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Radically Changing the Use of Stem Cells

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Best Practices in hMSC Culture

Maintaining consistent seeding density, recording harvest density, and keeping track of population doubling level (PDL)

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Learning Objectives

1. How cellular lifespan is measured in population doublings, and why passage number is a misleading measure of age
2. How to calculate the Population Doubling Level of a cell culture
3. The impact of seeding density and passage number on PDL of cell cultures

MSCs are Primary Cells and have a finite lifespan

- Cellular lifespan is measured by the number of times the cell population has doubled since isolation.
- Population Doubling Level, or “PDL” is the standardized way to communicate the age of a primary cell
- Since hMSCs are a rare population in bone marrow, by convention, PDL counting starts at the first passage *in vitro*.
- PDL is not designed to take into account the number of times these cells have divided *In Vivo*, and this is where donor age and health comes into play as an important variable to monitor.
- Senescence is when cell populations stop dividing, and is considered the “end” of a cell’s lifespan
- Regulatory agencies highly advise that PDL be closely monitored and documented for therapeutic cells, and PDL is thus becoming the standard in industry.

Regulatory guidelines recommend the use of PDL to estimate in vitro lifespan

"For diploid cell lines possessing finite in vitro lifespan, accurate estimation of the number of population doublings during all stages of research, development, and manufacturing is important."

– from the ICH Q5D Guidance

INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

DERIVATION AND CHARACTERISATION OF CELL SUBSTRATES USED FOR PRODUCTION OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Q5D

Current *Step 4* version

dated 16 July 1997

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

- Still, many will use Passage Number when reporting the age of their cell cultures used in experiments.

Passage Number is not a standardized means of reporting cell age

Many research publications use the less precise “passage number” to communicate cellular age, which can be misleading.

Why?

Seeding passaged cells at varying densities can dramatically impact population doublings per passage, and can vary from experiment-to-experiment and lab-to-lab.

Thus, not all Passage 5 cells are equal!

Let’s go through an example of how varying seeding density greatly impacts PDL per passage

Here is an equation from which you can calculate PDL of your cells

$$PDL = X + 3.322\ (\log Y - \log I)$$

Where:

X = initial population doubling level

I = Initial cell number seeded into your vessel

Y = final cell Yield, or the number of cells at the end of the growth period

A simple example:

•You seed 5,000 cells at a PDL of 2 into a vessel, and assume it doubles twice.

•5,000 X 2 **(first doubling)** = 10,000 cells; 10,000 cells X 2 **(second doubling)** = 20,000 cells

Current PDL = 2 + 3.322 (log20,000 – log5000) = 2 + 2 = 4 total PDL

Hopefully it makes sense that 5,000 doubling twice to 20,000

Thus, if you seed at 2,500 (half of 5,000), then you would get 3 doublings to make 20,000

http://www.wikihow.com/Calculate-Doubling-Time
http://www.atcc.org/Global/FAQs/B/C/Passage%20number%20vs%20population%20doubling%20level%20PDL-175.aspx

Assumptions and explanations for chart on next page

Assume a constant harvest density of ~20,000 cells/cm^2

Thus, if you seed at 5,000, a harvest of 20,000 = 2 PDL per passage

Seeding at 2500 (half of 5,000) with a harvest of 20,000 = 3 PDL per passage

Seeding at 1250 (half of 2500) with a harvest of 20,000 = 4 PDL per passage

Etc…..

On the next page, you will see this sequence continued down to a seeding density of 78 cells/cm^2

The lower the seeding density, the more population doublings per passage

Seeding Density	Harvest Density	PDL per passage	Starting Cell #	cm2 to seed	#T225s	Cells/Harvest	Passages till PDL 16
5000	20,000	2	1.00E+06	200	1	4.0E+06	8.0
2500	20,000	3	1.00E+06	400	2	8.0E+06	5.3
1250	20,000	4	1.00E+06	800	4	1.6E+07	4.0
625	20,000	5	1.00E+06	1,600	7	3.2E+07	3.2
313	20,000	6	1.00E+06	3,200	14	6.4E+07	2.7
156	20,000	7	1.00E+06	6,400	28	1.3E+08	2.3
78	20,000	8	1.00E+06	12,800	57	2.6E+08	2.0

Taking 3 examples from the above chart highlighted in yellow, you can see:

- Seeding cells at 5,000 per cm^2 achieves only 2 PDL per passage.
- Seeding at 1250 per cm^2 achieves 4 PDL per passage
- Seeding at 78 per cm^2 (similar to Prockop protocol) achieves 8 (!) PDL per passage

It takes only 2 passages at the lowest seeding density to get to a cellular age of 16 PDL, where it takes 8 passages at the highest density.

THUS: reporting passage level without seed and harvest densities is not a sufficient or accurate method of reporting cellular age.

How much can cumulative population doublings of hMSCs vary depending on seeding density?

Quite a bit!

Passage Number	Cumulative Population Doubling Level at varying seeding densities with harvest density of 20,000 cells/cm2		
	78 cells/cm2	1250 cells/cm2	5000 cells/cm2
0	0	0	0
1	8	4	2
2	16	8	4
3	24	12	6
4	32	16	8
5	40	20	10
6	48	24	12
7	56	28	14
8	64	32	16
9	72	36	18

Take Home Messages

1. Always keep track of total viable cells seeded and harvested, and keep a running population doubling level for your cell cultures.

2. Population doubling level is a more precise method than Passage Level to report when publishing.
   - Eg. “All experiments were performed with hMSCs of PDL 12-16” is more precise than “All experiments were performed with hMSCs of passages number 4 to 6.”

3. Low seeding densities can yield many more population doublings per passage than higher seeding densities.

4. Cell experiments will be more reproducible if you perform them at consistent PDL within each donor.

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