ml. Expected yield is about 5 mg/L. The molecular weight of Notch1-RAMANK(1760–2126) after TEV cleavage is 40.7 KDa. The molecular weight of the GST-fusion is 69.2 KDa.

4.4. Protocol for bacterial expression and purification of CSL-6H

1. Plasmid: pET28a, Kanamycin resistant, with insert encoding human CSL(9–435)-6H (see Note 19).
2. Transform competent Rosetta2(DE3)pLysS cells with CSL plasmid and grow on LBKC plates (LB agar with 50 mg/L kanamycin, 34 mg/L chloramphenicol).

¹⁵Notch1-RAMANK is proteolytically sensitive. It is important to work fast and keep protease inhibitors in buffers until the protein is pure.

¹⁶Sonicator 3 times for 30 seconds with 30 seconds on ice between sonication steps is usually sufficient.

¹⁷This protocol describes removal of the GST-tag by protease cleavage. For purification of the GST-Notch1-RAMANK fusion, skip the TEV cleavage step and elute with 20 mM glutathione in wash buffer.

¹⁸Removal of TEV protease is not necessary if following with anion exchange.

¹⁹The protein yield of CSL-6H is low after expression and purification. With largescale expression (6–12 L), yields can reach 0.5 mg/L, but may be lower for smaller scale preparations.

3. Grow overnight cultures from a single colony in LBKC media (LB with 50 mg/L kanamycin and 34 mg/L chloramphenicol).
4. Inoculate each 2 L flask with 20 ml overnight culture, grow at 37°C until culture reaches OD₆₀₀ of 0.3–0.5 AU, then lower temperature to 18°C and allow density to reach OD₆₀₀ of 0.7–1.0 AU.
5. Induce protein expression with 0.5 mM IPTG and grow overnight at 18°C.
6. Harvest cells by centrifugation in 1 L bottles at 4000 g for 25 minutes (see Note 20).
7. Resuspend cell pellet in 40–80 ml Lysis buffer (50 mM Tris pH 8.5, 500 mM NaCl) and freeze at –80°C.
8. While thawing cells, add 5 mM β-mercaptoethanol, and protease inhibitors.
9. Sonicate thoroughly to lyse cells (*e.g.* 3 times 30 sec, cooling between sonication steps).
10. Centrifuge at 14,000–18,000 g for 30 minutes and collect supernatant.
11. Batch bind to 5 ml Ni-NTA agarose resin (pre-equilibrated with lysis buffer), 4°C, 1 hour.
12. Wash with 20–50 column volumes wash buffer (50 mM Tris pH 8.8, 0.5 M NaCl, 5 mM βME, 10 mM imidazole).
13. Elute with 2–5 column volumes of Elution buffer (50 mM Tris pH 8.8, 0.5 M NaCl, 5 mM βME, 250 mM imidazole) (see Note 21).
14. Prepare buffers for anion exchange: Buffer A (20 mM Tris pH 8.8, 5 mM DTT) and Buffer B (20 mM Tris pH 8.8, 1 M NaCl, 5 mM DTT). Wash and equilibrate column with 15% B (150 mM NaCl).
15. Dilute protein solution with Buffer A to reduce the NaCl concentration to 150 mM and load this sample onto the column immediately. Elute with a 20 column volume gradient to 40% B (400 mM NaCl).
16. Buffer exchange into Storage buffer (50 mM Tris pH 8.5, 0.5 M NaCl, 5 mM DTT) by size-exclusion chromatography (Superdex 200) if further purification is needed or in a 30 KDa molecular weight cutoff spin concentrator.
17. Store at 1 mg/ml or less, at –80°C in 20% glycerol for long term storage or at 4°C for short term storage (up to a several months if the protein is free of protease contamination). Expected yield is low, up to 0.5 mg/L. The molecular weight of CSL(9–435)-6His is 49.3 KDa.

²⁰CSL yield is very low. An induced band is often not visible on a Coomassie gel.

²¹CSL protein is not very pure at this stage and benefits greatly from a further step of purification.

