About Lambda:

Lambda (λ) is a large (48,502 bp GenBank Accession # NC_001416), *E. coli* bacteriophage with a linear, largely double-stranded DNA genome. At each end, the 5′ strand overhangs the 3′ strand by 12 bases. These single-stranded overhangs are complementary and anneal to form a *cos* site following entry into a host cell. Once annealed, the genome is a circular, completely double-stranded molecule which serves as a template for rolling-circle replication.

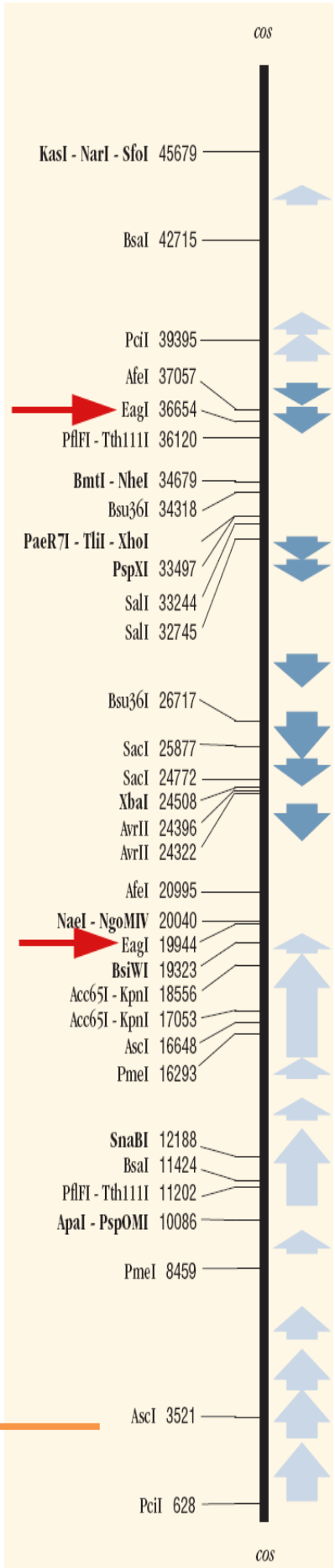
Numbering of the genome sequence begins at the first (5′-most) base of the left end (bottom of diagram to the right). Enzymes with unique restriction sites are shown in **bold** type and enzymes with two restriction sites are shown in regular type. Restriction site coordinates refer to the position of the 5′-most base on the top strand in each recognition sequence.

The *cos* site sequence is:

```
5′-GGG CGG CGA CCT----DS-DNA---                -3′
3′-                 ------------CCC GCC GCT GGA-5′
```

Making Lambda fit in the MiniTweezers:

The MiniTweezers typically has a wiggler range of about 10 μm, and since the contour length of 48502 bp λ-DNA is about (ass. B-DNA rise/bp 3.4 Å) 16.5 μm, it is too long to stretch efficiently. Therefore we need to cut it using an appropriate restriction enzyme. This protocol is based on Wei Cheng's ½-Lambda protocol from 2006, where *XbaI* was used to cut lambda in half. Our restriction enzyme of choice is *EagI*, which cuts λ-DNA at two sites, 19944 and 36654. This generates three DNA fragments (F1, F2, and F3). The reason that we are using *EagI* instead of *XbaI* is that it gives us fragments of DNA that are more homogeneous. F1 gives us a GC rich (GC = 57%), and F3 gives us an AT rich (GC=47%) fragment of reasonable homogeneity. Figure 1 illustrates the homogeneity of the fragments by showing the GC content of the lambda genome in a sliding window of 100 bases. More homogenous fragments should give us a better understanding of the effect of base composition on the physical properties of the DNA. For instance, does the force needed for overstretching depend on the base composition of the DNA? A set of homogenous samples should answer this question.



