KrosFlo®
Implant Membrane

Spectrum Product Instruction Manual

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I. Introduction

The KrosFlo® Implant Membranes, made from modified Polyvinylidene Difluoride (mPVDF), represent a revolutionary advance in separation technology. This unique pigmented hollow fiber with the ability to heat seal is ideal for controlled release, cell culture, encapsulation and implantation research. The membranes are hydrophobic and resistant to a wide variety of organic solvents as well as most aqueous acids and bases. They also can be heat sealed and autoclaved without affecting the pore size or changing the membrane performance. The Molecular Weight Cut Off (MWCO) rating is 500,000 Daltons.

The surface of the KrosFlo® Implant Membranes has been shown to be biocompatible in various animal models for periods exceeding 14 days[2,4]. Multiple cell lines have been grown within the KrosFlo® Implant Membranes both in vitro and in vivo[1,2,4]. Cell lines grown within KrosFlo® Implant Membranes, which have been implanted into a host animal, are not subject to immunological attack by the host animal. KrosFlo® Implant Membranes containing cancer cell lines have been implanted into mice and used to screen for compounds having anti-cancer activity[2,4]. Similarly, KrosFlo® Implant Membranes containing HIV infected cell lines have been implanted into mice and used to screen for compounds having anti-HIV activity[3,4].

II. Summary of KrosFlo® Implant Membrane Hollow Fiber Assay:

- Flush fibers with cold medium immediately prior to adding cells.
- Fill the fibers with the test cells, heat seal the ends and divide the fibers into 2-cm long samples.
- Implant the samples into mice (ip and sc).
- Administer test agent to the mice at the desired dose and schedule.
- Remove the samples from mice after 7 to 8 days.
- Assess cell viability (stable endpoint MTT assay).
- Serum samples and fibers may be assessed for p24 antigen concentration or reverse transcriptase activity.
- Compare the results in samples from diluent-treated mice to those obtained from test agent treated mice.

III. Methods and Procedures

1. KrosFlo® Implant Membrane Preparation Materials

- Bacteriological incinerator
- Sterile drapes
- Sterile syringes with 20 gauge needles
- Sterile stainless steel work surface
- Sterile gloves
- Sterile scissors and forceps
- KrosFlo® Implant Membrane
- Smooth-jawed needle holders
- RPMI 1640 with 20% FBS
- Autoclavable pipette pan with cover (Nalgene)

2. Procedures

A. Prewetted Membrane Procedure

Note: The KrosFlo® Implant Membranes are packaged into double bags. The inner bag has been autoclaved and sealed. Tear open the inner bag and drop plastic tubes containing membranes onto a sterile surface.

1. Using gloves, remove fiber tube-holders from the outer and inner pouch container and place tube holders on sterile work surface.
2. Using sterile gloves, remove the end cap from the tube holder and deposit the wet, autoclaved membrane onto a sterile surface.
3. Cut the fibers into 12-15 inch lengths with sterile scissors so they will fit into the pipette trays.

Caution: Do not allow the fibers to dry out

Caution: All work with the membranes must be conducted under sterile conditions using sterile technique.

4. Don sterile gloves and prepare a sterile work field in the BSC.
5. Deposit sterile fiber tube onto work surface.
6. Slide the hollow fiber over the PTFE catheter or needle on the syringe containing the cell suspension and fill with the cell suspension being careful to exclude air bubbles (refer to Figure 1).
7. Place the needle holder into the incinerator and heat for a few seconds (generally 3-5 seconds is adequate). Heat seal the loose end of the hollow fiber by briefly clamping the end with the needle holder. Heat seal the end of the fiber attached to the catheter and lay the fiber down.
8. Cover the fibers with cold medium to prevent them from drying out.

9. Heat seal each fiber at 2 cm intervals. Cut the individual samples apart in the center of the heat seal. The heat seals should be clear, not white, in appearance. The desired length of a heat seal is 3-4 mm so that when the samples are cut apart each sample has a 1.5-2 mm sealed “flap”. If necessary, the seal can be reheated until the desired effect is achieved. KrosFlo® Implant Membranes heat seals well when wet or when dry. For the purpose of cell samples it is important that the membrane not be allowed to dry.
10. Transfer to medium-filled dishes and incubate 1 to 2 days for cell growth assays or implant immediately for virus-inhibition assays (refer to Figure 2 & 3 for ip and sc implantation).

B. Dry Membrane Procedure

1. Wet the membrane in 100% ethyl alcohol for 30 minutes.
2. Rinse in water or buffer to remove alcohol.
3. Autoclave the membranes
4. Once the membranes are wetted do not allow them to dry out.
5. Continue with steps 3 - 10 from prewetted membrane preparation.

