Labeling and Hybridization of genomic DNA (DamID PCR products) to NKI Oligo arrays

Maartje Vogel version 11/05/2005

Please check http://www.nki.nl/nkidep/vansteensel for updated versions of this protocol.

Measure concentration

Measure the concentration of the cleaned DamID PCR products on the Nanodrop spectrophotometer.

DNA sample	Final DNA concentration (ng/ul)

Labeling for Oligo-microarray hybridization

Genomic DNA can be labeled with a simple random-priming protocol based on Gibco/BRL's Bioprime DNA Labeling kit. This kit is a convenient and inexpensive source of random octamers, reaction buffer, and high concentration Klenow. Do not use the dNTP mix provided in the kit; prepare yourself using PCR grade dNTPs.

10X dNTP mix (store at -20°C):

1.2 mM each dATP, dGTP, and dTTP
0.6 mM dCTP
$T_{10}E_1$ (10 mM Tris pH 7.5, 1mM EDTA, filtered)

eppie	DNA name	… ul (=…ug)	+ …ul ddH_2O	…ul Cy5/Cy3
1				
2				
3				
4				
5				
6				
7				
8				

- Add 2 ug DNA of the sample to be labeled to a PCR tube.

(Note: for For DamID with lentiviruses add 1 ug.)

- Add ddH_20 to bring the total volume to 21 ul.

- Then add 20 ul of 2.5X Random Primers Solution (from the Bioprime kit Lot no:……)
- Boil 5 min (in PCR machine), and place directly on ice.
- On ice, add 5 ul 10X dNTP mix.
- Add 3 ul Cy5-dCTP or Cy3-dCTP (Amersham, 1 mM stocks).

Cy5 dCTP Lot no	**Cy3** dCTP Lot no

- Add 1 ul Klenow Fragment (40 U/ul from the Bioprime kit, Lot no:……, Exp. Date:……).
- Incubate 1.5 hours at 37°C in PCR machine.
- Keep samples on ice.

DNAse treatment of labeled DNA

For hybridization to the NKI 70mer oligoarrays the DNA (usually DamID PCR product which ranges in size from ~200 to 2000 bp) needs to be broken down to fragments < 200 bp. To accomplish this (random) fragmentation the DNA is incubated with DNAse

10x DNAse buffer (make aliquots and store at -20 ^{o}C):

100mM	Tris-HCl pH7.5
25mM	$MgCl_2$
5mM	$CaCl_2$

- Make a fresh 1:1000 dilution of the 2U/ul DNAse stock (aliquots in –70^{o}C freezer van Steensel-lab, after opening store at -20 ^{o}C);

100	ul	10x DNAse buffer
1	ul	DNAse (2U/ul)
899	ul	ddH_2O
1	ml	

- Make 2x DNAse Buffer containing 0.025U DNAse:

10	ul	10x DNAse Buffer
12.5	ul	DNAse (0.002U/ul)
27.5	ul	ddH_2O
50	ul	

- Add 50ul of 2x DNAse Buffer containing 0.025U DNAse to the pcr tube with labeled DNA (total volume is 100□l).
- Incubate at 25^{o}C for 6 minutes.
- Place the tubes on ice and add 10 ul of 0.5 M EDTA pH 8.0 (stopbuffer from the Bioprime kit).
- Mix and incubate at 75^{o}C for 10 minutes to inactivate the DNAse.
- Reduce the volume by speedvac from 110ul to ≤ 35ul (~40-45 minutes at medium heat in H6 speedvac).

Pre-hybridisation (part 1)

Pre-hybridisation buffer (5x SSC, 0.1x SDS and 1% BSA)
(Make a 10% BSA stock, filter and store at –20°C, thaw only once)

For 30 ml:	7.5 ml	20x SSC (filtered)	(60 ml:	15 ml
	150 ul	20% SDS (filtered)		300 ul
	3 ml	10% BSA		6 ml
	19.35 ml	ddH2O		38.7 ml)

- Warm pre-hyb buffer to 42°C prior to use.
- Add the buffer to the 'pre-hyb chamber' (plastic box) and place the slide in the chamber, making sure the array is submerged.
- Pre-hybridize the microarray at 42°C for at least 1 hour up to 3 hours.

