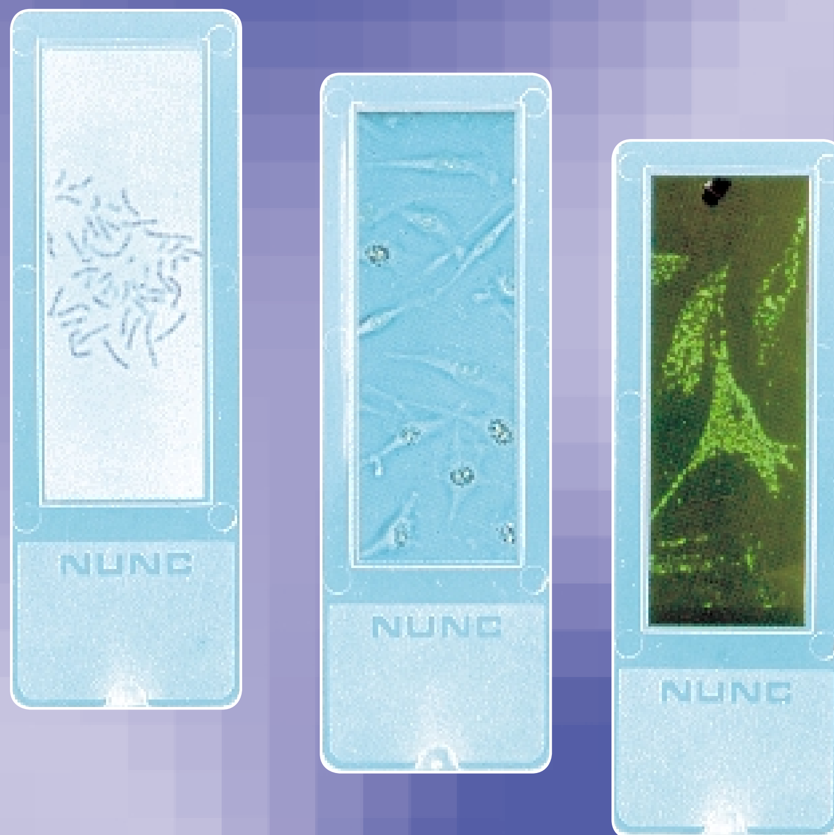
SlideFlask

Procedures



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Peter Esser, M.Sc., March 1999

NUNC SlideFlask Procedures

Introduction

The SlideFlask is for cell culture, cell preparation and subsequent close examination of single cells under high power microscopic magnification. In order to prevent individual cell images from obscuring each other, cells are not to be grown too densely before fixation.
This is important especially for chromosome preparation that includes hypotonic spreading of the chromosomes from mitotic cells.

In general it is recommended that cell density is kept below 25% of confluence (Fig.1).

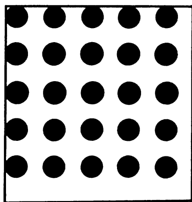

Fig 1. Dot density illustrating 25% of confluence.

Typical SlideFlask applications are:

- karyotyping
- single cell autoradiography
- single cell immunofluorescence

The procedures will be described in the following.

Karyotyping of Human Amniotic Fluid Cells

High quality preparations of bandstained chromosomes for reliable karyotyping require a delicate technique which must be adjusted for each individual cell type.

The Chromosome Laboratory of the State University Hospital in Copenhagen uses the following procedure for karyotyping human amniotic fluid cells.

Cell Culture (aseptic conditions)

1. 15 ml amniotic fluid is divided into two 7.5 ml portions that are processed in parallel (but separately). Processing is done at room temperature prior to incubation.

2. Each 7.5 ml portion is centrifuged at 800 rpm for 10 minutes.

3. The supernatants are removed by aspiration, leaving 0.5 ml in which the cells are gently resuspended.

4. 5 ml medium, RPMI 1640 + Ham F10 = 1+1, supplemented with 15% AmnioMAX C100, 2 mM L-glutamine and 50 IU/50 µg pen/strep per ml (all from Gibco), is mixed with each of the cell suspensions, which are then transferred to separate 25 cm^2 Nunc TC-flasks. Alternatively, one of the cell suspensions is mixed with 3 ml medium and transferred directly to a SlideFlask for a quick answer (cf. step 7).

