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14. ABSTRACT

The purpose of this project is to identify breast cancer antigens and autologous antibodies that breast cancer patients make to those antigens. The scope of this project is the development of new targeted therapeutics to treat breast cancer. Screening over a million clones from a patient tumor cDNA expression library with autologous serum has identified over 440 possible positive proteins revealing autoantibodies to atleast six SEREX antigens and at least 28 other proteins that represent novel antigens. PCR amplification of heavy and light chains for construction of a phage displayed scFv library from tumor indicates the presence of B cells in this patient's tumors. These results have significance for the development of new therapeutic targets and antibody therapies against those targets in patients with breast cancer.

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Introduction

The subject of this research project is the humoral immune response to breast cancer. The purpose of this research project is to identify breast cancer specific protein fragments from a patient and identify autologous antibodies that bind to those fragments. The scope of this project is to develop a method to utilize a patient's own immune system to make targeted therapeutics.

Body

An update on progress for the previous year is described in Table 1. Last year completion of several project milestones were described in the submitted annual summary. Briefly, Task One was completed for one patient, labeled 09-067-1, and methods were being developed to accomplish Task Two using the library that was created from this patient's tumor. Tissues were being processed from more patients to accomplish these tasks and to develop antibody libraries and test them, as outlined in Tasks Three through Five. A protocol for antibody library construction was described as developed by a colleague with more expertise, and I was accepted into a Phage Display course held at Cold Spring Harbor Laboratory conducted by experts in the field to learn how to apply these techniques to my own research. This report describes efforts completed within the last twelve months to complete this goal and builds upon progress from the previous two years.

Table 1. Summary of Overall Project

Phase	Task	Statement of Work Projected Completion	Status
1	1	Months 1-6	Complete
	2	Months 6-12	In progress
2	3	Months 12-16	In progress
	4	Months 16-20	Not initiated
	5	Months 20-34	Not initiated

Phase One. Generation of breast cancer phage displayed library and identification of autologous antibodies to phage displayed breast cancer proteins.

Task 1. Create cDNA phage library from a patient with breast cancer.

Progress related to this task was reported in both the 2009 and 2010 Annual Reports. In summary, the creation of cDNA libraries from patients with breast cancer has evolved over the three year course of this project based on my level of experience and expertise.

Obtain tumor specimens

In my 2009 Annual Report, I noted that we had developed methods for obtaining tissue from patients in the hospital and further honed these techniques to provide optimal material for each step in the cDNA synthesis reaction. By the time I finished my 2010 Annual Report, our laboratory had standardized a

protocol for sample procurement using core biopsy punches. I described step by step actions from operating room to surgical pathology and finally to the laboratory in obtaining these tissues (summarized on Appendix 6: Slides 17-18). Tissues have been collected for four patients and recruitment recently commenced for six more patients. A list of some of the tissues collected is on Appendix 6: Slide 19. There was a small delay to determine which method of blood procurement would yield the highest quality RNA (see Task 3 for more information).

Isolate RNA

As mentioned earlier, much effort has gone into developing a method to obtain human tissues from the operating room to preserve high quality RNA. In my 2009 Annual Report, I described obtaining de-identified tissue and isolating RNA from it. Based on these experiments, I learned that I needed to thoroughly homogenize tumor tissue using experimental conditions that protect RNA in these tissues from being degraded and that necrotic tumor will yield very low quality RNA. Appendix 5 Slide 16 shows capillary electropherograms demonstrating high quality RNA derived from study specimens and compares them to cell line RNA.

Since this time, I have further developed methods for RNA isolation on other study specimens. Since the aim of these experiments was to develop antibody libraries, I will elaborate further in Task 3.

Create cDNA library

The actual creation of high quality cDNA libraries from tumor specimens has taken a considerable amount of time over the course of this project. In my 2009 Annual Report, I described synthesizing a library from a de-identified tumor specimen using the originally proposed method cited often in the older literature (Jäger et al., 2001). At that time, I had difficulty synthesizing enough cDNA from the small quantity of RNA I was able to obtain from tumor specimens to successfully insert it into phage. This method is highlighted in Appendix 5 Slide 17 and Appendix 6 Slide 22. In 2010, I began using a newer technique that amplified full length cDNAs in the second strand reaction using PCR (SMART cDNA Synthesis Kit, Clontech) enabling libraries to be created from smaller amounts of RNA. In my 2010 Annual Report, I described several libraries I had synthesized using this method: hPlacental (using RNA provided from Clontech), cell line RNA (SK-BR-3), de-identified breast tissue (188-6), and one study specimen. The first three libraries served as testing grounds for purification and ligation into a lambda expression vector, and I described experiments to remove small cDNAs via size fractionation in the 2010 Annual Report.

In the past year more tumor libraries have been prepared from other patient tissues. The library reported last year from patient 09-067-1 had an appropriate titer (>1 x 10^6) but was only 54% recombinant. Although the number of recombinant clones was still over one million, I chose to create a library from a different patient's tumor (09-067-3) because I wanted the most optimal conditions possible for screening in Task Two. This library contained over one million clones and was greater than 90% recombinant; therefore this library possessed characteristics more similar to the libraries created with control materials and de-identified tissues described last year (Table 2). Another attempt was made to create a library from patient 09-067-1, and it resulted in much higher cDNA insertion rates, too. First

and second strand synthesis reactions were run on an agarose gel to check insert lengths (Appendix 5 Slide 18).

Table 2. Description of SMART cDNA expression libraries created using various sources of RNA.

Library	Source	Size	cDNA insertion rate
SK-BR-3	breast cancer cell line	$1.63x10^6$	> 90%
188-6	fresh frozen tumor	$1.08x10^6$	86-96%
hPlacental	purchased, Clontech	$4.80x10^6$	80%
09-067-1	fresh frozen tumor	$5.71x10^6$	54%
09-067-3T	fresh frozen tumor	$1.43 x10^6$	90-95%
09-067-1T	fresh frozen tumor	$2.15x10^6$	91-94%

Appendix 6 Slide 24 shows similar results but raised an issue. It was brought up during my thesis committee meeting (Appendix 6 represents the entire presentation) that size range of my libraries may not be very representative of the mRNA repertoire of the cell (i.e. the cDNA library inserts are smaller than most cellular mRNAs). Since this meeting, I have rerun these samples on an agarose gel for a longer and can demonstrate that the cDNA inserts range from 500bp to >2500bp (Figure 1).

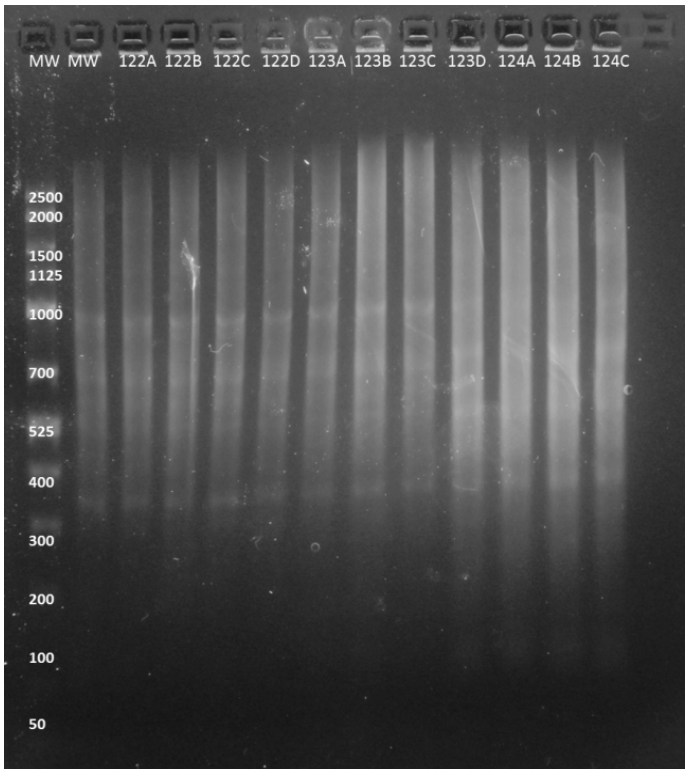


Figure 1. Agarose gel of second strand cDNA synthesis reactions, all samples are derived from 09-067-3 tumor specimens.

Outcome: A cDNA expression library from a breast cancer patient

Task 2. Select phage displayed breast cancer antigens that bind to immobilized antibodies from the same breast cancer patient's serum.

In the 2010 Annual Summary, I described methods developed during my first attempt to screen a patient's library. Since these methods were slightly different from those initially proposed, the Statement of Work has been revised and accepted.

Obtain patient serum

As noted earlier, samples have been collected for four patients and I anticipate collecting samples from six more patients in the future.

Plate expression library on agarose plates and perform plaque lift and Expose nitrocellulose membranes to patient serum and identify binding proteins

Since the 2010 Annual Summary was submitted, I have completed primary screening the tumor cDNA expression library (09-067-3T, synthesis described in Task 1) with autologous serum. Briefly, I plated the entire cDNA expression library on 127 agarose plates and transferred the plaques on these plates to nitrocellulose membranes. The membranes were exposed to the same patient's serum and binding areas were revealed using an alkaline phosphatase conjugated secondary antibody to human IgG (Türeci, Usener, Schneider, & Sahin, 2005)(Chen, Gure, & Matthew J Scanlan, 2005). More than one million clones were screened during the primary round and roughly 500 plaques were identified as positive clones (see Figure 2A for an example and Appendix 4 for all membrane data).

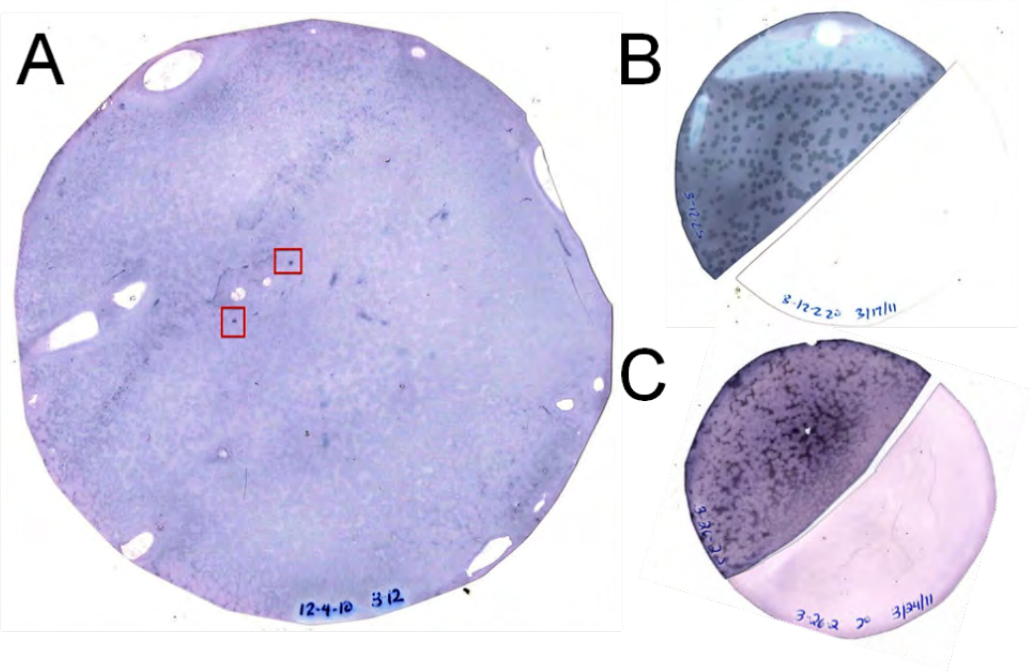
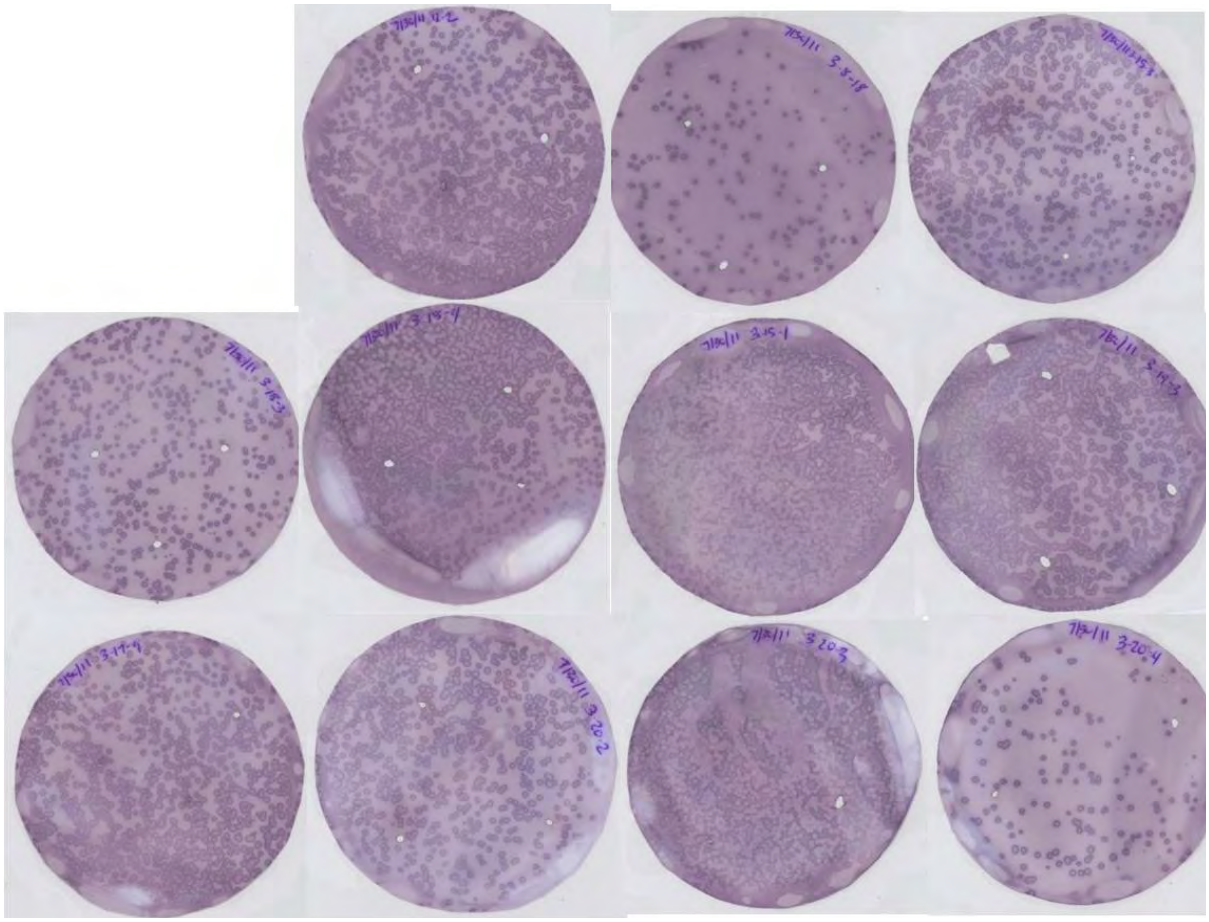


Figure 2. Samples from screening (A) Primary immunoscreening plaque lift with red squares indicating positive clones (B) Secondary screening with positive clones and (C) negative clones.

Verify protein binding with a secondary screen

Secondary screening for this project has proven to be more difficult than primary screening. Plugs of the corresponding areas on primary screening membranes were taken from the agarose plate and the clones were plated again for secondary screening (Chen et al., 2005). Homogenous concentrations of primary clones were plated on agarose. The same protocol for primary screening was completed for these membranes (see Figures 2B and 2C for examples, data for all screening completed can be found in Appendix 8); however, it was more difficult to gauge whether clones were positive. Roughly 30 clones are screened at a time, so clones can be compared with others within the batch. In contrast to the heterogenous population of clones represented on the primary membrane, no proper internal control existed homogenous population of clones on the secondary membranes.

Regardless, many membranes did appear to exhibit a positive response to the patient's serum (Figure 3),



and the clones associated with these were sequenced and characterized (Appendix 9).

Figure 3. Membranes of positive clones that encode identified proteins from secondary screening.

