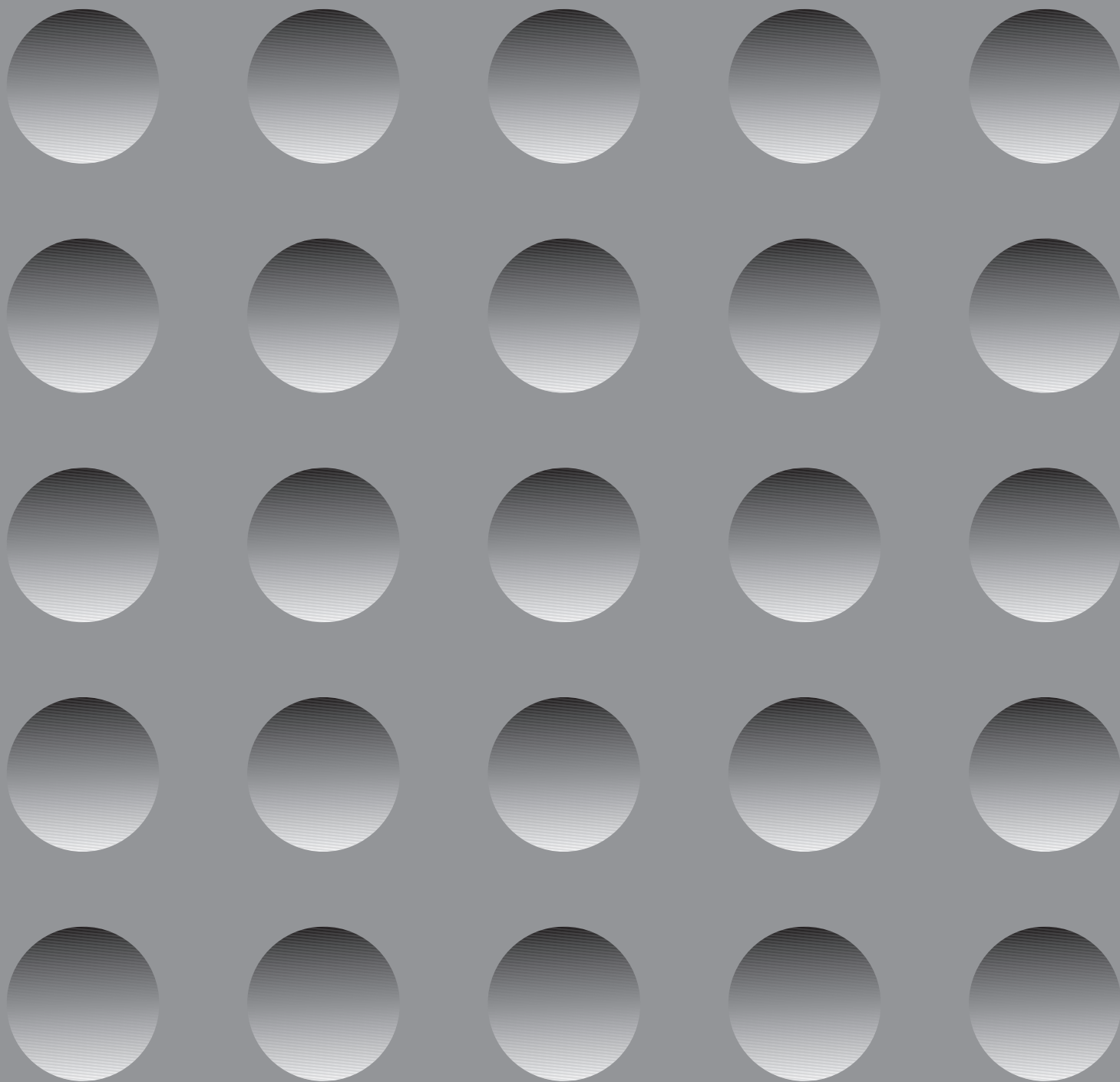


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Oocyte Preparation



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Table Of Contents

1	Materials	1
2	Oocyte Removal	3
3	Defolliculation	3
4	Selecting Good Oocytes	4
5	Plating Oocytes	4
6	Washing Oocytes	5
7	Sources of Supply	7

1 Materials

Recommended products are listed under "Sources of supply".

