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# 🌐 Preparation of Nucleic Acid Solutions for Endonuclease Induced Modification of the Mouse or other Mammalian Genomes

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Transgenic Animal Mod...



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**We use this protocol and it's working**

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## Abstract

The Transgenic Core has used Cas9, ZFNs, and TALENs, to target more 500 genes in mice and rats. In the current era, all projects involve gene knockins, such as floxed alleles, recombinase knockins, reporter knockins, cDNA knockins to the ROSA26 locus. Simple gene knockouts are not in demand as they were when CRISPR/Cas9 technology was reported in 2013. It is essential to protect sensitive RNA components from degradation during zygote microinjection and/or electroporation. All materials mixed with gRNA or mRNA components, such as Cas9 protein (used to prepare ribonucleotide protein complexes), DNA donors (such as oligonucleotides, long single stranded DNA donors, and double stranded circular plasmids), and AAV donors must be resuspended in a buffer that is free from endonucleolytic activity. Fine particulate contaminants can be reduced in microinjection solutions by judicious use of 0.02  $\mu$ m filters. The suggestions below are based on our successful experience in the production of genetically engineered mice, rats, and cultured cells.



# 1 Table 1. List of Materials for RNase Free Microinjection Buffer.

| A                                                                                  | B |
|------------------------------------------------------------------------------------|---|
| Guide RNA produced by commercial synthesis.                                        |   |
| Guide RNA produced by in vitro transcription.                                      |   |
| Other mRNA molecules such as Cas9 mRNA, ZFN mRNA, or TALENs mRNA.                  |   |
| Oligonucleotide donor resuspended in RNase free microinjection buffer.             |   |
| Long single stranded donor resuspended in RNase free microinjection buffer.        |   |
| Double stranded DNA donor plasmid resuspended in RNase free microinjection buffer. |   |
| AAV virus preparation resuspended in RNase free                                    |   |



| A                                                | B                                                               |
|--------------------------------------------------|-----------------------------------------------------------------|
| microinjection buffer.                           |                                                                 |
| Pipetman.                                        |                                                                 |
| RNAse Free Pipet Tips.                           |                                                                 |
| Sterile 1.5 ml Microtubes.                       |                                                                 |
| Sterile 15 ml tubes.                             |                                                                 |
| Sterile 50 ml tubes.                             |                                                                 |
| Sterile 10 ml pipets.                            |                                                                 |
| Sterile 10 ml syringes.                          |                                                                 |
| Sterile 15 cm diameter Petri dishes.             |                                                                 |
| Whatman 0.02 um Anotop 10 Syringe Filters.       | Whatman Cat. No. 6809-1002, SigmaAldrich Cat. no. WHA68091002.  |
| Millipore MCE Membrane Filter, pore size 0.05 um | Millipore Cat. no. VMWP02500, Sigma Aldrich Cat. no. VMWP02500. |
| 1 M Tris-HCl, pH 7.4.                            | SigmaAldrich Cat. no. T2663.                                    |
| 0.5 M EDTA.                                      | SigmaAldrich Cat. no. E7889.                                    |
| Nuclease Free Water.                             | igmaAldrich Cat. no. W4502.                                     |

- 2 Table 2. Prepare Reagent: RNase Free Microinjection Buffer - 10 mM Tris-HCl, pH 7.4, 0.25 mM EDTA.

| A                                                                                                                               | B                |
|---------------------------------------------------------------------------------------------------------------------------------|------------------|
| For 25 ml<br>Mix<br>Together.                                                                                                   |                  |
| Water                                                                                                                           | 24.7 ml          |
| Tris-HCl,<br>pH7.4                                                                                                              | 0.25 ml          |
| EDTA                                                                                                                            | 0.125 ml         |
| Wash 0.02<br>µm syringe<br>filter with 1<br>ml buffer.                                                                          | Discard<br>wash. |
| Push<br>remaining<br>buffer<br>through<br>filter for use<br>as dialysis<br>buffer or for<br>use as<br>microinjection<br>buffer. |                  |
| Store at<br>room<br>temperature<br>in 50 ml<br>tube.                                                                            |                  |

nota bene: Do not filter nucleic acid solutions through the Anotop filters, the 0.02 µm pores will trap nucleic acids and remove them from solution.

- 3 Procedure: Prepare mRNA/sgRNA/oligo/plasmid for Microinjection.

Materials for microinjection are commercially sourced or prepared in house. For example, Cas9 protein, mRNA and sgRNA can be transcribed in vitro with kits, etc. Microinjection buffers must be prepared with nuclease free stock solutions.

### 3.1 Plasmid DNA donor preparation.

Kits that are based on columns often produce particulate contaminated DNA as shown by Montigny et al. (2003). Particulates clog glass microinjection needles. Clogged

needles decrease microinjected zygote survival and lead to lower yields of genetically modified mice or rats. The following steps will reduce but not eliminate particulates. Montigny WJ, Phelps SF, Illenye S, Heintz NH. 2003. Parameters influencing high-efficiency transfection of bacterial artificial chromosomes into cultured mammalian cells. *Biotechniques*. 35:796-807.

- 3.2 Purify plasmids with endotoxin free kits.
- 3.3 Pre-filter kit buffers and elution buffers with 0.02  $\mu$ m syringe filters.
- 3.4 When possible, the final elution step should be in filtered RNA microinjection buffer or water.
- 4 Oligonucleotide donor preparation.
  - 4.1 Oligos are subjected to spot dialysis to remove embryotoxic chemicals.
  - 4.2 Follow standard guidelines for working with RNA to protect against later RNA degradation.
  - 4.3 Resuspend the lyophilized oligo in filtered RNA microinjection buffer.
  - 4.4 Fill a 15cm Petri dish with 200 ml nuclease free microinjection buffer. 
  - 4.5 Place a Millipore dialysis filter on the surface of the buffer so that it floats (place the filter shiny side up).
  - 4.6 Carefully spot the resuspend oligo solution into the center of the filter. 
  - 4.7 Replace the Petri dish lid.
  - 4.8 Dialyze for 30-60 minutes.

- 4.9 Up to 200 ul can be placed on a filter without losing it to the buffer.
- 4.10 Leave the dialysis to proceed quietly without any shaking or movement.
- 4.11 Do not let the dialysis to go more than 3 hours otherwise the drop might begin to evaporate.
- 4.12 Carefully Pipette off the solution: Place the tip in the middle of the droplet and carefully aspirate as much as possible without stopping.
- 4.13 Transfer the nucleic acid solution to a sterile microtube.
- 4.14 Quantitate and store at - 80°C.
- 4.15 Recoveries between 50-70% of the original volume are normal.
- 4.16 The rest of the solution remains attached as a very thin liquid layer onto the surface of the filter and is difficult to pipette off.
- 5 Cas9 protein resuspension.  
Resuspend lyophilized Cas9 in filtered RNA microinjection buffer to the desired concentration and store at -20°C.
- 6 sgRNA, tracrRNA, crRNA resuspension.
  - 6.1 Resuspend lyophilized RNA species in filtered RNA microinjection buffer to the desired concentration and store at -20°C.
  - 6.2 Optional step: subject resuspended DNA to microdialysis.
- 7 Microinjection or electroporation mixture preparation.



- 7.1 Mix together nucleic acids in filtered RNA microinjection buffer to the desired concentrations.
- 7.2 Prepare aliquots in 1.5 ml microtubes.
- 7.3 Mixtures may be used fresh or stored at -80°C prior to microinjection or electroporation.