High-Throughput Screening of Cell Lines Expressing Monoclonal Antibodies

Development of an Immununoprecipitation-Based Method

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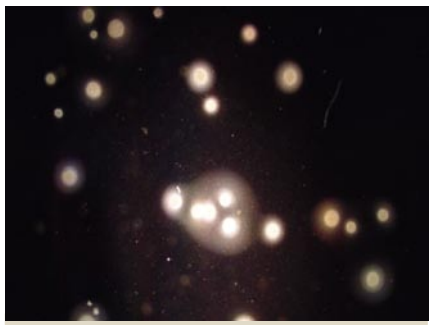
Recombinant proteins (r-proteins) are an important class of therapeutic agents. Monoclonal antibody technology has grown tremendously in the biopharmaceutical industry over the past two decades. With improvements in host-cell engineering, vector systems, media composition, and bioprocess optimization, productivity of mammalian cells can reach as high as grams per liter in bioreactor processes, an increase of more than 100-fold over titers reported for similar systems in the 1980s. Opportunities for improvement in expression systems still exist through further advancement in the technology of high-throughput screening.

Obtaining a stable clone for recombinant protein production usually requires cell transfection with an expression vector containing a gene of interest and a dominant genetic marker. Typically, a selectable marker such as a chemical resistance gene is transfected with a target gene of interest. Selection then is carried out in the presence of the specific toxic chemical. Cells that have taken up the expression-vector DNA survive in the corresponding selection media.

Currently, cloning stably transfected cells relies on performing a series of limiting dilution procedures, a time-consuming and labor-intensive process. For example, many commonly used mammalian expression systems are based on stably transfected Chinese hamster ovary (CHO) cells. Transfection efficiencies in this system range from 10% to 60% of cells taking up the vector DNA. However, because expression of a transfected gene is differentially modulated depending on the site of chromosome integration, there is wide variation in recombinant gene expression among clones that stably incorporate foreign DNA. Many hundreds, even thousands of transfected clones are typically screened for high producers because of the random variation in recombinant protein production. In many cases, therefore, screening to find high producers has been one of the rate-limiting procedures in developing cell lines expressing r-proteins because of the huge number of cells to screen and the complicated assays to perform.

Soluble proteins interact with their corresponding antibodies to form a precipitate in solid or semisolid substrates such as agarose (**1**). When mammalian cells that secrete soluble recombinant proteins are grown in semisolid agarose plates, precipitates formed by secreted r-proteins and their corresponding antibodies deposit around colonies, forming a halo-like structure. This characteristic has been used to identify high-expressing mammalian cell lines based on the assumption that the halo size may be positively correlated to the amount of secreted recombinant protein (**2**).

Several difficulties, however, were reported previously when this semisolid agarose technique was used for screening high-expressing clones. For example, poor mammalian cell growth is caused by using the incorrect temperature to seed cells while agarose cools. Another common problem is difficulty viewing the halo in agarose media even under a microscope. It is also difficult to obtain a distinct halo for size correlation to the level of protein secretion (**2**).



Picture of halo-producing clones at 10× magnification (PHARMACEUTICAL DEVELOPMENT, CENTOCOR R&D, WWW.CENTOCOR.COM)

Accordingly, improved and/or modified screening methods are needed to overcome or substantially ameliorate one or more of these and other problems. Here we describe the advantages and automation of a new immunoprecipitation method using methylcellulose-based semisolid medium for high-throughput screening of cell lines expressing monoclonal antibodies.

Materials and Methods

Preparing Methylcellulose-Based Semisolid Growth Medium with Capture Antibody: Medium containing methylcellulose was obtained from StemCell Technologies

Inc. (ClonaCell-TCS medium, Catalog #03814, www.stemcell.com). This medium contains 40 mL of methylcellulose, 30 mL of fetal bovine serum (FBS), 10 mL of bovine serum albumin (BSA), 0.1 mL of 2-mercaptoethanol (10-1 M), and 1 mL of L-glutamine (200 mM). Alternatively, the same semisolid medium can be prepared using MethoCult (Stem Cell Technologies, Inc. Catalog #03134 or equivalent), L-glutamine, and FBS without the BSA supplement and mercaptoethanol (10-1 M).

A 20-mL working solution contains 13 mL of ClonaCell-TCS medium or Methylcult, 1 mL of capture antibody (20 mg/mL), and 6 mL of cell suspension at 12,000 to 100,000 cells total. Capture antibody used in this procedure was rabbit antihuman IgG heavy and light (H&L) from Jackson ImmunoReseach Laboratories, Inc. (Catalog #309-001-0039, www.jacksonimmuno.com). This antibody recognizes both chimeric and human IgG.