To address the discrepancy in secondary screening, I have tried two different approaches. The first was the plate the clones in an array format instead of on individual plates. This approach has two main benefits: negative clones could be included on the array and provide an opportunity for internal controls during batch screening and more clones may be evaluated at one time, since more than one clone was evaluated with each membrane. I tried to develop a protocol for this technique, based on another publication (M J Scanlan et al., 2001), but could not obtain consistent results after two months (see Appendix 7 Slide 12 for an example of this approach).

The second approach is to specifically plate a negative clone with the experimental clone (Chen et al., 2005). So I plated two clones, one identified previously as negative and one I wanted to test and completed our screening process. Preliminary results have been inconsistent. In the next month, I plan to alter the protocol again slightly. The SMART cDNA synthesis library comes with a control insert to test packaging extracts and cloning efficiency. Essentially, this control insert is ligated to the vector and packaged into lambda phage in parallel with the cDNA library. The size of the control insert library and cloning efficiency are used as a positive control to ensure that these steps were completed correctly. However, this component may be useful for our negative control experiments because we can consistently incorporate it with experimental clones and ensure that no antibodies exist against it by subtracting serum against it in a uniform manner.

Sequence peptides

I have sequenced some of the positive clones obtained during secondary screening (full data set can be found in Appendix 9). I anticipate sequencing remaining clones after developing a better protocol to screen secondary membranes.

Characterize peptides using online databases and literature

Several of these proteins have been identified in a previous SEREX analysis, which most likely confirms that our technique is successful (see Appendix 9). Others are sequences that don't correspond to known proteins. These clones may represent novel proteins to which the patient has experienced an antibody response. More information regarding these clones can be found in Appendix 10.

Anticipated Outcome: isolation and identification of breast cancer protein fragments (deliverable) to which patients have pre-existing antibodies.

Phase Two. *Ex vivo* identification and amplification of patient antibodies that bind to breast cancer protein fragments

Task 3. Generate phage display antibody library from the patient's B-cells.

In October 2010, I attended a Phage Display course at Cold Spring Harbor Laboratory to familiarize myself with techniques for constructing scFv antibody libraries(Barbas, Burton, Scott, & Silverman, 2001). While there, I created a phage displayed library from chicken RNA and practiced panning with both this library and two other libraries (see Appendix 6 Slide 42 for an example).

Obtain B-cell mRNA from patient serum, sentinel nodes, and primary breast cancer and

Since that time, I have processed the tissues needed to make a library from patient 09-067-3 (which I am currently screening for autoantibodies). RNA has been obtained from tumor, sentinel nodes, and an adjacent non-sentinel lymph node (Appendix 7 Slide 40). A modified technique is now used to extract RNA from blood with a QIAGEN kit and is incorporated into the tissue procurement methods.

Isolate variable portion mRNA from patient's IgG

In September 2011, I began constructing antibody libraries from these materials based on techniques developed by Barbas et al. I first practiced these techniques with healthy blood and then moved on to the tissues collected for this study. Tissue RNA was converted to cDNA using the SMART method previously used in Task One. I chose to use this method (as opposed to that suggested by Barbas et al) because I could generate libraries from small quantities of tissue, such as sentinel node scrapings. When checked for quality on agarose gels, the cDNA appeared as smears, as shown in Figures 1 and 4, demonstrating successful synthesis.

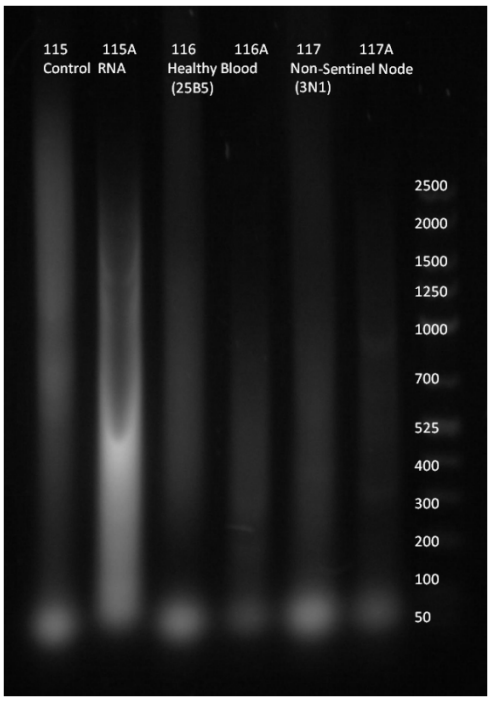
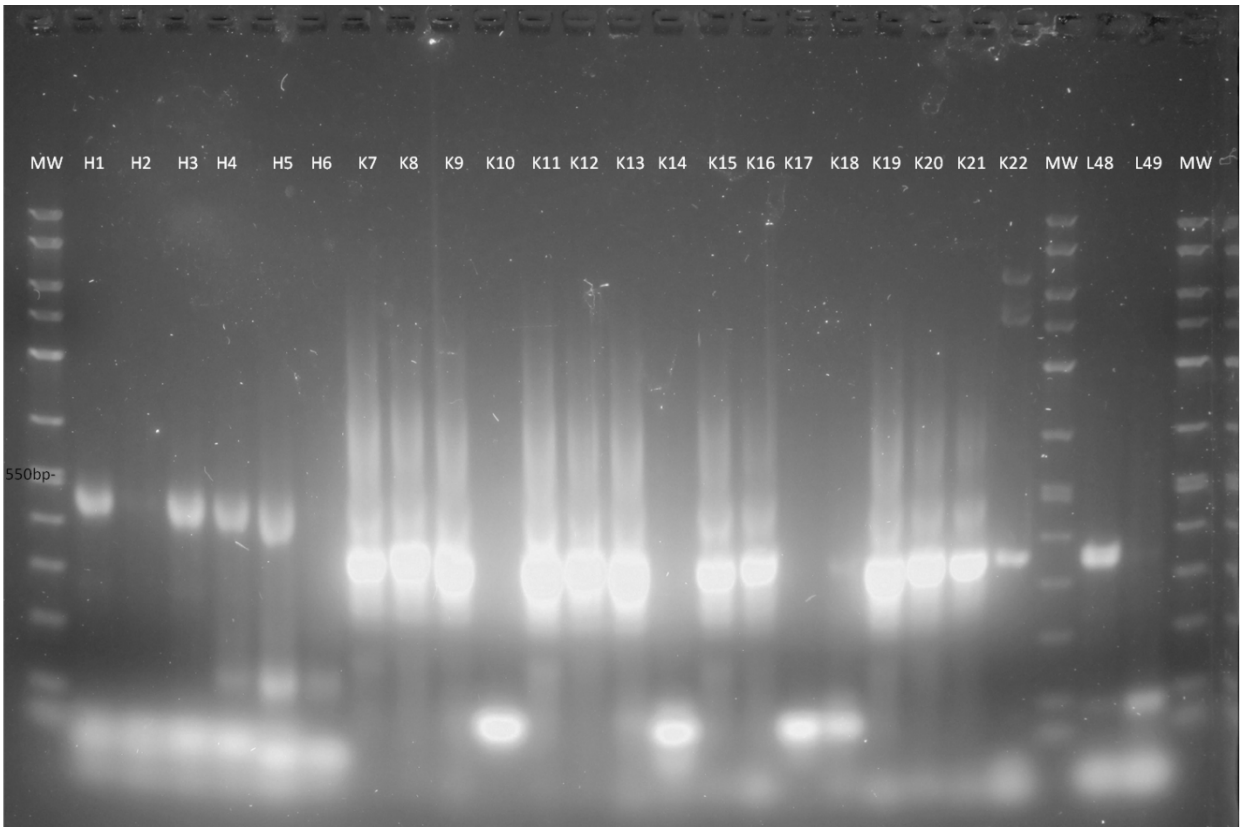


Figure 4. First and second strand cDNA synthesis reactions of source material used to make antibody libraries.

Create antibody libraries using phage display

I completed forty-nine PCR reactions based on a protocol and primers used successfully by another member of the laboratory (described in 2010 Annual Report), and I checked the quality of these reactions on an agarose gel (Figure 5 the reactions for the tumor sample). Since these reactions were completed at the end of September, we anticipate successful completion of the library in the next month followed soon after by panning experiments.



Anticipated Outcome: three phage display antibody libraries per patient derived from B-cells recovered from the blood, lymph nodes, and tumor

Task 4. Select phage displayed antibodies that bind to selected breast cancer proteins

There is no progress to report on Task 4.

Task 5. Determine sensitivity and specificity of individual phage antibody clones for binding to breast cancer.

There is no progress to report on Task 5.

Key Research Accomplishments

- Screening over a million clones from a patient tumor cDNA expression library with autologous serum has identified over 440 possible positive proteins. Although still in progress, this breast cancer patient developed antibodies to atleast six SEREX antigens (including one previously identified in a breast cancer analysis) and at least 28 other proteins.
- PCR amplification of heavy and light chains for construction of a phage displayed scFv library from tumor indicates the presence of B cells in this patient's tumor.

Reportable Outcomes

Abstracts (2011 Only)

Novinger L, Shukla GS, Pero S, Krag DN. Characterization of tumor associated antigens using a recombinant expression patient derived breast cancer cDNA library and autologous serum. UVM COM Graduate Research Day. October 14, 2010.

Novinger L, Shukla GS, Pero S, Krag DN. Characterization of tumor associated antigens using a recombinant expression patient derived breast cancer cDNA library and autologous serum. Vermont Annual Breast Cancer Conference. October 15, 2010

Novinger L, Shukla GS, Pero S, Krag DN. Characterization of tumor associated antigens using a recombinant expression patient derived breast cancer cDNA library and autologous serum. AACR Annual Meeting. April 4, 2011.

Novinger LJ, Pero S, Shukla GS, Krag DN. Characterization of tumor-associated antigens using a recombinantly expressed patient derived breast cancer cDNA library and autologous serum. Department of Defense Era of Hope Meeting. August 4, 2011

Presentations (2011 Only)

Utilizing the Patient Immune Response to Develop Customized Cancer Therapies. UVM Cell and Molecular Biology Seminar. November 30, 2010.

Crossing the Moat To Get To the Castle: Innovative Drug Delivery Mechanisms to Treat Glioblastoma. UVM Surgical Research Seminar. March 10, 2011.

Characterizing the Humoral Response to Breast Cancer in an Individual Patient. UVM MD/PhD Research Day. July 22, 2011.

Conclusions

Studies show that a dynamic relationship exists between the humoral immune response and breast cancer cells (Spaner & Bahlo, 2011); however it is unclear as to whether B cells contribute by eliminating tumors (Li et al., 2011) or promoting their growth (Olkhanud et al., 2011). B cells from different sources *within the same body* must be analyzed to resolve the question of where anti-tumor B cells reside. This project has identified tumor derived antibody targets using the SEREX method and aims to study phage displayed antibodies derived from various tissue sites within the same patient to better characterize the humoral response to breast cancer antigens. Specifically, this project addresses questions regarding this response from two different approaches: the B cell target and the antibody binding to this target.

In Phase 1 of this project, I began to characterize the patient-specific immune profile with SEREX to confirm the existence of corresponding B cells in that patient. Tumor associated antigens that bind to the patient's antibodies are currently being divided into previously characterized SEREX antigens, novel

antigens, and unknown proteins. Over one million clones have been screen with patient serum and 440 have been identified as potentially positive. Of these, 54 have been tested again and sequenced. Several clones that react with patient serum were previously characterized SEREX antigens may be utilized in Task 4 as targets of phage displayed antibody libraries. Twenty eight other clones are proteins newly identify as antigenic and may provide information for new therapeutic targets or biomarkers.

In addition to serum, the role of the immune response by B cells has been investigated with breast cancer using tumor, sentinel node (SN), and peripheral blood lymphocytes (PBL) obtained from different patients but never from all of these tissues in the same patient. Phage displayed antibodies that bind to SEREX antigens will be isolated from antibody libraries currently being constructed from primary tumor, SN, NSN, and PBL of this patient. Given evidence for the oligoclonal response in some breast tumors (Coronella-Wood & Hersh, 2003), these experiments will also test the hypothesis that SEREX antigen binding B cells will be located in the tumor and tumor draining lymph nodes. Construction of a phage displayed scFv library from tumor is currently underway and first round PCR reactions indicate the presence of B cells in this patient's tumor via amplification of heavy and light chains from this tissue source.

In summary, the results of this project may impact the list of potential breast cancer targets for therapeutic application and provide insight to the relationship of B cells within breast tumors, nearby lymph nodes, and the blood stream. It may address the conflicting data that result from studying different sources in different patients due to limited access to tissues by restricting the study of this complex relationship to one immune system at a time.

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Türeci, O., Usener, D., Schneider, S., & Sahin, U. (2005). Identification of tumor-associated autoantigens with SEREX *Methods in molecular medicine*, *109*, 137–154.

Appendix 1: Bibliography of all publications and meeting abstracts

ABSTRACTS/POSTERS (National Meetings Only)

Novinger L, Shukla GS, Pero S, Krag DN. Characterization of tumor associated antigens using a recombinant expression patient derived breast cancer cDNA library and autologous serum. AACR Annual Meeting. April 4, 2011.

Novinger LJ, Pero S, Shukla GS, Krag DN. Characterization of tumor-associated antigens using a recombinantly expressed patient derived breast cancer cDNA library and autologous serum. Department of Defense Era of Hope Meeting. August 4, 2011

PRESENTATIONS

Construction of a Protein Expression Library From Human Breast Cancer Tissue. UVM MD/PhD Seminar. November 13, 2008

Construction and Applications of a Protein Expression Library from Human Breast Cancer Tissue. UVM Cell and Molecular Biology Program Seminar. December 2, 2008

Construction and Application of a Protein Expression Library from Human Breast Cancer Tissue. UVM Graduate Research Day. April 16, 2009

Creation of a Phage Displayed Breast Cancer cDNA Library. UVM MD/PhD Research Day. July 17, 2009

Characterization of Patient Autoantibodies to Breast Cancer Using Phage Display. UVM Cell and Molecular Biology Program Seminar. December 15, 2009.

Tumor Autoantibodies: Potential Therapeutic or Irrelevant Phenomenon? UVM Surgical Research Seminar. May 12, 2010.

Characterization of tumor associated antigens using a recombinant expression of a patient derived breast cancer cDNA library and autologous serum. UVM MD/PhD Research Day. July 23, 2010.

Utilizing the Patient Immune Response to Develop Customized Cancer Therapies. UVM Cell and Molecular Biology Seminar. November 30, 2010.

Crossing the Moat To Get To the Castle: Innovative Drug Delivery Mechanisms to Treat Glioblastoma. UVM Surgical Research Seminar. March 10, 2011.

Characterizing the Humoral Response to Breast Cancer in an Individual Patient. UVM MD/PhD Research Day. July 22, 2011.

Appendix 2: List of Personnel receiving pay from the research effort

Leah Novinger

Appendix 3: Revised Statement of Work

This training plan is divided into two research phases, each with specific tasks directly related to the objectives of the project.

Phase 1. Generation of breast cancer phage displayed library and identification of autologous antibodies to phage displayed breast cancer proteins.

Task 1. (0 - 6 months) Create cDNA lambda expresion library from a patient with breast cancer

- Obtain tumor specimens
- Isolate RNA
- Create cDNA library

Outcome: a library of protein fragments from the primary breast cancer expressed in bacteria

Task 2. (6 -12 months) Select breast cancer antigens that bind to antibodies from the same breast cancer patient's serum

- Obtain patient serum
- Plate expression library on agarose plates and perform plaque lift
- Expose nitrocellulose membranes to patient serum and identify binding proteins
- Verify protein binding with a secondary screen
- Sequence peptides
- Characterize peptides using online databases and literature

Outcome: isolation and identification of breast cancer protein fragments (deliverable) to which the patient has pre-existing antibodies

Amplification and identification of protein fragments represents a milestone that will allow progression onto Phase 2 of the project.

Phase 2. *Ex vivo* identification and amplification of patient antibodies that bind to breast cancer protein fragments

Task 3. (12 – 16 months) Generate phage display antibody library from the patient's B-cells

- Obtain B-cell mRNA from patient serum, sentinel nodes, and primary breast cancer
- Isolate variable portion mRNA from patient's IgG
- Create antibody libraries using phage display

Outcome: Successful completion of this objective will result in three phage display antibody libraries (deliverable) derived from B-cells recovered from the blood, sentinel nodes, and primary breast cancer.