4.5. Protocol for bacterial inclusion body expression and purification of MAML1

1. Plasmid: pRSETa, ampicillin resistant, with insert encoding 6H-TEV-MAML(13–74).
2. Transform BL21(DE3)pLysS with plasmid (or streak from glycerol stock).
3. Grow 50 ml overnight culture from a single colony in LBAC, in 100 ml flask.
4. Inoculate 2×2L with 20 ml overnight culture, grow at 37°C to OD₆₀₀ 0.8 AU (about 6 hours).
5. Induce with 0.5 mM IPTG for 4 hours.
6. Recover cells by centrifugation and resuspend in 40 ml lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 6 M guanidinium•HCl, 5 mM βME, with 150 μM PMSF).
7. Sonicate 3 times 30 sec, cooling between sonication steps.
8. Centrifuge at 14,000 *g* for 30 minutes.
9. Batch bind to 5 ml Ni-NTA agarose resin (pre-equilibrated with lysis buffer), 4°C, 1 hour.
10. Wash with 10 column volumes wash buffer (50 mM Tris pH 8, 150 mM NaCl, 6 M guanidinium•HCl, 5 mM βME, 10 mM imidazole).
11. Elute with 1 column volume elution buffer (50 mM Tris pH 8, 150 mM NaCl, 6 M Guanidinium•HCl, 5 mM β-mercaptoethanol, 250 mM imidazole).
12. Dialyse against 5% acetic acid for 24 hours and lyophilize at least 24 hours.
13. Dissolve in 1–2 ml water.
14. Slowly adjust pH to 6.2 with phosphate buffer (50 mM Na Phosphate, pH 6.2, 5 mM DTT, 1 mM EDTA).
15. Slowly add TEV protease, cleave overnight at room temperature.
16. (Optional) Remove TEV protease by applying to 0.5 ml Ni-NTA agarose (TEV is tagged with 6His) for one hour and collecting the supernatant (see Note 22).
17. (Optional) Purify on cation exchange column: Buffer A: (20 mM Na Phosphate buffer, pH 6.8, 5 mM DTT, 1mM EDTA), Buffer B (20 mM Na Phosphate buffer, pH 6.8, 2 M NaCl, 5 mM DTT, 1mM EDTA)
18. Important **Note**: Test fractions for binding activity because there is a frequent contaminant that has the same apparent molecular weight as the MAML1 peptide but does not bind Notch and CSL. EMSA is a good method for this test.
19. Store at a concentration of 5–10 mg/ml at –80°C. Expected yield is 5–10 mg/L. The molecular weight of MAML1(13–74) after TEV cleavage is 7.5 KDa.

²²Removal of the TEV protease is not necessary if following with another column purification step.

References

1. Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. *Science* (New York, NY). 1999; 284:770–776.
2. Bray SJ. Notch signalling: a simple pathway becomes complex. *Nature reviews Molecular cell biology*. 2006; 7:678–689.
3. Kopan R, Ilagan MXG. The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*. 2009; 137:216–233. [PubMed: 19379690]
4. Kovall RA, Blacklow SC. Mechanistic insights into Notch receptor signaling from structural and biochemical studies. *Current topics in developmental biology*. 2010; 92:31–71. [PubMed: 20816392]
5. Tamura K, Taniguchi Y, Minoguchi S, et al. Physical interaction between a novel domain of the receptor Notch and the transcription factor RBP-J kappa/Su(H). *Current biology : CB*. 1995; 5:1416–1423. [PubMed: 8749394]
6. Kovall RA, Hendrickson WA. Crystal structure of the nuclear effector of Notch signaling. CSL, bound to DNA, *The EMBO journal*. 2004; 23:3441–3451.
7. Nam Y, Sliz P, Song L, et al. Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. *Cell*. 2006; 124:973–983. [PubMed: 16530044]
8. Wilson JJ, Kovall RA. Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. *Cell*. 2006; 124:985–996. [PubMed: 16530045]
9. Weng AP, Nam Y, Wolfe MS, et al. Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Molecular and cellular biology*. 2003; 23:655–664. [PubMed: 12509463]
10. Fryer CJ, Lamar E, Turbachova I, et al. Mastermind mediates chromatin-specific transcription and turnover of the Notch enhancer complex. *Genes & development*. 2002; 16:1397–1411. [PubMed: 12050117]
11. Wallberg AE, Pedersen K, Lendahl U, et al. p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains in vitro. *Molecular and cellular biology*. 2002; 22:7812–7819. [PubMed: 12391150]
12. Kuroda K, Han H, Tani S, et al. Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. *Immunity*. 2003; 18:301–312. [PubMed: 12594956]
13. Oswald F, Kostezka U, Astrahantseff K, et al. SHARP is a novel component of the Notch/RBP-Jkappa signalling pathway. *The EMBO journal*. 2002; 21:5417–5426. [PubMed: 12374742]
14. Kao H-Y, Ordentlich P, Koyano-Nakagawa N, et al. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes & development*. 1998; 12:2269–2277. [PubMed: 9694793]
15. Zhou S, Fujimuro M, Hsieh JJ, et al. A role for SKIP in EBNA2 activation of CBF1-repressed promoters. *Journal of virology*. 2000; 74:1939–1947. [PubMed: 10644367]
16. Hsieh JJ, Zhou S, Chen L, et al. CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. *Proceedings of the National Academy of Sciences of the United States of America*. 1999; 96:23–28. [PubMed: 9874765]
17. Taniguchi Y, Furukawa T, Tun T, et al. LIM protein KyoT2 negatively regulates transcription by association with the RBP-J DNA-binding protein. *Molecular and cellular biology*. 1998; 18:644–654. [PubMed: 9418910]
18. Tun T, Hamaguchi Y, Matsunami N, et al. Recognition sequence of a highly conserved DNA binding protein RBP-J kappa. *Nucleic acids research*. 1994; 22:965–971. [PubMed: 8152928]
19. Jarriault S, Brou C, Logeat F, et al. Signalling downstream of activated mammalian Notch. *Nature*. 1995; 377:355–358. [PubMed: 7566092]
20. Wu L, Aster J, Blacklow S, et al. MAML1, a human homologue of Drosophila Mastermind, is a transcriptional co-activator for NOTCH *Nature genetics*. 2000

