NF-kB ELISA protocol

Kit: TransAm p65 ELISA, Cat# 40096
Nuclear extract kit: Cat # 40010
Recombinant NF-kB Cat # 31102

Day 1

1) **Make Buffers for Nuclear Lysate Preparation Using the Nuclear Lysate Kit**
- Fast Cool the microfuge for use during the cytoplasmic fraction portion (4ºC)
- Make a chart for volume of buffers needed for each sample (1X HB and CLB)

cell #	1X Hypotonic Buffer (HB)(microliters)	Complete Lysis Buffer(CLB)(microliters)	Detergent (microliters)
$1x10^6$	150	15	15
$5x10^6$	250	25	25
$1x10^7$	400	40	40
$2x10^7$	500	50	50

Table 1: Use this as a reference for how much buffer is to be used. Note the pellet size when removing it from the freezer. Adjust buffer according to size and color. If pellet is small and red, use less volume than the table calls for. *It is better to have a sample too concentrated than too dilute!*
-Determine total buffer needed and use table 2 as a reference for ingredients

CLB

Component	25 microliters	50 microliters	100 microliters
10mM DTT (10x)	2.5	5	10
Lysis Buffer AM1	22.25	44.5	89
Protease Inhibitor (100x)	0.25	0.5	1

1X HB

component	250 microliters	500 microliters	1000 microliters
10X Hypotonic Buffer	25	50	100
Distilled H_20	225	450	900

Table 2: Buffer components

2) **Make Cytoplasmic Fraction**
- *If you need to make a positive and a negative control, use $1x10^7$ cells of HDLM2 as the positive control and $1x10^7$ cells of Jurkat cells as the negative control. Follow the exact same protocol for making the nuclear lysates.*
- Always keep pellets on ice
- Make sure the microfuge is cooled to 4ºC
- Gently resuspend pellet in volume of 1X Hypotonic Buffer (100ul-500ul—see table 1) and incubate on ice for 15 minutes. Make sure pellet is completely homogenized in buffer.
- Label eppendorfs during the incubation for the cytoplasmic lysate. Label with sample ID, date, exp #, cytoplasmic fraction NF-kB. Pre chill these tubes.

- Add determined volume of detergent (table 1) and vortex sample for 10s (only) on setting 8.5.
-Quickly centrifuge the sample at 14,000g for 35s (the 35s doesn't start until the centrifuge has reached 14,000g)
- After spin you should notice a small, clear, jelly-like pellet in the eppendorf (this can be hard to see sometimes).
- Remove supernatant (this is your cytoplasmic fraction) and put into pre-chilled eppendorfs. BE CAREFUL....the pellet is not really stuck to the eppendorf wall and can be easily removed if not careful. Be sure to remove as much of the supernatant as possible.
- Put in -80º and note location in freezer book and in lab book (VERY IMPORTANT).

3) **Make Nuclear Fraction**
- Add determined volume of CLB to pellet and break up by pipetting (not vortexing). The pellet is difficult to break up. Try smashing it up with the tip, but be careful when pipetting up and down, the CLB foams very easily.
- Vortex the sample for 10s at highest setting
- Incubate samples on ice for 30 minutes using a rocking platform set at 100rpm.
- Label eppendorfs for the Nuclear lysate portion (Sample ID, date, exp #, nuclear lysate NF-kB) and pre-chill; label another set of eppendorfs for OD determination for each sample.
- After incubation vortex sample for 30s on highest setting
- Centrifuge samples for 10 minutes at 14,000g in the microfuge at 4ºC.
- Transfer 10 microliters of supernatant to one of the pre-chilled, labeled eppendorfs and the rest of the supernatant to the other. If you have limited sample, you can set aside 2.5 ul for each OD
- Put in -80º and note location in freezer book and in lab book (VERY IMPORTANT).

