



# The Clonogenic Assay

*A guide to understanding and performing a clonogenic assay*

*Dr. Jenna Bleloch*





## The Clonogenic Assay

Written by: Dr. Jenna Bleloch

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CytoSMART.com

CytoSMART Technologies B.V.  
Emmasingel 33  
5611 AZ Eindhoven  
Netherlands

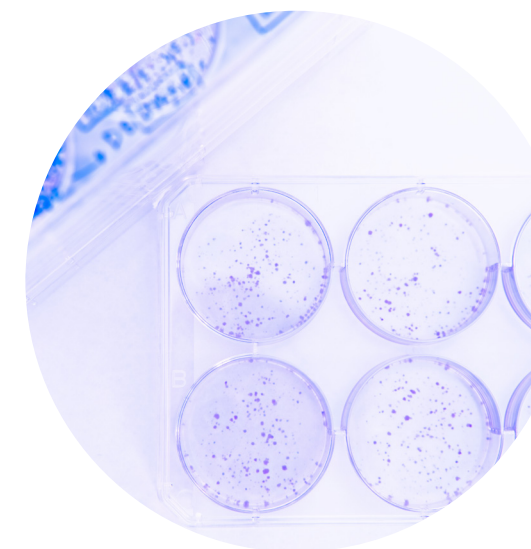
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eBook Layout by: Cescilia Araujo

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# Introduction

The clonogenic assay, also known as the colony formation assay, is a widely used *in vitro* cell survival assay that assesses the ability of single cells to survive and reproduce to form colonies<sup>1</sup>

## Defintions

**Clone:** n. A group of cells or organisms that are descended from and genetically identical to a single progenitor.

**Clonogenic:** adj. Giving rise to a clone of cells.

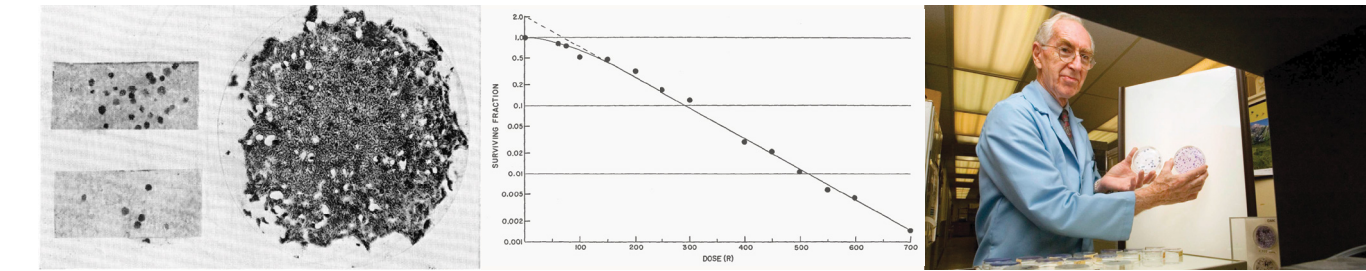
**Clonogenicity:** n. The ability of a cell to clone itself and grow into a full colony of cloned cells.

**Assay:** n. An investigative procedure in laboratory medicine for qualitatively assessing or quantitatively measuring the presence, amount, or functional activity of a target entity.

**In vitro:** adj. (Of a process) performed or taking place in a test tube, culture dish, or elsewhere outside a living organism.

The clonogenic assay was first described in the 1950s by Philip Marcus and Theodore Puck from the University of Colorado Medical Centre, Denver where it was used to study the effects of radiation on cancer cell survival and growth and has subsequently played an essential role in radiobiology ever since.<sup>2,3</sup>

Today the clonogenic assay is widely used in the field of cancer research as the formation of clones is interpreted as a trait of cancer cells with tumour initiating capabilities. It has become a standard tool to evaluate cellular reproductivity in response to cytotoxic or genotoxic effects of various therapies with potential clinical application.<sup>4,5</sup> Clonogenic growth is also used for evaluating the stemness as stem cells are long-living cells with the potential for ongoing proliferation and is thus a technique often used to evaluate and characterise haematopoietic stem cells.<sup>6,7</sup>



HeLa cell colonies produced from the first successful clonogenic assay performed by Marcus and Puck in 1955.

The first dose-response survival curve of irradiated HeLa cells performed by Marcus and Puck in 1956.

Marcus holding up stained cell colonies from a clonogenic assay.

# Types of *in vitro* colony forming assays

It is important to know the different types of *in vitro* colony forming assays that are available and their respective applications.<sup>6-8</sup>

Assay	Clonogenic Assay	Soft agar colony formation assay	Colony-forming cell (CFC) assay
Cells	Normal and cancer cell lines	Cancer cell lines	Haematopoietic stem cells
Measurements	2D adherent colony growth.	3D anchorage-independent colony growth in soft agar.	Proliferation and differentiation of 2D (small colonies) and 3D (large colonies) in a semi-solid media in response to cytokine stimulation.
Description	The original clonogenic assay where cells adhere to the surface of the culture dish/well and demonstrate anchorage dependent monolayer colony growth. Normal and cancer cells can demonstrate this. <sup>6</sup>	This evolved from the traditional clonogenic assay. Cells are grow in a layer of soft agar mixed with medium. This is the ultimate indicator of tumorigenesis as only cancerous cells display anchorage-independent colony growth. <sup>7</sup>	The CFC assay is used in the study of hematopoietic stem cells. It is based on the ability of hematopoietic stem cells to proliferate and differentiate into haematopoietic progenitor cells that can be morphologically characterised. <sup>8</sup>

The focus of this eBook is on the 2D adherent-cell clonogenic assay.

# The clonogenic assay today

The clonogenic assay is an essential tool in radiobiology since it was first described in the 1950s and has also become a powerful assay in the greater field of cancer biology.<sup>9</sup> The assay requires cells to be seeded at very low densities (before or after treatment) and left for a period of 1-3 weeks for colonies to form from the individual single cells. Colonies are then fixed, stained with crystal violet to make them visible, counted and analysed<sup>1,3</sup>. The assay measures the ability of cells to retain their reproductive integrity over a prolonged period of time. This is an important feature as it reveals phenotypic effects that require time and possibly several cell divisions to develop. When analysing the efficacy of potential therapeutics for clinical application this is particularly important as therapeutic resistance cannot be identified using short-term cytotoxicity assays such as the colorimetric based MTT, MTS and XTT cytotoxicity assays that use metabolic activity as a biochemical marker for cell viability.<sup>10-12</sup>

Today the clonogenic survival assay is considered as the “gold standard” in measuring cellular reproductivity as it measures the sum of all modes of cell death, encompassing both early and late events. Technological advancements in computing power and image analysis can now remove counting bias and human error; count colonies in a fraction of the time; remove the need to even stain colonies; and offer more high throughput experimental setups. See the section on the Evolution of Colony Analyses for more on this.