5. The flasks are gassed with a mixture of 5.3% CO_2, 6.9% O_2 and 87.8% N_2 through a sterile filter for about 5 seconds, then closed and incubated at 37°C in 5% CO_2.

6. After 5-6 days' incubation, the medium is removed by aspiration and replaced by aliquots of fresh medium, prewarmed to 37°C. The flasks are gassed and reincubated as in step 5.

7. After another incubation period, when at least 5 substantial colonies have appeared, the SlideFlask (cf. step 4) is processed from step 11.

8. Whereas, from the 25 cm^2 flask the medium is removed by aspiration, and the cells are detached with freshly diluted Trypsin-EDTA (Gibco), 0.5 mg trypsin + 0.2 mg EDTA per ml HBSS, prewarmed to 37°C: 3 ml solution is added to rinse the cells and aspirated, then 2 ml is added for a quick washing and aspirated leaving a few drops, and after 3-5 minutes the cells are detached by bumping the flask against the flat of the hand.

9. 3 ml medium is added to the 25 cm^2 flask, and the cell suspension is transferred to 1-2 SlideFlasks, depending on the cell density. The medium is made up to a total of 3 ml per SlideFlask.

10. The SlideFlasks are gassed and incubated as in step 5 for 1-3 days until the appropriate cell density is obtained.

11. To each SlideFlask is added 10 µl colcemid, 10 µg/ml (Gibco), per ml medium, then the flasks are reincubated as in step 5 for 45 minutes. As colcemid is a metaphase-arresting drug, this treatment is applied to increase the number of cells in metaphase, the mitotic sub-phase presenting the most distinct chromosome appearance.

Hypotonic Treatment and Fixation (room temperature)

1. The colcemid medium is poured off, and the slides are broken off and immediately transferred to a slide preparation dish with 100 ml 60 mM KCl. This volume and the following liquid volumes may be adjusted according to the size of the applied preparation dish. The slides are left in KCl for 15 minutes.

2. 20 ml freshly made fixative (CH_3COOH+ C_2H_5OH = 1+3) is added to the edge of the dish and left for 10 minutes.

3. 30 ml liquid is removed by aspiration, and 30 ml fixative is added to the edge of the dish and left for 10 minutes.

4. 50 ml liquid is replaced by 50 ml fixative (as in step 3) and left for 10 minutes.

5. All the liquid is replaced by 100 ml fixative (as in step 3) and left for 10 minutes.

6. The fixative is replaced by another 100 ml fixative (as in step 3) and left for 30 minutes.

7. The slides are removed from the dish, dabbed on their edge against filter paper and left on filter paper to dry in 40-50% relative humidity for at least one day.

G-Band Staining

1. The slides are placed in freshly made 2 x SSC-buffer (0.3 M NaCl + 0.03 M sodium citrate dihydrate) prewarmed to 60°C in a slide preparation dish in a water bath - and left for 2 hours at 60°C.

2. The dish is transferred to a refrigerator and left for 30 minutes.

3. The buffer is replaced by water twice, leaving the slides in water. Further procedures are performed at room temperature.

4. The slides are transferred from the water to a preparation dish with freshly thawed 0.25% trypsin solution (0.25 g trypsin (Difco) per 100 ml freshly made PBS (see step 5), filter-sterilized and frozen) - and left for 90 seconds (variable).

5. The slides are carefully washed twice in freshly made PBS (one PBS buffer tablet (Oxoid) per 100 ml dist. H_2O)

6. The slides are transferred to a preparation dish with freshly made staining solution, i.e. 155 ml freshly made Gurr-buffer (one Gurr-buffer tablet (BHD) per 1000 ml dist. H_2O, pH 6.8) + 45 ml freshly filtered Leishmann stock staining solution (0.2 g Leishmann staining powder (BDH) per 100 ml dried methanol (Merck), stirred magnetically for about 2 days until the majority is dissolved). The slides are left in staining solution for 90 seconds.