Task 4. (16- 20 months) Select phage displayed antibodies that bind to selected breast cancer proteins

- Prescreen antibody libraries against plastic and other non-specific binders
- Biopan antibody libraries against tumor protein lysates bound to a plate
- Test antibody hits for significant binding using an ELISA

Outcome: isolation of patient antibodies (deliverable) that bind to breast cancer proteins.

Task 5. (20-34 months) Determine sensitivity and specificity of individual phage antibody clones for binding to breast cancer.

- Amplify antibodies identified in Task 4
- Sequence antibodies
- Test antibodies for sensitivity to tumor protein fragments with an ELISA
- Immunofluorescence microscopy evaluation of clones for binding to patient tumor
- Test for localization of binding to the cancer cell membrane using confocal microscopy
- Immunofluorescence microscopy evaluation of binding to patient normal tissue, other breast cancers, other nonbreast epithelial cancers, a panel of normal tissues, and a panel of 60 cancer cell lines (NCI 60).

Outcome: a set of antibodies derived from a patient that specifically binds to the same patient's breast cancer (deliverable). Binding profile of these breast cancer binding antibodies will be established to a wide range of cancer types and normal tissue (deliverable).

Appendix 4: 09-067-3 Primary Screening Membranes

See attached

Appendix 5: Seminar Presentation Dated November 30, 2010

See attached (6 pages)

Appendix 6: Thesis Committee Presentation Dated May 11th, 2011

See attached (8 pages)

Appendix 7: Presentation for MD/PhD Research Day Dated July 22, 2011

See attached (3 pages)

Appendix 8: Secondary Screening Results from SEREX Analysis of Breast Cancer Patient's Tumor (09-067-3)

See attached (14 pages)

Appendix 9: Secondary Clone Sequencing Information from SEREX Analysis of Breast Cancer Patient's Tumor (09-067-3)

See attached (10 pages)

Appendix 10: Protein Identification Data from SEREX Analysis of Breast Cancer Patient's Tumor (09-067-3)

See attached (2 pages)

Results: Primary Screen
09-067-3T

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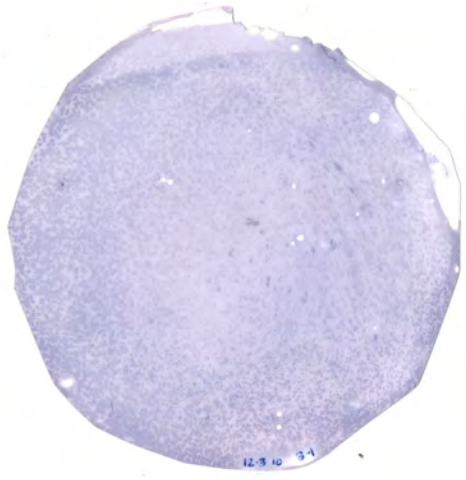

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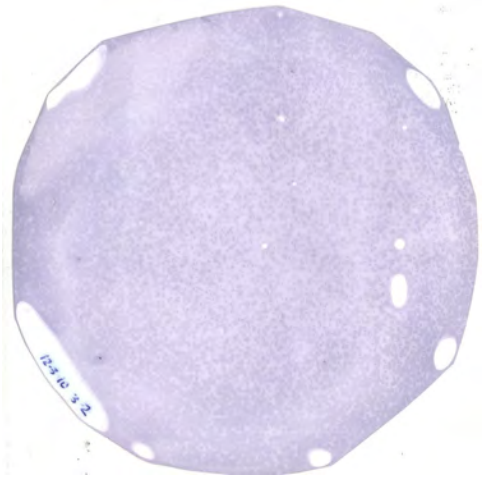

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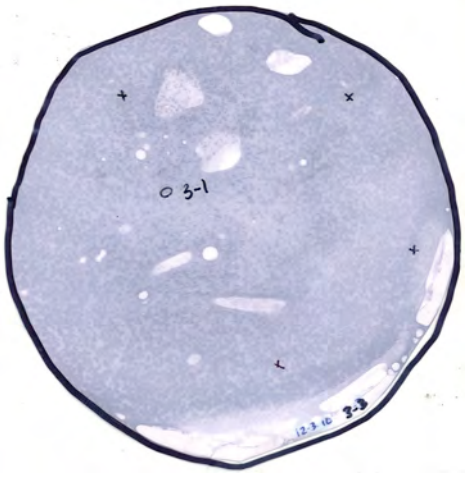

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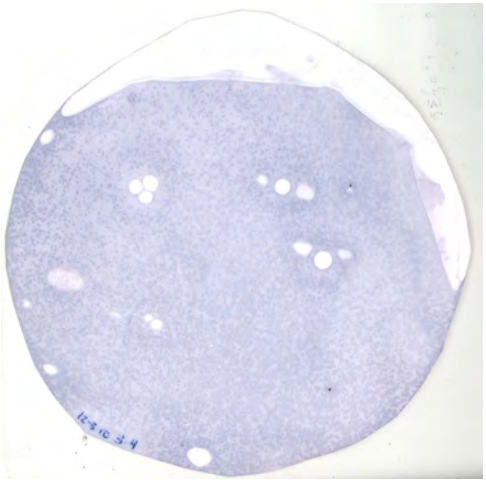

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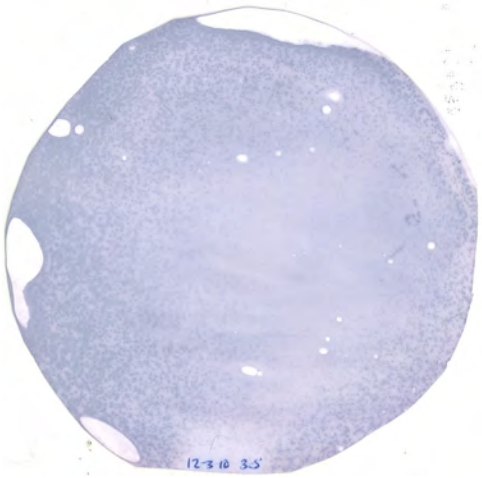

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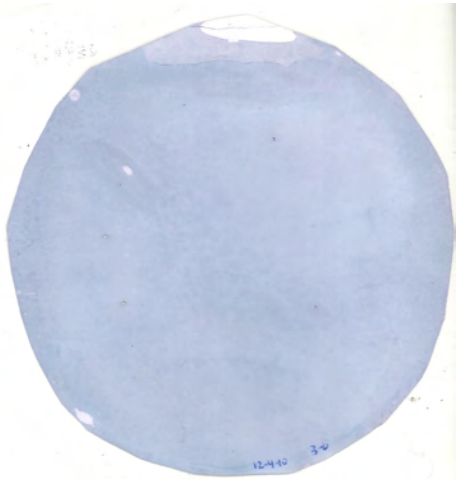

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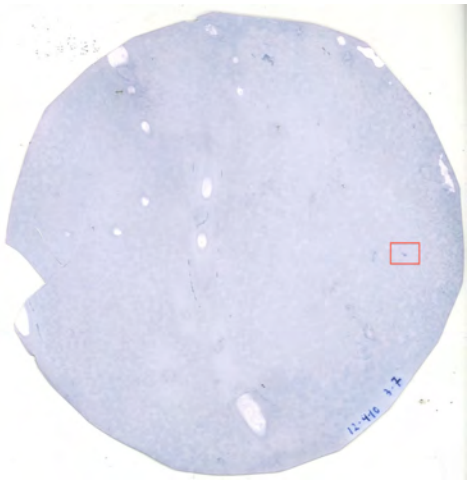

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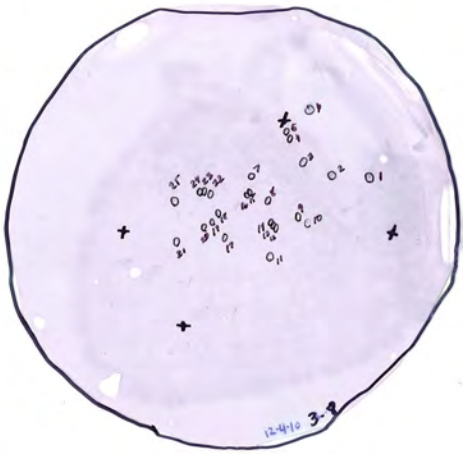

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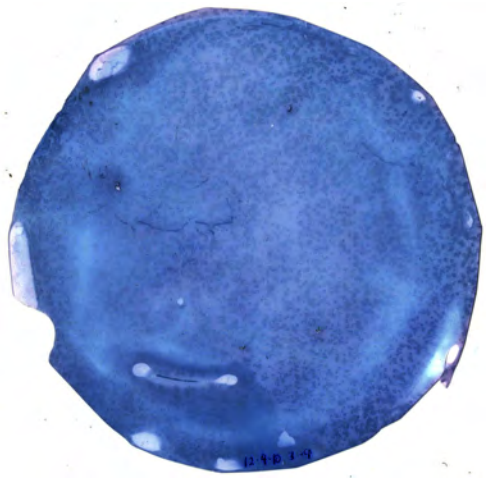

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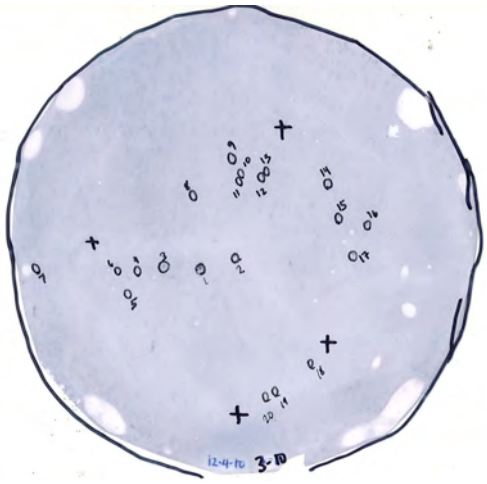

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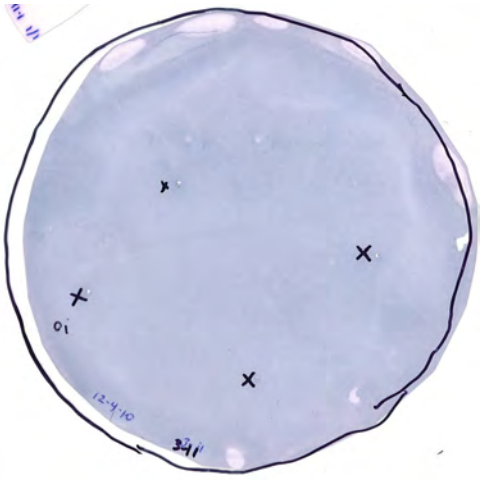

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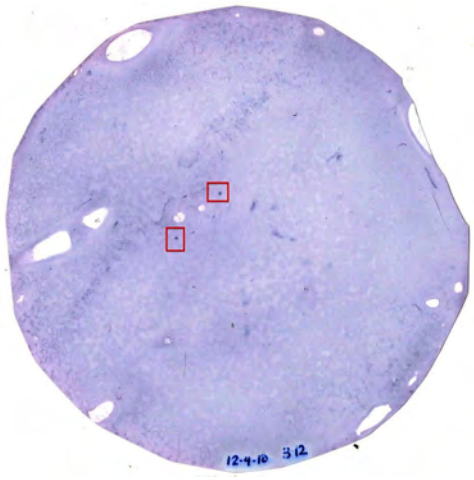

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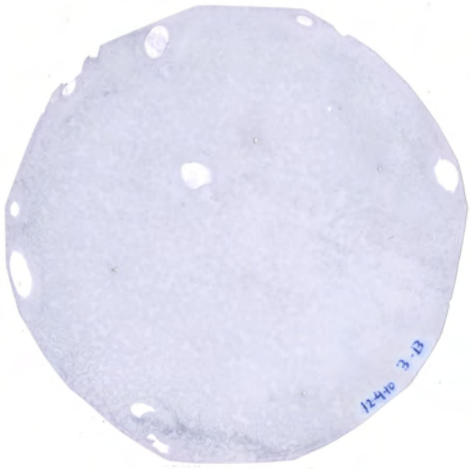

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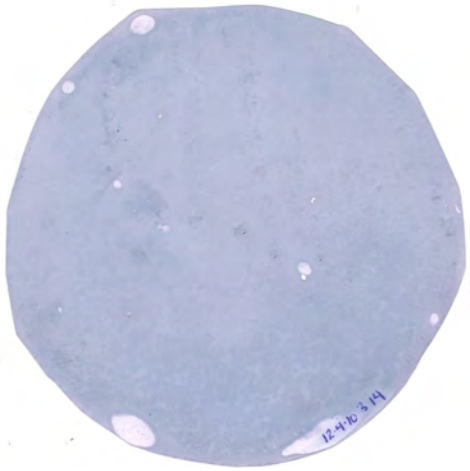

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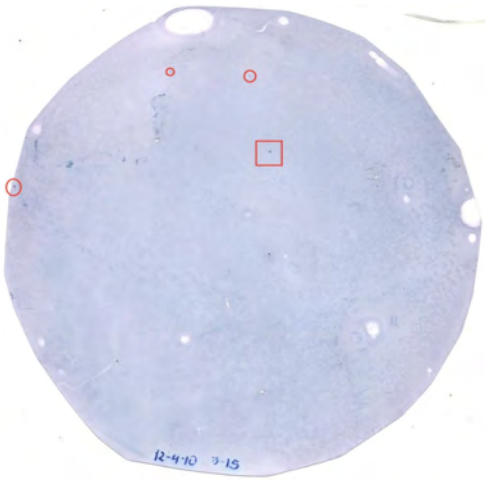

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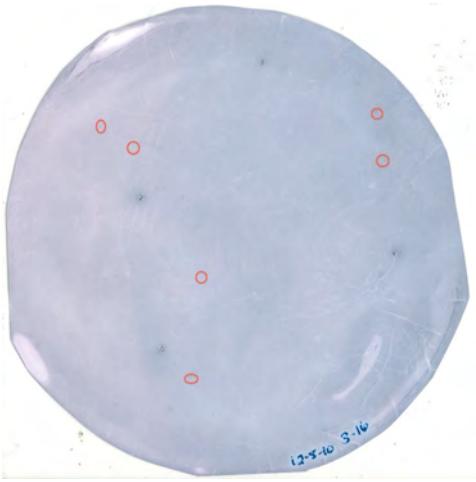

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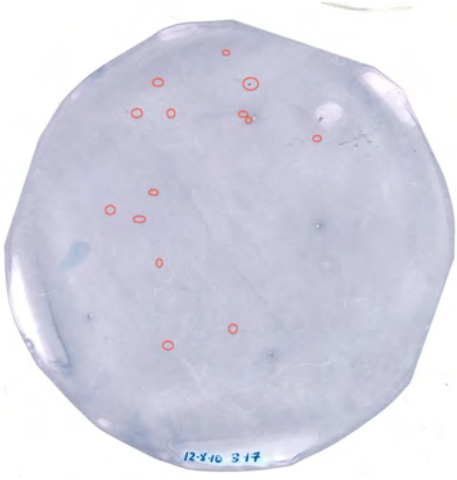

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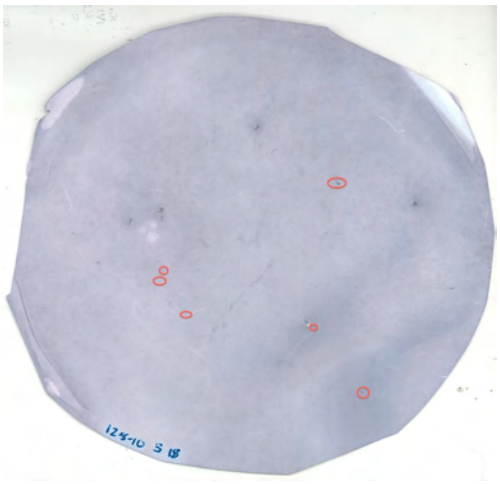