Our working solution was placed in a proper container (such as a 50-mL conical centrifuge tube), then mixed or vortexed vigorously for 30 seconds. After mixing, the tube sat at room temperature for 5–10 minutes to allow air bubbles to disappear. Next, the entire 20-mL working solution was evenly dispensed into a six-well plate (about 2.8 mL per well). That plate was incubated in a 37 °C CO_2 incubator. We optimized the procedure's sensitivity by changing the volume of ClonaCell-TCS and the concentration of capture antibody, e.g., by lowering capture antibody concentrations and using less volume of ClonaCell-TCS to achieve better sensitivity of IgG detection.

Immunoprecipitation and Halo Detection: As described above, the capture antibody was incorporated into a methylcellulose base growth medium (final concentration 1 mg/mL) with appropriate cell densities. Cultures in methylcellulose plates then were incubated in a CO_2 incubator at 37 °C. For our initial experiments, the plates were examined daily under a phase microscope for signs of precipitation. However, once the method became routine, plates were left undisturbed in the incubator for at least eight days.

Transfection and Subcloning: Myeloma cells adapted to grow in a chemically defined medium were transfected with expression vectors coding for monoclonal antibodies (IgG) by electroporation as published (**3**). The heavy-chain and light-chain expression plasmids were linearized by restriction enzyme digestion. About 10 µg of each plasmid was used for electroporation. Following that, the cells were grown in T-flasks for 48 hours. After recovery, cells were selected with mycophenolic acid in T-flasks for several passages. Cells from that continuously selected culture were plated in methylcellulose plates containing appropriate capture antibody. Plates were incubated at 37 °C, 5% CO_2 for at least eight days before being examined for colony and halo formation.

Results and Discussion

Myeloma cells that produce monoclonal antibodies were used for these experiments. After plating, most cells were individually separated when examined under a microscope. Multiplication was observed within 20 to 24 hours after plating, and colonies were formed within 10 days.

The specific interaction between secreted IgG and capture antibody for this method was demonstrated by the following steps. First, nontransfected host cells were plated in methylcellulose with a capture antibody. No precipitate or halo-like structure was found with this treatment (Photo 1). Then IgG producing cells were plated in methylcellulose plates without the capture antibody. No precipitate or halo-like structure was found here as well (Photo 2). Third, IgG producing cells were seeded in methylcellulose plates containing capture antibody. A precipitate-like structure was found around cells in five days. That structure became halo-like under a phase microscope in nine days (Photo 3). These results clearly indicate the specificity of this method.

To expand the colonies with halos, single colonies were pippetted from the methylcellulose under a microscope and grown in multiple well plates with proper growth medium. Colonies with distinct halos were picked no later than day 12. They were grown in multiple well plates first, then expanded to T-flasks and spinners for studying cell growth and IgG production.

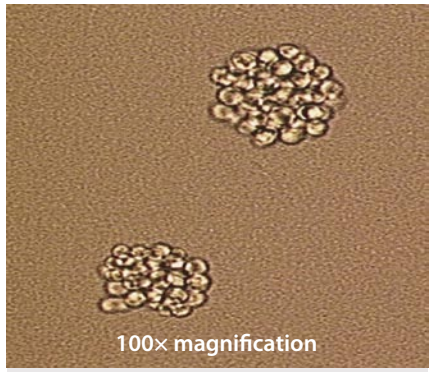


Photo 1: Myeloma host cells in methylcellulose with capture antibody; no precipitation is formed around the cells.

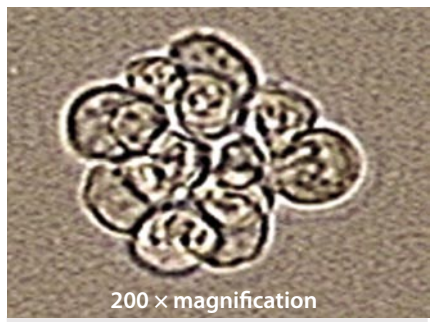


Photo 2: IgG-expressing cells in methylcellulose without capture antibody; no precipitation is formed around the cells.

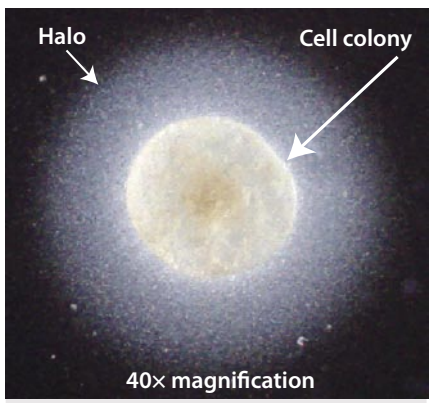


Photo 3: IgG-expressing cells in methylcellulose with capture antibody; the secreted protein and capture antibody complex formation is visualized using a phase microscope as a halo-like figure around the colony after nine days.