21. Nam Y, Weng AP, Aster JC, et al. Structural requirements for assembly of the CSL-intracellular Notch1-Mastermind-like 1 transcriptional activation complex. *The Journal of biological chemistry*. 2003; 278:21232–21239. [PubMed: 12644465]
22. Nam Y, Sliz P, Pear WS, et al. Cooperative assembly of higher-order Notch complexes functions as a switch to induce transcription. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104:2103–2108. [PubMed: 17284587]
23. Arnett KL, Hass M, McArthur DG, et al. Structural and mechanistic insights into cooperative assembly of dimeric Notch transcription complexes. *Nature structural & molecular biology*. 2010; 17:1312–1317.
24. Fried M, Crothers DM. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic acids research*. 1981; 9:6505–6525. [PubMed: 6275366]
25. Garner MM, Revzin A. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the Escherichia coli lactose operon regulatory system. *Nucleic acids research*. 1981; 9:3047–3060. [PubMed: 6269071]
26. Buratowski, S.; Chodosh, LA. Mobility shift DNA-binding assay using gel electrophoresis. In: Ausubel, Frederick M., et al., editors. *Current protocols in molecular biology*. Vol. Chapter 12. 2001.
27. Hellman LM, Fried MG. Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nature protocols*. 2007; 2:1849–1861.
28. Berger R, Duncan MR, Berman B. Nonradioactive gel mobility shift assay using chemiluminescent detection. *BioTechniques*. 1993; 15:650–652. [PubMed: 8251167]
29. Pagano JM, Clingman CC, Ryder SP. Quantitative approaches to monitor protein-nucleic acid interactions using fluorescent probes. *RNA (New York, NY)*. 2011; 17:14–20.
30. Rye HSH, Drees BLB, Nelson HCH, et al. Stable fluorescent dye-DNA complexes in high sensitivity detection of protein-DNA interactions. Application to heat shock transcription factor. *The Journal of biological chemistry*. 1993; 268:25229–25238. [PubMed: 8227088]
31. Jing D, Agnew J, Patton WF, et al. A sensitive two-color electrophoretic mobility shift assay for detecting both nucleic acids and protein in gels. *Proteomics*. 2003; 3:1172–1180. [PubMed: 12872218]

Figure 1.

Assembly of the Notch Transcription Complex. (A) Domain organization of human Notch1, MAML1, and CSL proteins and schematic drawing of the core components of the NTC. The initial and terminal residues of the constructs described in this protocol are numbered and the binding regions and domains are colored: Notch1 RAM (blue), ANK repeats (cyan), CSL N-terminal domain (NTD), β -trefoil domain (BTB), and C-terminal domain (CTD) (shades of green), and MAML1 (red). (B) The X-ray crystal structure of the human NTC,

colored as in (A), showing a complex of CSL, Notch1 RAM, Notch1 ANK, and the MAML1 polypeptide bound to a segment of the *HES1* promoter.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

A 5' -AGTTAC**TGTGGGAA**AGAAAGTTTGGGAAGT**TTACACACG**AGC
 TG**ACACCCTT**TCTTTCAAACCCTTCA**AAGTGTGC**TCGGCAAG-5'

Figure 2.

Electrophoretic Mobility Shift Assay illustrating cooperative dimerization of Notch Transcription Complexes on SPS DNA. (A) Human *HES1* promoter oligonucleotide duplexes used for EMSA. (B) EMSAs performed on *HES1* SPS element using wild-type Notch1-RAMANK or mutant R1984A form (Prior literature refers to this mutant as R1985A. The change to R1984A reflects current Genbank numbering for human Notch1). A low (50 nM) and a high concentration (200 nM) of CSL were incubated with labeled DNA (40 nM), with an excess of wild-type or mutant Notch1-RAMANK (1.2 μ M) in the presence or absence of an excess of MAML1 (2.6 μ M). In the absence of MAML1, wild-type and the mutant form of Notch both supershift CSL–DNA complexes. In the presence of MAML1,

wild-type Notch1-RAMANK supershifts to a higher order dimeric complex and the R1984A mutant does not. The gel is a 10% acrylamide gel made as described in Table 1.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Table 1

Preparation of polyacrylamide gel mixture for one 16×16 cm gel.

	acrylamide		4%		8%		10%	
	bis-acrylamide		0.10%	0.20%	0.20%	0.25%	0.25%	
ddH ₂ O			21.17	15.67	12.92			(ml)
10× Running buffer			3	3	3		3	
30% acrylagel			4	8	10			
2% bis-acrylagel			1.5	3	3.75			
10% APS (0.1g/mL)			0.3	0.3	0.3		0.3	
TEMED			0.03	0.03	0.03		0.03	