About lambda & EagI: www.neb.com
Wei Cheng's ½- Lambda Protocol: http://tweezerslab.unipr.it/cgi-bin/mt/documents.pl/Show?_id=ab03&sort=DEFAULT&search=&hits=17

EagI recognition site:

```
5′...CGGCCG...3′
3′...GCCGGC...5′
```

```
F1: 19.9kb
5′-GGGCGGCGACCT----//----C    -3′
3′-            ----//----GCCGG-5′

F2: 16.7kb
5′-GGCCG----//----C    -3′
3′-    C----//----GCCGG-5′

F3: 11.8kb
5′-GGCCG----//----            -3′
3′-    C----//----CCCGCCGCTGGA-5′
```

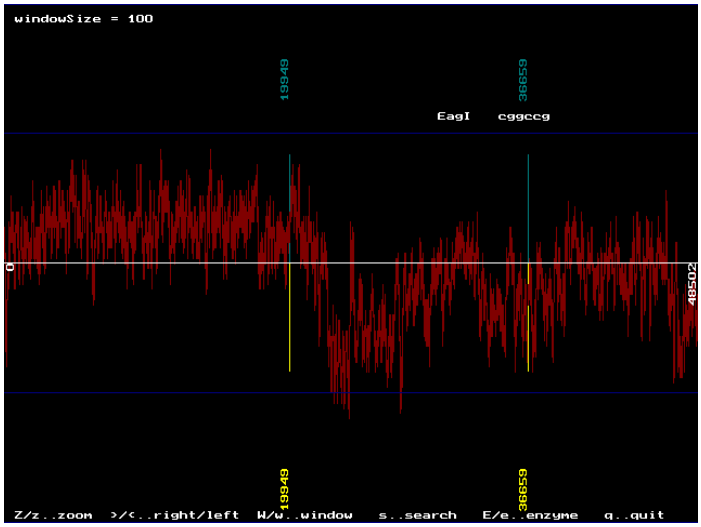


Figure 1 - GC content of lambda genome in a sliding window of 100 bases, the scale goes from 0% GC at the bottom of the graph to 100% at the very top. The positions of the EagI cut sites are shown as vertical yellow/cyan colored lines.

Attaching the DNA to beads

In order to use the DNA in the MiniTweezers, we need to attach the ends of the DNA to beads. Here we will modify the ends with digoxygenin and biotin. This means that we can use anti-digoxigenin- and streptavidin coated beads for the force-manipulation in the Minitweezers micro chamber.

The biotin modification is done by letting a Klenow fragment (3'->5' exo-) fill in the cos-sites of lambda DNA with biotin-dCTP, which gives us at the most 6(F1) or 4(F3) biotins. The F2 fragment does not get filled in since we do the Klenow fragment step before we cut with *EagI*.

```
F1: 19.9kb
5′-GGGCGGCGACCT----//----C    -3′
3′-CCCGCCGCTGGA----//----GCCGG-5′
   ||| || |
   BBB BB B

F2: 16.7kb
5′-GGCCG----//----C    -3′
3′-    C----//----GCCGG-5′

F3: 11.8kb
                      B  B  BB
                      |  |  ||
5′-GGCCG----//----GGGCGGCGACCA-3′
3′-    C----//----CCCGCCGCTGGA-5′
```