Probe cleanup

TE = $T_{10}E_1$ (10mM TrisCl pH7.5, 1mM EDTA) filtered.

Probe clean up on chromaspin column:

- Pre-spin chromaspin column for 3 minutes at 700g (in eppendorf 5417) (see manufactures protocol for instructions). Immediately continue with the next step do not let the column dry out!
- Place a fresh tube below the chromaspin column. Load the sample to the top of the column, make sure that you pipette the probe slowly in the center of the column material, which may come loose from the inside of the column because of spinning.
- Spin column for 3 min at 700 g.
- Collect tube and mark it properly, keep eluate as dark as possible (probes can be stored at -20˚C)
- Use ~1.5 ul of each eluate to measure the amount of DNA and frequency of dye incorporation on the nanodrop.
- Pool the (experimental and control) Cy3 and Cy5 reactions.

OPTIONAL
Analyze the fragmentation of the labeled DNA on a 4% agarose gel:

- Prepare a 4% agarose gel with Ethidium Bromide in 1xTAE.
- Prepare pcr tubes with 0.8-1.0 ug of each probe.
- Heat 2 mins to 99˚C and snap cool on ice (to denature the DNA).
- Add loading buffer to the samples and run the gel (include a 100 bp DNA marker).

Continue the probe clean up using microcon columns:

- Mark a microcon column and add 450 ul TE to the column. Pre-spin 5 min at 13.000 g (11.063 rpm) to wash column, discard flow trough. Check if column surface is still wet (if not use new column).
- Apply 200 ul TE to Microcon column.

- Add 64 ul of blocking solution to the Microcon column.
- Optional: Add 25 ug pre-digested competitor plasmid to the Microcon column.
- Load the eluate from the chromaspin column to the Microcon column.

Blocking solution (store at -20˚C):

4 ul	20 ug poly (A) (5 mg/ml Roche)
20 ul	100 ug yeast t-RNA (5 mg/ml stock Life)
40 ul	40 ug human Cot-1 DNA (1 mg/ml Invitrogen)

- Spin Microcon column for 5 min at 13.000g (11.063 rpm) (discard flow trough).
- Add 300 ul TE to top of column and spin 4 min at 13.000 g (discard flow trough).
- Add another 300 ul TE to top of column and spin 5 min at 13.000 g.
- Spin to reduce the volume to <22.5 ul (judge by eye; when half of the column surface it wet, the volume is <10 ul). Inspect the color of the labeled DNA by eye.
- Collect probe by inverting the column in a fresh tube (cut of the lid and keep it to seal afterwards) and spin 2 min at 13.000 g.
- Measure the amount of probe and adjust to 22.5 ul with TE.

Eppie (array number)	
	……ul probe + ……ul TE = 22.5ul
	……ul probe + ……ul TE = 22.5ul
	……ul probe + ……ul TE = 22.5ul
	……ul probe + ……ul TE = 22.5ul
	……ul probe + ……ul TE = 22.5ul
	……ul probe + ……ul TE = 22.5ul

- Heat probe for 2 minute at 100 ˚C (dry heat block) and snap cool on ice for 2 minutes. Store the probe for at least 5 minutes at 42˚C.
- Make fresh 2x F-hybridization buffer, heat the buffer to 42˚C for at least 5 minutes and add 30 ul to the probe, mix the 2x F-buffer well before adding. Keep at 42˚C.
- Thaw an aliquot of chemical block mix (=“Kreablock”) and add 7.5 ul to the probe. Keep at 42 ˚C for at least 5 minutes. (Note: 1/8 of the total volume under the lifter slip should be chemical block mix).