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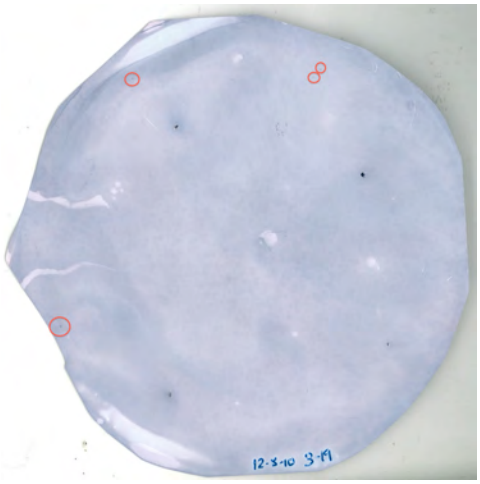

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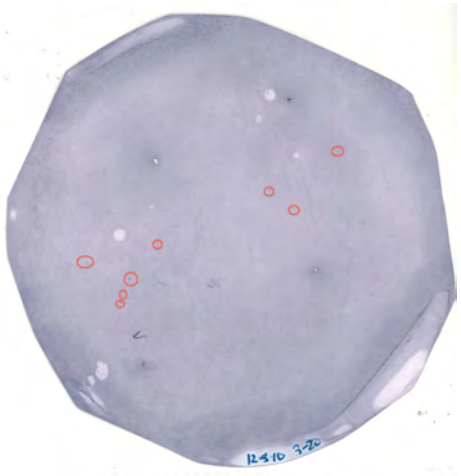

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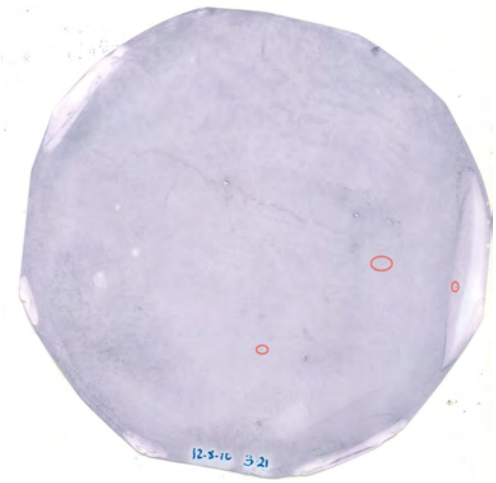

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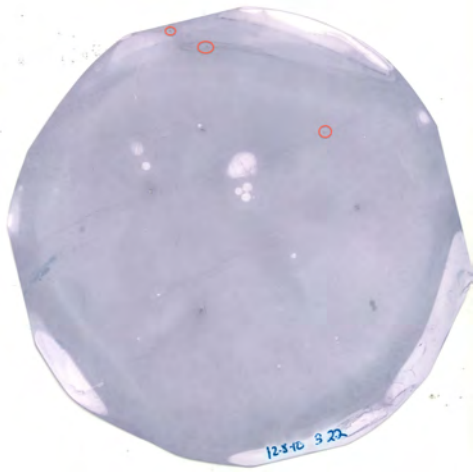

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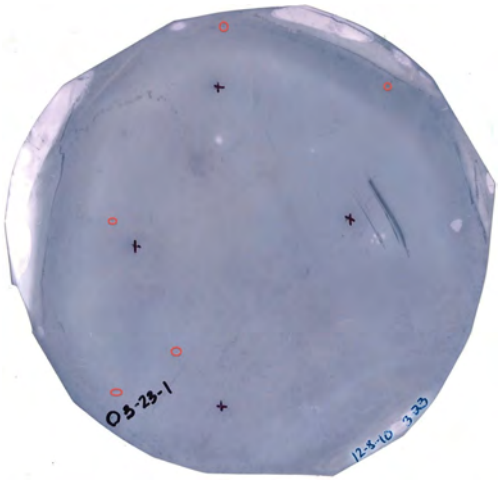

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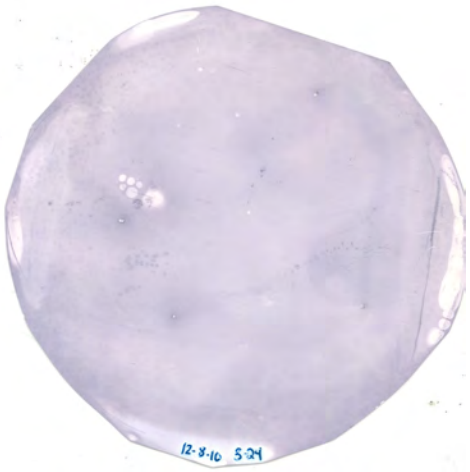

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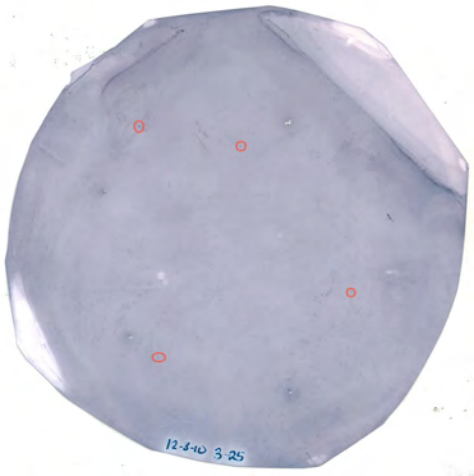

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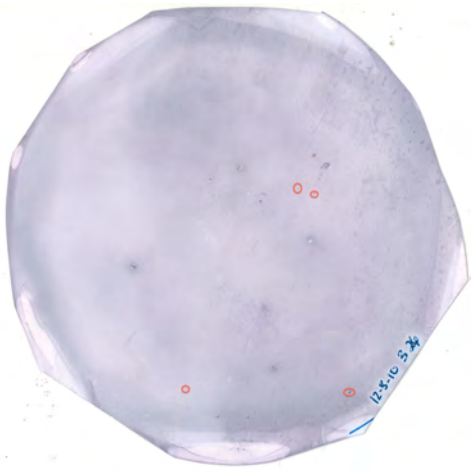

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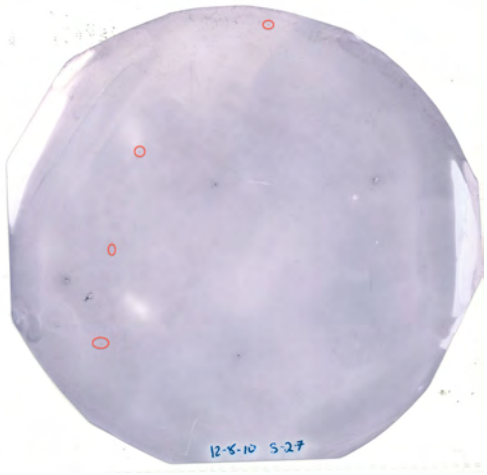

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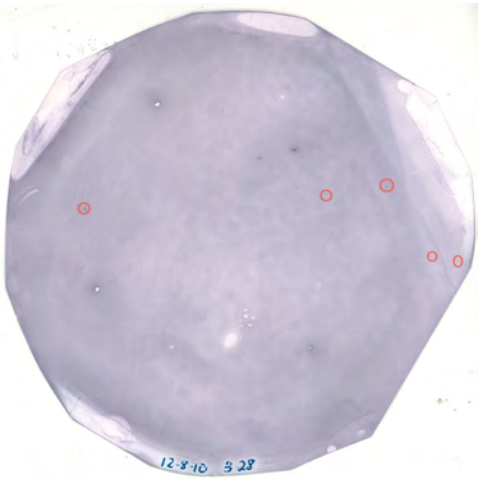

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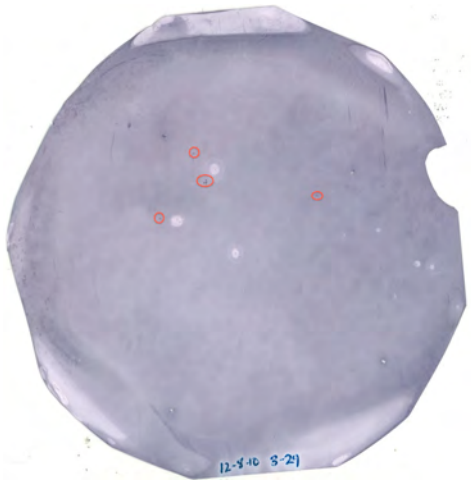

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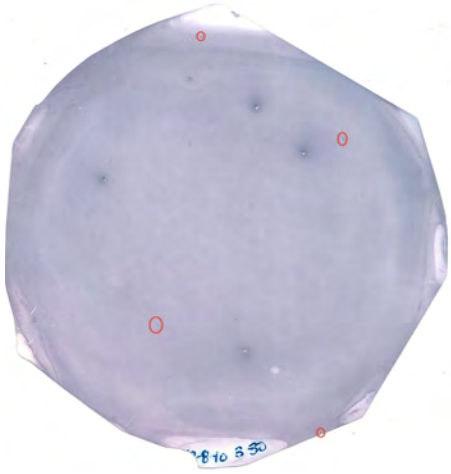

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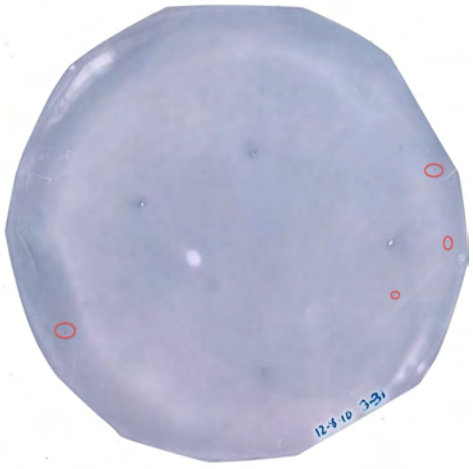

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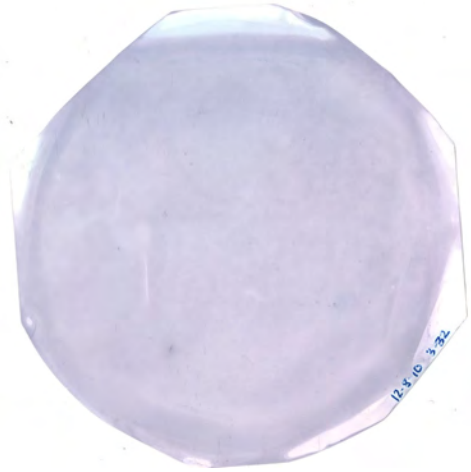

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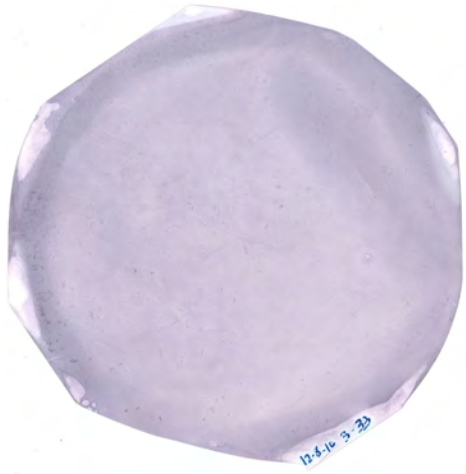

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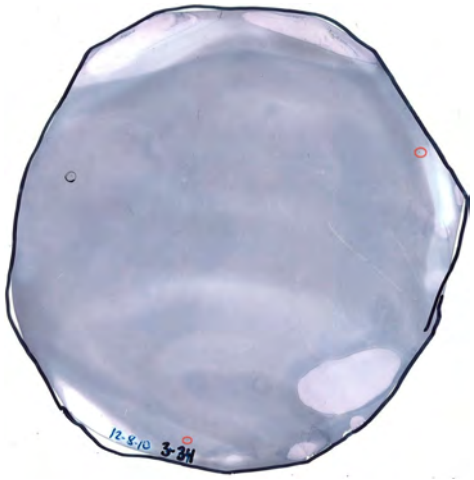

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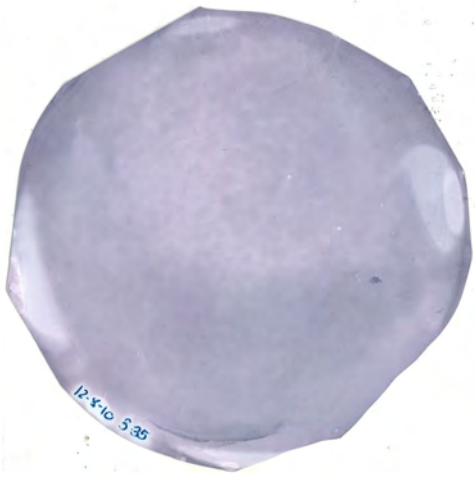

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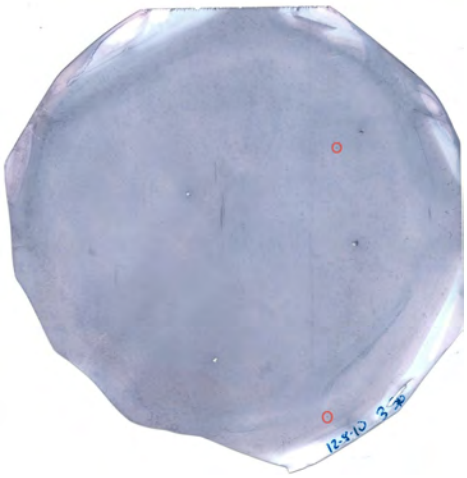

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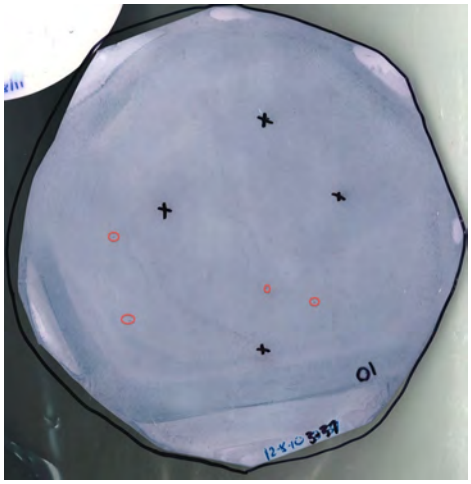

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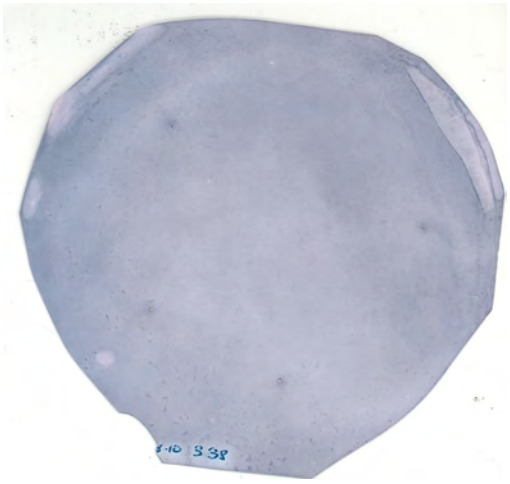

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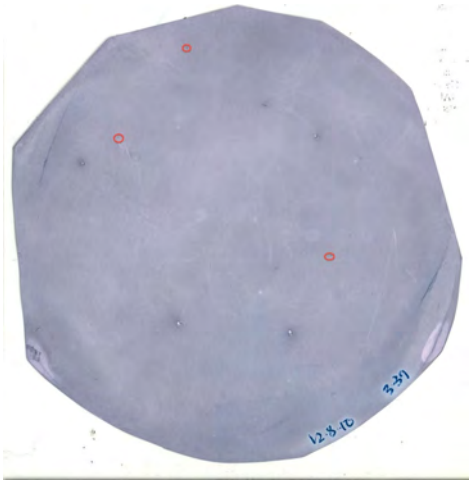

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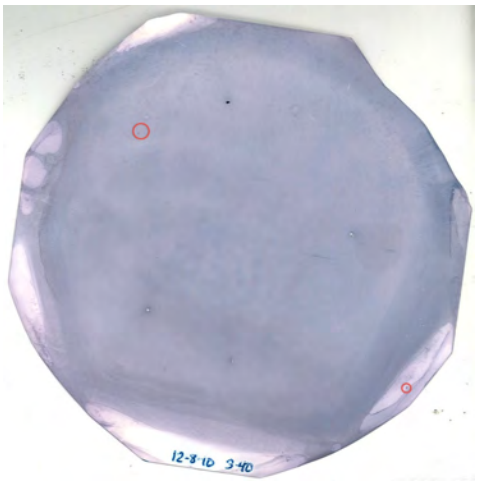