3. Stable Endpoint MTT Assay

Solutions and Reagents

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide)

Stock Solution

MTT	500 mg (Sigma M2128)
PBS	100 ml

Mix with vortex for 20 minutes, then sterile filter. Wrap bottle in aluminum foil or paper to block out light as MTT is light sensitive. Store at 4°C. Prepare fresh stock each month.

Working Solution

MTT stock solution	10 ml
RPMI 1640 + 20% FBS	40 ml

Prepare just prior to use and pre-heat to 37°C before use. To use add 1 ml of working solution to 2 ml of RPMI 1640/20% FBS containing the hollow fiber samples.2.5% Protamine sulfate (P/S)

Protamine sulfate	2.5 gm (Sigma P4380)
Normal saline (0.95)	100 ml

Add P/S slowly to saline while stirring vigorously. Stir for 20 minutes or longer if particles are still present. Sterile filter and store at room temperature. Prepare fresh stock monthly.

Procedure (Alley et al. 1991 Cancer Res 51: 1247-1256).

1. Transfer fibers for MTT assay into individual wells of 12 well plates containing 2 ml of RPMI 1640 with 15% FBS and 2mM L-glutamine, 50 µl/ml gentamycine and 50 U/ml penicillin.
2. Incubate plates for 30 minutes in a humid 5% CO2 atmosphere at 37°C.
3. Add 1 ml of MTT working solution (pre-warmed to 37°C) and incubate for four hours at 37°C in a 5% CO2 atmosphere.
4. Aspirate MTT solution and add 2.5% protamine sulfate (pms) to twice the MTT volume.
5. Store at 4°C for 24 hours.
6. Aspirate protamine sulfate and add 1ml of protamine sulfate for a second wash.
7. Store at 4°C for at least four hours. The samples can be stored for >2 weeks which allows an entire experiment to be batch tested.
8. Wipe each fiber with gauze to remove any stained debris from the exterior of the fiber, transfer to a 24-well plate and air dry for 24 hours.
9. Add 250 µl of DMSO (Burdick & Jackson) and incubate at room temperature for four hours protected from light (MTT is light sensitive).
10. Transfer 150 µl of each sample to 96-well plates and read the plates at 540-570 nm in a standard microliter plate reader (Figure 4).

4. Membrane Preparation for RT and p24 Ag Assays

1. Randomly separate the encapsulated membrane samples following collection into groups for MTT assay and RT and p24 antigen assay.
2. Place each fiber to be assayed for RT activity and/or p24 antigen into a 1.5 ml Eppendorf safe lock tube and store at-20°C until all fiber collection timeouts are complete so all the fibers for a single experiment can be batch tested.
3. To assay, thaw the Eppendorf tubes and add 250 µl of lysing buffer [0.02 M tris-HCI (pH 7.5), 0.05M NaCl, 0.5% Triton X-100] to each tube.
4. Cut the encapsulated membrane in the tubes using curved tip scissors.

5. Incubate at room temperature for 30 minutes.
6. Transfer 150 µl of the lysate into the well of a 96-well round-bottom microliter plate. This serves as the source of sample for the RT and p24 antigen assays.

RT Assay Procedure

Materials

- Tritiated thymidine triphosphate (specific activity: 2.5 mCi/ml in 1.5 M Tris buffer (NEN)
- rAdT stock [0.5 mg/ml poly rA (Pharmacia) and 1.6 units/ml oligo dT Pharmacia]
- 5X RT reaction mix (prepare fresh daily):

125 µl 1MEGTA	50 µl 1M Tris (pH 7.4)
125 µl dH20	50 µl 1M DTT
125 µl 20% Triton X-100	40 µl 1 M MgCl2

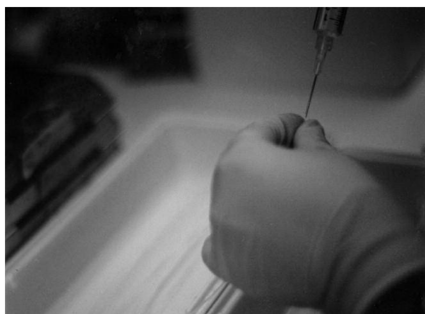
- Whatman DE 81 filter paper

Procedure

1. Prepare the complete RT reaction mixture by mixing 1 part tritiated thymidine, 2.5 parts rAdT, 2.5 parts 5X reaction mix and 4 parts water.
2. Place 10 µl complete RT reaction mixture into each well of a 96-well round-bottom microliter plate.
3. Add 15 µl of fiber lysate to the test wells and mix.
4. Incubate samples for 1 hour at 37°C.
5. Spot sample onto DE81 filter paper.
6. Wash filters 6 times (5 minutes each) in 5% sodium phosphate buffer.
7. Wash filters 2 times (1 minute each) in distilled water.
8. Wash filters 2 times (1 minute each) in 95% EtOH.
9. Place filters into plastic scintillation vials and dry.
10. Add 3 ml of Opti-Fluor O (Packard) or other scintillation fluid to each vial and quantitate the radioactivity in a liquid scintillation counter.