The digoxigenin modification is done by PCR over an *EagI* cut site on lambda DNA where Dig-dUTP is incorporated during the reaction. We have chosen to amplify the region around the cut site at 19944, starting at 224 bases upstream and 266 bases downstream.

```
                                                    19721 GAAAGCCAGA CGTAACAGCA
    19741 CCACGGTGGT GGTGAACACG GTGGGCTCAG AGAATCCGGA TGAAGCCGGG CGTTACAGCA
    19801 TGGATGTGGA GTACGGTCAG TACAGTGTCA TCCTGCAGGT TGACGGTTTT CCACCATCGC
    19861 ACGCCGGGAC CATCACCGTG TATGAAGATT CACAACCGGG GACGCTGAAT GATTTTCTCT
    19921 GTGCCATGAC GGAGGATGAT GCCCGGCCGG AGGTGCTGCG TCGTCTTGAA CTGATGGTGG
    19981 AAGAGGTGGC GCGTAACGCG TCCGTGGTGG CACAGAGTAC GGCAGACGCG AAGAAATCAG
    20041 CCGGCGATGC CAGTGCATCA GCTGCTCAGG TCGCGGCCCT TGTGACTGAT GCAACTGACT
    20101 CAGCACGCGC CGCCAGCACG TCCGCCGGAC AGGCTGCATC GTCAGCTCAG GAAGCGTCCT
    20161 CCGGCGCAGA AGCGGCATCA GCAAAGGCCA CTGAAGCGGA AAAAAGTGCC 20211
```

```
Forward Primer:  5'-GAAAGCCAGA CGTAACAGCA
Backward Primer: 5'-GGCACTTTTT TCCGCTTCAG
PCR gives 490 bp fragment, after digestion with EagI we get two fragments 224 bases
(D1) and 266 bases (D2)

D1:|------224 b------|
5'-GAAAGCCAGA---//---C    -3'
3'-CTTTCGGTCT---//---GCCGG-5'

D2:|--------266 b--------|
5'-GGCCG---//---AAAAAGTGCC-3'
3'-    C---//---TTTTTCACGG-5'
```

After purification, the product is cut with *EagI*, and thus gives us two digoxigenin modified handles (D1 & D2) of similar size which can be ligated to the biotin modified F1 and F3 fragments. Before the ligation we treat the Dig-handles (D1 & D2) with Antarctic phosphatase to dephosphorylate them. This will when we ligate them together with the biotinylated fragments (F1 & F3) introduce a nick (v or ^ below) which gives us the possibility of producing ssDNA when we stretch the fragments. This however requires high force (~140 pN) for an extended period of time (seconds). The Klenow fill in of biotin-dCTP gives us biotins that are in close proximity of each other, which means that we need a bead with a high surface density of streptavidin in order to reliably be able to produce ssDNA. An alternative method to create a biotin handle for lambda is to use terminal transferase to extend an oligomer complementary to the *cos*-site with biotin-dCTP, but that is another protocol.

```
F1: 19.9kb + Dig-handle ~20 kb (Left, should have overstretching region of 4.7 um)
                          v
5'-GGGCGGCGACCT----//----CGGCCG----Dig-modified---3'
3'-CCCGCCGCTGGA----//----GCCGGC-------handle------5'
   ||| || |
   BBB BB B

F2: 16.7kb + 2x Dig-handle ~17 kb (does not hook up to streptavidin bead)
                                        v
5'---Dig-modified----CGGCCG----//----CGGCCG----Dig-modified---3'
3'------handle-------GCCGGC----//----GCCGGC-------handle------5'
                     ^

F3: 11.8kb + Dig-handle ~12 kb (Right, should have overstretching region of 2.8 um)
                                       B  B  BB
                                       |  |  ||
5'---Dig-modified----CGGCCG----//----GGGCGGCGACCA-3'
3'------handle-------GCCGGC----//----CCCGCCGCTGGA-5'
                     ^
```

The things you need and where to get them

Description	Company	Cat#	Price
Digoxigenin-11-dUTP, 25 nmol (25 µL)	Roche	#11093088910	$196
Deoxynucleotide Solution Set, 25 µmol of each (A, T, G, C)	NEB	#N0446S	$150
EagI restriction enzyme, 500 units	NEB	#R3505S	$58
Lambda DNA, 250ug	NEB	#N3011S	$58
Klenow Fragment (3'->5' exo-), 200 units	NEB	#M0212S	$56
Quick Ligation Kit, 30 reactions	NEB	#M2200S	$95
Taq DNA Polymerase with Standard Taq Buffer, 400 units	NEB	#M0273S	$58
Antartic Phosphatase, 1,000 units, 5,000 units/mL	NEB	#M0289S	$58
QIAquick PCR Purification Kit (50)	Qiagen	#28104	$96
Biotin-14-dCTP, 50nmol	Invitrogen	#19518-018	$273
Micro Bio-Spin 6 Columns	Bio-rad	#732-6221	$76
Primers: 5'-GAAAGCCAGA CGTAACAGCA, 5'-GGCACTTTTT TCCGCTTCAG	IDT	-	$98
Sterilized H2O	-	-	-
EDTA 0.5M	-	-	-

