**Plasmid DNA preparation for mouse Oocyte Pronuclear Microinjection**

**Mouse Genome Engineering Facility**

**NCBS Animal Care and Resource Center**

**Important considerations before you start:**

The quality of DNA fragments to be microinjected into mouse oocytes is of crucial importance. To maximize the success rate of obtaining transgenic animals; the purified DNA fragment needs to be prepared by taking into account the following parameters:

1. It is highly recommended that you design your constructs in such a way that the injected fragment be digested out with minimal remaining flanking plasmid sequences.
2. The purified DNA needs to be free of salts, organic solvents or of any traces of agarose as these are toxic for the embryos.

Since autoclaved glassware may come with possible detergent residues; please try to maximize the use of disposable CLEAN/STERILE plasticware or extensively pre-rinse your autoclaved glassware with filtered ddH2O immediately before use.

1. Solutions used in DNA purification steps and any prepared required buffers should be made using fresh & high quality sterile reagents. (endotoxin free – embryo tested reagents are highly recommended). All stock solutions and buffers should be autoclaved and sterile filtered before use.
2. In all filtration steps – please do NOT USE filters which have been sterilized with ethylen oxide.
3. It is also important to avoid contamination of any particles that could clog the injection needles (their tip is less than 1m in diameter). Please therefore try to work in a clean – dust free environment.

And it is strongly recommended to use powderfree gloves when handling DNA preparation to avoid potential clogging of injection needles.

1. The Injection DNA fragment solution should be pure and intact without any degradation products (not sheared, or nicked and with no visible smear when checking on gel) to avoid integration of partial constructs.
2. The injection fragment DNA stock concentration must be precisely measured so that the facility can make a final injection working solution of 1-3ng/l.

The DNA concentration is a crucial factor to the success of experiments. Excess DNA is toxic and results in developmental arrest. Too diluted DNA will decrease the number of transgenic founders. Errors in DNA concentration can significantly affect transgenic production. Please follow the protocol below to accurately quantify your DNA stock solution.

1. A minimum of 1-5g of total purified injection DNA fragment at high concentration (50-200ng/l) needs to be delivered to the facility lab at least one week before the assigned microinjection session. The facility will double check for DNA quantity and purity.
2. The facility will ask that you prove that your construct is expressed in vitro by an appropriate cell line. If this has not been shown for your construct, please highlight this in your application.

**What needs to be handed over to the facility at least ONE WEEK before scheduled micro-injection date:**

1. Filled the MGEF Mouse Transgenesis Project Request form.
2. A CLEARLY labelled 1.5ml tube (with project ID - date - DNA concentration) containing 1-5g of purified DNA fragment at a final minimum concentration of 50ng/l or above in embryo tested filtered H20 (Sigma W1503).
3. Picture of RECENT dated test agarose gel attesting the quality and quantity of DNA fragment (see protocol below for details).

FYI: the facility with also test your DNA quality by making sure that the OD 260nm/280nm ratio is above 1.8 & that the OD 260nm/230nm ratio is above 2.

The MGEF can do the final digested DNA purification for you upon request and at a cost of 1000 Rs per sample.

**Plasmid DNA preparation protocol:**

The Mouse Genome Engineering Facility recommends you strictly follow the following guidelines to prepare your DNA destined to pronuclear microinjection.

1. Perform restriction digest of 25-30 g endotoxin free prepared plasmid DNA to excise the required “injection” fragment.

(minimum of 3-4 h digest in 100l final digest volume is recommended).

1. test run 1l of digest on minigel to check that all the plasmid is digested and that the digest gives expected bands free of any DNA degradation products.
2. The DNA digest solution can be stored at -20C at this point or at 4C in stop/gel loading solution containing EDTA.
3. Run rest of (prewarmed) digest on freshly prepared long (around 0.8% depending on your fragment size) agarose gel (using clean tank, fresh running TBE or TAE buffer, minimal amounts of Ethidium Bromide (less then 0.5g/ml) & high purity agarose).
4. To avoid clogging of the gel purifying columns and gel DNA overload: run the 100l digest in 3-4 different large wells - NOT in a single small well - (10g of DNA max per large well).
5. A slow / low voltage gel run is recommended (this may take several hours to overnight).
6. Once run is complete (= desired fragment reached mid gel length) - Delicately place gel on CLEAN UV transilluminator (use clean saran wrap on transilluminator to minimize the risks of contaminating your DNA with whatever maybe on that equipment).

Using adequate UV protective gear: Visualize DNA using low UV intensity or long wave UV light and minimizing UV exposure time to prevent photochemical damage (less than 1 minute exposure). Cut out desired DNA bands with sterile scalpel taking care to remove all excess of non-DNA containing gel slices.