P24 Antigen Assay

The p24 antigen assay is conducted following the manufacturer's protocol. Such can be purchased from Coulter Corporation (Hialeah, F1).



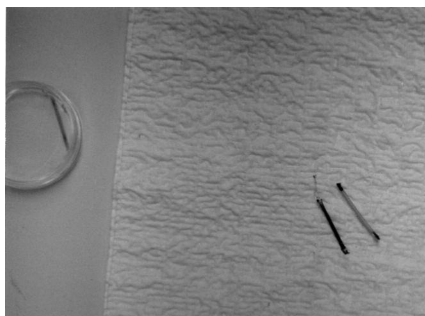
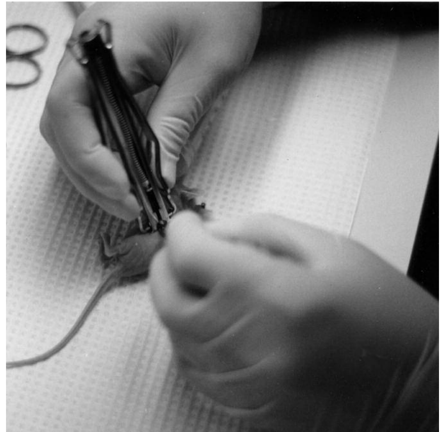
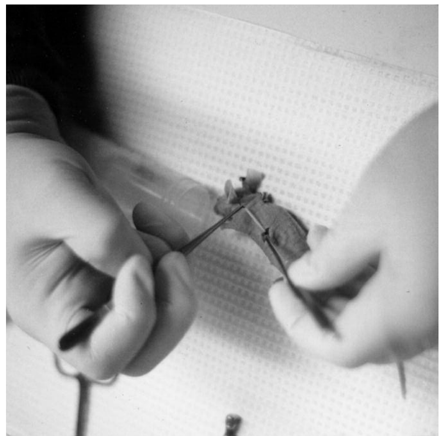


Figure 1: Filling of the equilibrated KrosFlo® Implant Membrane hollow fibers. A syringe, filled with the cell suspension, is attached to a hollow fiber by a needle, PTFE catheter or pipet tip. The fiber is flushed with the cell suspension. The fiber is sealed on each end with hot smooth-jawed needle holders and the individual samples are prepared by cutting at each heat seal. The prepared samples are transferred to culture dishes containing growth medium for incubation.

Figure 2: Intraperitoneal implantation in methoxyflurane anesthetized mice is accompanied via a dorsal incision. A 0.5 cm skin incision exposes the abdominal musculature which is incised 1/2 cm to allow passage of the hollow fiber into the abdomen. The hollow fibers are inserted and deposited along the lateral abdominal wall. The abdominal wall is closed using skin staples.





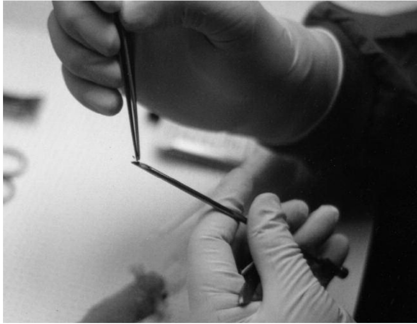
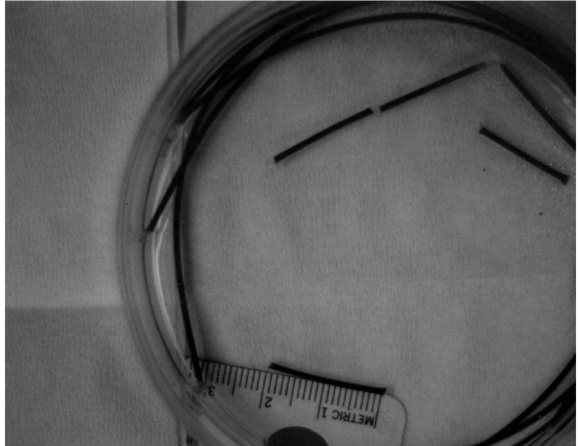
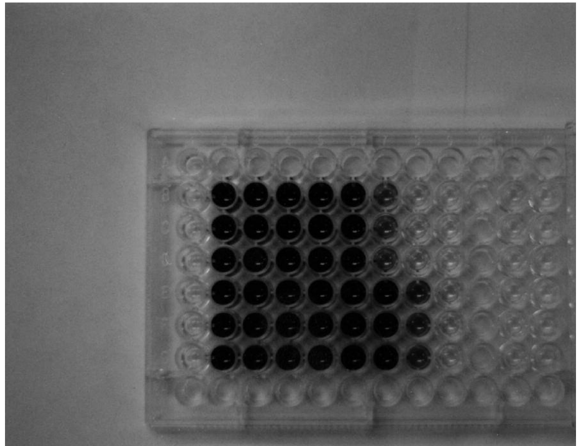