Biological materials

- Female frogs of *Xenopus laevis*

Technical Equipment

- Shaker for the tubes (during defolliculation)
- Stereo microscope (or checking and selecting oocytes)
- (Optional) Tecan Columbus Microplate Washer
- 96 well plates, non-treated polystyrene, conical bottom

It is very important that the well plates are produced carefully and have minimum variations. Do **not** use coated plates, because oocytes will not adhere to the well bottom of coated plates.

Check each single plate before use. The plate should be even and it should not be distorted in any way.

Note: If you use warped plates, you will encounter problems during injection or recording. Check each plate carefully before use.

- Oocyte filter

For a coarse selection of oocytes according to the size:

Remove the bottom of a 50 ml Falcon tube. Place a mesh with an 800 μm grid over the cut end and fix it with glue.

- Oocyte transfer pipette
- Pipette for handling oocytes
- Large petri or cell culture dishes, 100 mm
- Petri dishes, 60 mm
- Beaker, 100 ml
- Razor blade
- Forceps
- Parafilm
- General laboratory equipment

Roboocyte - Preparation of Xenopus Oocytes

Chemicals

- **Collagenase**

For defolliculation:

Fresh 1.5–2 mg/ml collagenase in Barth's solution without Calcium (concentration has to be optimized according to the collagenase batch and experimental conditions, see also chapter "Defolliculation"). Do not prepare solutions in advance as collagenase activity may decrease rapidly even if the solution is stored at –20 °C.

Collagenase from *Cl. histolyticum* ca. 0.17–0.28 U/mg lyophilized

- **Gentamicin**

Stock solution: 50 µg/ml gentamicin (free base) in Barth's solution. 1 ml Aliquots with 50 mg/ml gentamicin (free base) are stored at –20 °C

Working solution: Dilute 1 ml of gentamicin stock in 1 l Barth's solution.

Gentamicin sulfate salt, potency approx. 600 µg Gentamicin per mg

- **Barth's solution**

pH 7.4 (with NaOH)

88 mM NaCl

2.4 mM NaHCO₃

1 mM KCl

0.33 mM Ca(NO₃)₂ * 4 H₂O

0.41 mM CaCl₂ * 2 H₂O

0.82 mM MgSO₄ * 7 H₂O

5 mM Tris/HCl

- **Barth's solution without Ca²⁺**

pH 7.4 (with NaOH)

88 mM NaCl

2.4 mM NaHCO₃

1 mM KCl

0.82 mM MgSO₄ * 7 H₂O

5 mM Tris/HCl

- **Frog Ringer's solution** (for perfusion)

NaCl 115,0 mM

KCl 2.5 mM

CaCl₂ 1.8 mM

HEPES 10 mM

pH=7.2 / Osmolarity: 240 mOsm/kg

2 Oocyte Removal

1. Remove the appropriate amount of ovarian tissue surgically from one side of the frog. Please refer to standard protocols on this subject.
2. Immediately transfer the portion of removed oocytes to a petri dish containing Barth's solution without Ca^{2+} .

3 Defolliculation

Isolated oocytes are enveloped in a tough follicle cell layer. The follicle cell layer should be removed completely by collagenase digestion. It does not disturb the recording, but it causes trouble when plating oocytes into well plates. Remaining pieces of follicular tissue causes oocytes to stick to the walls. Oocytes will not move into correct positions in the middle of a well by themselves.

The whole procedure should be completed after about 2–2.5 hours. Please adjust the collagenase concentration if this is not the case.