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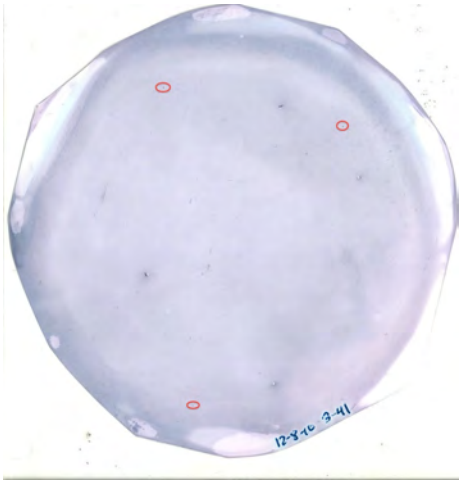

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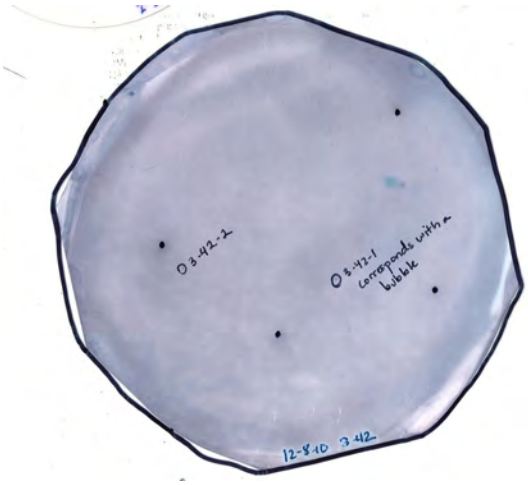

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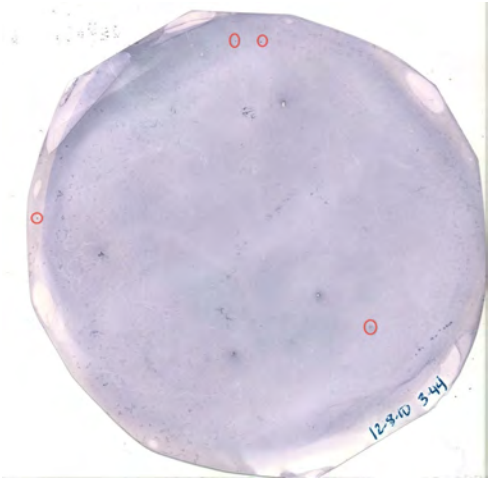

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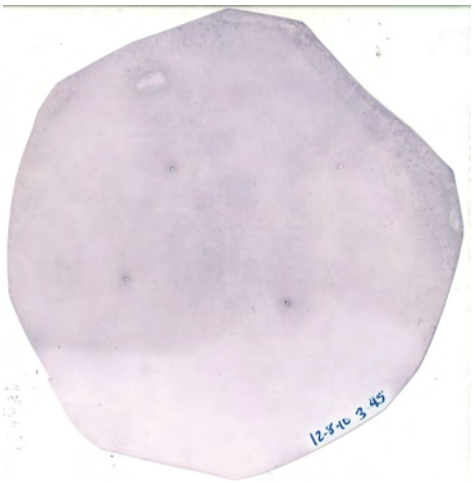

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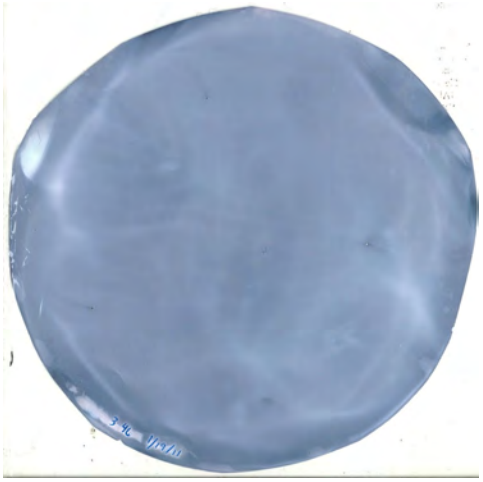

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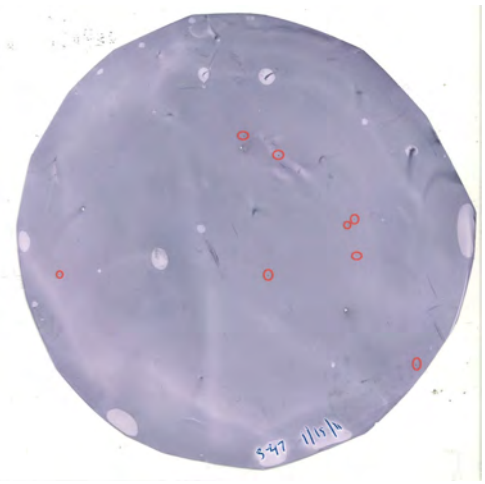

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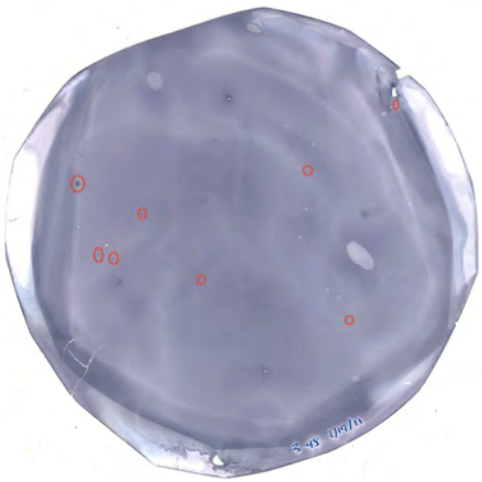

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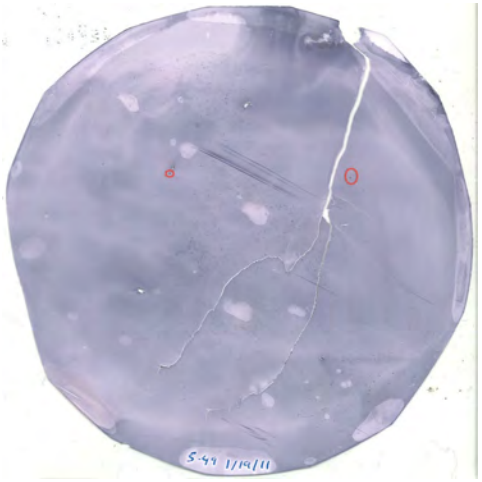

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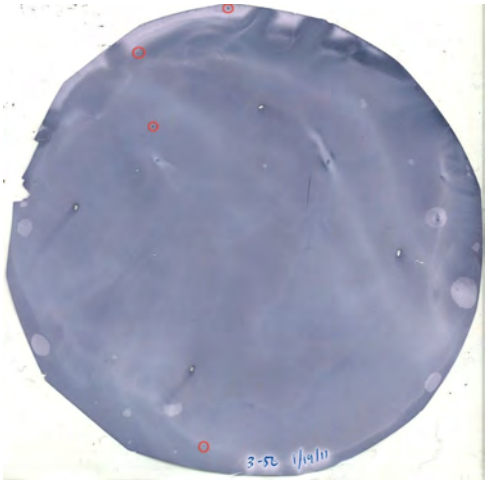

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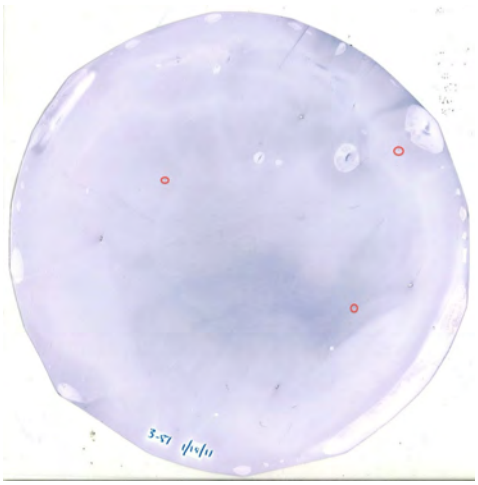

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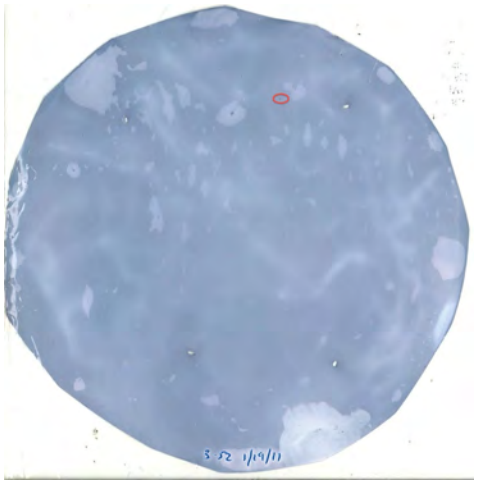

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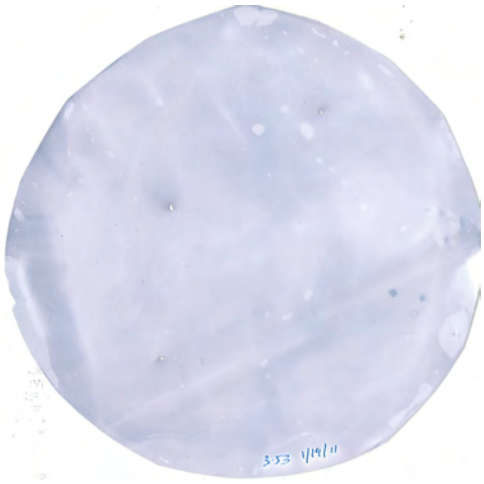

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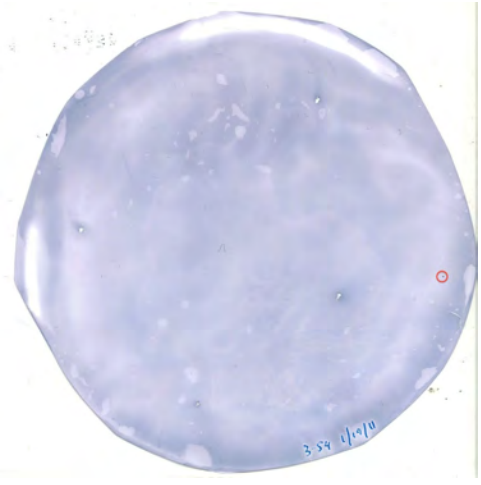

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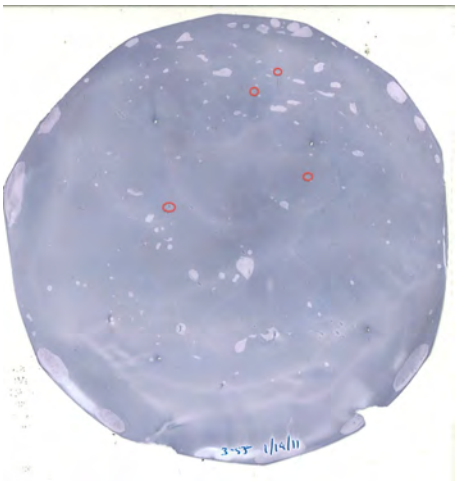

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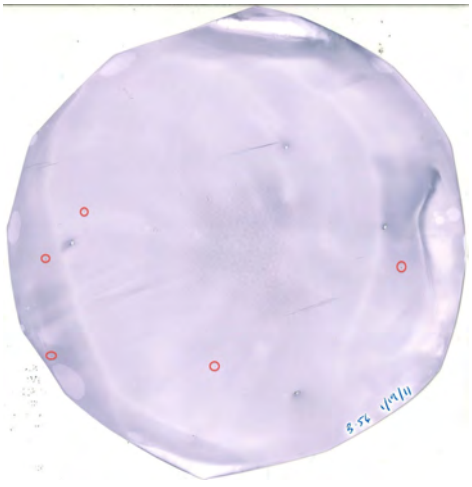

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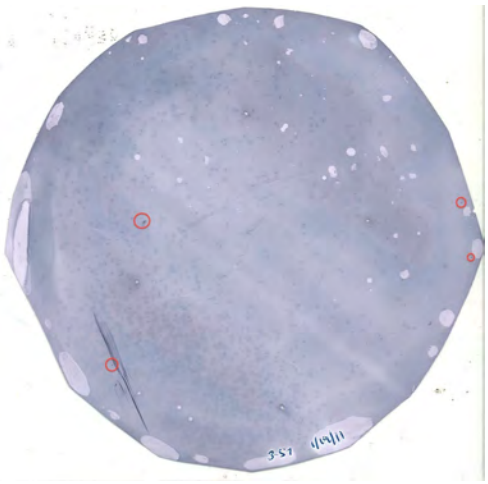

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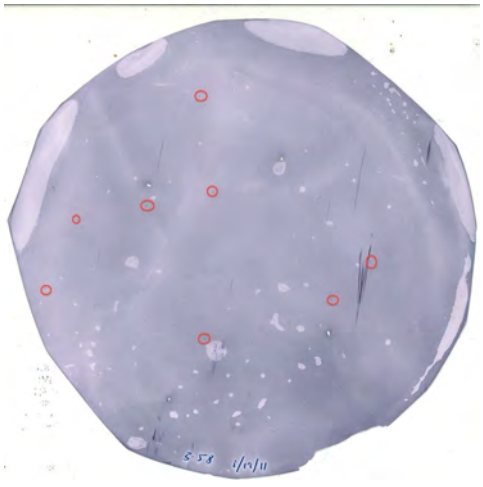

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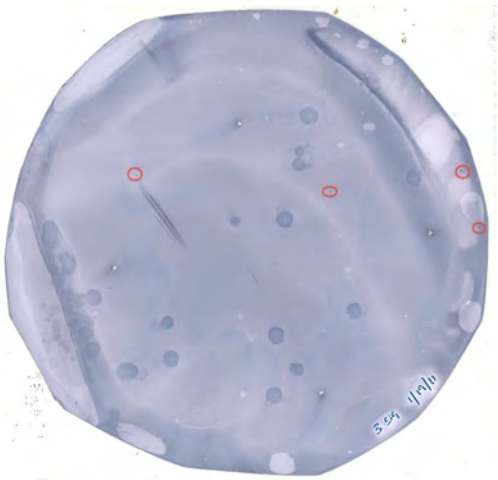

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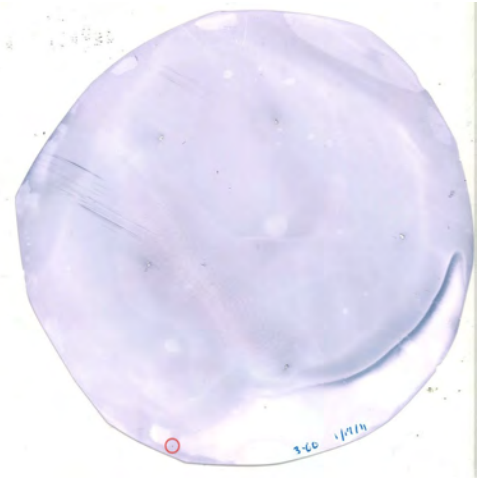

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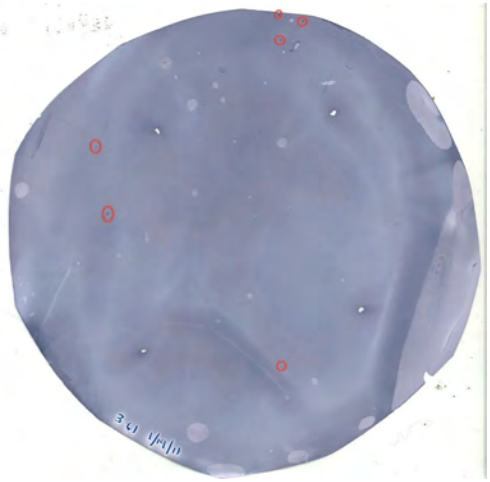