7. The slides are washed twice in freshly made Gurr-buffer and once in dist. H_2O.

8. The slides are air-dried on filter paper and mounted with cover glasses (Cat. No. 171862) using copaiba balsam as mounting medium (see Appendix II).

Karyotyping

1. The slides are scanned for metaphase figures in a photomicroscope with a 16 x objective, and the chromosomes are closely examined using a 100 x objective and immersion oil.

2. Selected, complete sets of well separated chromosomes are photographed through the 100 x objective in green light on a fine-grained black and white film (Agfa Ortho).

3. Enlarged paper prints are made from the exposures, and the pictures of the individual chromosomes are cut out and arranged in numbered pairs according to authorized characterization rules based on size, location of centromere and number and location of bands (Fig. 2).

In some labs, including the State Hospital Chromosome Laboratory in Copenhagen, the microscopic image is now computer-processed, allowing the search for metaphase figures and the arrangement of chromosomes (Fig. 3) to be performed more or less automatically, thus circumventing the cumbersome procedure described above.

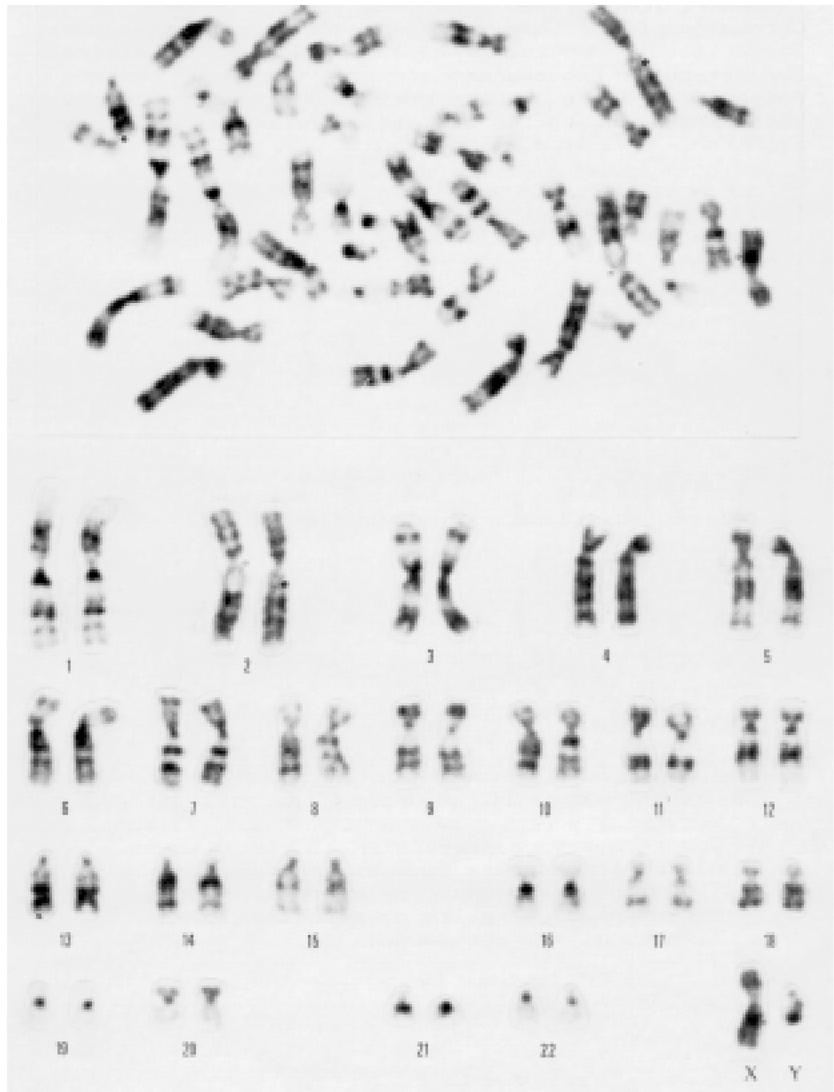

Fig. 2. Photograph of the chromosomes and manual arrangement of the individual chromosome pictures from a human amniotic fluid cell representing a normal male karyotype of 22 pairs of autosomes and an XY-pair of sex chromosomes.