1. Transfer the ovarian lobes into a new large petri or cell culture dish (for example 100 mm Falcon) filled with Barth's without Ca^{2+} .
2. Divide the tissue with a razor blade and a forceps into smaller parts (approximately 0.5 mm^3).
3. Put the clumps into 50 ml Falcon tubes with collagenase in Barth's without Ca^{2+} . A volume of up to 7.5 ml of tissue can be put into a single tube. For more tissue, use an additional tube. Otherwise, it would take too much time to separate the oocytes by collagenase digestion.
4. Put the tubes onto the mixer and let them shake gently for 120 minutes at room temperature. Check the progress after 90 min (then every 15 min) and shake the tube vigorously to accelerate the process.
5. If **all** oocytes are isolated and the first of them are already defolliculated, wash them extensively with Barth's solution (minimum of 5 times with 30 ml). If **not**, put them back onto the mixer for up to 30 min.
6. Then fill up the tube (approx. to 45 ml) and put it back onto the mixer for 10 minutes.
7. Change the solution to Barth's without Ca^{2+} and put it onto the mixer again for approx. 10 minutes.
All oocytes should be defolliculated now. Shake the tube vigorously to remove the follicle cells completely, if necessary.
8. Wash the oocytes with Barth's solution (2 x 30 ml).

4 Selecting Good Oocytes

Rough selection by filtration

1. Fill 50 ml of Barth's solution into a 100 ml beaker. Place the oocyte filter into the beaker. The filter should be immersed in the fluid.
2. Pipette an amount of oocytes onto the filter. Approximately half the filter should be covered with oocytes. Too many oocytes on the filter will lead to an inefficient filtration.
3. Gently move the filter about two centimeters up and down (in the fluid) to separate the oocytes by size.
4. Use the transfer pipette to place the residual oocytes into a 60 mm petri dish filled with Barth's + gentamicin.
5. The filtered oocytes are incubated at 19 °C for 1 h.

Note: The incubation step is necessary for identifying damaged oocytes in the next step.

Fine selection

→ Use a stereo microscope and the provided pipette to check each single oocyte for the criteria mentioned in the following.

Outer form:

- No visible damage of the cell
- Two-colored (dark and light brown), well separated colors
- No residues of follicular tissue

Size:

- About 1.2 mm

Note: Selecting oocytes is an important step. Perform it very carefully to obtain best results.

5 Plating Oocytes

You need a well plate filled with Barth's + gentamicin (see "Washing Oocytes").

1. Aspirate an amount of oocytes by the provided transfer pipette.
2. Drop one oocyte in each of the wells of the plate carefully. The oocytes should settle on the well bottom with the animal pole up.
3. Check the position of each oocyte to complete the preparation. Correct it carefully by using a pipette, if necessary. A manual correction should be necessary for less than five percent of the oocytes, if the follicle cells have been removed completely.
4. Seal the well plate with Parafilm and incubate it at 19 °C until use. Sealing with Parafilm is necessary to avoid evaporation of the liquid. The oocytes will have adhered to the well bottom after about 2 to 3 hours. Do not use or wash the cells before. Best results are obtained if oocytes have been incubated over night before use.

6 Washing Oocytes

Wash the oocytes approximately every second day for best performance. For more convenience, oocytes can be washed automatically by a cell washer.

Pre-fill the wells with about 200 μ l Barth's + gentamicin before plating the oocytes.

In the following, the parameters for use with the Tecan Microplate washer are provided. Refer to the Tecan user manual for more information.

Note: First, you have to define the plate-type specific parameters according to the plate type you use. Choose **Flat** as bottom form. Refer to the Tecan user manual to do so. Replace the parameters *Plate No.* and *Plate Name* in the programs below accordingly.

Hint: It is not necessary to wash oocytes directly before starting a recording, because the well content is exchanged by the perfusion anyway.

Program for filling the well plate

Use this program to fill the well plate with Barth's + gentamicin before plating the oocytes.