You will also need a PCR thermocycler, a table-top centrifuge capable of 13000 rpm, a spectrophotometer, and a heat block.

Protocol Outline

- **Make biotin lambda fragments**
  1. **Quench lambda *cos*-sites**
  2. **Biotin fill in by Klenow fragment**
  3. **Purify using Bio-rad spin column**
  4. **RE digest using EagI-HF**
  5. **Determine concentration**
- **Make Dig-handles**
  1. **Pre-mix dNTP cocktail**
  2. **PCR of lambda EagI site with Dig-dUTP**
  3. **Purify using Qiagen PCR purification kit**
  4. **RE digest using EagI**
  5. **Dephosphorylation using Antarctic phosphatase**
  6. **Purify using Qiagen PCR purification kit**
  7. **Determine concentration**
- **Mix and ligate biotin labeled fragments and Dig-handles**

Protocol

Making the Dig-handles by PCR incorporation of Dig-dUTP at lambda Eag-I site

1. Make the following dNTP-cocktail

What to mix:	Add vol.	Final conc.
1 mM Dig-dUTP (Roche, #11093088910)	9.9 µL	0.33 mM
10 mM dTTP (diluted from 100 mM stock)	2.1 µL	0.66 mM
10 mM dATP (dNTPs from NEB #N0446S)	3.0 µL	1 mM
10 mM dCTP ----------//----------	3.0 µL	1 mM
10 mM dGTP ----------//----------	3.0 µL	1 mM
MQ water, sterilized	9.0 µL	
Total:	**30.0 µL**	**5x**

2. Make the following PCR reaction mix

What to mix:	Add vol.	Final conc.
0.5 µg/µL Lambda DNA (NEB #N3011S)	3.0 µL	10 ng/µL
~30 µM (31.2 µM) Eag-I Left primer (IDT)	2.5 µL	0.5 µM
~20 µM (23.1 µM) Eag-I Right primer (IDT)	3.7 µL	0.5 µM
10x Standard Taq reaction buffer (NEB #B9014S)	15.0 µL	1x
50 mM MgCl2 (Extra, Taq buffer contains 15 mM)	1.5 µL	2 mM, Mg++
5x dNTP-cocktail from previous step	30.0 µL	200 µM ea. dNTP
5U/µL Taq polymerase	2.0 µL	10 U
MQ water, sterilized	93.8 µL	
Total:	150 µL	

Split the 150 µL into suitable PCR tubes if needed. (We used 3x50 µL).

Run the PCR reaction according to the following protocol. This step takes about 2h. Tests concluded that more than 20 cycles where unnecessary as the reaction runs out of dNTPs.

Step		Temperature	Duration
1.	Initial Denaturation Step	95 °C	6 min
2.	Annealing	54 °C	45 s
3.	Extension	72 °C	90 s
4.	Denaturation	95 °C	45 s
5.	Repeat steps 2-4 19 times		
6.	Terminal Extension	72 °C	10 min
7.	Store at 4°C	4 °C	Forever

3. Purify the PCR product using QIAgen PCR purification kit (Qiagen, #28104). Pool the product to avoid dilution. Follow the kit instructions.