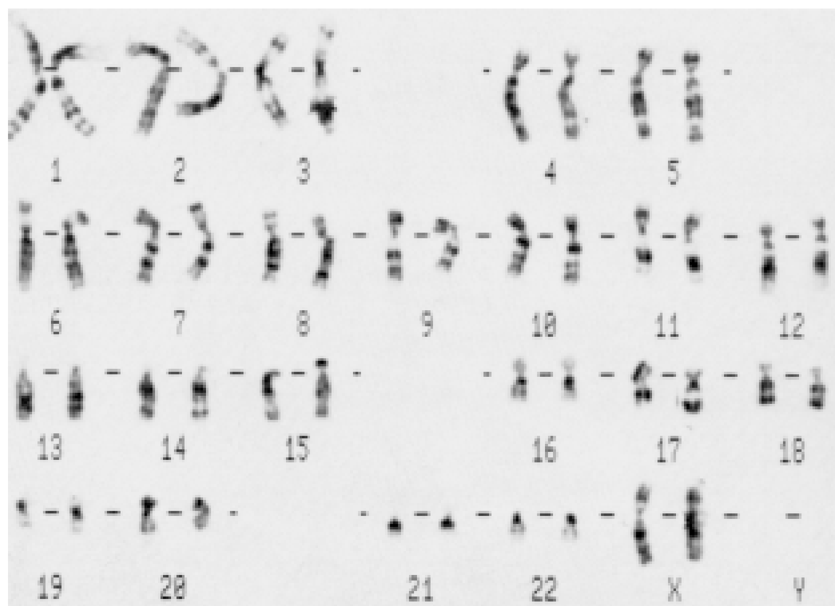

Fig. 3. Computer-screen projection and arrangement of the chromosomes from a human amniotic fluid cell, representing a normal female karyotype of 22 pairs of autosomes and an XX-pair of sex chromosomes.

Autoradiography of L-Cells Pulse-Labelled with Tritiated Thymidine

Incorporation in exponentially growing cells of a radiolabelled DNA-precursor (most often tritiated thymine deoxyribose, ^{3}H-TdR), followed by single cell autoradiography, is commonly used for cell cycle studies, e.g. drug interference with the normal cell cycle transit.

The cell cycle parameter most easily determined in this way is the labelling index (LI), i.e. the percentage of DNA-synthesizing cells in a population. The LI is obtained by "pulse-labelling" of the cells with ^{3}H-TdR (i.e. offering the cells ^{3}H-TdR for a short period compared to the cell cycle time) just before fixation. The percentage of labelled cells (nuclei) is equal to the percentage of cells in the DNA-synthesis phase, as only DNA-synthesizing cells incorporate thymidine.

Cell Culture, Pulse-Labelling and Fixation

1. Approximately 9 x 10^4 L-929 mouse fibroplasts (Gibco) in 3 ml Eagles E-MEM (Flow), supplemented with NEAA (Flow), 2 mM glutamine (Flow) and 10% FCS (TCS) are seeded in a SlideFlask (9 cm^2) thus presenting an initial density of approximately 10^4 cells/cm^2.

2. The SlideFlask is incubated with the cap loosened in a 5% CO^2 incubator for 2-3 days until an appropriate cell density is obtained, i.e. the cells should be amply preconfluent and in exponential growth.

3. 1 µCi ^{3}H-TdR (Amersham, 5 Ci/mM) per ml medium is added to the SlideFlask, which is then incubated as described above for 15 minutes. (Note: L-929 cells have a cycle transit time of about 20 hours).