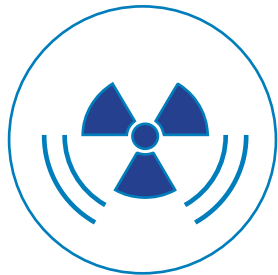
## Clonogenic assay applications

The clonogenic assay is used to assess the sensitivity of cancer and normal cells to radiotherapy, chemotherapy, and targeted therapy alone or in combination to provide an indication of the clinical efficacy of a treatment regimen. Drug combinations and multimodal therapy can offer additive and synergistic effects providing more efficacious treatment options<sup>13</sup>.



## Radiotherapy

The clonogenic assay is an essential tool in radiobiology. Radiation survival curves are used to identify appropriate radiation dosages and identify radioresistance. The assays are often used to identify compounds that induce radiosensitivity for more effective treatment.<sup>5</sup>



## Chemotherapeutics

Chemotherapy is a standardised form of anti-cancer treatment, however cells vary greatly in their susceptibility to treatment. Clonogenic assays are used to identify lethal doses, assess the efficacy of various combinations of chemotherapeutics and identify novel lead compounds with clinical application.<sup>12</sup>



## Targeted Therapies

The field of targeted therapies is growing rapidly as our biological understanding of cancer deepens. Clonogenic assays are a useful tool to assess the effect of these therapies on long-term cellular reproductivity. Synergistic effects are often observed when combined with other modes of therapy.<sup>13</sup>



## Genetic Modification

Clonogenic assays can be used to assess the effect of gene knock-out/down or overexpression on cellular reproductivity. This can provide functional insight and identify novel therapeutic targets that can be used on their own or in combination with other treatments.<sup>6</sup>



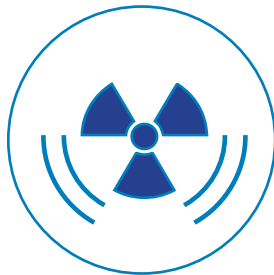
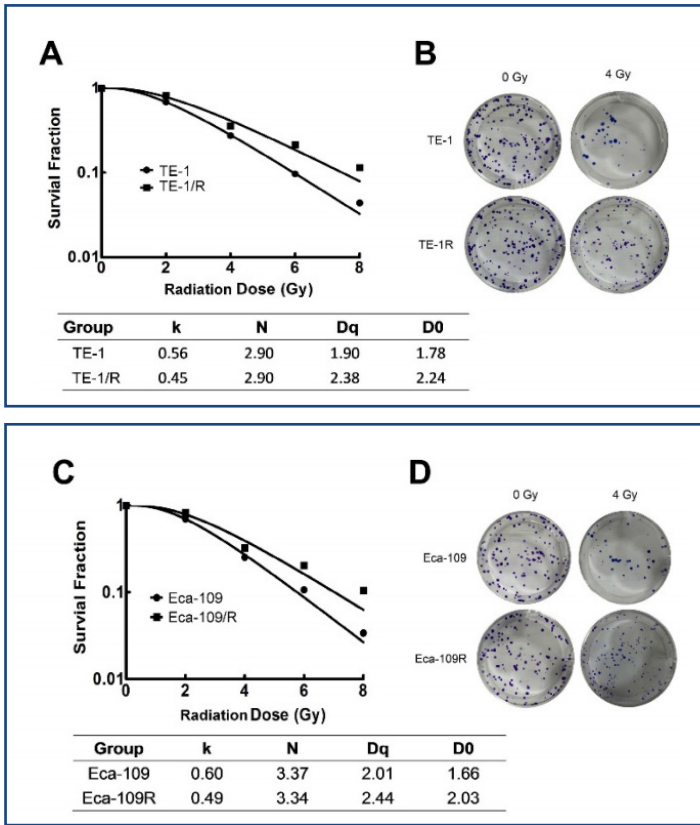


# Clonogenic assay examples

## Testing the radiosensitivity of cancer cells

**Case Study:**  
Luo et al. (2017) perform clonogenic assays to investigate radioresistance in oesophageal cancer cell line variants (TE-1/R and Eca-109/R) compared to parent cells (TE-1 and Eca-109) after 0, 2, 4, 6, 8 Gy irradiation. Survival curves and images of colonies clearly show a level of radioresistance. This triggers further investigation into the mechanism of action responsible for this.

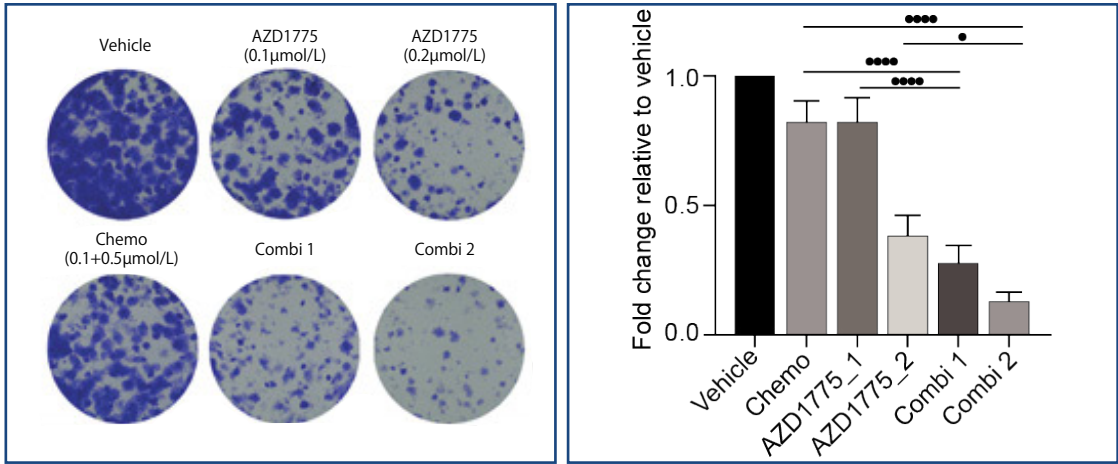
**Methodology:**  
Cells were seeded into six-well plates at 200-6000 cells/well, depending on the dose of radiation, 24 hours before irradiating with doses of a single fraction of 0, 2, 4, 6 or 8 Gy X-ray irradiation. After 10–14 days, colonies were stained with crystal violet, colonies counted and the survival fraction calculated.