4) **Determine Protein Concentration (RC/DC Method)**
- Make standards for standard curve using BSA 10mg/ml and a serial dilution. Label eppendorfs 10, 5, 2.5, 1.25, 0.6125, and 0.3mg/ml. Add 10µL of stock BSA to eppendorf labeled 10mg/ml. Add 5µL of water to the rest of the eppendorfs. Transfer 5µL of the 10mg/ml tube to the 5mg/ml tube. Mix. Add 5µL of the 5mg/ml tube to the 2.5mg/ml tube and mix. Continue this serial dilution until the 0.3mg/ml is made. Transfer 2.5µL of each standard to a labeled eppendorf.
- Make sure there is a blank with 2.5µL **ddH_20**? Are you using the nuclear lysate buffer as the blank
    -
    - Remove 2.5 ul of nuclear lysate from OD eppendorf. Add to 22.5 ul dd H2O
- Add 125µL of BioRad reagent I to each eppendorf, vortex and incubate for one minute.
- Add 125µL of BioRad reagent II to each eppendorf, vortex and spin at 14,000g for 4.5 minutes.
- After centrifugation dump liquid from tubes, place tubes in the vSpeed-vac (located in the room where the -80 is) for 7 minutes on medium heat setting. Don't go longer than 7 minutes.

- Make reagent A'. Make enough to use 130µL per eppendorf (always make a little extra). For every mL of reagent A use 20µL of reagent S, and this will make A'. Mix gently using the vortexer on a low setting.
- Remove eppendorfs from Speed-vac and add 127µL of reagent A', vortex, and incubate for 5 minutes at room temperature.
- Add 1 mL of reagent B to each eppendorf, vortex, and incubate for 15 minutes.
- Turn on spectrophotometer in Plon lab. Go to *Applications* then to *Standard Curve* on the machine. Hit *New* and enter settings. Wavelength should be 750nm, the number of standards will be 6, the number of duplicates will be 1. Enter in the standard concentrations..
- Transfer eppendorf content to cuvettes—use plastic microcuvettes.
- Place the blank in the blue cuvette holder. The cuvettes should placed in their holders such that their arrows point clockwise. Place the standards in next as you are now making the standard curve. Go counterclockwise from the blank and place the standards in the holders by increasing concentration. Lowest first and highest last.
- Shut top and use the green button to scan. Press the green button for every reading you take
- Leave blank in blue holder for every reading, even samples
- Remove standards
- Record the r^2 value, the slope and the intercept
- Determine the absorbances of the samples the same way as you did the standards. MAKE sure you leave the blank in the blue holder for all measurements.
    - Record all absorbances and concentration values.
    - Plot standard curve on Excel if unable to print from machine.

5) **Make Buffers for the ELISA Using the ELISA Kit**

- Determine how many wells you are going to use, always add 8 more wells (or one strip) for the Standard Curve
- Also, make sure to include in your CLB buffer volume that you need to use CLB for making up the standards. This usually requires about 200µL for each standard including the blank.
- Determine total volume needed of each buffer and use this chart to make up the necessary volumes

CLB

Components	for 1 well (ul)	for 1 strip (8 wells)	for six strips
1M DTT	0.1	0.9	5.4
Protease Inhibitor	0.23	1.8	10.8
Lysis Buffer AM2	22.5	177.3	1.064

1X Wash buffer (WB)

components (mL)	for 1 well (ml)	for 1 strip (8 wells)	for six strips
10X Wash Buffer	0.225	1.8	10.8
Distilled H_20	2.025	16.2	97.2

Complete binding buffer (CBB)

component (µL)	for 1 well (ul)	for 1 strip (8 wells)	for six strips

1M DTT	0.07	0.54	3.2
Herring Sperm DNA	0.34	2.7	16.2
Binding Buffer AM3	33.4	267	1.6

1X Antibody Binding Buffer

component (µL)	for 1 well (ul)	for 1 strip (8 wells)	for six strips
10X Antibody Binding Buffer	22.5	180	1080
Distilled H_20	202.5	1620	9720

Table 3: Use this as a guide to making the buffers.

6) **Determine How Much Lysate is Needed to Get 10µg of Protein in the Well**
- Use the OD's to determine the volume of sample that is needed to get 10µg of protein (don't forget to determine this for the positive and negative controls)
- Then calculate how much CLB is needed to bring the sample up to 20µL. If preparing a lot of samples, make an Excel spreadsheet of protein and CBB volumes.

