Making 19x601 (1xbio), 19x601 (2xbio) and 1x 601

Starting with pAS696 (Guse…Straight, Nature, 2011 https://www.nature.com/articles/nature10379), which contains 19 copies of the 601 nucleosome positioning sequence (Thastrom et al., J Mol Biol, 1999 https://www.sciencedirect.com/science/article/pii/S0022283699926864) integrated into the EcoRV site of pUC18, one can generate 19x601 arrays with different ends that allow labelling with biotinylated or fluorescent nucleotides. One can also generate 1x601 fragments with 25 bp linker DNA on either end.

Array preps

Restriction digests

The 19x601 array can be liberated with EcoRV (generating blunt ends, make sure to use the HF version of EcoRV because regular EcoRV isn't buffer compatible with the other enzymes) or with EcoRI/XbaI (allowing the generation of fragments biotinylated on both ends) or with EcoRI/HindIII (allowing the generation of fragments biotinylated on only one end. Make sure to use HindIII-HF because regular HindIII isn't buffer compatible with the other enzymes).
In addition, it is necessary to digest the vector backbone into small fragments (the reason will become apparent later). This is accomplished by addition of DraI and HaeII restriction enzymes.

pAS696	~600ng/µl final
BSA	50µg/ml final (use NEB stock as 200x)
Buffer 4	add to 1x
HaeII, DraI, EcoRI, XbaI	Add at 1:100
or	
HaeII, DraI, EcoRI, HindIII-HF	Add at 1:100
or	
HaeII, DraI, EcoRV-HF	Add at 1:100

Leave at 37C o/n

PEG purification

Next, we need to separate the ~3kb 19x601 fragment from the small vector fragments. This is done by PEG precipitation (larger DNA fragments are less soluble in solutions containing PEG than small DNA fragments). Add NaCl to 0.5M, take Input sample (1µl in 10µl TE or so). You may have to split the sample at this point into several 1.5ml or 2ml centrifuge tubes.
Add PEG 6000 to 4% (stock is 20 % PEG 6000, 0.5 M NaCl). Vortex, spin down (13 krpm, 10 min, RT). Save pellets. Add PEG to 5% (1/19 vol), repeat spin. Do this for two more increments. An alternative way to spin is 20 min at max speed in a 15 ml Falcon tube in the table top Eppendorf Falcon tube centrifuge.
Then, add 200µl of 1xTE per pellet, leave at RT for ca. 20min, then resuspend thoroughly, combine pellets of each PEG percentage, take sample (0.5µl in 30µl TE). Run on gel. Combine pellets of the best fractions & repeat PEG precipitation. Finally, combine the best fractions, EtOH precipitate the DNA, proceed to Klenow fill-in.

Klenow fill-in
(Note: Obviously, if you do not need to couple to beads, skip this step)
For 1xbio (E/H) fragments, use thio-dTTP, thio-dGTP, dATP & biotin-14-dCTP
For 2xbio (E/X) fragments, use thio-dTTP, thio-dGTP, biotin-14-dATP, dCTP
Make a master mix of fill-in reactions then split into 100µl aliquots and transfer to PCR strips.

DNA	0.9µg/µl final
NEB buffer 2	to 1x
Klenow	0.4u/µl final
Nucleotides	to 35µM final (thio-dNTPs & dNTPs are 10mM stock, biotin-14-dNTPs are 400µM stock). Note: You can also substitute biotinylated dNTPs with fluorescently labelled dNTPs if you want to visualise the arrays on a gel or by microscopy.

Incubate at 37C for 3.5hrs.

Nick column purification
Follow instructions. Use 1x TE as buffer. If you have a large sample volume, take two columns and re-use them. Wash with two times 3.5ml of 1x TE in between samples to remove dNTPs from the column.
EtOH precipitate, dry well, resuspend in 1x TE at a volume to give you ~2-4µg/µl. Resuspend well. Spin 5min full speed. Take sup, measure DNA concentration.

Making 1x601

Restriction digest

Digest pAS696 with AvaI (this cuts between each of the 601 units but doesn't cut the vector backbone). Do this in 400 ng/µl final, using buffer Cutsmart and 1:40 AvaI. Leave at 37C over night. Check digest before proceeding to PEG precipitation.

PEG precipitation

As opposed to the 19x601 array, here we are pelleting away the vector backbone, and we will keep the supernatant, which contains the 601 monomer. Start by adding NaCl to 0.5 M, then PEG/NaCl to 5%, (stock is 20 % PEG 6000, 0.5 M NaCl). Vortex, spin down (20 min at max speed, 22C, in a 15 ml Falcon tube in the table top Eppendorf Falcon tube centrifuge.). Add PEG to 6% (1/19 vol), repeat spin. then do 1% increments until ~9%. Check supernatants. If good, precipitate with EtOH.