## Supplementing standard chemotherapy with a synergistic targeted therapy

**Case Study:**  
Xu et al. (2019) identify WEE1 in a CRISPR screening as a targeted therapy that is effective in combination with standard chemotherapy in malignant pleural mesothelioma. WEE1 inhibitor AZD1775 in combination with standard chemotherapy shows a very impressive synergistic effect in the aggressive chemoresistant malignant pleural mesothelioma cells.

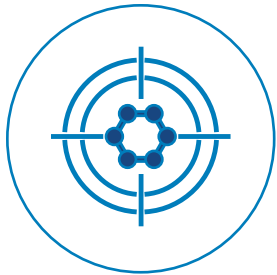
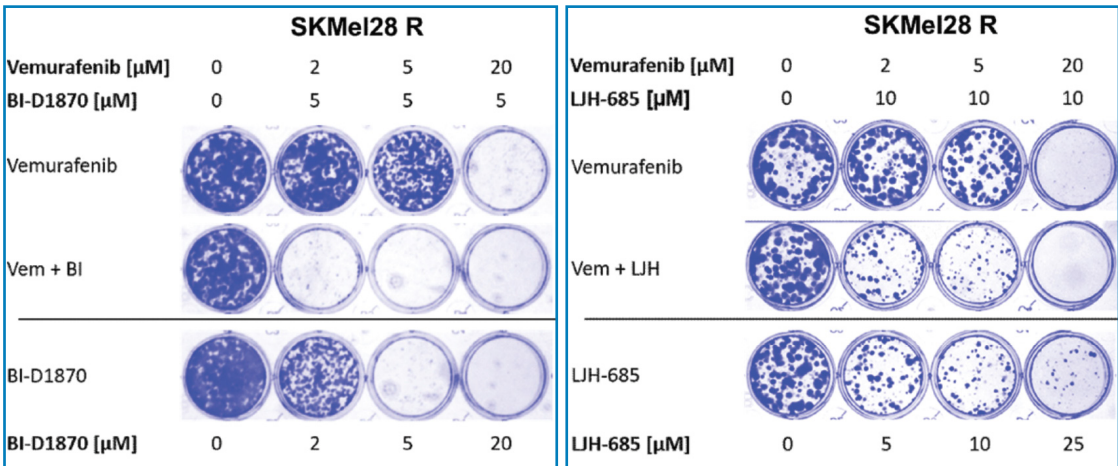
**Methodology:**  
Cells were seeded in 6-well plates (1000–2000 cells/well) and treated with drugs for 3 days and cultured without drugs for 10 to 14 days depending on growth rate. Colonies were stained with crystal violet which was then eluted and absorbance readings were taken.



## Investigating the effect of alternative targeted therapies alone or in combination with current therapies in drug resistant cancer cells.

**Case Study:**  
Kosnopfel et al. (2017) show that BRAF inhibitor (vemurafenib) resistant melanoma cells (SKMel28 R) are sensitive to treatment with RSK inhibitors, BI-D1870 and LJH-685. Interestingly, the RSK inhibitors are not only effective on their own, but when treated in combination with vemurafenib there is an impressive synergistic effect.

**Methodology:**  
200 cells/well were seeded in 12-well plates and then treated with signalling pathway inhibitors. Colonies were left to form for 7–10 days and then stained with Coomassie Brilliant Blue solution.



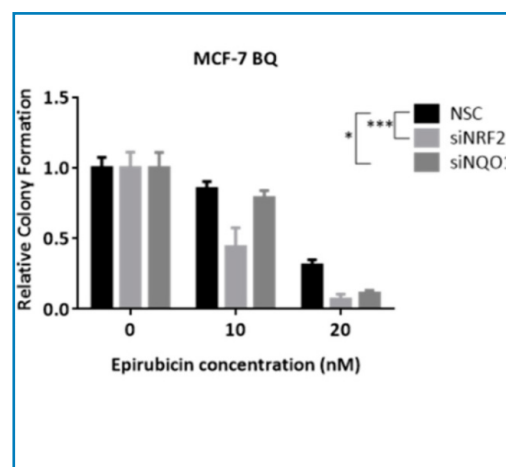
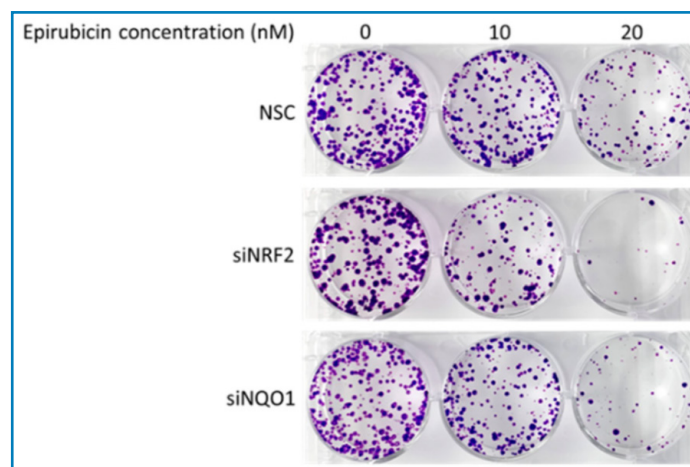
## Investigating the effect of alternative targeted therapies alone or in combination with current therapies in drug resistant cancer cells.

### Case Study:

Leung et al. (2020) show that the increased NRF2 and NQO1 transcriptional activity observed in Epirubicin resistant (EpiR) breast cancer cells (MCF-7) plays a role in their chemoresistance. siRNA knockdown of NRF2 or NQO1 re-sensitised the resistant breast cancer cells to Epirubicin treatment.

### Methodology:

Cells were transfected with an siRNA to NRF2 or NQO1 for 24 hours with non-targeting siRNA (NSC) used as control. 1000 cells per well were then seeded in 6-well plates and after attachment cells were treated with increasing concentrations of Epirubicin for 14 days. Colonies were stained with crystal violet which was then dissolved and absorbance was measured to quantify relative colony formation.



## Performing a clonogenic assay

### Clonogenic assay methods

There are two different ways to perform a clonogenic assay. The first method involves seeding cells at low densities and then treating them for a period of time (hours, days or for the full duration of the assay). The second method involves pre-treating cells at a higher confluency (60-80%) and then re-seeding them at low densities in treatment-free medium.