Figure 3: For subcutaneous implantation, the KrosFlo® Implant Membrane are loaded into the end of a trocar (11-16ga.). A craniodorsal skin incision (1/2 cm) allows entry of the trocar which is passed caudally through the subcutaneous tissues. The fibers are deposited during withdrawal of the trocar. The skin is closed with suture or a wound clip.

Figure 4: Samples (in vivo or in vitro) are assayed for viable cell mass by a stable-end-point MTT dye conversion assay. The cells are exposed to MTT for 4 hours without removal from the hollow fibers. After MTT exposure, the samples are incubated in two 2.5% protamine sulfate washes to stabilize the MTT formazan. The formazan is extracted from the hollow fibers with DMSO and 150 µl aliquots of the extract are transferred to 96-well trays for determination of the optical density at the 540 nm wavelength (OD540)





5. Tumor types and cell line applications in KrosFlo® Implant Membrane

Many different cancer cell lines, including some poorly tumorigenic lines, have been grown in KrosFlo® Implant Membrane hollow fibers:

Tumor Type	Cell Line	Tumor Type	Cell Line	Tumor Type	Cell Line
Lung	A549/ATCC	**Colon**	Colo-205	**Lymphoid**	CCRF-CEM
	NCI-H23		HCC-2998		CEM-S(HIV screen)
	NCI-H226		HCT-15		HL-60 (TB)
	NCI-H460		HCT-116		K-562
	NCI-H522		HT29		MOLT-4
			SW-620		RL
					U937 (HIV screen)
Prostate	DU-145	**Breast**	MCF-7	**Melanoma**	LOX IM VI
	JCA-1		MDA-MB-231		SK-MEL-5
	PC-3		MDA-MB-435		SK-MEL-28
	PC-3 (M)		MDA-N		UACC-62
					UACC-257
CNS	SF-295	**Ovarian**	IGROV1	**Renal**	A498
	SNB-75		OVCAR-3		CaKi-1
	U251		OVCAR-5		RXF-393
			SK-OV-3		SN12C

The Spectrum KrosFlo® Implant membrane in vivo model has shown positive effects, at both IP and SC sites, when using anticancer test compounds:

Compound (dose)	Cell Line	IP-% of control	SC-% of control
Mitomycin C	H23	2	25
2.5 mg/kg/dose	SN12C	55	52
Q2D X 3, IP	SK-MEL-28	61	81
Cyclophosphamide	H23	83	61
60 mg/kg/dose	SN12C	69	38
Q2D X 3, IP	SK-MEL-28	105	64
Doxorubicin HCL	H23	20	82
4 mg/kg/dose	SN12C	79	103
Q2D X 3, IP	SK-MEL-28	96	108
BCNU	H23	72	86
10 mg/kg/dose	SNIZC	59	52
Q20 X 3, IP	SK-MEL-28	96	66
Actinomycin D	LOX	1	80
0.2 mg/kg/dose	U251	5	93
Q2D X 3, IP	SW-620	20	127

IV. References

1) Casciari et al., Jnl. Nati. Cancer, Inst. 86, 1846-1852 (1994)

2) Hollingshead et al., Life Sci. 57, 131-141 (1995)

3) Hollingshead et al., Antiviral Research 28, 265-279 (1995)

4) Hollingshead, unpublished

V. Ordering Information

KrosFlo® Implant Membrane

Packaged wet in DI water and autoclaved or dry

Length: 34 cm

Quantity: Three fibers per package

ID: 1.0 mm, OD: 1.2 mm

MWCO	Product Numbers			
(Daltons)	white	blue	yellow	green
500,000, wet	M138615	M138616	M138617	M138618
500,000, dry	S9320101	S9320102	S9320104	S9320103

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