1. **Mix 5 volumes (750 µL) of Buffer PB to 1 volume (150 µL) of PCR product, and load half of it in a Qiaquick column. Do step 2. Then add the other half, and proceed with step 2 again.**
2. **Centrifuge: 60 s @ 13000 rpm, discard flow-through**
3. **Wash by adding 750 µL of Buffer PE and centrifuge: 60 s @ 13000 rpm, discard flow-through**
4. **Centrifuge: 60 s @ 13000 rpm, discard flow-through**
5. **Place your Qiaquick column in a 1.5 ml eppendorf spin-tube, add 50 µl Eluation Buffer EB (10 mM Tris, pH 8.5) to the middle of the column.**
6. **Centrifuge: 60 s @ 13000 rpm, this time save the flow-through (~50 µl) as it contains your purified DNA.**

4. Cut the purified PCR product with EagI-HF (NEB #R3505S)

What to mix:	Add vol.	Final conc.
#Purified PCR product (all of it)	50 µL	
10x NE Buffer 4	5.8 µL	1x
20 U/µL EagI-HF	2.0 µL	0.69 U/µL
Total:	58 µl	

- Incubate overnight @ 37°C. Inactivate enzyme using heat: 20 min @ 65°C.

5. Dephosphorylate the product using Antarctic phosphatase. (We saved half of our product (~28 µL), hence the 30 µL in the protocol. Should be ok to scale.)

What to mix:	Add vol.	Final conc.
#EagI cut purified PCR product	30 µL	
10x Antarctic phosphatase buffer	3.5 µL	1x
5 U/µL Antarctic phosphatase (NEB, #M0289S)	1.5 µL	0.21 U/µL
Total:	35 µL	

   - Incubate for 1 h @ 37°C. Inactivate enzyme using heat: 5 min @ 65°C.
6. Purify using QIAquick PCR purification kit. Follow the kit instructions. See step 3.
7. Determine concentration using absorption spectroscopy at 260 nm. ε_{260}=6600 M^{-1} cm^{-1}.

Making biotin labeled lambda fragments using Klenow fragment fill in

1. Heat 48 µL 500 ng/µL Lambda-DNA (NEB, #N3011S) for 5 min @ 65°C to melt the *cos*-sites, then put immediately on ice to quench. The *cos*-sites now form hairpins and are ready to be filled in by the Klenow fragment.
2. Klenow fill in with biotin-dCTP

What to mix:	Add vol.	Final conc.
0.5 µg/µL Quenched Lambda DNA (NEB #N3011S)	48.0 µL	348 ng/µL
10 mM dATP	0.5 µL	72 µM
0.4 mM Biotin-14-dCTP (Invitrogen #19518-018)	10.0 µL	58 µM
10 mM dGTP	0.5 µL	72 µM
10 mM dTTP	0.5 µL	72 µM
10x NE Buffer 2	7.0 µL	1x
5U/µL Klenow exo- (NEB #M0212S)	2.5 µL	0.18 U/µL
Total:	69 µL	

   - Carefully mix using a pipette tip, incubate for 1 h @ 37°C. Kill the enzyme by adding 1.2 µL 0.5 M EDTA and heat 5 min @ 70°C.
3. Purify the biotin labeled lambda-DNA using a Bio-Rad P6 spin column (#732-6221, 6kD cut off).
   - First exchange the buffer of the column to 1x NE Buffer 4. We do this to ensure that the EagI-HF restriction enzyme will work efficiently. Then add sample and centrifuge.

1. **Snap the tip of a P6 column and place it in a 2 mL collection tube that comes with the kit. Let the column drain by gravity until it stops dripping (~2 min). Centrifuge for 2 min @ 1000 g, discard the flow-through.**
2. **Make 1.5 mL of 1x NE Buffer 4 from the supplied 10x stock.**
3. **Add 0.5 mL 1x NE Buffer 4 to the P6 column, centrifuge for 1 min @ 1000 g and discard the flow-through. Repeat two more times, then place the column in a 1.5 mL collection tube. The P6 column now contains 1x NE Buffer 4.**
4. **Pipette the biotinylated Lambda DNA to the center of the P6 column. Centrifuge for 4 min @1000g. The flow-through (~72 µL) contains the DNA in 1x NE Buffer 4.**

4. Cut the purified biotinylated Lambda DNA with EagI-HF. This generates three fragments 5'-F1 - F2 - F3-3', where only fragments F1 and F3 are biotinylated.