There is no rule for which method to use and there is also very little data on the experimental outcome differences between these two methods. A recent study showed that pre- and post-irradiation clonogenic assays yielded negligible treatment sensitivity differences.<sup>13</sup> In general, the first method is often used for assessing sensitivity and efficacy of a treatment on clonogenicity, while the second method is more commonly used to investigate cell damage repair and treatment resistance. Depending on your research question and treatment type, concentration and duration, the method to use may be obvious or experimentally favourable. Checking the literature for clonogenic assays performed with similar experimental elements is a good starting point. It is also important to keep in mind that if you want to compare your results to similar studies in the literature or to your colleagues it is important for the methods to be the same. If you are struggling to get the one method to work, you can always try the other.<sup>1</sup>

### Choosing the right cell culture plate

Clonogenic assays are typically performed in 6-well plates (12-well and 24-well plates are also commonly used) as the bigger well size is easier for staining colonies and removing excess stain. It is easier to achieve sparseley and evenly plated cells on a larger surface area. Clonogenic assays are traditionally not high throughput so there hasn't been an urgent need to miniturize the assay. When deciding on the plate format to use, it is useful to know your experimental design.<sup>1,4</sup>

For example, if I am testing up to four concentrations of a drug with two controls, a 6-well plate is perfect (see the clonogenic assay overview schematic on the next page). One would ideally perform the experiment in triplicate so you would have three 6-well plates. If you are testing more concentrations and drug combinations with more control samples using a 12-well or 24-well plate would be more favourable. With the development of automated colony counting software and stain-free clonogenic assay analysis, having a more complex experimental setup with multiple treatment combinations, concentrations, and even cell lines is more accesible.



## Seeding cells sparsely

For isolated colonies to form, cells need to be seeded sparsely and evenly. Therefore, it is important to optimise your seeding density for your chosen cell line and well size before performing your clonogenic assay. A good starting point is to look in the literature to see if any studies have performed clonogenic assays with your cell line and then test this seeding density and one or two higher and lower densities.<sup>1,4,5</sup>

During this seeding optimisation process, it is also important to consider your experimental end-point (e.g. 7 days, 14 days or 21 days) and cell growth rate as you don't want merging colonies, which will interfere with colony quantification and analysis.

## Cell treatment

Treatment can refer to radiation, drugs including chemotherapeutics and/or targeted therapies, genetic modifications, or a combination of any of the above. Optimising treatment conditions is a critical preparation step for performing clonogenic assays. Whether pre-treating and then seeding at low densities or seeding at low densities and then treating it is important to optimise your conditions and these will differ depending on which approach you go with. Any available data in the literature on your cell line, treatment methods and strategies and drug concentrations will be helpful in making informed experimental decisions.

## Experimental controls

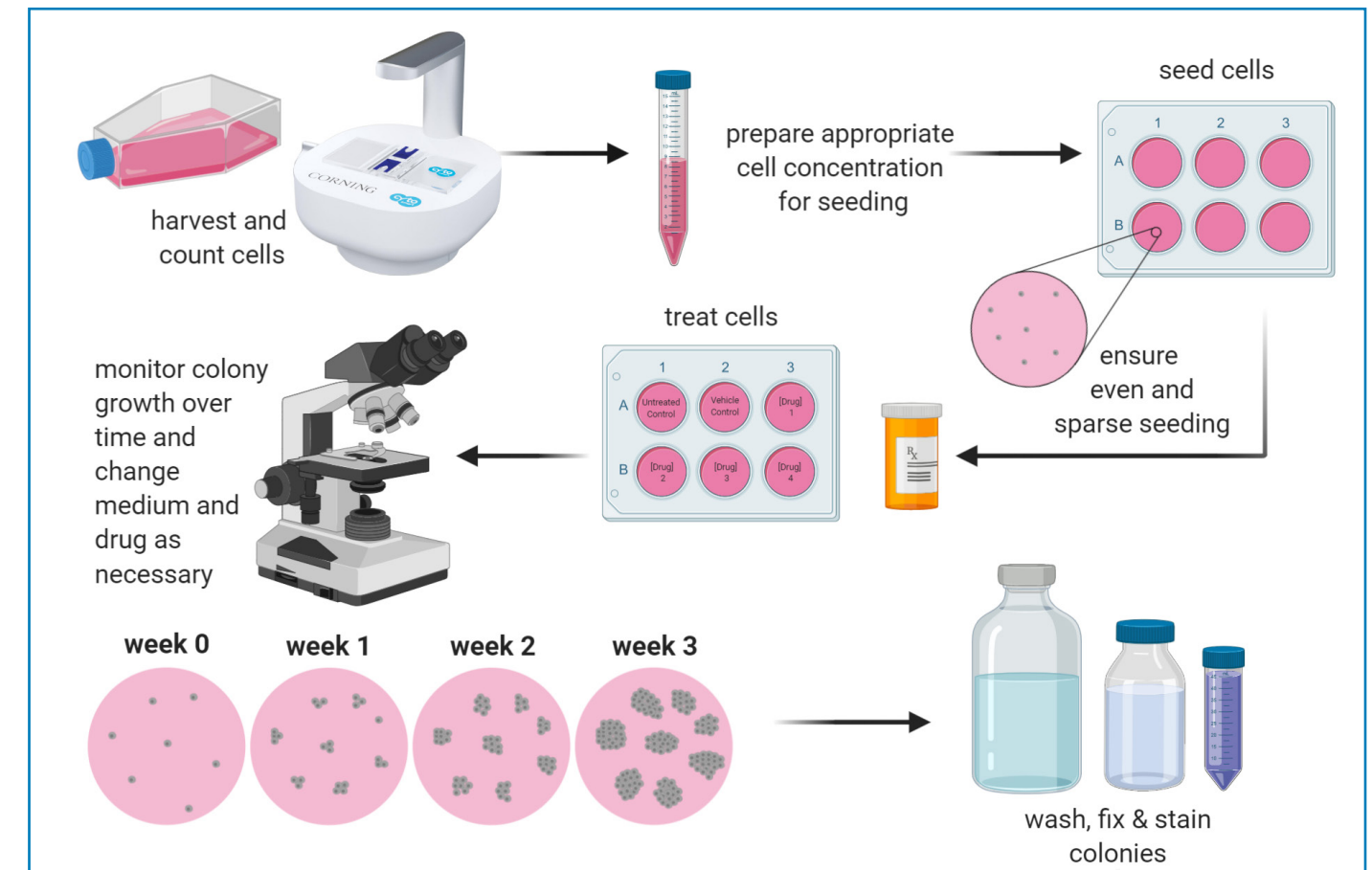
Using the right controls is critical for the analysis stage. One should always use an untreated control. In the case of drug treatment this would be a vehicle-only control (e.g. DMSO, PBS, or whatever solvent your treatment is dissolved in). For a genetic modification this could be a control siRNA (for transient transfection) or a stable cell line with an empty vector to compare against the stable knockdown or overexpression of the gene of interest. Each control and experimental condition should be performed in triplicate and data should be pooled from at least 3 independent repeats.