4. The medium is poured off, and the cells are washed briefly in cold 0.5 M perchloric acid (Merck, 70%).

5. The SlideFlask is emptied, and the slide is broken off and fixed in absolute ethanol in a slide preparation dish at room temperature for 10 minutes.

6. The slide is placed on filter paper to air-dry.

Autoradiography and LI-Determination

1. The slide is dipped into Ilford K.2 Nuclear Research Gel Emulsion, air-dried in horizontal position and placed in absolute darkness in a refrigerator for one week's exposure.

2. The slide with emulsion exposed is processed according to the enclosed Ilford instructions.

3. The slide may be stained in undiluted, freshly filtered Giemsa solution (Merck) at room temperature for 1 minute, then washed 3 times in distilled H_2O and air-dried.

4. The LI is determined in a microscope with a 40 x objective (Fig. 4) by differential counting of labelled and unlabelled cells to a total of e.g. 1000 cells. The viewing field is displaced in a meandric pattern as shown in Fig. 5.

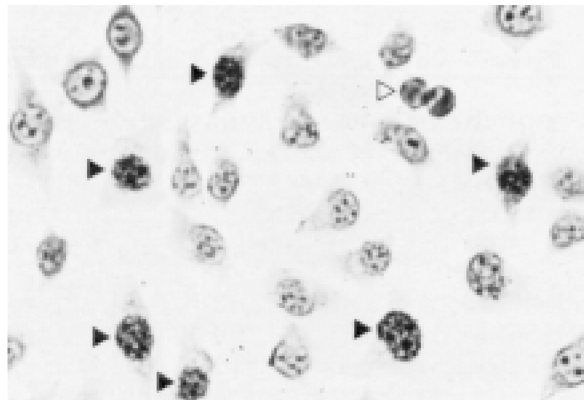

Fig. 4. Microphotograph of a Giemsa-stained autoradiogram of L-929 cells, pulse-labelled with tritiated thymidine. Labelled cells are marked with filled arrows. Note the late mitosis (open arrow) among the unlabelled cells. See text for details.

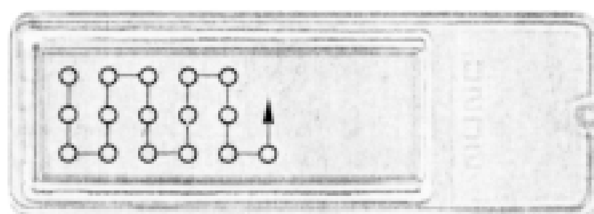

Fig. 5. Meandric displacement of viewing field.

Immunofluorescence Detection of Mycoplasma hyorhinis in Sheep Brain Endothelial Cell

Cell Culture & Fixation

1. Approximately 3 x 10^5 sheep brain endothelial cells derived from lamb choroid plexus are seeded in a SlideFlask in 3 ml medium 199 supplemented with 10% FCS, 2 mM glutamine and 25 mM HEPES buffer (Flow).

2. The SlideFlask is closed and incubated at 37°C until the appropriate cell density is obtained. The HEPES buffered medium makes it unnecessary to gas the flask with CO_2 or to incubate in CO_2 atmosphere.

3. The medium is poured off, and the slide is broken off, fixed in cold 20% acetone fixative (acetone + PBS = 1+4) in a refrigerator for 10 minutes and air-dried. As opposed to pure acetone, diluted acetone will not harm the polystyrene-based slide (cf. Appendix I). Acetone fixative is used on a routine basis for immunological mycoplasma detection because it is claimed to improve the access of immuno-reactants to the membrane-attached mycoplasma particles (Fig. 6).