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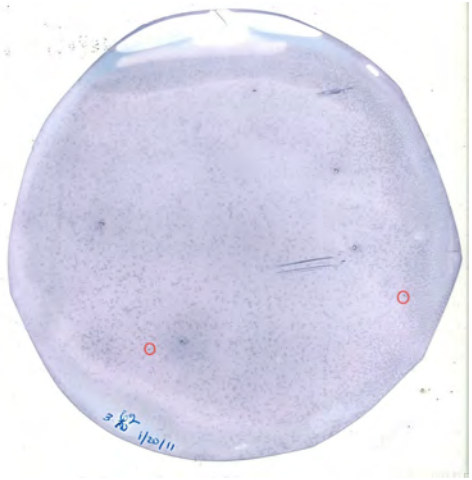

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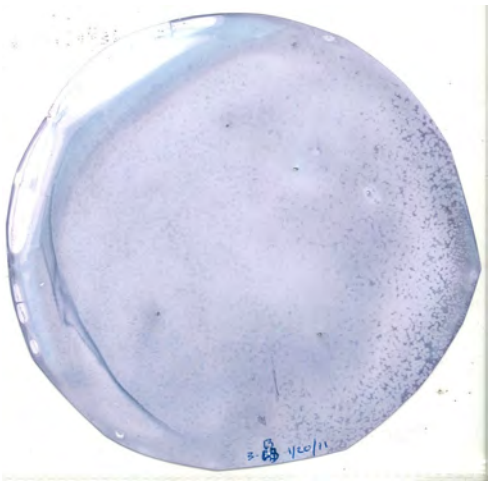

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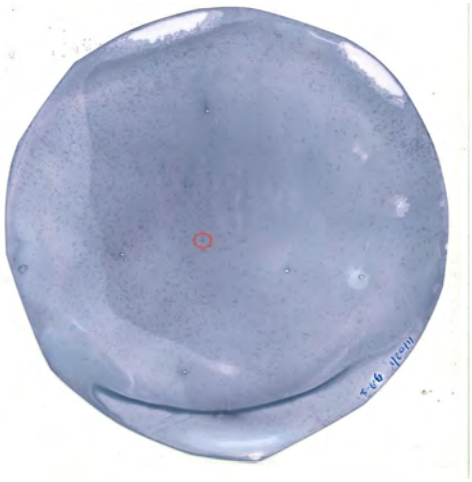

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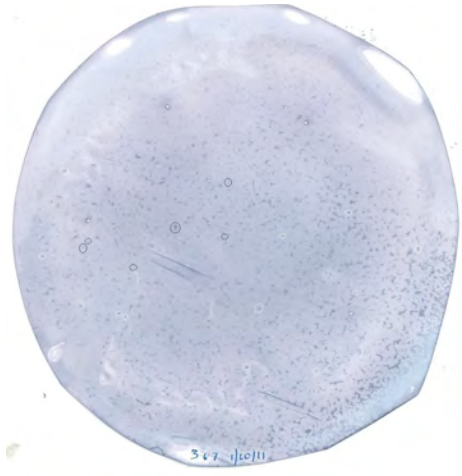

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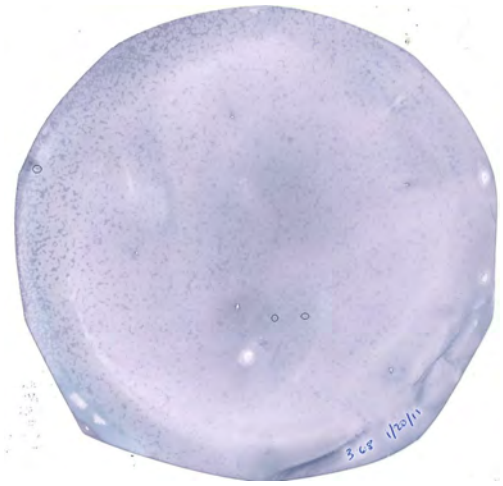

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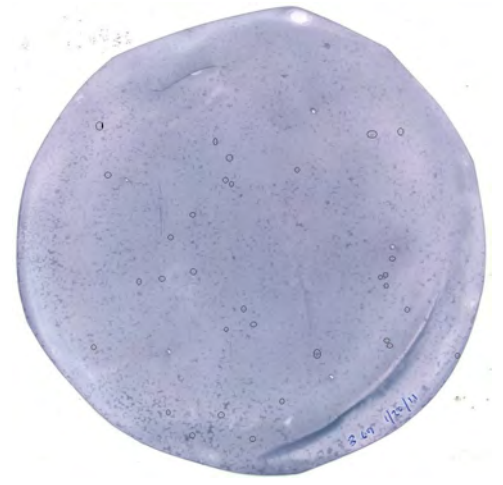

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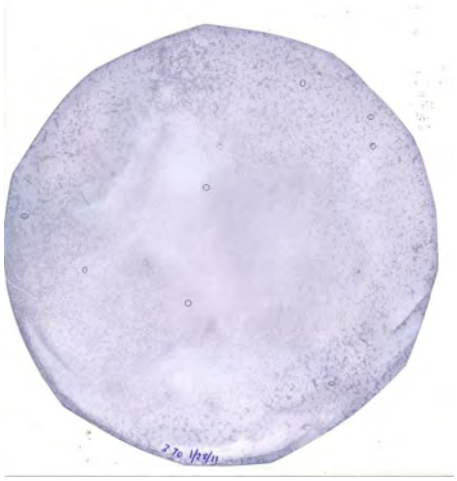

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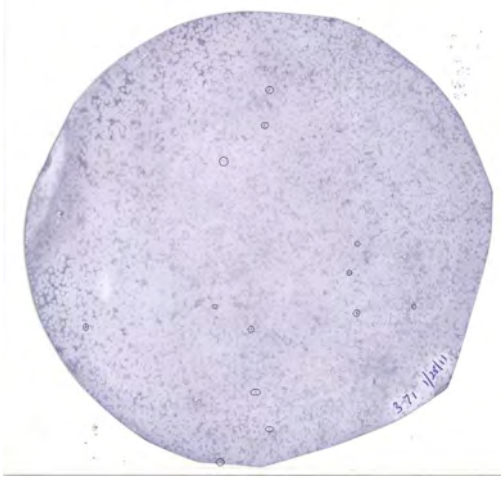

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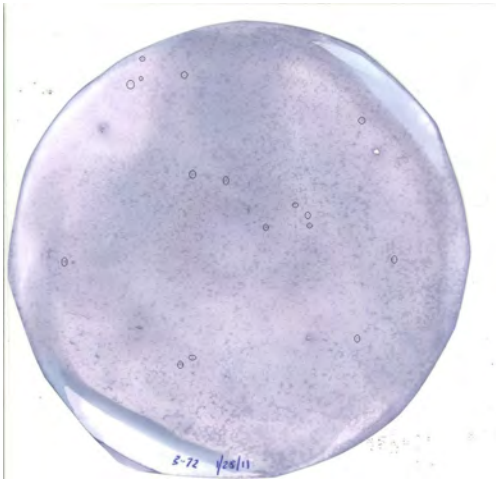

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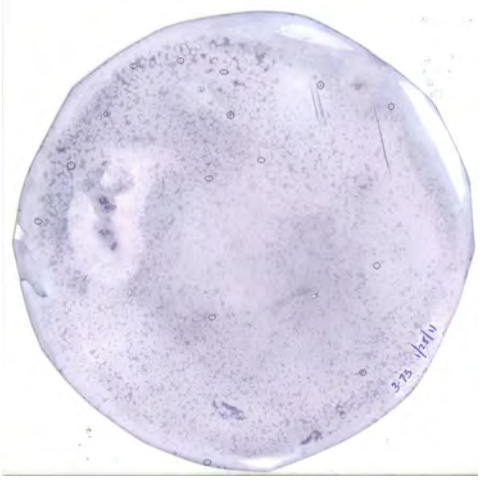

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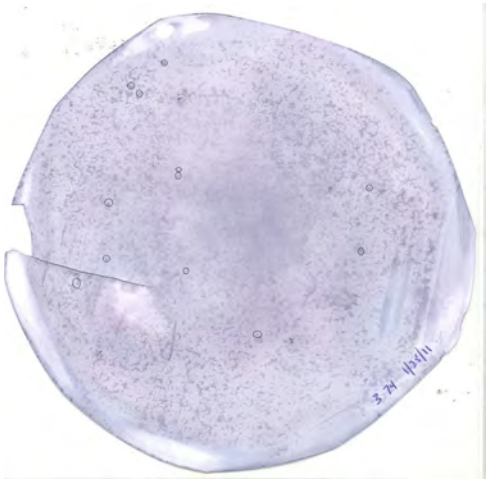

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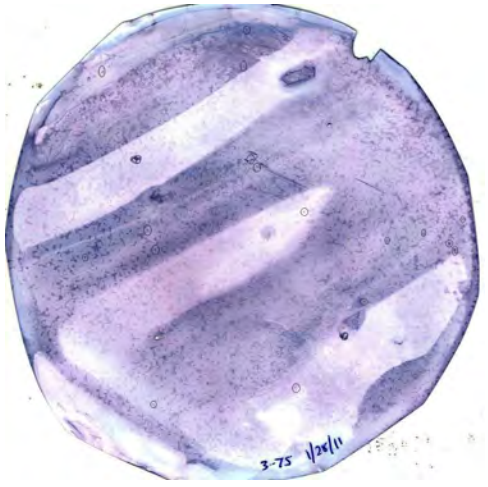

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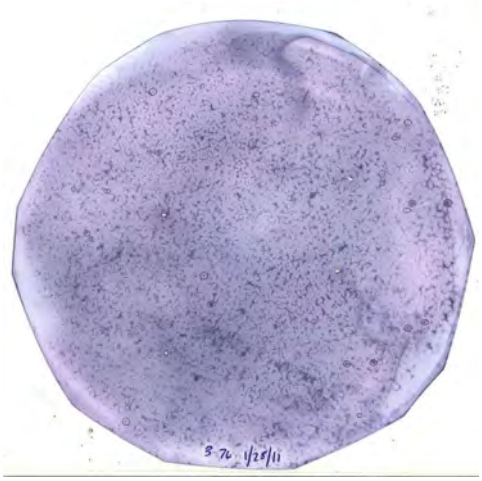

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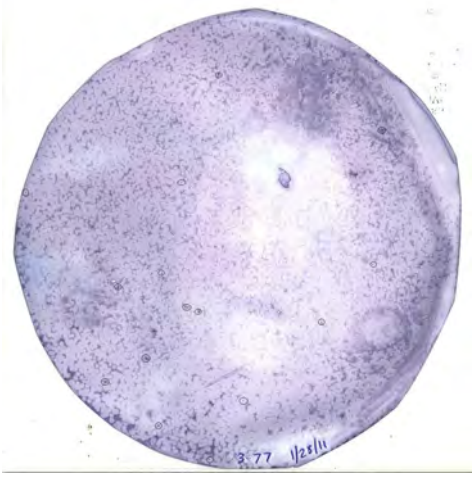

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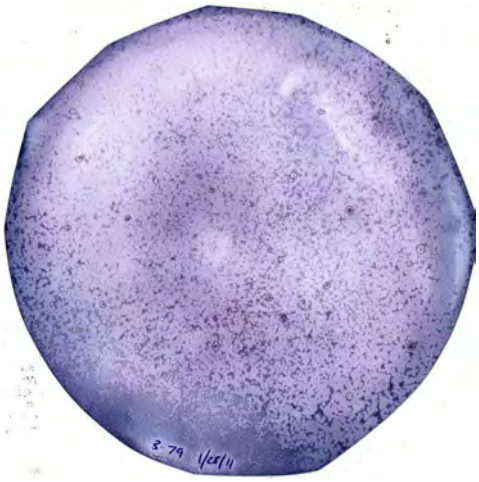

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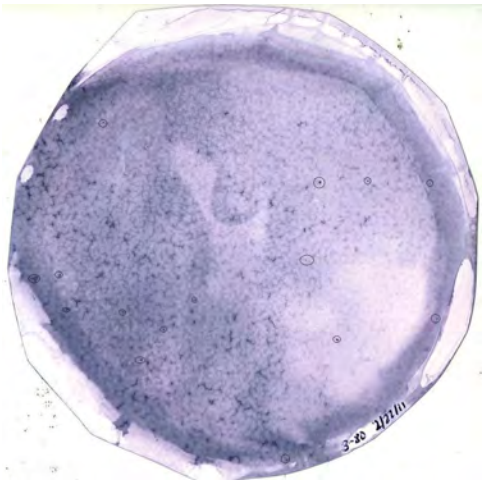

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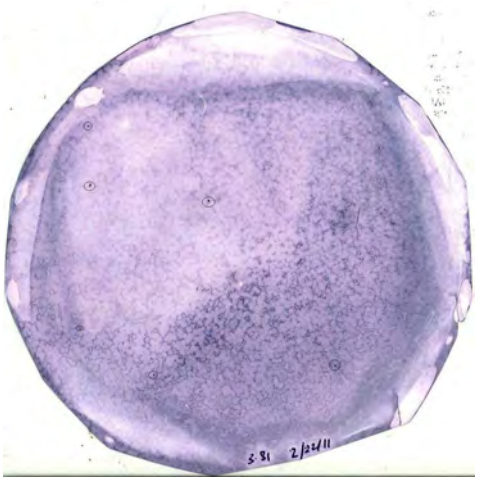

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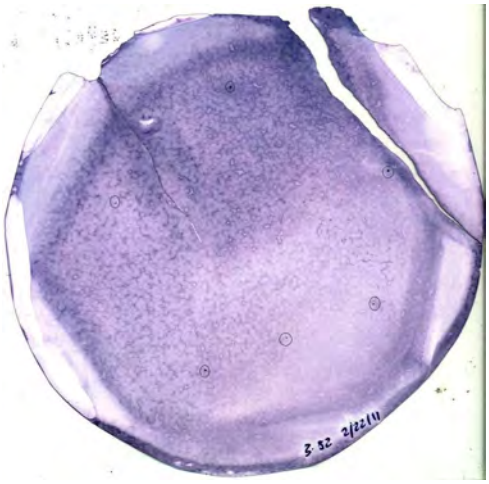

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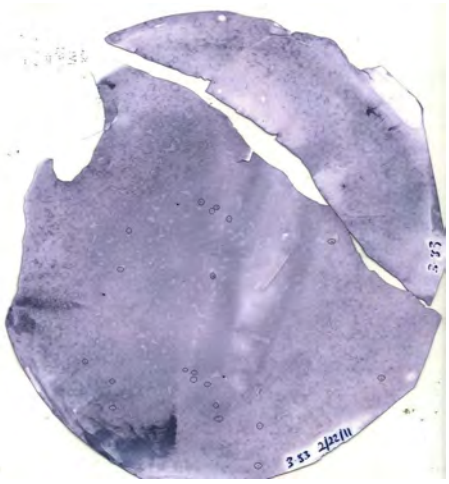

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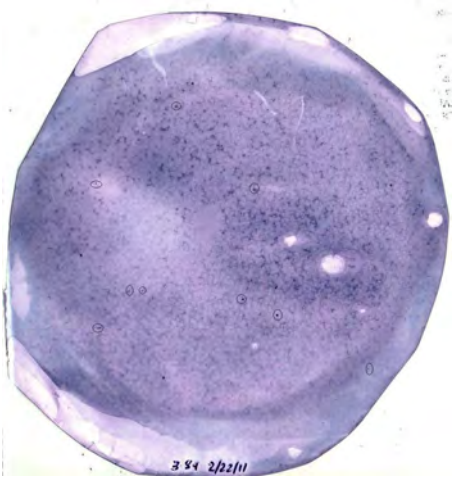

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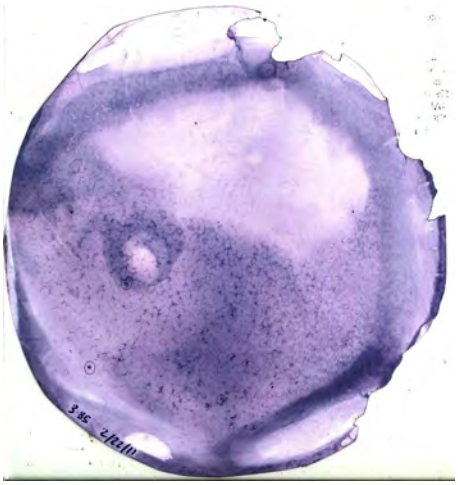