## Colony monitoring

During the colony formation phase you will need to monitor colony growth under all treatment conditions as colonies will be growing at different rates depending on the effect of treatment. Control colonies usually grow the fastest so using the control wells as an indicator for your experimental end-point can be helpful as you don't want merging colonies that can't be counted. A colony is considered to be 50 cells or more and they are only visible under a microscope. As this assay typically runs over a long period, you will need to change your cell media to ensure the cells always have the required nutrients and are under optimal culture conditions - you don't want to accidentally starve your cells as it will affect your clonogenic assay results.

Once you have reached your experimental end-point, cells need to be washed gently with PBS (add the PBS to the side of the well to not disrupt the colonies), fixed, and stained with the DNA intercalating dye, crystal violet (0.5% w/v) for at least 30 minutes. Removing the excess stain can be messy. The best technique is to gently dunk the plates in beakers immersed with water until all excess stain has been removed and you are only left with bright-purple colonies. Stained colonies can be counted up to 50 weeks after staining. Take high resolution pictures of your wells to use for analysis, presentations and publication figures.<sup>1,4,5</sup>

## A schematic overview of the clonogenic assay

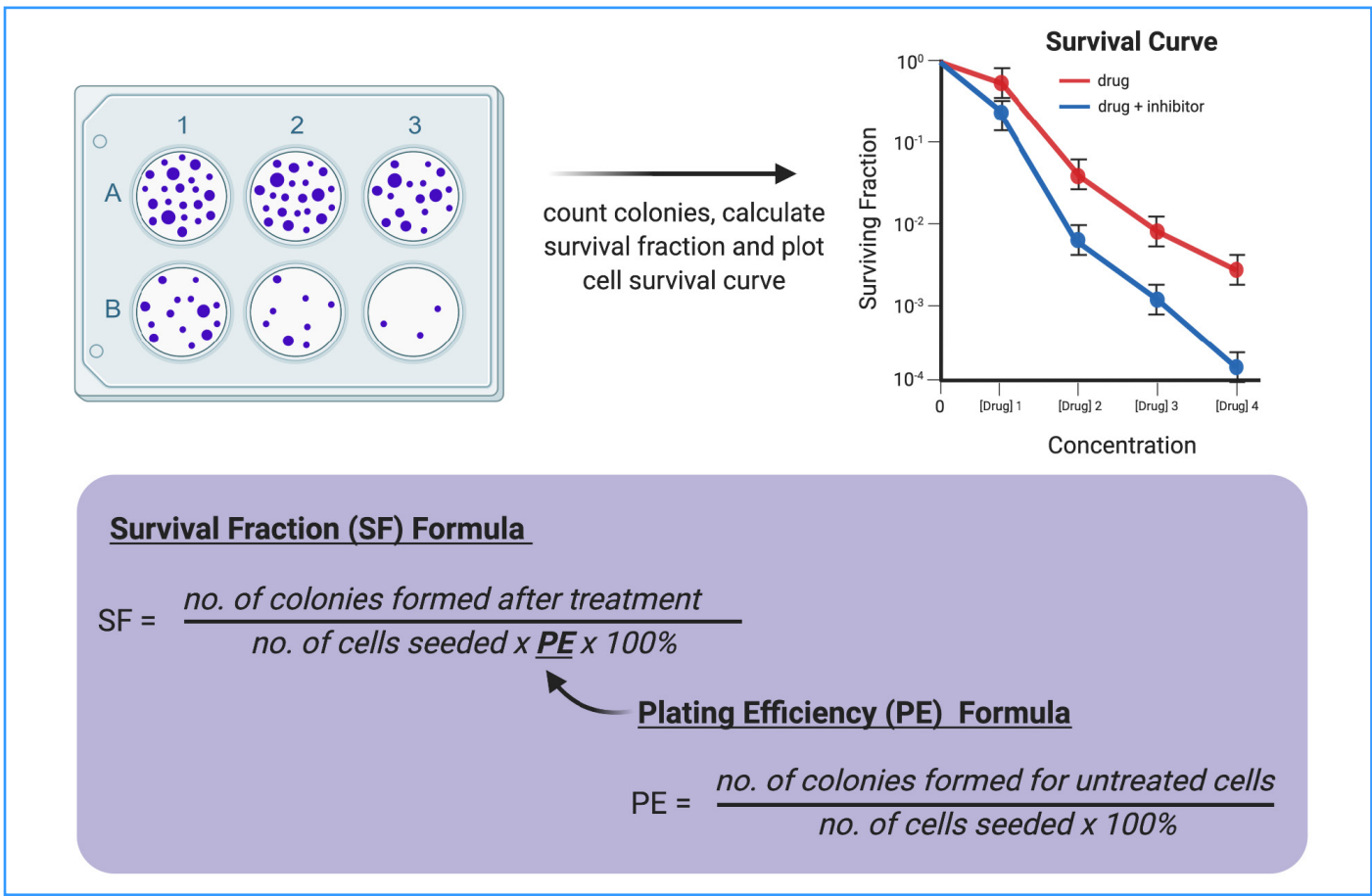


## Analysing your clonogenic assay

It really is as simple as counting your colonies (manually or using colony counting software) for each treatment condition and representing the data in a survival curve.

A survival curve requires the surviving fraction (SF) of treated cells (this includes a ratio of colonies formed to cells seeded) to be calculated and plotted against treatment dose. Before you can calculate the SF, the plating efficiency (PE) of your cells needs to be determined, as different cell lines have different plating efficiencies and this affects the survival fraction calculation. PE is the ratio of the number of colonies to the number of cells seeded in your untreated cells.<sup>1</sup> The diagram below provides the formulae to calculate SF and PE and shows an example of what a survival curve should look like. Statistical analyses should be performed on at least three biological repeats to determine statistical significance.