Correlation Between Halo Size and IgG Production: Previously, one major obstacle of semisolid subcloning and immunoprecipitation for high-production cell line development was a poor correlation between halo size and actual protein production. The new

Table 1: Correlation between halo size and overgrow titer (mg/L)

Halo Size		
Large	Small	No
48.7	20.72	30.16
47.25	5.33	30.56
26.93	26.33	18.13
33.41	19.41	17.37
34.55	37.4	7.02
34.26	29.1	23.14
33.41	19.59	4.12
35.4	42.29	18.72
13.81	27.36	22.94
22.37	22.37	22.71
44.04	23.38	3.43
52.02	16.2	4.68
54.44	26.64	4.43
26.93	26.64	2.99
30.84	23.35	5.76
34.83	23.23	3.23
22.37	22.17	7.39
33.69	31.98	19.56
47.84	49.62	4.56
40.27	10.7	died
Average 35.9	**Average** 25.2	**Average** 13.2

method dramatically improves halo formation, making correlation of halo size to protein production more accurate and reliable.

To correlate halo size with protein production, 20 colonies from each of the following categories were picked and grown in 24 well plates in the proper growth medium. Halo size was defined as follows:

Large Halo: The width of the halo greater than the radius of the colony that the halo surrounds.

Small Halo: The width of the halo is equal to or less than half of the radius of the colony that the halo surrounds.

No Halo: No surrounding halo.

Colonies from each category were grown for 12 days. Supernatant from these cultures was then collected for IgG quantitation by nephelometry. IgG titers from halo different sizes were analyzed statistically (T test and F test).

Our results clearly show strong positive correlation between halo size and IgG production (Table 1). The confidence level of obtaining high-expressing clones using halo size was >95%.

Table 2: Development timelines and improvement of IgG production using the halo procedure as a selection method

Host	Selection Method	Time to Reach Expected Titer	Remarks
Sp2/0 GPT	ELISA	52 weeks	10–50 ELISA plates per screening
Sp2/0 GPT	Halo	24 weeks	Plate unlimited number of cells[a]
NS0 GPT	Halo	24 weeks	
NS0 GS	Halo	16 weeks	
CHO GS	Halo	16 weeks	

[a] Every surviving colony has been screened for growth and expression

From Clones to Cell Lines: Transfected cells from the chemically selected culture were screened for colonies with surrounding halos in methylcellulose plates containing capture antibody. The total number of halo colonies from each transfection varied from a few colonies to several hundred depending on the constructs and chemical selection. Routinely, more than 70% of picked colonies survived in 96-well plates. The majority of surviving clones produced >10 µg/mL of IgG in 24 well plates after cultures became spent. Many clones were found to produce 30 µg/mL or more.

The methylcellulose plates also were used in subcloning higher IgG-expressing clones based on the size and intensity of produced halos. After subcloning, a significant increase in average IgG production was observed. We found the new level of IgG production to be adequate to support early phase clinical trials in several cases. Further improvement by another round of subcloning succeeded in increasing titer in some cases but not others. Each round of subcloning took about a month to complete.

In several projects, this method identified rare high expressing clones from large numbers of cells. So it increases the efficiency and reliability of obtaining high-expressing cell lines, thus dramatically shortening cycle times for cell-line development. As shown in Table 2, using the Sp2/0 host with ELISA screening resulted in a one-year timeline to achieve the desired titer. But applying the halo procedure to that same host and selection system shortened the timeline to about six months. Applying the halo procedure to yet other hosts and selection systems has generated cell lines with the expected titers in 16 to 24 weeks.

Automation of Halo Procedure

Although many advantages were observed after implementing the halo procedure into cell-line development processes, better titers require an even higher throughput in colony screening. Automating the colony-picking procedure can screen hundreds of thousands of transfectants for clones with even higher productivity because of the random variation in recombinant protein production. In addition, excessive manual picking of colonies causes eye fatigue and dizziness, which is easily remedied by automation.

An automated colony picking machine called ClonePix (Genetix, www.genetix.com) contains software to identify and rank halo areas surrounding colonies. It then can pick these colonies according to preestablished criteria. It can pick 300–400 clones in one hour compared with 30–40 colonies sorted manually.

References

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2 French D., et al. Somatic Cell Genetic Analysis of Myeloma and Hybridomas. *Methods Enzymol.* 151, 1987: 54–60.

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4 *Clonal Cell TM-TCS* (Technical Manual). Transfected Cell Selection Kit, StemCell Technologies; www.stemcell.com.

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