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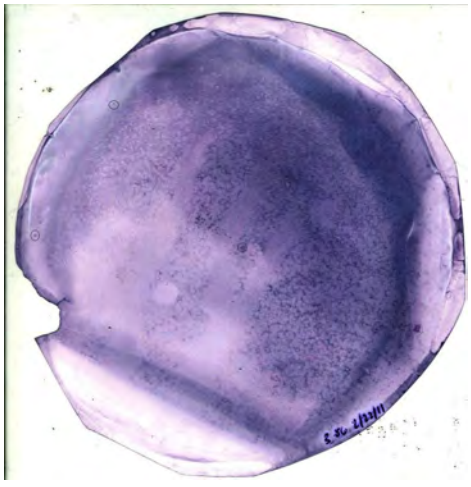

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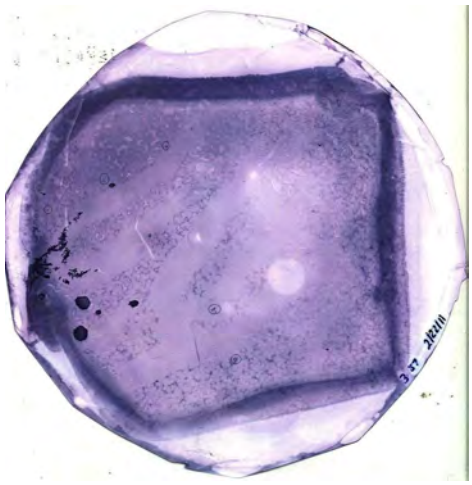

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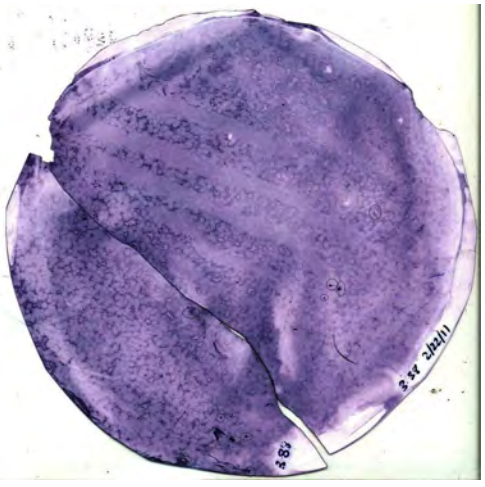

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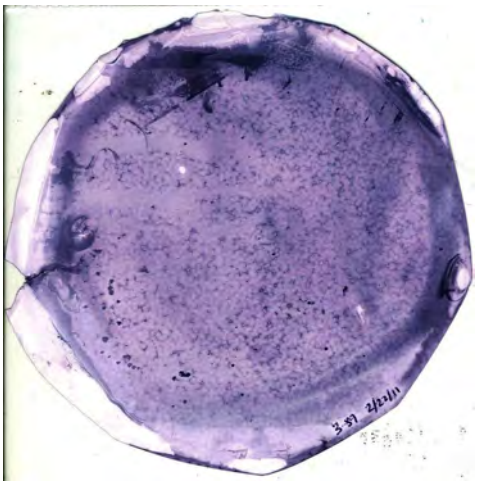

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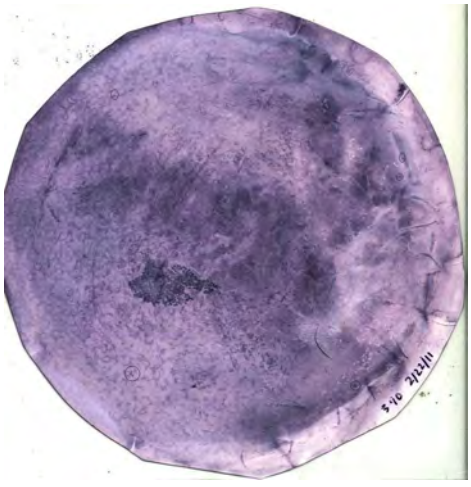

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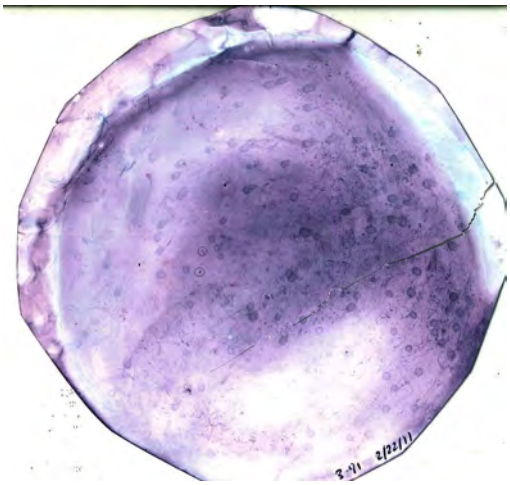

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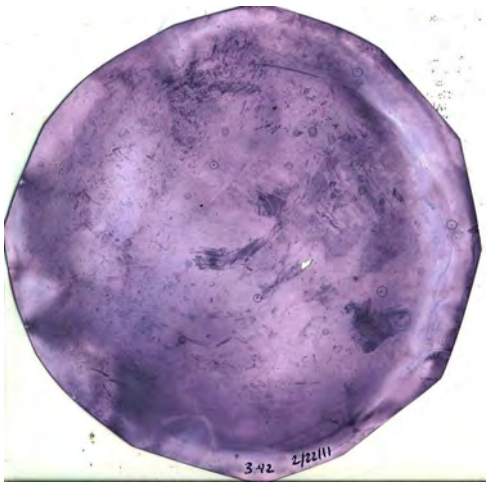

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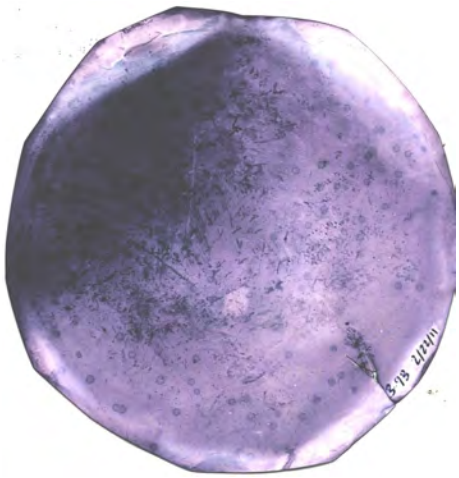

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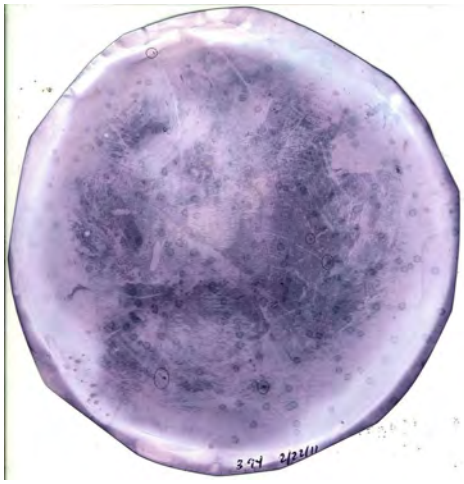

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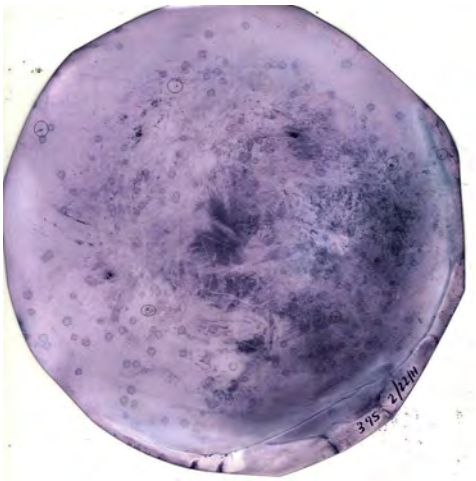

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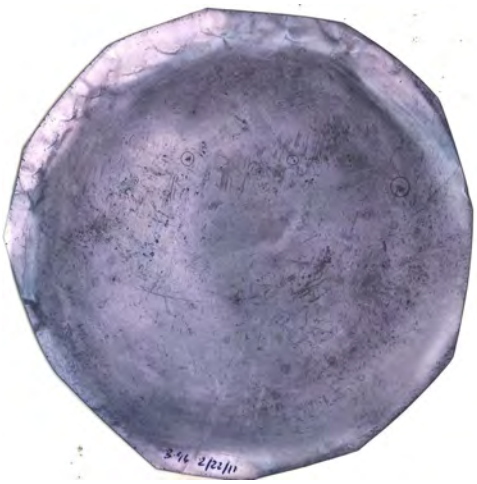

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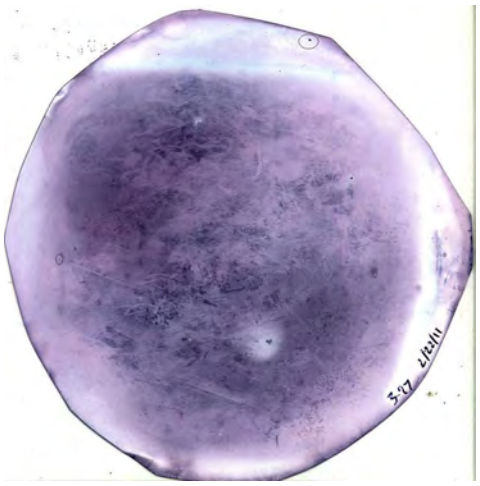

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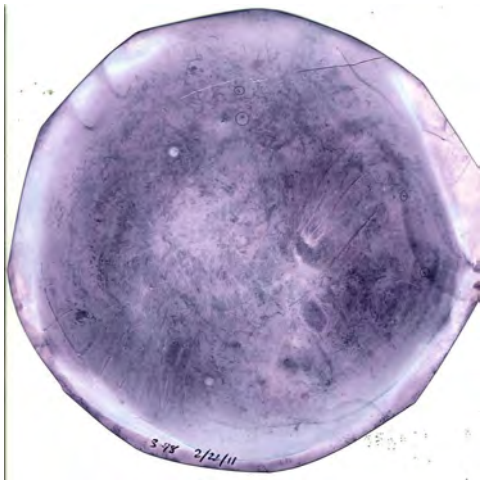

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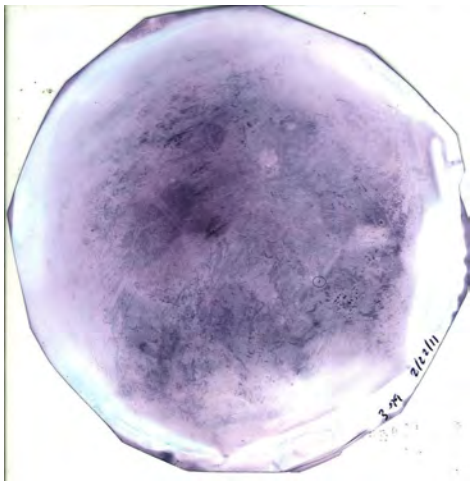

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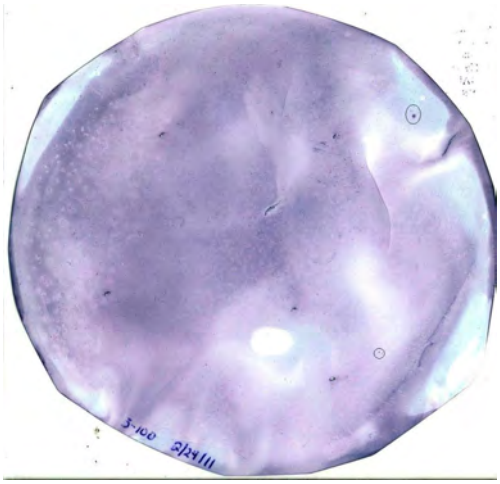

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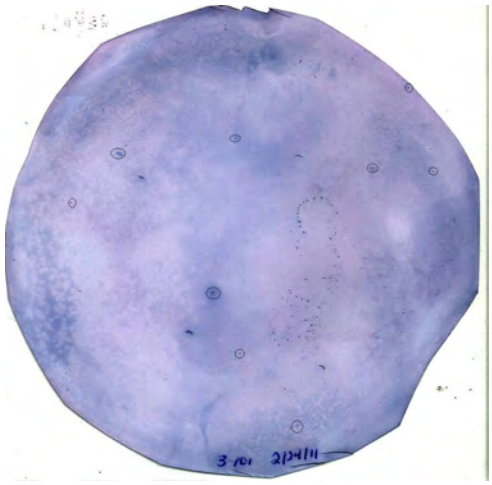

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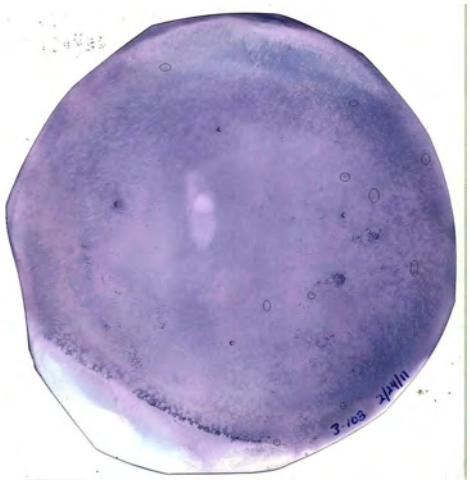

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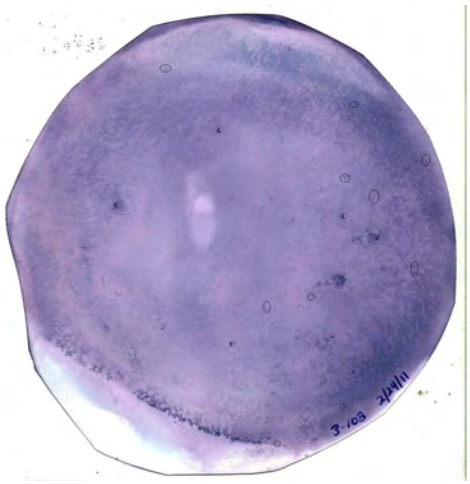

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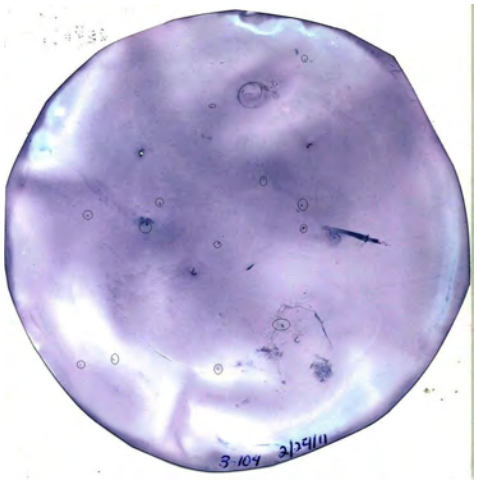

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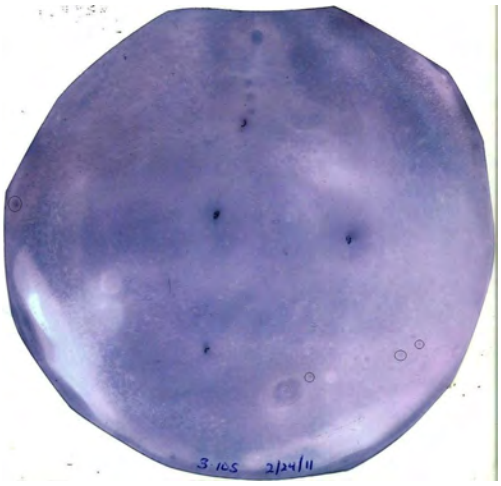