Plotting dose-response survival curves for clonogenic assays is routine in radiobiology, however there are other ways in which clonogenic data can be represented such as relative colony count as a percentage of the control.



# Experimental design example

## HEP-G2 Cells treated with Doxorubicin

Below is an example of a clonogenic assay experimental plan. The aim of this particular clonogenic assay experiment was to test the long-term cell viability of HEP-G2 human liver cancer cells after treatment with the chemotherapeutic drug, doxorubicin. The chosen approach was to pre-treat cells and then seed at low densities in drug-free medium to assess long-term viability. The plan includes trialing different seeding densities and using a drug treatment range obtained from the literature. Note that this is one way of doing a clonogenic assay, there are different ways

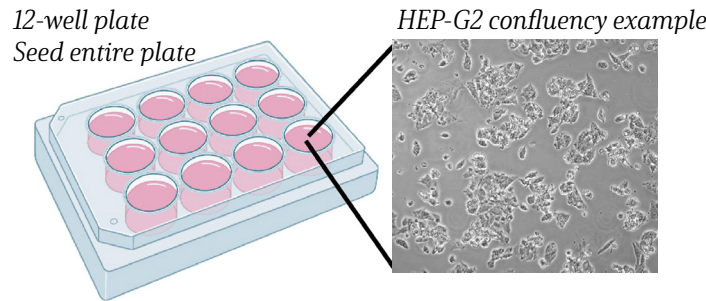
The schematic shows the key steps involved in setting up this clonogenic assay:

1. seeding cells for drug treatment
2. treating cells
3. re-seeding cells at low densities.

Each section includes typical lab book notes on how to plan the detail of your experiment before you begin. This includes literature searches, experimental layout, calculating seeding densities and drug concentrations, and other useful tips and tricks.

### 1) Seed cells for drug treatment

Achieve 60% confluency 24h after seeding. Important for cells to be evenly spaced for equal drug exposure (i.e. not clumping together or too confluent).



#### Lab book notes

**Literature starting point:**  
1x10<sup>5</sup> HEP-G2 cells/well in a 12-well plate (Chen et al., 2018)

**Try a few densities:**  
1x10<sup>4</sup> cell/wells  
1x10<sup>5</sup> cells/wells  
1x10<sup>3</sup> cells/wells



**Even Plating**  
Shift plate W-to-E a few times and then N-to-S a few times in a linear motion. Avoid swirling as cells tend to settle towards the centre of the well. For cells that like to clump, this can be done every 5 minutes for the first 15-30 minutes after seeding.

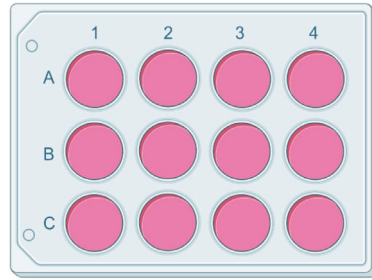




## 2) Drug treatment

Determine the range of drug treatments from the literature or from previous experiments.

Treat cells for 48 hours



A1: vehicle control  
A2: vehicle control  
A3: 0.05μM  
A4: 0.05μM  
B1: 0.1μM  
B2: 0.1μM  
B3: 0.2μM  
B4: 0.2μM  
C1: 0.4μM  
C2: 0.4μM  
C3: 0.8μM  
C4: 0.8μM

Doxorubicin



### Lab book notes

Doxorubicin (CAS: 25316-40-9)

MW+ 579.98g/mol

10mg powder + 2mL sterile H<sub>2</sub>O = 8.621mM solution

**1mM stock:** 884μL 1XPBS + 1116μL Doxorubicin

**10μM stock:** 990μL 1XPBS + 10μL stock

Make up 4mL for each concentration using 10μM stock

0.05μM: 3.98mL medium + 20μL Dox stock

0.1μM: 3.96mL medium + 40μL Dox stock

0.2μM: 3.92mL medium + 80μL Dox stock

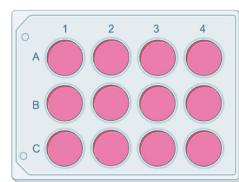
0.4μM: 3.84mL medium + 160μL Dox stock

0.8μM: 3.68mL medium + 80μL Dox stock

Vehicle ctrl: 3.68mL medium + 80μL 1XPBS



Ensure to mix drug solutions well



Remove medium from cells and add fresh drug medium at different concentrations

Label wells clearly  
Treat cells for 48 hours

## 3) Re-seeding at low densities

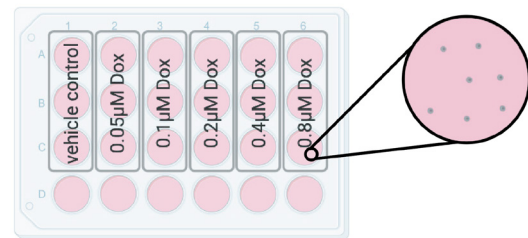
Trypsinise and count cells and seed very low densities for clonogenic assay.



Serial dilution for accurate seeding densities



Very important to mix cells well to achieve even single cell seeding. Gentle resuspend with pipette to mix and break up clumps of cells.