Program parameters:

Program Name: FILL

Program Locked: Yes

Manifold: 8

Plate: Nr: 1

Aspirate Rate: 1

Dispense Rate: 1

Crosswise: No

Mode: Strip Mode

Select Strips: Yes

Printout: Yes

Plate Name: 1

Bottom form: Flat

Final Aspirate: No

Roboocyte - Preparation of Xenopus Oocytes

Program-Steps:

- 1: Cycle Begin: 1
- 2: DISP Overflow CH:2 250µL
- 3: Cycle Repeat: 1
- 4: END

Program for washing oocytes

Use this program to wash the oocytes with the Tecan washer approximately each second day with Barth's + gentamicin.

Note: Do not wash the oocytes before they have attached to the well bottom, that is, after an incubation of at least two to three hours, recommended over night. Otherwise, they will be washed away and may block the manifold of the washer. **Few** oocytes that have not attached properly to the well bottom may be washed away during the procedure. This is okay, because these oocytes would not give good results.

Program Parameters:

Program Name: WASH

Program locked: Yes

Manifold: 8

Plate: Nr: 1

Crosswise: No

Mode: Strip Mode

Aspirate Rate: 1

Dispense Rate: Drip

Select Strips: Yes

Printout: Yes

Plate Name: 1

Bottom form: Flat

Final Aspirate: No

Program-Steps:

- 1: Cycle Begin: 1
- 2: ASP 2sec 8mm/s
- 3: WASH Bottom CH:2 400µL 8mm/s
- 4: WASH Bottom CH:2 400µL 8mm/s
- 5: WASH Overflow CH:2 200µL 8mm/s
- 6: Cycle Repeat: 1
- 7: END

7 Sources of Supply

We recommend the use of the products tested with the Roboocyte system. You can use any equivalent equipment as well.

Well plates

Product	Product Number	Description	Supplier
PS-Microplate, 96 Well V-Shape	651101 651161	Well plate, clear polystyrene, non-treated, conical	Greiner Bio-One GmbH www.greinerbioone.com
Nunc MicroWell™ Plates	249570 non-sterile 249662 sterile	96 MicroWell™ Plate, Polystyrene, clear, conical bottom, non-treated	Nunc www.nuncbrand.com
Well plate Greiner with cut open well	WPG	PS-Microplate, 96 Well V-Shape from Greiner, well H12 is cut open	Multi Channel Systems MCS GmbH www.multichannelsystems.com Please contact your local retailer.
Well plate Greiner with cut open well	WPN	96 MicroWell® Plate from Nunc, well H12 is cut open	
Adjustment device for Greiner plates	ADG	For adjusting the Roboocyte, for PS-Microplates from Greiner	
Adjustment device for Nunc plates	ADN	For adjusting the Roboocyte, for 96 MicroWell™ Plate from Nunc	

Roboocyte - Preparation of Xenopus Oocytes

Oocyte preparation

Product	Product Number	Description	Supplier
Oocyte filter		For selecting oocytes	Multi Channel Systems MCS GmbH www.multichannelsystems.com Please contact your local retailer.
Vari-Mix Aliquot Mixer, Type 48700		(for shaking the tubes during defolliculation)	Barnstead International www.barnsteadthermolyne.com Please contact your local retailer.
Olympus SZH Zoom Microscope		Magnification range 7.5x to 64x (or checking and selecting oocytes)	Olympus www.olympus.com
Tecan Columbus Microplate Washer	Tecan washer	Tecan 96-well plate washer, with drip mode option and 8 channel manifold (for automated oocyte washing)	Multi Channel Systems MCS GmbH www.multichannelsystems.com Please contact your local retailer.
BD Falcon™ Style Standard Dishes	353003	100 x 20 mm	BD Biosciences www.bdbiosciences.com
BD Falcon™ Conical Centrifuge Tubes	352098	50 ml, high clarity polypropylene	
Collagenase NB4	17454	From <i>Cl. histolyticum</i> , lyophilized (for defolliculation)	SERVA Electrophoresis GmbH www.serva.de
Gentamicin sulfate salt	G3632	Potency: approx. 600 µg gentamicin base per mg	Sigma www.sigmaaldrich.com