7) **Make Standard Curve Using NF-kB**
- Label 8 tubes: 30ng, 15ng, 7.5ng, 3.75ng, 1.875ng, 0.94ng, 0.47ng, and 0ng (blank).
- Add 1.5µL of NF-kB (located in the -80, box 8) to 98.5µL of CLB in the eppendorf labeled "30 ng"
- Mix gently and keep all these tubes on ice while making the standards
- Next add 50µL of CLB to each eppendorf (except 30ng).
- Serially dilute the standards: use 50µL of 30ng and add it to the tube labeled 25ng. Next, add 50µL of 15ng to the 7.5ng tube and so on. Change tips for each tube. When mixing, do so carefully so as not cause bubbles.
- Keep on ice until ready to use

8) **Make CLB and Lysate Mixture (20µL)**
- Label eppendorfs for each sample to be used
- Add the determined volume (from step 6) of each lysate to the corresponding eppendorf (keep on ice)
- Add CLB to each eppendorf and bring the nuclear lysate mixture up to 20µL. Mix carefully, try to avoid bubbles. If there are bubbles, you can briefly spin at 14,000g to remove.

9) **Begin ELISA**
- Remove the ELISA plate from the refrigerator
- Use only the strips you need, put the others back in the refrigerator
- Note in your book the location of all the samples and the standards on the plate
- Add 30µL of CBB to each well that is to be used
- Add 20µL of the standards and the blank, change tips.
- Add 20µL of the lysate mixtures to their corresponding wells, change tips between each sample.
- Place adhesive cover on top of the plate and incubate on a rocking platform (100rpm) for 1 hour.

10) **Add Primary NF-kB Antibody**

- Ten minutes before the end of the incubation make the primary antibody. Make a 1:1000 mixture. Use the frozen NF-kB p65 antibody located in -20degree (1 part) and the 1X Antibody Binding Buffer (1000 part). Make enough to be able to add 100µL to each well: standards, blanks, and samples. Always make a little more than you think you need.
- Remove the plate from the shaker
- Wash each well three times with 200µL of 1X Wash Buffer. Change tips for each well (it is best to use a multipipetter). Add it to the side of the well, at an angle, so you don't remove the NF-kB bound to the bottom of the well. Never add anything straight to the bottom of the well.
- Add 100µL of the primary antibody to each well, change tips.
- Place adhesive cover on plate and incubate for 1 hour without agitation.

11) **Add Secondary Antibody**

- Ten minutes before end of incubation make the secondary HRP antibody. Get the HRP antibody from the -20 freezer. Make a 1:1000 dilution in the same way that you made the primary antibody. Make enough to add 100µL to each well.
- Wash each well three times with 200µL of 1X Wash Buffer. (it is best to use a multipipetter). Add it to the side of the well, at an angle, so you don't remove the NFkB bound to the bottom of the well. Never add anything straight to the bottom of the well.
- Add 100µL of HRP 1:1000 antibody to each well.
- Cover and incubate for 1 hour.
- Take out Developing solution from refrigerator and leave out at room temperature for one hour.

12) **Final Wash and Read Plate**

- Take everything over to the Plon lab where the Tecan reader is.
- Set up the plate reader. First access the computer next to the plate reader. Open an excel file called XFluor.xls. Go to the tab *XFluor*. Then go to *Connect*. The plate reader should open up. Next, go to *XFluor* tab and go to *Load Measurement Parameter*. Load Will's Test file. Then go to *XFluor* tab again and go to *Edit Measurement Parameter*. You can adjust the plate reader settings this way. You can adjust how many wells or strips the Tecan reads. Adjust it so that it reads to the bottom of your last strip.
- After incubation wash the wells four times with 200 ul 1x Wash Buffer.
- Add 100µL of developing solution to each well and incubate in the dark for about five minutes. Do not overdevelop.
- After five minutes add 100µL of Stop solution to each well. Be quick to get the plate to the plate reader. The color holds only for a short while, maybe 7 minutes.
- Place the plate in the Tecan with the letters and numbers facing you, ie. you can read them, they're not upside down
- Go to the *XFluor* tab and go to *Start Measurement*

-Plot Standard curve in Excel. Draw a linear regression and determine the equation (in Excel; use the “left-click” button).

-Determine if any points fall below the level of sensitivity (2x background) or if any point is above the saturation point of the assay.
-Determine NF-kB concentration for all samples using the equation from the standard curve. If samples done in duplicate or triplicate, plot average with standard deviation (or range if in duplicate.)
-Plot each sample using a bar graph (Y axis: ng NF-kB/ug protein, X axis sample)
Make sure to include the experiment # in both titles of graph and in data files. Store data files on S drive under ELISA data and Experiment number.