24-well plate  
Seed treatments in triplicate  
**Monitor colony formation**

### Lab book notes

#### Literature starting point:

800 HEP-G2 cells/well in a 6-well plate (Chen et al., 2018)

#### Convert to 24-well plate:

6-well plate = 9.5cm<sup>3</sup> growth area

24-well plate = 1.9cm<sup>3</sup>

800/5 = 160 cells/well in 24-well plate

#### Try a few densities:

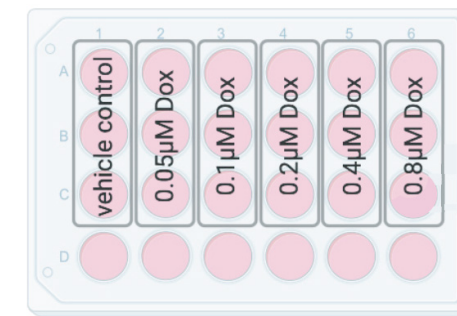
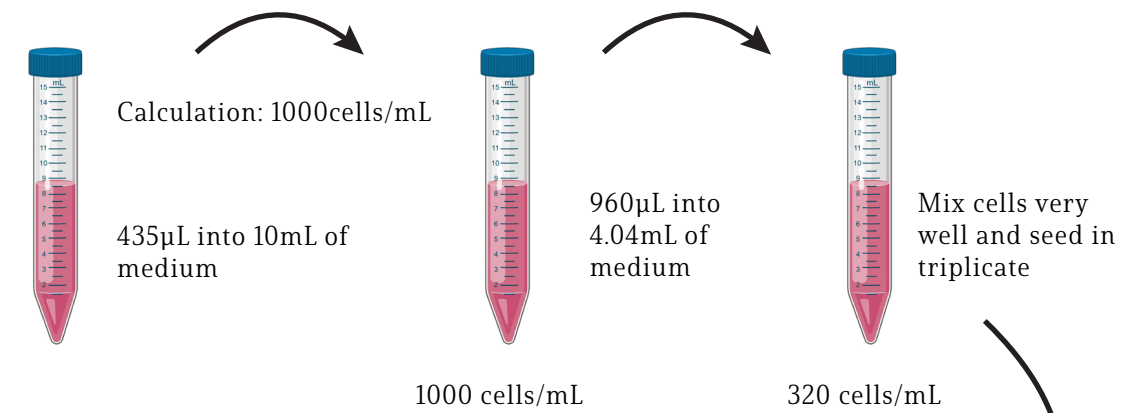
160 cells/well  
300 cells/well  
500 cells/well



#### Dilution Example

Count 2.3x10<sup>4</sup> total cells harvested in 2mL medium for vehicle control cells

**Want 160 cells per well in 500μL** which is a concentration of 320 cells/mL



Once all wells seeded shift plate W-E and N-S as described above to help with even plating.

# Clonogenic assay FAQs

## How do I figure out what drug concentration to use?

To determine your drug concentration you firstly need to decide if you are pre-treating your cells before the clonogenic assay (where they will be treated at ~ 60% confluency and then re-seeded at a low density) or treating them during the assay (very low density seeding) and then decide how long treatment will be for. A good starting point is to look in the literature to find any similar studies to yours e.g. similar drug types, treatment durations and experimental designs.

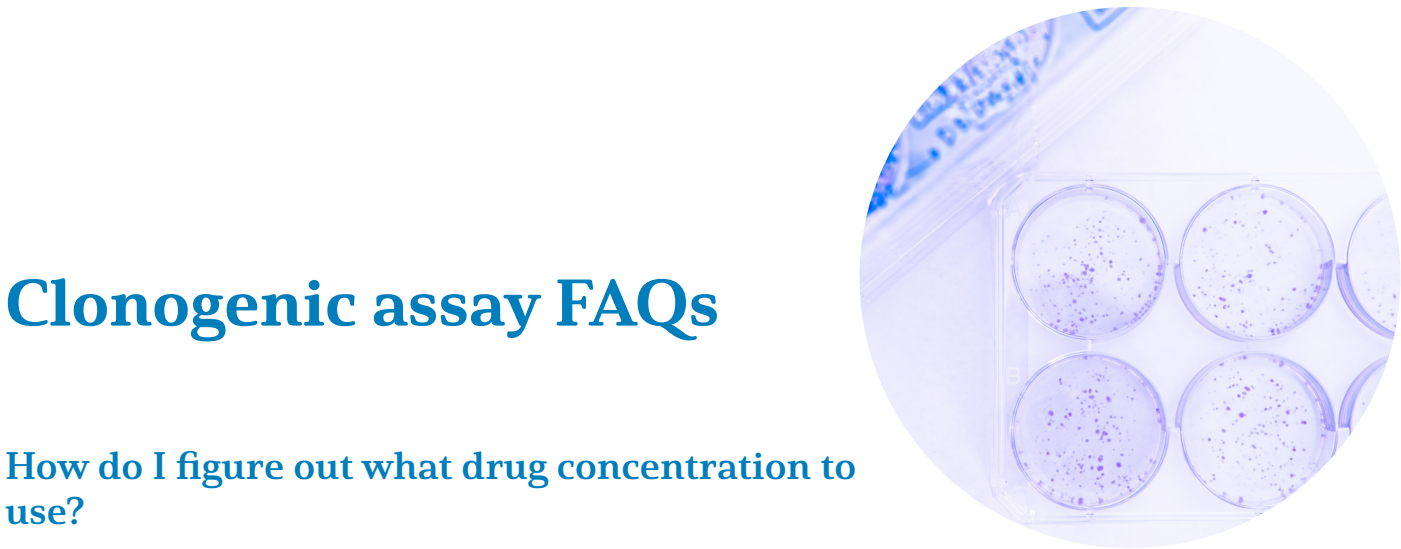
If you know the IC50 of your treatment in the cell type of interest (determined from a short-term cytotoxicity assay or found in the literature) this will give you a good idea of the range to work with (e.g.  $\frac{1}{4}$  IC50 to 2X IC50). Alternatively you will have to perform some pilot clonogenic assay experiments to determine the best concentration range and treatment duration.

## How often must the medium be changed for a clonogenic assay?

In normal cell culture, the medium is changed every 2-3 days depending on growth rate and confluency of the cells. A clonogenic assay typically runs for 7 to 21 days so it is important for the medium to be changed, however, this does not need to occur as regularly as every 2-3 days initially as the cells are seeded very sparsely. It is important to monitor the cells and as the colonies grow and the cells become more confluent the medium needs to be changed more regularly.

## How do I know when to end my clonogenic assay?

A clonogenic experiment is usually 7 to 21 days long. Typically your control dish should have the fastest growing colonies, so it is a good indicator of when to end the experiment. A colony is defined as > 50 cells and the speed of colony formation will vary from cell type to cell type as it depends on growth rate. Colonies can grow to much more than 50 cells (more impressive visual staining results with larger colonies) but ensure to end your experiment before colonies start merging as it then becomes very difficult to quantify them.



## My cells keep dying during my clonogenic assay, what should I do?

Cell death could be due to a number of factors:

1. Different cell types have different sensitivities to being seeded sparsely. You could try seeding a higher density of cells, as long as they are still sparse enough to form isolated colonies.
2. Sparsely seeded cells are naturally stressed. If you are changing the medium very often this could be disrupting the cells and causing more stress and then add the stress of your treatment this could take them over the edge. Try changing the medium less regularly or you could even replace half the medium at a time for less disruption.
3. Your treatment could be too harsh. Although the treatment concentrations might be fine for other experiments, treatment followed by low-density seeding could be too stressful causing your cells to die. Try lower concentrations.