What to mix:	Add vol.	Final conc.
#Purified biotinylated lambda (all of it)	72 µL	
10x NE Buffer 4	1.0 µL	1x
20 U/µL EagI-HF	2.0 µL	0.53 U/µL
Total:	**75 µl**	

   - Incubate for 2 h @ 37°C, inactivate by adding 1.5 µL 0.5 M EDTA and heat to 70°C for 20 min.
5. Determine concentration by absorption spectroscopy, ε_{260}=6600 M^{-1} cm^{-1}. (May want to clean first to get better accuracy, but this will give us a rough estimate that will be sufficient.)

Mixing and ligating biotinylated lambda fragments with Dig-handles

Both the biotinylated lambda DNA fragments and the Dig-handles should be ready for ligation without further treatment. Mix molar ratio; Dig-handle : Lambda fragment = 4 : 1. In our case:

What to mix:	Add vol.
315 ng/µL ⅓ Lambda, biotinylated, EagI-HF digested	5.0 µL
78.5 ng/µL Dig-handles, EagI-HF digested, dephosphorylated	1.0 µL
2x Quick Ligation Reaction Buffer (NEB, #M2200S)	6.0 µL
Quick T4 DNA Ligase (NEB, #M2200S)	1.5 µL
Total:	**13.5 µL**

- Incubate for 20 min @ room temperature, Inactivate the ligase by adding 2 µL 0.5 M EDTA and heat to 65°C for 10 min. Store @ 4°C.

What you can expect to see when stretching ⅓ λ-DNA.

The following experiments were performed in 0.5 M NaCl. The figures below show Force-Extension curves of ⅓ λ-DNA, acquired using MiniTweezers. Figure 2 shows a typical right end (F3) which is ~12kb, and has a contour length of ~4 µm. We can see that the overstretching region is the expected ~2.8 µm (=0.7*4µm), but also that the AT-rich right end easily forms ssDNA when overstretching. Figure 3 shows a typical left end (F1) which is ~20kb, and has a B-form contour length of ~6.8 µm. The overstretching region corresponds to the expected ~4.7 µm. The more GC-rich left end does not as easily form ssDNA when overstretched, and has an overstretching plateau that is consistently about 2 pN lower than the AT-rich right end.

