Nuclear Run-On Protocol

- modified by Robida from Nickenig and Murphy 1993

***** Use RNase Free Technique (check your Rnases at the door). Be sure to remove all Rnases from your solutions by treating with DEPC (0.1% DEPC final) overnight. Autoclave these solutions the next day to remove DEPC. This cannot be done with Tris-HCl/Base buffers. Treat all plastic ware used in process with Ambion RNA removal reagent.**

Day 1
Isolation of Nuclei

1) Grow up cells to confluence in a P150mm dish. Change to serum free media 24 hr prior to stimulation
2) Treat cells as needed for a given time (stagger plates 5-10 minutes apart)
3) Aspirate media and add 10 ml of ice-cold Solution 1 (150 mM KCl, 4 mM MgOAc, 10 mM Tris-HCl pH7.4) let sit at 4oC for 5 minutes
4) Scrape off cells into a 15 ml Fisher blue-top tube (rinse 10 ml several times)
5) Pellet at 4C at 1200 rpm for 5 minutes in Beckman GS-GR tabletop centrifuge
6) Remove supernatant, re-suspend in Solution II (Solution 1 + 0.5% NP40)
7) Let sit on ice for 10 minutes
8) Pellet nuclei through a 4 ml cushion of 0.6 M sucrose (in fresh fisher tube) at 2000 rpm 4C in same centrifuge for 10 minutes
9) Remove all but 200 ul of supernatant and add 2 ml of Solution 1 and resuspend the pellet
10) Spin at 2000 rpm 4oC for 5 minutes
11) Remove supernatant, re-suspend in 100 ul Solution III (40% glycerol, 50 mM Mg2Cl2, 0.1 mM EDTA)
12) Store on ice until start of run on reaction (alternately, freeze in liquid nitrogen until thawed for use later)

Run-On Reaction

1) Prepare 5X run on buffer:

	For 500 ul
200 ul	DEPC Q H20
20 ul	0.5 M Tris pH 8.00
10 ul	1 M Mg2Cl2
200 ul	1.5 M KCl
25 ul	200 mM DTT (0.0308 g/ 1 ml)
5 ul	Rnasin (40 U/ul)
10 ul	**EACH OF** 100 mM rNTPs (ATP, CTP, GTP)
10 ul	2 mM COLD rUTP

2) Calculate how much 5X buffer you need (will depend upon volume of nuclear suspension)

For example, a 100 ul nuclei sample gets:
100 ul nuclei
25 ul 32P-UTP (3000 Ci/mmol of 10mCi/ml; **NEN #:BLU007H**)
25 ul 5X run-on buffer

3) Incubate at 30C for 30 minutes in a heat block
4) Add 800 ul trizol reagent to stop reaction
5) Isolate RNA according to standard RNA isolation protocol (**EXCEPT ADD 1/10 VOLUME OF RNase FREE 3M NaOAc PRIOR TO ADDITION OF ISOPROPANOL - INVERT TO MIX**)
6) Dry final RNA pellet for 10 minutes, Re-suspend in 50 ul DEPC QH20. Just prior to adding to hybridization solution, the RNA should be heated to 95C for 10 min to denature.

Plasmid Slot Blots

1) Need 5 ug of DNA per blot, bring to volume of 180 ul with DEPC QH20
2) Add 80 ul of 4N NaOH to denature, sit at room temperature for 15 minutes
3) Add 800 ul of ice cold 2M NH4OAc, sit samples on ice until loading
4) Treat the slot-blot apparatus with Ambion RNase Away reagent, and pre-wet 2 BioRad filter paper 60 pads with 2XSSC
5) Prewet Hybond-N (neutral nylon membrane Amersham Life Science)in QH20 and 2XSSC
6) Place two gel blotting pads on grid, and then place Hybond membrane on top (tighten screws, turn on vacuum and tighten screws again ¼ turn)

*** be sure to test to ensure that there are no liquid leaks between each slot

7) Pre-wet lanes with 1M NH4OAc (apply vacuum, then turn off)
8) Load 400 ul of each DNA sample (apply vacuum, then turn off)
9) Rinse each lane with 1M NH4OAc (apply vacuum, then turn off)
10) Carefully remove cover top and cut the nylon membrane into appropriate sections (usually 1.5 cm X 4 cm - this size should fit into a 12 X 75 mm tube)
11) Cross-link the DNA to membrane by using Stratalinker (Press autolink, start)
12) Pretreat 12 X 75 mm polypropylene tubes with Ambion RNase Away and carefully insert a slot blot into each tube.
13) Preheat ULTRAhyb (Ambion) to 68C, swirl to redissolve precipitated material and then add 3 ml to each polypropylene tube and cap tightly. Incubate to 68C for 30 min.
14) Add 50 ul of labeled RNA (usually 2 to 5 million cpm per sample) and incubate at 68C for 12-16 hr using a rotary incubator.

Day 2: Blot Washing Protocol

1) Remove membranes and place in 50 ml of 2XSSC. Place on shaker for 5 minutes, and then replace 2XSSC solution (pour waste in hot waste container) incubate for an addition 10 minutes
2) Remove 2XSSC solution, and replace with 50 ml of 2XSSC and 0.1%SDS at 50C. Incubate for 5 minutes then replace solution as before and incubate for an additional 10 minutes
3) Rinse membranes in 2XSSC and then place on film or phosphorimager cassette for 1-2 days

* Stringency can be increased by performing last rinse with 0.5X to 1X SSC/0.1% SDS at 50C