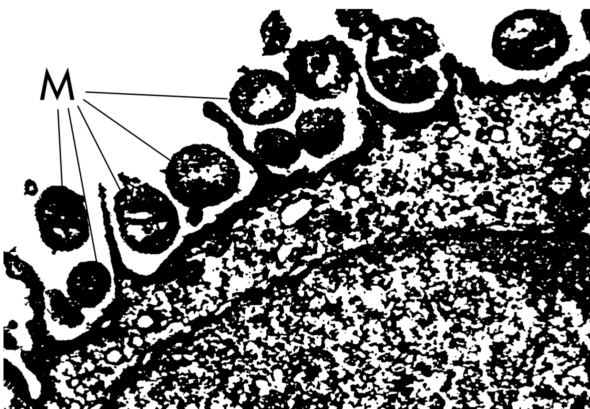

Fig. 6. Electron microphotograph of a mycoplasma infected HeLa cell. Note the multiple mycoplasma particles (M) attached to the cell membrane (from M.F. Barile).

Immunofluorescence Preparation and Detection (room temperature)

1. Two drops of rabbit antiserum to Mycoplasma hyorhinis, diluted 300 x in PBS, are spread on the culture area of the slide. The slide is then incubated in a moisture chamber for 30 minutes.

2. The slide is washed twice in PBS for 10 minutes and the PBS is drained off.

3. Two drops of FITC-conjugated swine anti-rabbit IgG (Dako), diluted 80 x in PBS, are spread on the culture area of the slide, which is then incubated in a moisture chamber for 30 minutes.

4. The slide is washed twice in PBS for 10 minutes and the PBS is drained off.

5. The slide is mounted with barbitone buffered glycerol (glycerol + barbitone buffer, pH 8.6, = 9+1) under a coverglass. For proper FITC fluorescence it is necessary that the preparation is buffered to a basic pH.

6. The slide is examined using blue illumination in a fluorescence microscope with a 40 x objective for possible green fluorescent cells indicating Mycoplasma hyorhinis infection.

Appendices

I. Fixation Conditions

Table 1. Various polystyrene-compatible and non-compatible fixation conditions when using the aggressive solvents acetone and chloroform. Nunc SlideFlask slides were immersed in the indicated fixation mixtures (by volume) at the indicated temperatures for 15 minutes, and then air-dried at RT. Only "unaffecting" conditions can be recommended. It should be noted that pure methanol, ethanol, acetic acid, formalin, and mixtures of these are all polystyrene-compatible as fixation agents.

Fixative			Temperature 4°C	
Acetone + PBS	1+4	—	0	0
	2+3	—	0	0
	3+2	—	0	0
	4+1	—	+	+
Acetone + methanol	1+4	—	0	0
	2+3	—	0	0
	3+2	—	0	+
	4+1	—	+	++
Acetone + ethanol	1+4	0	0	0
	2+3	0	0	+
	3+2	0	+	++
	4+1	++	++	++
Chloroform + methanol	1+4	0	0	—
	2+3	+	+	—
	3+2	++	++	—
	4+1	++	++	—
Chloroform + ethanol	1+4	0	0	—
	2+3	++	++	—
	3+2	++	++	—
	4+1	++	++	—

0 = unaffecting + = affecting ++ = destroying — = not tested

II. Mounting Media

Table 2. Polystyrene-compatible media for coverglass-mounting of Nunc SlideFlask slides. The media are selected for their polystyrene-compatibility only, their possible influence on the specimen preparation itself has not been taken into consideration.

Mounting medium	Hardening
Aquamount[a] Glycerol-gelatine[b]	Quick-hardening (hours)
Canada balsam in octanol[c] Copaiba balsam, nat.[d]	Slow-hardening (days)
Immersion oil, PCB-free Glycerol	Non-hardening

a) BDH Chemicals Ltd., Poole, England.

b) 50% (vol.) glycerol in 14.3% (weight) gelatine solution, heated to 60°C.

c) 70% (weight) Canada balsam in n-octanol.

d) Fa. Caesar & Loretz, Hilden, Germany.