## How do I quantify my colonies if my cell line does not produce nice visible colonies?

Some cell lines are more migratory than others and may form more of a 'lawn' than beautiful colonies as cells start moving apart from one another. This makes it tricky to count your colonies. A possible solution is to not use colony count as an output, but rather colony area. A useful tool is the ColonyArea plugin for ImageJ which helps you calculate the area of the dish/well covered by colonies as well as a readout for staining intensity. Another option is using a matrigel which helps keeps colonies together.

## When I treat my cells before performing the clonogenic assay and they are not seeded at the right density can I just wait until they are confluent enough for treatment?

It is generally accepted that cells should be treated *in vitro* at 60% confluency with even dispersal. Consistency with this will enable reproducibility with your experimental results. While it is the easy option to wait for your cells to reach confluency, some cell lines tend to clump together more and thus cells do not all receive equal drug exposure which could interfere with your experimental results. Some cell lines do take longer to adhere to the cell culture dish and require more time before treatment. Whatever your method is before treatment, the key is to remain consistent. If you seed and treat your cells 24 hours at 60% confluency, then stick with that. If you seed less and treat 48 hours later at 60% confluency, then stick with that.

# The Evolution of Colony Analyses

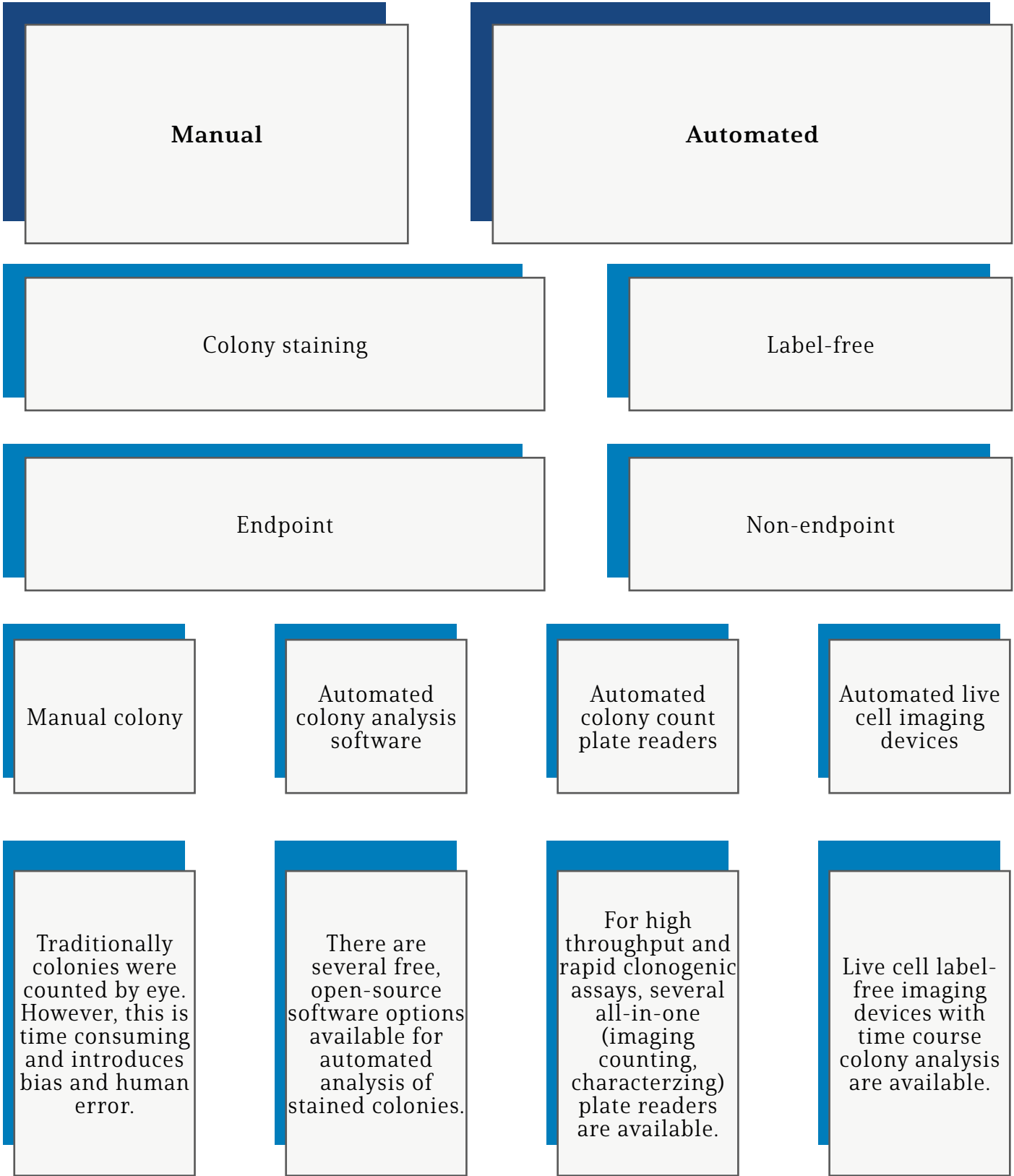
Traditionally, the clonogenic assay requires cells to be stained with a dye such as crystal violet so colonies are visible for manual counting. This is tedious and prone to bias and human error. With advancement in computing power and image analysis principles, stained colonies can now be automatically counted and analyzed resolving the key issues associated with manual counting. This enables faster, more high-throughput and reproducible results.

To further reduce the time of clonogenic assays, colonies can now be automatically counted without colony staining (label-free). Label-free analyses enable the movement away from fixed endpoint experiments which only provide information for a “snapshot” in time. Label-free, live monitoring of colony formation over time has the potential to provide much richer information on the entire colony formation process potentially revealing earlier and later-stage treatment effects and insight into cellular kinetics. Combined with sophisticated colony analysis algorithms, label-free live automated imaging is a step into the future of biological assays.

## Automated Colony Analyses Software

Clono-Counter	Colony Area	Colony CountJ	CountPHICS
2007	2014	2017	2019
Colony count	Total area covered by colonies.  Staining intensity (representative of cell density)	Colony count.  Colony area percentage.  Average colony size.  Average colony staining intensity.	Colony count.  Individual colony size distribution
Greyscale images of stained colonies in culture dishes or plates.	Images of stained colonies in 6-well to 24-well plates.	Images of stained colonies in any culture dish or plate D area of interest selected for measurement.	Images of stained colonies in any culture dish or a 6-well plate.

# Colony Analyses Evolution



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**CytoSMART Technologies**

Emmasingel 33  
5611 AZ Eindhoven  
Netherlands  
+31 88 2032 200  
[info@cytosmart.com](mailto:info@cytosmart.com)