2x F-hybridization buffer (pipet in the following order):

500 ul 100% formamide
500 ul 20x SSC
10 ul 20% SDS

1x F-hyb buffer:

dilute 2x F-buffer with ddH_2O

- Place a clean lifter slip on an untreated object glass slide. Determine the volume under the lifter slip with ddH_2O. Adjust the probe volume to the volume under the lifter slip with 1xF-buffer.

Pre-hybridisation (part 2)

- Take the arrays from the pre-hyb buffer, dip them in ddH_2O and collect them in a glass color box with ddH_2O.
- Wash of the pre-hyb buffer for 10 mins in the glass color box.
- Transfer the arrays to a second distilled water bath for another 5 mins.
- Spin dry the arrays for ~4 mins at 400 rpm. Make sure that the time between the drying and the actual hybridization is not longer than 15 minutes.

Hybridization

- Clean the lifter slip with soap, plenty of water, ethanol and acetone. Dirty lifter slips give high background. (When the slips are not used before: start to wash with acetone.)
- Place the array in the chamber with the array site up; make sure that the array does not touch the inner sides of the chamber.
- Put the lifter slip on the array with the with Teflon linings facing down, so the lifter slip is not directly touching the glass.

The next 3 steps should be performed as quickly as possible to prevent evaporation of the probe.

- Spin probe for 1min at max speed to get rid of floating precipitates.
- Apply ~40 ul 1x F-hybridization buffer to the far ends of the chamber.
- Adjust probe to array, the probe will be sucked under the cover slide, due to capillary force. (Inject the probe slowly from one corner of the lifter slip until array is thinly covered – the pipette tip should be as close to the slip as possible without touching it)
- Close the hyb chamber, keep it horizontally, move it only slowly and make sure that the array does not move in the chamber.
- Incubate at 42˚C (in a stove) for 1x o/n.

Washes

Prepare the washing solutions, the first three washing solutions are kept at 42˚C, solution 4 and 5 are kept at RT.

WASH 1: 5x SSC / 0.1%SDS (149 ml H_2O +50 ml 20xSSC + 1 ml 20% SDS)
WASH 2: 2x SSC / 0.1%SDS (179 ml H_2O +20 ml 20xSSC + 1 ml 20% SDS)
WASH 3: 1x SSC (190 ml H_2O +10 ml 20xSSC)
WASH 4: 0.2x SSC (198 ml H_2O +2 ml 20xSSC)
WASH 5: 0.05x SSC (200 ml H_2O +0.5 ml 20xSSC)

- Take the array from hyb chamber and submerge it as quickly as possible in the first wash solution. (Process – put washing buffer 1 in a 50 ml falcon tube, submerge array standing upright, lifter slip will slide off after a while without scratching the array.)
- Wash the array in solution 2 for about half a minute, plunging gently.
- Wash the array in solution 3 for about 5 mins, also plunging gently. (Arrays can be collected in a rack in solution 3).
- Wash the array(s) in solution 4 for about 2 mins, again plunging gently.
- Wash array(s) in solution 5 for 20 seconds without plunging and spin dry quickly (~4 minutes 400 rpm). Be careful to use a good counter weight. DO NOT LET SLIDES AIR DRY. Keep the dry arrays dark.

- Scan the array (arrays can be stored for three to four days in the dark before scanning.

General notes

Concentration measurements are done on the nanodrop spectrophotometer

Ordering information

Labeling and microarray hybridization:

Clontech	Adv. cDNA polymerase mix	100 rxns	639105
Invitrogen	BioPrime DNA labeling System	30 rxns	18094-011
Invitrogen	yeast tRNA	25 mg	15401-011
Amersham	Cy3-dCTP	25 nmol	PA53021
Amersham	Cy5-dCTP	25 nmol	PA55021
Roche	Poly [A]	100mg	108626
Millipore	Microcon YM-30 filters	100	42410
Invitrogen	human Cot-1 DNA	500ug	15279-011
Clontech	Cromaspin +TE-30 columns	50	636069
Cambrex	Metaphore agarose	25g	50181
Kreatech	KreaBlock	only available as part of kits	
Worthington	DNAse		LS006333