III. Product List

Cat. No·	Description	Culture area/volume	Material	Quality	Units per sleeve/case
170920	SlideFlask	9 cm²/3 ml	N	SI	5/50
177453	Flaskette	10 cm²/3 ml	G	SI	8/96
163371	Flask with angled neck	25 cm²/7 ml	N	SI	20/160
136196	Flask with angled neck and filter cap	25 cm²/7 ml	N	SI	20/160
156340	EasyFlask angled with vent/close cap	25 cm²/7 ml	N	SI	10/200
156367	EasyFlask angled with filter cap	25 cm²/7 ml	N	SI	10/200

Cat. No.	Description	Dimensions	Material	Quality	Units per sleeve/case
171862	Cover Glass	18x50 mm	G	–	100/1000

KEY to SYMBOLS: N=Nunclon Delta polystyrene. G=Glass. SI=Sterilized by irradiation.

Other products

Lab-Tek™ CC - Chamber Slides - Sterilized

Cat. No.	Number of wells	Slide material	Suggested working volume	Culture area, well	Units per tray/pack/case
177372	1	Glass	4.0 - 5.0 ml	9.4 cm2	8/16/96
177410	1	Permanox™	4.0 - 5.0 ml	9.4 cm2	8/16/96
177380	2	Glass	2.0 - 2.5 ml	4.2 cm2	8/16/96
177429	2	Permanox™	2.0 - 2.5 ml	4.2 cm2	8/16/96
177399	4	Glass	0.7 - 1.0 ml	1.8 cm2	8/16/96
177437	4	Permanox™	0.7 - 1.0 ml	1.8 cm2	8/16/96
177402	8	Glass	0.3 - 0.45 ml	0.8 cm2	8/16/96
177445	8	Permanox™	0.3 - 0.45 ml	0.8 cm2	8/16/96
178599	16	Glass	0.3 ml	0.4 cm2	8/16/96

Lab-Tek™ Chambered Coverglass - Sterilized

Cat. No.	Number of wells	Slide material	Suggested working volume	Culture area, well	Units per tray/pack/case
136307	1	Glass	4.5 ml	9.4 cm2	8/16/96
178565	2	Glass	2.3 ml	4.2 cm2	8/16/96
136420	4	Glass	0.8 ml	1.8 cm2	8/16/96
136439	8	Glass	0.4 ml	0.8 cm2	8/16/96

Lab-Tek™ II - Chamber Slides - Sterilized

Cat. No.	Number of wells	Slide material	Suggested working volume	Culture area, well	Units per tray/pack/case
154453	1	Glass	4.0 - 5.0 ml	8.6 cm2	8/16/96
154461	2	Glass	2.0 - 2.5 ml	4.0 cm2	8/16/96
154526	4	Glass	1.0 - 1.3 ml	1.7 cm2	8/16/96
154534	8	Glass	0.5 - 0.6 ml	0.7 cm2	8/16/96

Lab-Tek™ II Chambered Coverglas - Sterilized

Cat. No.	Number of wells	Slide material	Suggested working volume	Culture area, well	Units per tray/pack/case
155360	1	Polystyrene	4.0 - 5.0 ml	8.6 cm2	8/16/96
155379	2	Polystyrene	2.0 - 2.5 ml	4.0 cm2	8/16/96
155382	4	Polystyrene	1.0 - 1.3 ml	1.7 cm2	8/16/96
154409	8	Polystyrene	0.5 - 0.6 ml	0.7 cm2	8/16/96

Lab-Tek™ II CC² - Chamber Slides - Sterilized

Cat. No.	Number of wells	Slide material	Suggested working volume	Culture area, well	Units per tray/pack/case
154739	1	Glass	4.0 - 5.0 ml	8.6 cm2	8/16/96
154852	2	Glass	2.0 - 2.5 ml	4.0 cm2	8/16/96
154917	4	Glass	1.0 - 1.3 ml	1.7 cm2	8/16/96
154941	8	Glass	0.5 - 0.6 ml	0.7 cm2	8/16/96

SonicSeal Slide Wells - Removable chamber - Sterilized, flat bottom

Cat. No.	Material	Well size m	Working volume	Culture area, cm2	Units tray/pk./case
138121	Permanox™	15 mm dia., 17mm hgt.	1.0 - 2.0 ml	1.54	8/16/96

For futher information see the Nunc Catalogue page 26-28

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