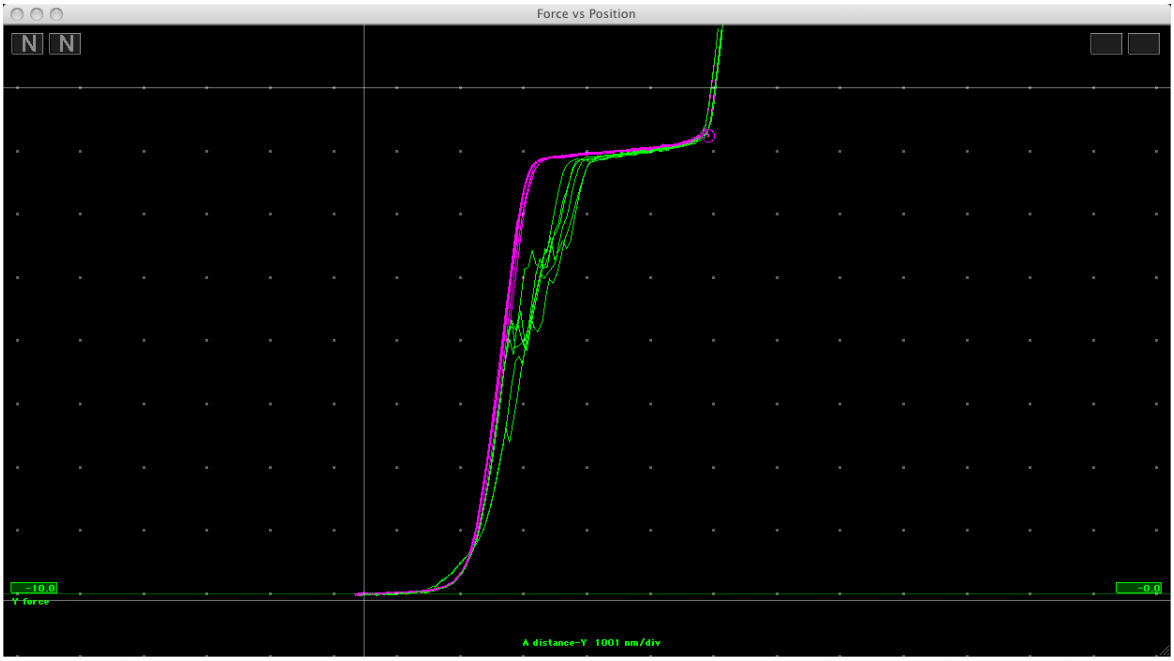


Figure 2 - Force-extension curve of typical right end of ⅓-lambda.

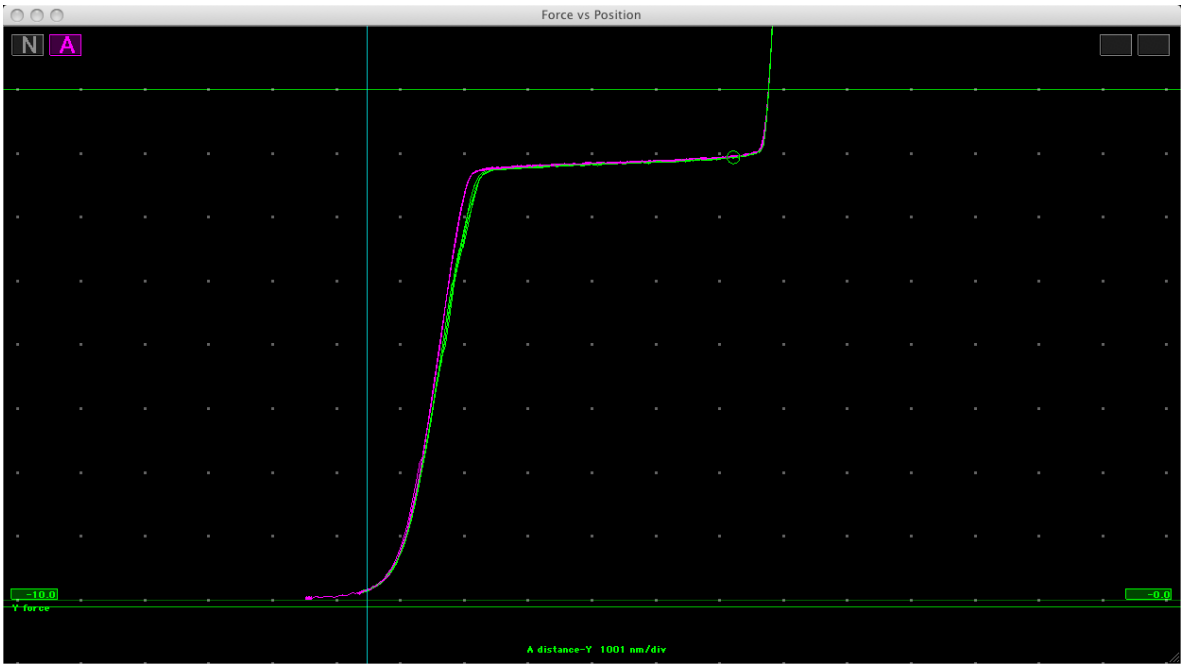


Figure 3 - Force-extension curve of typical left end of ⅓-lambda.