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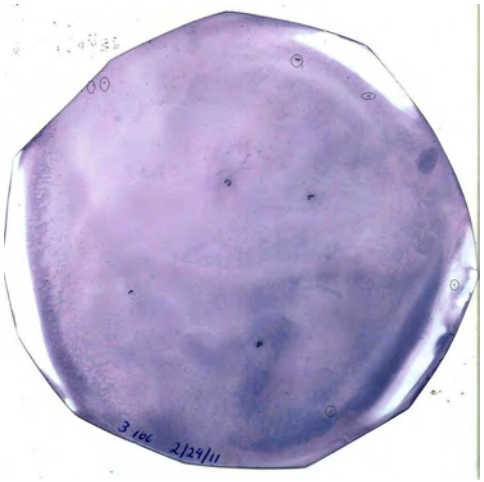

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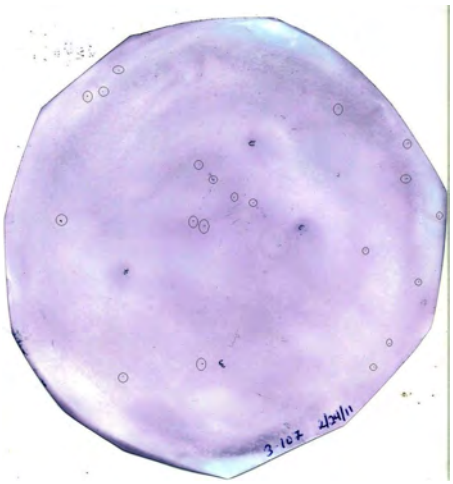

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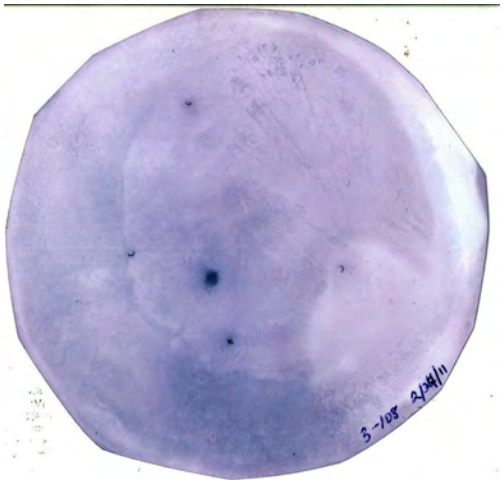

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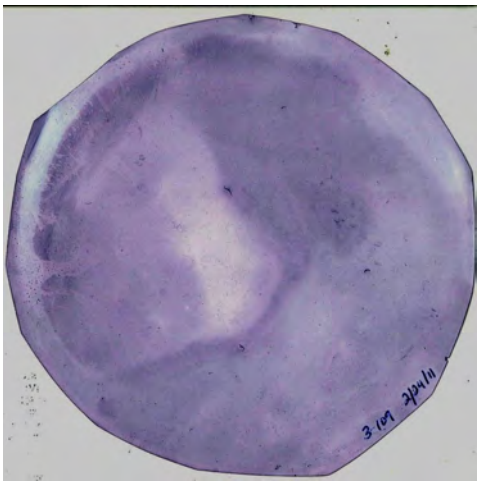

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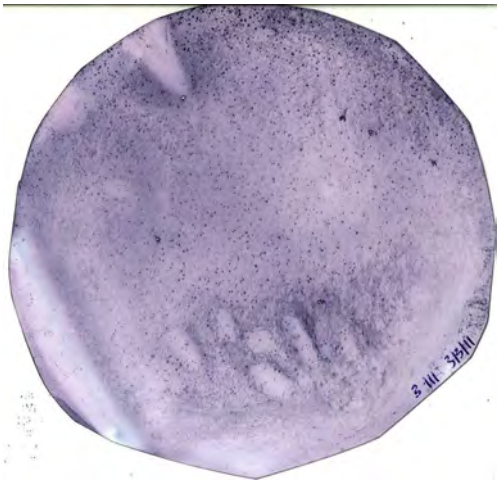

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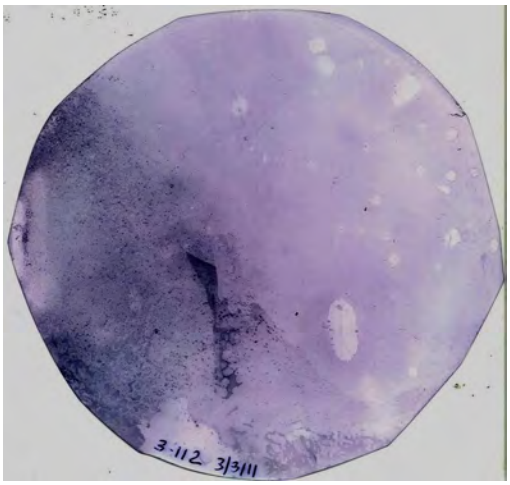

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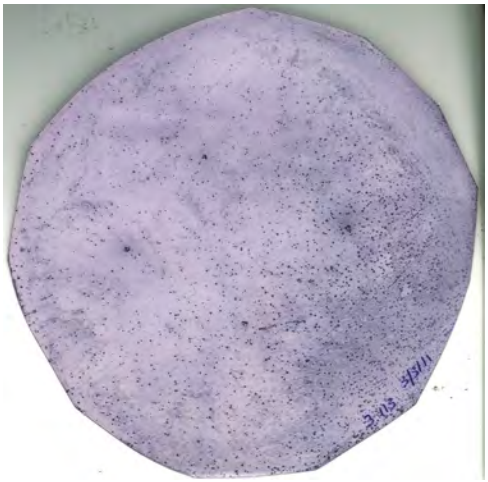

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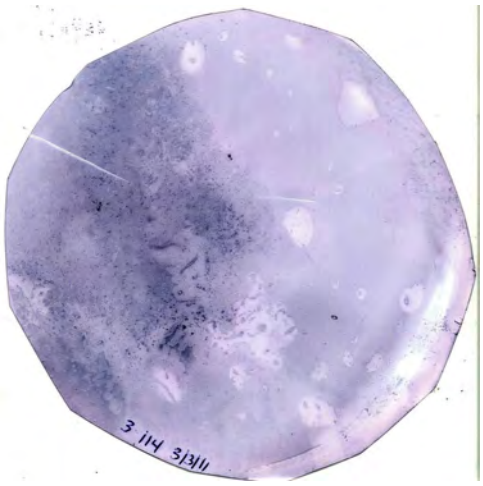

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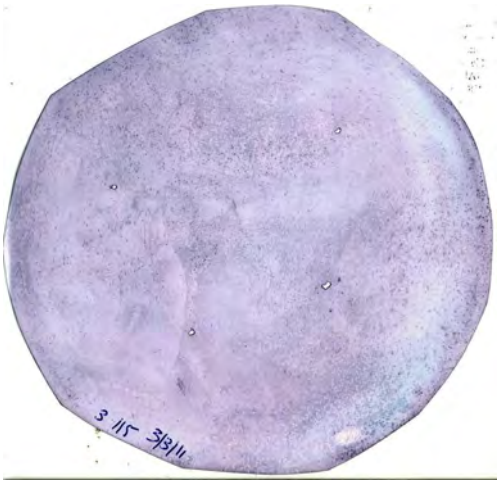

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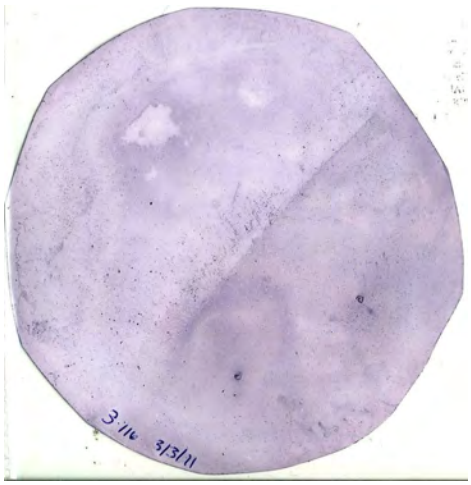

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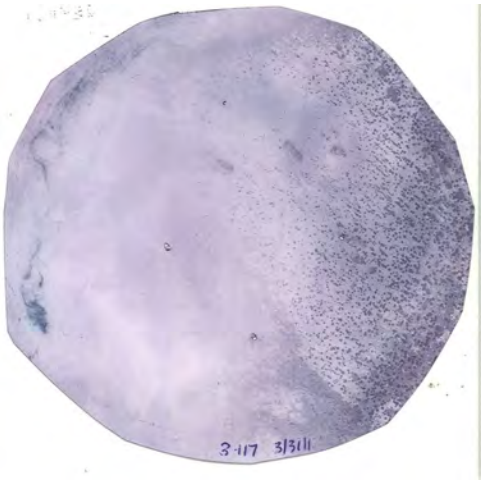

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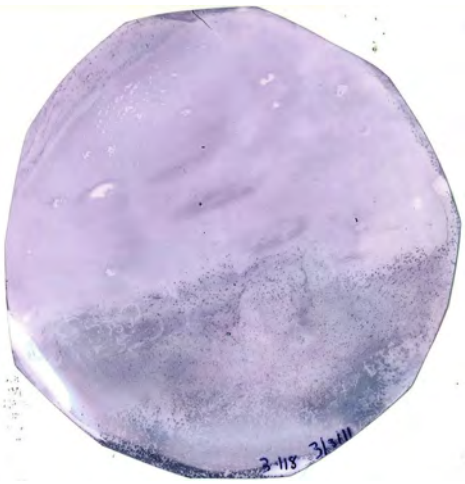

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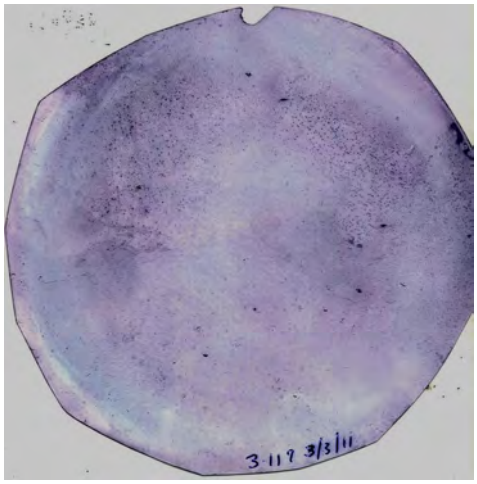

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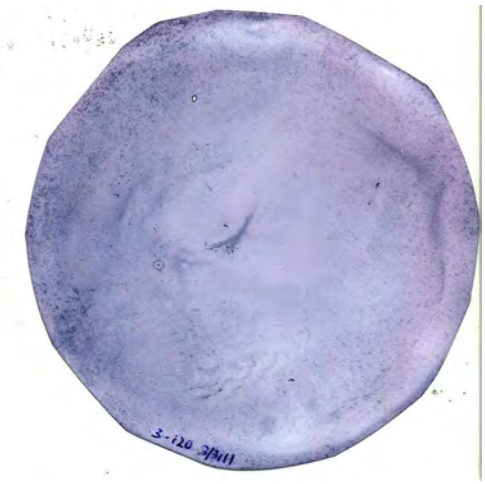

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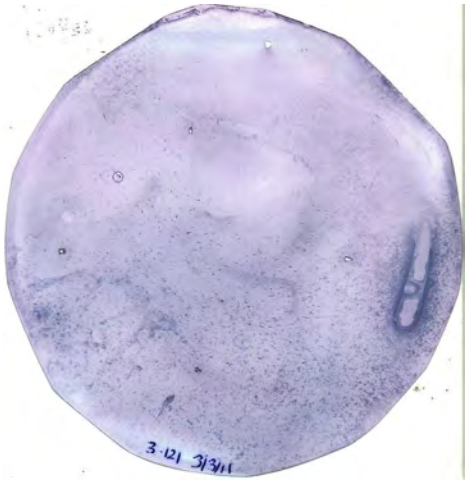

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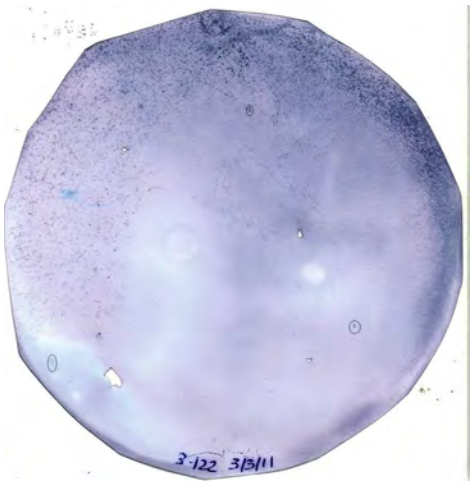

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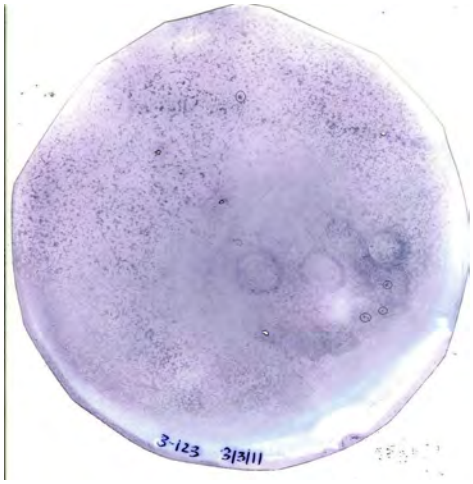

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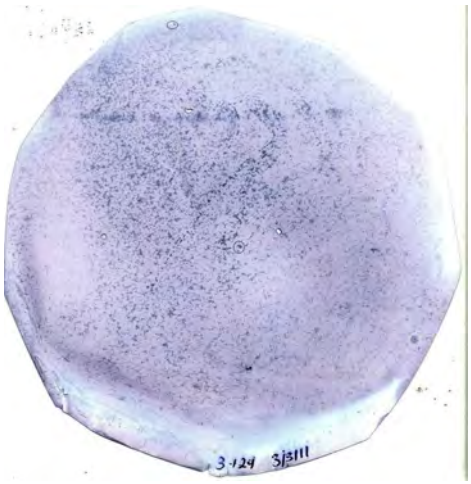

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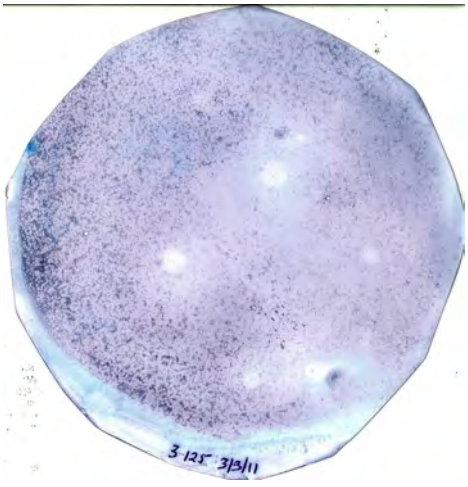

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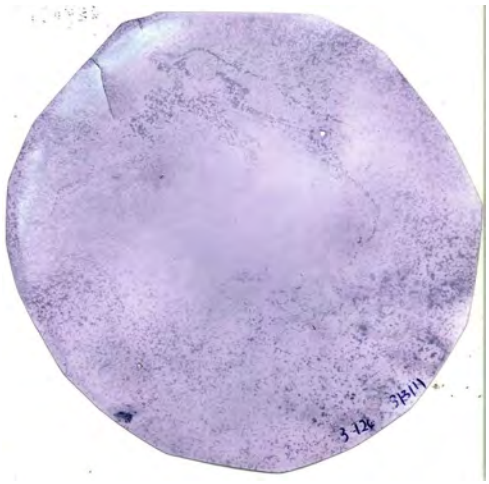

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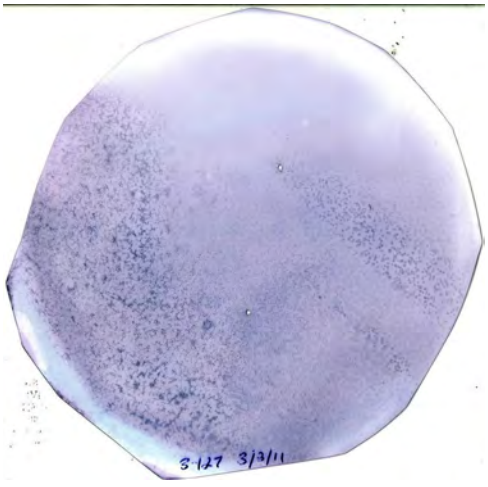

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