Note: Increments do not make too much sense for this. So you could also just start with 9% straight away.

Nucleosome reconstitution

Note: This protocol is based on the paper by Guse…Straight, Nature, 2011 (https://www.nature.com/articles/nature10379). Also see Guse…Straight, Nat Protoc, 2012 (https://www.nature.com/articles/nprot.2012.112)

Nucleosome formation by gradient dialysis

We usually do 50µl reactions of 0.2µg/µl DNA (=10µg total). Because each unit in the array is ca. 200bp, this comes down to a general concentration of positioning sequence of ca 1.6µM. We use H3/H4 tetramers at this same concentration (ie, 3.2µM of dimer) but usually add ~10% excess of H2A/H2B (~3.5µM of H2A/H2B dimer). However, measuring concentrations of histones can be somewhat error prone (they absorb poorly in UV & if you have some contaminant that absorbs well, even if it's a minor species, that can really mess up your measurement). So in practice, every time you use newly made histones (and/or DNA), you need to test a few different concentrations/ratios of H3/H4 and H2A/H2B to find optimum conditions. Ask me for what I currently use.

Add to your tube in this order:
DNA, 1xTE (calculate to bring final volume up to 50µl), 20 µl 5M NaCl, mix, add H3/H4, mix, next sample, add H3/H4 mix, etc., add H2A/B, mix, next sample, add H2A/B, mix. etc.
Then fill up dialysis buttons (Hampton Research HR3-326) & cover with a pre-wetted dialysis membrane.

Add dialysis buttons to 500ml of High Salt Buffer. Exchange into 2 litres Low Salt Buffer I over a 36-48 h long gradient in the cold room (two pump lines, one pumps buffer out into waste, the other pumps buffer in)

Move to a new beaker with ~0.2l Low Salt Buffer II, dialyse 3-4 hrs. Remove dialysate with syringe & pipette with gel loading tip. Proceed to AvaI digest. Keep some of the Low Salt Buffer II from the dialysis beaker (to be used for concentration measurements later).

AvaI digests

Between each of the units of the array there is an AvaI restriction site. This allows for convenient testing for how complete your chromatinisation reaction was.
Take 3µl of each reaction & digest with 1µl of AvaI in a total reaction volume of 15µl. Because the buffer that NEB supply with AvaI has too much $MgCl_2$ (which induced higher order structures in chromatin fibres and thus might interfere with AvaI accessibility), we use a home-made low Mg buffer (10x concentrated). I have some you can have.
In order to not stress the nucleosomes out too much, we do these digests at RT over night (I usually do them in the PCR machine set to 22ºC). Don't forget to do a control digest of naked array (about 150ng).

Native gel electrophoresis

By native gel electrophoresis, we can distinguish between naked array units, nucleosomal units and tetrasome units. Naked DNA runs fastest but tetrasomes and nucleosomes are increasingly

retarded in their electrophoretic mobility. A 5% PAA gel in 0.5x TBE is pretty good at resolving these species.

1x TBE	5.5ml
Water	3.55ml
Acrylamide mix	1.87ml
10% APS	110µl
TEMED	11µl

I usually use loading buffer without dye & for some reason I've settled on using sucrose instead of glycerol. Add ~1.7µl of 2M sucrose to each digest, load on gel. Run some 100bp ladder on the side. I usually label each well with the sample number that will go into it so as not to double fill any of the wells or omit them.
Use 0.5x TE as running buffer. Run for ca. 1hr at 10mA. When blue front from the ladder is run out, you can remove the gel from the glass plate and dump it into 1xTE with whatever dye you sue for visualizing DNA. Shake 30min. Put gel into document wallet & take a picture. Measure Abs_{260} to determine DNA concentrations in the samples that have good chromatin. Concentrations should be in the 50ng/µl (poor) to 150ng/µl (great) range.

Buffers:
High Salt Buffer
10mM Tris-Cl pH 7.5 @ 4ºC
1mM EDTA
2M NaCl
5mM BME (200µl for 500ml of buffer).
0.01% Triton X-100

Low Salt Buffer I
Just like High Salt Buffer but 50mM NaCl

Low Salt Buffer II
10mM Tris-Cl pH 7.5 @ 4ºC
0.25mM EDTA
100mM NaCl
1mM TCEP
(use un-pH'ed Tris to make this buffer & then titrate in HCl to reach appropriate pH).

10x AvaI low Mg buffer
50mM potassium acetate
20mM Tris-Acetate
0.5mM Magnesium acetate
1mM DTT
pH 7.9