PRIMACYT
Cell Culture Technology

Thawing and Culturing of Cryopreserved Primary Hepatocytes in 2D and Suspension

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Version 12

Required and recommended media and consumables

- Thawing and Plating Kit consists of
  - HTM: Hepatocyte Thawing Medium
  - HWM: Hepatocyte Washing Medium
  - Customised Plating Medium tailored for the different species:
    - HM-cryo (Rat, Minipig)
    - L-15-cryo (Fish)
    - HPM-cryo (all other species)
- For the use of cryopreserved hepatocytes in suspension only HTM and HWM are required. Both components may be purchased separately as Thawing Kit (TK-1).
- Culture media (HHMM, 3D-HMM or MHM) are not included in this kit
- Collagen coated cell culture plates (not included in this kit)
  - Necessary for most species
  - Please see donor datasheet for details about recommended culture plates

Tab. 1: Details for thawing and culture of cryopreserved hepatocytes

<table>
<tr><th rowspan="2">Category</th><th rowspan="2">Species</th><th rowspan="2">Application</th><th rowspan="2">Centrifugation steps</th><th colspan="2">Recommended media for</th></tr>
<tr><th>Plating and Suspension</th><th>2D/3D culture</th></tr>
<tr><td rowspan="6">Mammals</td><td>Human</td><td rowspan="3">Suspension, 2D/3D culture</td><td rowspan="3">1. 100 x g at 20 °C for 10 min
2. 50 x g at 20 °C for 5 min</td><td rowspan="3">HPM Cryo</td><td rowspan="3">2D: HHMM
3D: 3D-HMM</td></tr>
<tr><td>Cyno-molgus</td></tr>
<tr><td>Beagle</td></tr>
<tr><td>Minipig</td><td>Suspension, 2D culture</td><td>1. 100 x g at 20 °C for 10 min
2. 50 x g at 20 °C for 5 min</td><td>HM Cryo</td><td rowspan="3">HHMM</td></tr>
<tr><td>Landrace Pig</td><td rowspan="3">Suspension, 2D culture</td><td rowspan="3">1. 100 x g at 20 °C for 10 min
2. 50 x g at 20 °C for 5 min</td><td rowspan="3">HPM Cryo</td></tr>
<tr><td>Horse</td></tr>
<tr><td>Rabbit</td></tr>
</table>

Category	Species	Application	Centrifugation steps	Recommended media for	
				Plating and Suspension	2D/3D culture
Mammals	Sheep	Suspension	1. 200 x g at 20 °C for 10 min 2. 100 x g at 20 °C for 5 min	HPM Cryo	-
	Mouse	Suspension, 2D culture	1. 50 x g at 20 °C for 10 min 2. 50 x g at 20 °C for 5 min		HHMM
	Rat		1. 100 x g at 20 °C for 10 min 2. 50 x g at 20 °C for 5 min	HM Cryo	HHMM

- For 3D cultures: please see our separate manual entitled "**3D-Spheroid Culture of Cryopreserved Primary Hepatocytes**".

Tab. 2: Details for thawing and culture of cryopreserved hepatocytes from birds and fishes

Category	Species	Application	Centrifugation steps	Recommended media for	
				Plating and Suspension	2D culture
Birds	Chicken	Suspension	1. 200 x g at 20 °C for 10 min 2. 100 x g at 20 °C for 5 min	HPM Cryo	HHMM
	Turkey	Suspension	1. 100 x g at 20 °C for 10 min 2. 50 x g at 20 °C for 5 min		-
	Duck	Suspension, 2D culture			HHMM
Fishes	Rainbow Trout	Suspension	1. 100 x g at 20 °C for 10 min 2. 50 x g at 20 °C for 5 min	L-15 Cryo	-
	Atlantic salmon				-
	Common Carp	Suspension, 2D culture			L-15 complete

1. Arrival of the cryopreserved cells in your laboratory

- Place the cryogenic vial with frozen hepatocytes immediately into the gas phase of a liquid nitrogen tank or in a freezer at a temperature below -130 °C.

2. Thawing and Plating of primary hepatocytes

1. Warm water bath, HTM, and HWM to 37 °C. For thawing of fish hepatocytes use both media at 10-20 °C
   - Aliquot HTM into 41 mL per tube (in case it is provided in bottles)
2. Set plating medium or your preferred medium for suspension assays to room temperature
3. Remove the vial with hepatocytes form liquid nitrogen storage/-150 °C and place it immediately into the 37 °C warm water bath until the cell suspension is thawed (approx. 1-2 min)
4. Spray 70 % ethanol on the cryogenic vial for disinfection
5. Transfer the cell suspension into the tube with HTM
6. Wash the cryogenic vial with 0.5-1 mL HWM to remove the cells completely and combine it with the cells in the tube
7. Add HWM to a final volume of 50 mL
8. Rotate the tube slowly two or three times
9. Pellet the hepatocytes by 1. centrifugation step (spin time and speed as stated in Tab. 1-2)
10. Remove the supernatant, gently loosen the cells without adding any medium by gently agitating the bottom of the tube. Do not vortex or shake the cells.
11. Wash the loosen cells with 20 mL HWM followed by 2. centrifugation step according to Tab. 1-2
12. Remove the supernatant, gently loosen the cells without any additional medium by gently agitating the bottom of the tube. Do not vortex or shake the cells
13. Re-suspend the pellet in required Plating Medium (see data sheet for post-thaw yield per vial)
14. Determine cell viability and live cell number with the trypan blue exclusion test in a counting chamber (Neubauer or equivalent counting chamber). Do not use automated cell counter
15. Adjust cell suspension to the desired density for plating with required amount of plating medium
    - The actual seeding density may vary from lot to lot. The recommended seeding densities to reach confluent or nearly confluent plates and the density for suspension assays for each lot are stated in the accompanying data sheet.
    - Table 3 gives an overview about the recommended media volumes for plating, washing and culture of hepatocytes in the different plate formats
    - Further studies with fish hepatocytes should be performed within the temperature range of 10-20 °C (optimum is approx. 15 °C).
16. Plate the cells or perform suspension assays

17. Let the cells attach for at least 6-7 h at 37 °C and 5 % CO_2, do not let the cells attach overnight. **Cave:** Let Common Carp Hepatocytes attach overnight at approx. 20 °C

Tab. 3: Recommended volumes for plating, washing and culture of primary hepatocytes

Volumes per well	**6well**	**12well**	**24well**	**96well**
Plating Medium	2 mL	1 mL	0.5 mL	100 µL
Washing Medium (e.g. PBS)	2 mL	1 mL	0.5 mL	50 µL
Culture Medium	1 mL	0.5 mL	0.3 mL	50 µL

3. Culture of primary hepatocytes

- After attachment of cells: change medium to remove debris and/or dead and non-attached cells.
- Heat culture medium and PBS (optional) to 37 °C (no longer than 15 min); for Common Carp keep medium at RT
- Optional: Wash the cells with warm PBS (1-2 times)
- Replace the plating medium with the necessary culture medium (HHMM, MHM or L15 complete)
- Change the medium daily (especially when hepatocytes plated at high cell density)
- Change the medium quickly, do not let the cells dry

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