1. Dice each DNA-agarose block and place in one or more sterile 1.5ml or 2ml tube.
2. Purify desired DNA band on Qiagen qiaquick Gel extraction columns (cat# 28704) or similar gel extraction kit respecting all additional “microinjection quality purification steps & tips” mentioned in the Kit guidelines.

Use one column per well (i.e per gel slice) – max 10g DNA per column – if purified fragment is over 10Kb – then the fragment should be gel purified using the Qiaex II gel Extraction kit (cat# 20021)

1. Weigh each tube – (you should not have more than 400mg of agarose per tube (ie per column).
2. Follow kit instructions up to elution step. (with the following additional recommended steps:

a) after binding DNA to column - wash column again with 700l of binding buffer before proceeding to the ethanol based washing step

b) repeat ethanol-based washing step twice - and then spin column dry making sure to remove and evaporate all traces of Ethanol buffer before elution step.

1. Elute DNA from each column with 50l nuclease free H20 (embryo tested water (Sigma W1503) is even better – the facility can provide you with an aliquot of this) per column into fresh 1.5ml tube- let column rest with H20 for 5 minutes at room T°C before elution spin.

Pool all eluates of same DNA fragment in one tube.

1. Repurifying the eluate with a second round of DNA purification kit protocol is highly recommended.
2. Ethanol precipitate DNA by adding 1/10th of eluate volume of sterile sodium acetate - NaOAc (pH5.6), gently mix and add 2 volumes of 100% molecular biology grade ethanol (prechilled at -20°C).

Gently invert tubes several times and keep at -20°C for 15min to overnight.

1. Spin max speed (13K-14K rpm) for 30min at 4°C.
2. locate DNA pellet and carefully remove the supernatant (ethanol)
3. wash pellet with cold 75% ethanol - invert tube several times - (the pellet might detach from tube wall at this point and swirl in the ethanol - that is fine and actually allows to better wash the pellet ... but do not vortex! just gently finger mix or invert tube to wash pellet)

spin 15-20 minutes max speed

1. repeat steps 16-17
2. locate DNA pellet and slowly remove all remaining Ethanol and let pellet dry at room temperature by lying open tube flat on clean Kimwipe.
3. make sure all ethanol is evaporated before resuspending.
4. Fully resuspend DNA pellet in 20 l nuclease free embryo tested prefiltered H20 (Sigma W1503).

This is the concentrated stock of your microinjection DNA fragment.

(to be stored at -20°C until further use).

1. Quality test Run 1l and 0.5l of concentrated microinjection DNA fragment on a mini gel & estimate concentration by running next to a known DNA concentration scale series and compare staining intensities.
2. Provide the Quality test run gel picture to the facility to attest of DNA quality and concentration.

Quantifying via NanoDrop is not reliable so the gel run quality/quantification check is mandatory.

If checking on the NanoDrop: the OD 260nm/280nm ratio should be above 1.8 & 260nm/230nm ratio ideally above 2.

Store at -20°C as concentrated stock of microinjection fragment. This is the solution to be given to the facility - it should contain a minimum of 1g of purified DNA fragment at a final concentration of 50ng/l or above.

The following steps will be carried out at the facility on the day of injection:

1. On the day of microinjection - the facility will then dilute your DNA in prefiltered injection buffer (10mM Tris-HCl pH7.5, 0.15mM EDTA ; sterile filtered) to a concentration of 1-3ng/l (500-1000 copies/pl) in a volume of 100-200 l.
2. Spin diluted DNA solution and take supernatant in fresh sterile tube avoiding any precipitates at the bottom**. The collection tube and any tips to be used to manipulate the DNA from this point should be prewashed with injection buffer.**

Alternatively: if there are too much pipette clogging issues: this DNA solution can be refiltered by centrifugation in injection buffer pre-washed Costar Spin-x 0.45 m cellulose acetate column (2ml tubes). This diluted microinjection DNA solution can be used for up to two microinjection sessions and stored at 4°C for one week max.

Injection Buffer to be prepared with embryo tested water (Sigma W1503):

5-10 mM Tris pH=7.4-7.5; 0.1-0.25 mM EDTA

prepared from: 1M Tris-HCl pH7.5 autoclaved stock (Sigma T6066) and 0.5 M EDTA autoclaved stock (Sigma E6758)

e.g. for 10mM Tris-HCl pH=7.5; 0.15 mM EDTA for 50 ml:

500l 1M Tris-HCl pH7.5 + 15l 0.5 M EDTA + embryo tested water (Sigma W1503)

Sterile filtered (22M)